STERICALLY STABILIZED IMMUNOLIPOSOMES: FORMULATIONS FOR DELIVERY OF DRUGS AND GENES TO TUMOR CELLS *IN VIVO*.

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INTRODUCTION

Immunoliposomes represent a promising strategy to achieve targeted drug delivery for the treatment of cancers overexpressing specific surface receptors. Advances in immunoliposome design have been facilitated by independent progress in the areas of antibody-based therapeutics and liposomes, which can now be utilized for tumor-targeted drug delivery Park et al., 1997a).

A logical focus for the development of targeted cancer therapies is the HER2 receptor. The HER2 (C-ErbB-2, *neu*) protooncogene and its encoded $p185^{HER2}$(ErbB-2) receptor tyrosine kinase play an important role in the pathogenesis of breast and other cancers (for review, see Hynes and Stern, 1994). HER2 is overexpressed stably in a significant proportion of cancers, but in normal tissue it is expressed at much lower levels, if at all. As a cell surface receptor it is readily accessible to antibody-based therapeutics, and monoclonal antibodies directed against HER2 can inhibit tumor growth by affecting HER2 signal transduction. For example, muMAb4D5 and its humanized derivative, rhuMAbHER2, inhibit the growth of HER2-overexpressing breast cancer cells (Lewis et al., 1993) and enhance the efficacy of certain chemotherapy drugs (Shepard et al., 1991). In phase II clinical trials, treatment with rhuMAbHER2 alone (Baselga et al., 1996), or in combination with cisplatin chemotherapy (Pegram et al., 1996) was associated with encouraging antitumor activity against advanced breast cancer. rhuMAbHER2 is currently being evaluated in phase III clinical trials for the treatment of advanced breast cancer in conjunction with first-line chemotherapy.

Important new developments in liposomal drug delivery include "sterically stabilized" or "stealth" liposomes, which are more resistant to reticuloendothelial system (RES) clearance than so-called "conventional" liposomes (Papahadjopoulos et al., 1991). These developments

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produced substantially prolonged drug circulation with enhanced tumor accumulation (Papahadjopoulos et al., 1991; Huang et al., 1992a; Lasic and Martin, 1995). The coupling of anti-HER2 antibody fragments to sterically stabilized liposomes (SIL) represents a more recent design modification to further increase the therapeutic index of an encapsulated drug via selective delivery to HER2-overexpressing cancer cells (Park et al., 1995). The receptor-mediated internalization property of this targeted delivery vehicle also affords an opportunity to achieve tumor-specific uptake of novel therapeutics such as genes and oligonucleotides.

FORMULATION OF ANTI-HER2 IMMUNOLIPOSOMES

Immunoliposomes (SIL) targeted to the HER2 receptor, incorporate multiple design elements to optimize intracellular delivery of encapsulated agent to tumor cells. These include: (a) rhuMAbHER2 Fab' for reduced clearance and immunogenicity, and to provide internalization and antiproliferative activities; (b) PEG-Fab' linkage to facilitate immunoliposome binding and internalization; (c) sterically stabilized immunoliposomes for prolongation of circulation and selective tumor extravasation; and (d) encapsulated therapeutic agents (e.g.doxorubicin, nucleic acids) for enhanced therapeutic index via targeted intracellular delivery.

These designs were tested by constructing multiple versions of anti-HER2 immunoliposomes containing covalently linked rhuMAbHER2 Fab' (Genentech, Inc.) and poly(ethylene glycol) (PEG)-phosphatidylethanolamine (PE) in varying proportions (0-12 mol % of total phospholipid). SIL were prepared by conjugation of "conventional" (phosphatidylcholine + cholesterol; PC/Chol) or "sterically stabilized" (PC/Chol/PEG-PE) small unilamellar liposomes with rhuMAbHER2-Fab'. Initially, Fab' was conjugated to maleimido-phosphatidylethanolamine (M-PE), resulting in Fab' directly linked to the liposome surface, and hence in parallel with PEG. Recently, we have also prepared SIL with Fab' conjugated to maleimide-terminated PEG-PE (MMC-PEG-DSPE or MP-PEG-DSPE) (Kirpotin et al, 1997; O'Connell et al., 1993), resulting in Fab' linked to the distal end of PEG chains. Both procedures were highly efficient, typically yielding 50-100 Fab' fragments per liposome particle.

INTERACTION WITH CANCER CELLS *IN VITRO*

Studies of SIL binding, internalization and intracellular drug delivery were performed quantitatively with SIL containing a pH-sensitive fluorescent probe (1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS/pyranine). These studies demonstrated rapid uptake of SIL by SK-BR-3 cells into a neutral environment, with subsequent accumulation in an acidic intracellular compartment, consistent with surface binding followed by receptor-mediated endocytosis (Kirpotin et al., 1997a). Total uptake of SIL in SK-BR-3 cells reached 23,000 SIL/cell; while total uptake of non-targeted control liposomes was essentially undetectable. In addition, total uptake of SIL in non-HER2-overexpressing MCF-7 cells was 700-fold lower than in SK-BR-3 cells. With liposomes Fab'-linked SIL, but not with PEG-Fab'-linked SIL, high PEG content was associated with reduced binding affinity and endocytosis. Binding and endocytosis also depended on the quantity of conjugated Fab', reaching a plateau at ~40 Fab'/immunoliposome for binding, and ~10 Fab'/immunoliposome for internalization.

The tumor cell uptake of SIL containing colloidal gold particles was studied by electron microscopy (Park et al., 1995). SK-BR-3 cells treated with anti-HER2 SIL (0 mol% PEG) showed gold-loaded SIL at the cell surface and intracellularly in coated pits, coated vesicles, endosomes, multivesicular bodies, and lysosomes, consistent with internalization occurring via

the coated pit pathway. In addition, gold particles were noted free within the cytoplasm and not associated with membrane-bound organelles, indicating that delivery outside the endolysosomal pathway had also occurred.

PHARMACOKINETICS FOLLOWING I.V. INJECTION

Pharmacokinetic studies of doxorubicin (dox)-loaded anti-HER2 immunoliposomes following single i.v. injection were performed in normal adult rats. Plasma levels of dox following i.v. injection with either SIL or control (no Fab') sterically stabilized liposomes (SL) were similar, and indicated biexponential rate of clearance with terminal plasma half-lives of greater than 10 h and mean residence times of 16-24 h (Park et al., 1996). By comparison, levels of dox following injection of free dox at the same dose (800 mg) were undetectable at 5 min. The integrity of dox-loaded anti-HER2 SIL *in vivo* was assayed by two-component pharmocokinetic studies, in which plasma pharmacokinetics of dox and of rhuMAbHER2-Fab' were independently determined on identical plasma samples following single i.v. injection. The terminal plasma half-lives of rhuMAbHER2-Fab'(as measured by ELISA) and that of dox (as measured fluorimetrically) were both approximately 10 h, indicating negligible dissociation or drug leakage.

LOCALIZATION IN HUMAN TUMOR XENOGRAFTS IN MICE

Tumor localization of dox-loaded anti-HER2 SIL were evaluated in HER2-overexpressing tumor xenograft models, in which nude mice carrying established subcutaneous (s.c.) BT-474 tumor xenografts received a single i.v. injection of SIL (Park et al., 1996). The concentration of dox in tumor tissue still exceeded 1%/g of tissue 67 h after injection, which was significantly higher than dox levels in all other tissues except liver, the major site of liposome clearance. Tumor/blood and tumor/muscle ratios were both greater than 22-fold.

SIL localization in tumors was studied histologically in two tumor xenograft models, BT-474 and MCF7/HER2 (MCF7 cells stably transfected with HER2), following administration of SIL containing colloidal gold (Park et al., 1997). Gold-loaded SIL were administered i.v. every 48 h for 3 doses and subsequently visualized by light microscopy within tumor tissue using a silver enhancement technique (Huang et al., 1992). Silver grains indicating the presence of gold particles were observed throughout tumor tissue, both in perivascular areas and within cellular regions of the tumor. Importantly, silver grains were frequently observed within the cytoplasm of individual tumor cells, indicating intracellular delivery of gold particles. In contrast, treatment with control (no Fab') sterically stabilized liposomes (SL) showed silver grains accumulating in predominantly extracellular and perivascular spaces, consistent with previous reports of the tumor interstitial localization of sterically stabilized liposomes (Huang et al., 1992b). These control liposomes were not observed within individual tumor cells.

Intracellular localization of anti-HER2 SIL *in vivo* was confirmed using encapsulated ^{67}Ga-DTPA chelate administered by single i.v. injection in the MCF7/HER2 tumor xenograft model (Park et al., 1996). After 48 h, excised tumors were analyzed by electron microscopic autoradiography. Autoradiographic grains signifying ^{67}Ga emission resulting from immunoliposome delivery were frequently detected within tumor cells, at or near the cell surface, within the cytoplasm, and within the nucleus. In contrast, tumors from mice treated with ^{67}Ga-loaded control liposomes showed no intracellular localization of ^{67}Ga.

ANTI-TUMOR EFFICACY

Delivery of dox by anti-HER2 SIL may represent a particularly advantageous strategy for the treatment of HER2-overexpressing breast cancers, since these cancers appear to possess an especially steep dose-response relationship to dox-based therapy (Muss et al., 1994). In addition, the significant clinical problem of dox toxicity to myocardium and hematopoietic cells may be largely mitigated by anti-HER2 immunoliposome delivery, as HER2 expression is negligible in these cell types (Press et al., 1990). Finally, the antiproliferative effect of the anti-HER2 immunoliposome vehicle (i.e., the rhuMAbHER2 effect) may work synergistically with dox, since it is has been shown that rhuMAbHER2 augments the efficacy of dox in animal models (Baselga et al., 1994).

Studies with dox-loaded anti-HER2 SIL *in vitro* showed efficient and specific cytotoxicity against HER2-overexpressing breast cancer cells (Park et al., 1995). Treatment of SK-BR-3 cells for 1 h with dox-loaded anti-HER2 SIL yielded dose-dependent cytotoxicity (IC50 = 0.3 mg/ml) equivalent to that of free dox, indicating that SIL delivered dox as efficiently as the rapid diffusion of free dox into cells *in vitro*, and were up to 30-fold more cytotoxic than dox-loaded SIL bearing irrelevant Fab'. The specificity of targeting was further confirmed by treatment of WI-38 cells, a nonmalignant lung fibroblast cell line expressing minimal levels of HER2, wherein dox-loaded anti-HER2 SIL demonstrated cytotoxicity that was 20-fold less than free dox and equivalent to SIL with irrelevant Fab'.

Anti-tumor efficacy studies using dox-loaded anti-HER2 SIL in multiple HER2-overexpressing tumor xenograft-nude mice models showed a significant increase in the therapeutic index of dox due to targeted delivery (Park et al., 1996). In these studies, experimental treatment was initiated 1-2 wks after tumor implantation, at which time tumors were 200-1000 mm^3 in volume. SIL were administered at a total dox dose of 15 mg/kg, divided over three weekly i.v. injections. Therapeutic effects were compared to those of dox-loaded control (no Fab') SL at the same dose and schedule. Additional control arms include treatment with free dox, which was given at its maximum tolerated dose (MTD) in these animals (7.5 mg/kg), free rhuMAbHER2, and saline. In each of the four models (two independent sublines of BT-474, MCF7/HER2, and MDA-MB-453), treatment with SIL resulted in marked tumor growth inhibition and/or regression (including some cured animals) and was significantly superior to all other treatment conditions. Furthermore, SIL did not produce any apparent acute toxicities or significant weight loss in the nude mice. The MTD determined for SIL represents a 2.5-fold increase over that of free dox.

DELIVERY OF OLIGONUCLEOTIDES AND PLASMIDS

The results described above suggest that anti-HER2 SIL may be particularly advantageous for tumor-specific treatment with agents that require intracellular delivery. For example, anti-HER2 SIL could represent a gene therapy vector system that provides targeted delivery of therapeutic genes or oligonucleotides to tumor cells. To overcome existing obstacles for successful implementation of this strategy, SIL must be reconstructed to have the following properties: efficient packaging of DNA, favorable retention of drug delivery properties (such as stability, long circulation, minimal non-specific reactivity, and reduced immunogenicity), selective and efficient delivery of nucleic acids to tumor cells, and finally intracellular delivery that results in adequate gene expression.

A modification of anti-HER2 SIL for targeted gene therapy has been to include cationic lipids for efficient and high capacity packaging of DNA. Cationic liposomes, which readily form complexes with DNA molecules via electrostatic interactions and can mediate gene transfer to a variety of cell types, have previously attracted much attention as a gene therapy

vector system. However, cationic liposomes developed to date have been limited by poor stability, high non-specific reactivity, and lack of targeting. We recently reported cationic liposome-plasmid DNA complexes that are significantly more stable by manipulation of their lipid composition, inclusion of PEG-PE, and condensation of plasmid DNA with polyamines (Hong et al., 1997). These stable constructs show high gene transfer efficiency *in vitro* and in multiple tissue sites *in vivo* following i.v. administration. For targeting, rhuMAbHER2-Fab' fragments have recently been conjugated covalently to PEG-containing cationic liposomes, thus generating anti-HER2 cationic immunoliposome-DNA complexes. In these cationic SIL, PEG-PE provides the attachment site for Fab', and when present at appropriate concentration, minimizes non-specific reactivity. By this design, anti-HER2 cationic SIL mediate efficient transfection of SK-BR-3 cells *in vitro*: the addition of Fab' is associated with a 20-fold increase in reporter (firefly luciferase) gene expression as compared to cationic liposomes lacking Fab', with minimal transfection observed in non-HER2-overexpressing MCF7 cells (W. Zheng and K. Hong, unpublished results).

Anti-HER2 cationic SIL are also being developed for targeted delivery of oligonucleotides. In recent studies, we have been able to show that a new formulation of these SIL were found to be internalized in SK-BR-3 cells, resulting in rhodamine-labeled liposome accumulation within the cytosol, while the released FITC-labeled oligonucleotides accumulated within the nucleus (Meyer et al., 1998).

ENHANCED RATES OF RELEASE AND EXTRAVASATION

In an attempt to stimulate the extravasation and release of liposome material after localization in tumors, we have developed several different approaches, which are described below. Although these formulations are not targeted to tumor cells specifically, their application to this field could be substantial and could certainly include targeting.

Thermosensitive liposomes have been formulated to respond to an increase in temperature from 37 to 42 degrees C, by an increase in the rate of release of encapsulated drugs in the presence of plasma or serum (Gaber et al., 1995). *In vivo* studies with such liposomes have shown that externally applied local hyperthermia can not only increase the extravasation of intact lipoosomes into the tumor mass, but also increase drastically the rate of release of the encapsulated drug, doxorubicin (Gaber et al., 1996).

Increased extravasation of liposomes into specific tissues has also been observed after systemic administration of vasoactive agents such as substance P (Rosenecker et al., 1996). In such a system, there is a very large increase in the accumulation of liposomes along with their contents into the bronchial airways, the oesophagus and the urinary bladder (Rosenecker et al., 1996). This indicates the potential of increasing the permeability of specific tissues to liposomes by selecting specific ligands that have effects on the endothelial cells of such tissues. Recently we have been able to show that when such liposomes encapsulate β-adrenergic agonists, they can inhibit the effect of subsequent injections of substance P, indicating an anti-inflammatory effect (Zhang et al., 1998).

Finally, we have reported recently the synthesis of new conjugates of PEG-PE where PEG can be detached from liposomes under the influence of mild reducing agents (Kirpotin et al., 1997c). Such detachment is followed by aggregation and fusion, which may be useful for applications requiring intracellular delivery (Park et al., 1997b; Kirpotin et al., 1997a).

DISCUSSION

Anti-HER2 SIL represent a potentially powerful strategy for the treatment of HER2-

overexpressing cancers because of their ability to provide tumor-targeted intracellular drug delivery. Similarly to non-targeted sterically stabilized liposomes, SIL are stable and long circulating *in vivo*. Unlike liposomes, SIL bind to and internalize in target cells but are not reactive with normal cells. In HER2-overexpressing tumor xenograft-nude mouse models, anti-HER2 SIL localize selectively in tumor tissue, where they deliver encapsulated agents intracellularly. In each animal model tested, dox-loaded anti-HER2 SIL greatly extend the therapeutic index of dox, both by increasing its antitumor efficacy and by reducing the systemic host toxicity; SIL exhibited significantly superior efficacy as compared with either dox-loaded sterically stabilized liposomes or free dox.

The anti-HER2 immunoliposome strategy may also enable new therapeutic applications, such as gene therapy. Compared with cationic liposomes, anti-HER2 cationic SIL provided significantly higher levels of reporter gene expression in target cells, but not in non-target cells. Anti-HER2 cationic SIL have also been developed to deliver oligonucleotides to target cells, resulting in the internalization of the complex and nuclear accumulation of oligonucleotides.

The properties of anti-HER2 SIL described here make them a promising and novel strategy for tumor-targeted therapy. In particular, the development of immunoliposome constructs which are stable, long circulating, and able to deliver encapsulated agents intracellularly in tumor cells appears to be important for the success of this strategy. These properties may confer sufficient pharmacokinetic and pharmacodynamic advantages to overcome the previous limitations of monoclonal antibody therapy and liposomal drug delivery (Park et al, 1997b; Kirpotin et al, 1997b).

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Tumor targeting using anti-her2 immunoliposomes

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Abstract

We have generated anti-HER2 (ErbB2) immunoliposomes (ILs), consisting of long circulating liposomes linked to anti-HER2 monoclonal antibody (MAb) fragments, to provide targeted drug delivery to HER2-overexpressing cells. Immunoliposomes were constructed using a modular strategy in which components were optimized for internalization and intracellular drug delivery. Parameters included choice of antibody construct, antibody density, antibody conjugation procedure, and choice of liposome construct. Anti-HER2 immunoliposomes bound efficiently to and internalized in HER2-overexpressing cells in vitro as determined by fluorescence microscopy, electron microscopy, and quantitative analysis of fluorescent probe delivery. Delivery via ILs in HER2-overexpressing cells yielded drug uptake that was up to 700-fold greater than with non-targeted sterically stabilized liposomes. In vivo, anti-HER2 ILs showed extremely long circulation as stable constructs in normal adult rats after a single i.v. dose, with pharmacokinetics that were indistinguishable from sterically stabilized liposomes. Repeat administrations revealed no increase in clearance, further confirming that ILs retain the long circulation and non-immunogenicity of sterically stabilized liposomes. In five different HER2-overexpressing xenograft models, anti-HER2 ILs loaded with doxorubicin (dox) showed potent anticancer activity, including tumor inhibition, regressions, and cures (pathologic complete responses). ILs were significantly superior vs. all other treatment conditions tested: free dox, liposomal dox, free MAb (trastuzumab), and combinations of dox+MAb or liposomal dox+MAb. For example, ILs produced significantly superior antitumor effects vs. non-targeted liposomes (P values from <0.0001 to 0.04 in eight separate experiments). In a non-HER2-overexpressing xenograft model (MCF7), ILs and non-targeted liposomal dox produced equivalent antitumor effects. Detailed studies of tumor localization indicated a novel mechanism of drug delivery for anti-HER2 ILs. Immunotargeting did not increase tumor tissue levels of ILs vs. liposomes, as both achieved very high tumor localization (7.0–8.5% of injected dose/g tissue) in xenograft tumors. However, histologic studies using colloidal-gold labeled ILs demonstrated efficient intracellular delivery in tumor cells, while non-targeted liposomes accumulated within stroma, either extracellularly or within macrophages. In the MCF7 xenograft model lacking HER2-overexpression, no difference in tumor cell uptake was seen, with both ILs and non-targeted liposomes accumulating within stroma. Thus, anti-HER2 ILs, but not non-targeted liposomes, achieve intracellular drug delivery via receptor-mediated endocytosis, and this mechanism is associated with superior antitumor activity. Based on these results, anti-HER2 immunoliposomes have been developed toward clinical trials. Reengineering of construct design for clinical use has been achieved, including: new anti-HER2 scFv F5 generated by screening of a phage antibody library for internalizing anti-HER2

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phage antibodies; modifications of the scFv expression construct to support large scale production and clinical use; and development of methods for large-scale conjugation of antibody fragments with liposomes. We developed a scalable two-step protocol for linkage of scFv to preformed and drug-loaded liposomes. Our final, optimized anti-HER2 ILs-dox construct consists of F5 conjugated to derivatized PEG-PE linker and incorporated into commercially available liposomal doxorubicin (Doxil®). Finally, further studies of the mechanism of action of anti-HER2 ILs-dox suggest that this strategy may provide optimal delivery of anthracycline-based chemotherapy to HER2-overexpressing cancer cells in the clinic, while circumventing the cardiotoxicity associated with trastuzumab+anthracycline. We conclude that anti-HER2 immunoliposomes represent a promising technology for tumor-targeted drug delivery, and that this strategy may also be applicable to other receptor targets and/or using other delivered agents.

Keywords: Liposomes; Immunoliposomes; HER2; ErbB2

1. Introduction

We have developed anti-HER2 immunoliposomes (ILs) to combine the tumor-targeting properties of certain anti-HER2 monoclonal antibody (MAb) fragments with the pharmacokinetic and drug delivery advantages of long circulating liposomes. This approach has a number of potential advantages as compared with other immunoconjugate strategies. For example, in contrast to immunotoxins, liposomal agents can in principle be constructed so as to provide long circulation with minimal immunogenicity. Also, immunoliposomes can provide highly concentrated delivery of potent anticancer agents, and these encapsulated agents can possess their own inherent selectivity against cancer cells (e.g., accepted chemotherapeutic drugs), thus minimizing the potential for cytoxicity against normal tissues with low antigen expression. Finally, diffusion of drug released by immunoliposomes can effect bystander killing of nearby cancer cells, thus obviating the need for 100% targeting efficiency.

As a target antigen, HER2 is a readily accessible cell surface receptor, and, when overexpressed, provides a basis for selective immunotargeting of tumor cells. The *HER2* (c-*erb*B-2, *neu*) protooncogene encodes a 1255 amino acid, 185 kDa receptor tyrosine kinase (RTK) that is a member of the class I RTK family, along with the epidermal growth factor receptor (EGFR), HER3 (ErbB3), and HER4 (ErbB4) (for a review, see Refs. [1,2]). As an oncogene product, HER2 plays an important role in the development and progression of many breast and other cancers. HER2 is overexpressed in 20–30% of breast and ovarian cancers, most commonly via gene amplification, and overexpression is associated with poor prognosis in these patients [3,4]. In addition to breast and ovarian cancer, HER2-overexpression also occurs frequently in a number of other carcinomas (for a review, see Ref. [5]). In normal adult tissues, HER2 occurs only at low levels in certain epithelial cell types [6]. When present in cancer, HER2-amplification and overexpression typically display a relatively homogeneous distribution within primary breast tumors, and also appear to be retained or increased at metastatic sites, suggesting a continuous requirement for HER2-overexpression throughout the malignant process [7]. This is in contrast to most other tumor-associated antigens, which are often heterogeneously expressed and/or readily down-modulated in tumor cells. Other studies have also implicated ErbB2 as a direct contributor to metastasis [8–10] and angiogenesis [11], further raising the possibility that ErbB2 expression may be upregulated or selected for during metastatic progression. For example, one study suggests that ErbB2 expression is highly prevalent (67%) in bone marrow micrometastasis of breast cancer patients [12].

Monoclonal antibodies directed against HER2 offer one strategy for targeted anticancer therapy. One such antibody, muMAb 4D5, specifically binds HER2 in its extracellular domain (ECD), and inhibits the growth of HER2-overexpressing breast cancer cells in vitro and in animal models [13–15]. A humanized version of this antibody, rhuMAb HER2 (trastuzumab, Herceptin®), was developed to retain these properties while reducing the potential for immunogenicity [16]. In clinical studies in advanced breast cancer, trastuzumab induced antitumor responses as a single agent [17,18], and was par-

ticularly effective when combined with anthracycline- or taxane-based chemotherapy [19]. Based on these results, trastuzumab was approved by the FDA in 1998 for the treatment of advanced breast cancer, and thereby became the first MAb to achieve this milestone for solid tumor treatment. Next-generation strategies can be envisioned that combine the now validated approach of anti-HER2 MAbs with the drug delivery capability of liposomes.

Much like MAb technology, liposome technology required extensive optimization and clinical testing prior to attaining clinical validation. The first generation of liposomal pharmaceuticals, so-called 'conventional' liposomes, have been used to deliver a number of anti-cancer agents, sometimes resulting in reduced toxicity to normal tissues. However, a major limitation of conventional liposomes is their susceptibility to rapid clearance by the reticuloendothelial system (RES) [20]. The development of modified liposomes capable of prolonged circulation represents a significant advance in drug delivery. For example, so-called 'stealth' or 'sterically stabilized' liposomes (Ls) are small unilamellar particles of neutral charge, and contain an outer coat of inert polymers such as PEG (polyethylene glycol) [21,22]. Sterically stabilized liposomes undergo much slower clearance by the RES, resulting in significant prolongation of circulation and markedly altered biodistribution. Importantly, sterically stabilized liposomes have been shown to preferentially accumulate in tumor tissue, and can reach significantly higher levels in tumor than in normal tissues, as shown in both preclinical and clinical studies [23,24]. This property, an example of the 'enhanced permeability and retention (EPR) effect' [25], is believed to occur because of structural abnormalities in tumor-associated vessels that arise during tumor angiogenesis, which greatly facilitate liposome extravasation. These advances in liposome technology have generated renewed interest in liposomes as drug carriers [26]. Two liposomal agents (liposomal doxorubicin, Doxil®, liposomal daunorubicin, Daunosome®) have been approved by the US FDA for the treatment of AIDS-associated Kaposi's sarcoma. In addition, sterically stabilized liposomal doxorubicin recently received approval for the treatment of refractory ovarian cancer.

Although capable of enhanced accumulation in tumor tissue, existing liposomal chemotherapeutics do not interact directly with tumor cells in vitro or in vivo, and instead release drug for eventual diffusion into tumor cells. Indeed, the same modifications that enable long circulation (e.g., steric stabilization) prevent liposome–cancer cell interactions. Immunoliposomes (ILs) represent a logical strategy to achieve direct drug delivery to tumor cells, by linking liposomes to monoclonal antibody fragments against tumor-associated antigens (for a review, see Ref. [27]). Thus, we have developed anti-HER2 ILs for targeted, intracellular drug delivery.

2. Results and discussion

2.1. Preparation of anti-HER2 immunoliposomes (ILs)

Anti-HER2 immunoliposomes (ILs) were constructed by conjugation of anti-HER2 MAb fragments to sterically stabilized liposomes (Ls), to create a tumor-targeted drug delivery vehicle for the treatment of HER2-overexpressing breast cancers. Immunoliposome design involves multiple components [27], and ILs were constructed accordingly to optimize intracellular drug delivery.

Anti-HER2 ILs were prepared from small unilamellar liposomes containing 0–12 mol% PEG-phosphatidylethanolamine (PEG-PE; 0–12 mol% of total phospholipid; 0–7 mol% of total lipid) (Fig. 1). ILs were initially constructed with anti-HER2 MAb fragments conjugated to derivatized head groups of phosphatidylethanolamine on the liposome surface ('Ls-MAb linkage') as described [28]. However, ILs containing the Ls-MAb linkage and a high-density PEG coating showed reduced binding and endocytosis due to steric inhibition (see below and Refs. [28,29]). This problem was circumvented by constructing ILs with MAb fragments conjugated to the termini of PEG chains ('PEG-MAb linkage') [29]. Subsequently, a third conjugation strategy was developed, using micellar incorporation into preformed liposomes lacking functional sites for conjugation (described below).

MAb fragments were either rhuMAb HER2-Fab' [28,29]; scFv C6.5, an anti-HER2 single chain Fv cloned from a human antibody-phage display library

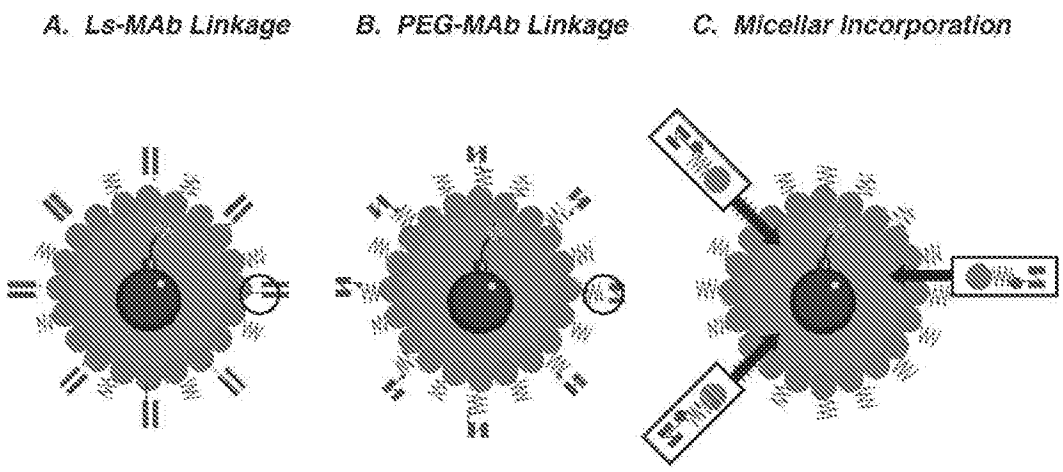

Fig. 1. Conjugation strategies used for construction of anti-HER2 immunoliposomes: schematic representation. Immunoliposomes (ILs) were prepared using small unilamellar liposomes (70–100 nm) consisting of hydrogenated soy phosphatidylcholine/cholesterol (HSPC/Chol, 3:2 molar ratio) and polyethylene glycol (PEG_{2000})-derivatized distearoylphosphatidylethanolamine (PEG-PE). Anti-HER2 MAb fragments consisted of rhuMAb HER2 (trastuzumab)-Fab′, scFv C6.5, scFv F5, or variants of these. MAb fragments all contained a C-terminal cysteine for covalent conjugation. Methods included: (Left) Ls-MAb linkage. MAb fragments were conjugated to maleimide-terminated phosphatylethanolamine (M-PE) at the liposome surface. (Middle) PEG-MAb linkage. MAb fragments were conjugated to maleimide-terminated PEG-PE (M-PEG-PE), resulting in MAb fragments at the distal ends of PEG chains. (Right) Micellar incorporation. Preformed liposomes lacking functional sites for conjugation were converted into immunoliposomes by incorporation of modified MAb fragments. In this method, MAb fragments are coupled to M-PEG-PE, forming micelles which incorporate into liposomes at high efficiency under controlled heating.

[30]; or scFv F5, another novel anti-HER2 scFv directly selected via internalization assay [31]. In addition to these three MAb fragments which bind distinct epitopes, higher affinity versions scFv ML3-9 and diabody ML3-9 were derived from scFv C6.5 by affinity maturation [32], and were also used in immunoliposomes to evaluate the effect of MAb affinity on immunoliposome behavior. Controls consisted of non-targeted liposomes, identically prepared but with omission of MAb fragment conjugation. In addition, irrelevant immunoliposomes were constructed using rhuMAbH52-Fab′, a humanized Fab′ that differs from rhuMAb HER2-Fab′ only in the antigen-binding loops and shows no detectable binding to any known antigen [33]. A final control consisted of inactivated scFv C6.5, in which the scFv was subjected to prolonged reducing agent exposure, resulting in reduction of an internal disulfide in the antigen-binding domain required for binding activity. All conjugations were highly efficient, typically resulting in 30–50 MAb fragments per liposome (70–90% of added MAb).

2.2. Binding of anti-HER2 ILs

Specific binding of anti-HER2 ILs to HER2-overexpressing cancer cells in culture was evaluated in a series of studies involving flow cytometry, competitive binding analysis, and spectrofluorometry. Flow cytometric assay demonstrated strong binding of anti-HER2 ILs to cells with HER2-overexpression (BT-474 and SK-BR-3 breast cancer cells; ~10^6 receptors/cell, '3+' immunohistochemical (IHC) score), but no detectable binding to cells lacking HER2-overexpression (MCF-7 breast cancer cells, ~10^5 receptors/cell, '1+' IHC score) [28]. Control liposomes, prepared identically but either lacking MAb fragments or conjugated to irrelevant Fab′, showed negligible binding.

2.3. Internalization and intracellular disposition of anti-HER2 ILs in vitro

Certain anti-HER2 MAbs, including trastuzumab,

are readily internalized in HER2-overexpressing tumor cells via receptor-mediated endocytosis [14]. To assess whether anti-HER2 ILs internalize within target cells in vitro, a series of studies using conventional and confocal fluorescence microscopy of rhodamine-labeled ILs was performed [28]. SK-BR-3 cells treated with rhodamine-PE-containing anti-HER2 ILs (Ls-MAb linkage, 0 mol% PEG) demonstrated intense foci of fluorescence both at the cell surface and intracellularly by 5 min, indicating rapid internalization. Treatment with anti-HER2 ILs containing high PEG concentrations (≥4 mol%) resulted in decreased intracellular fluorescence, indicating that the rate of internalization was inhibited by the PEG component of ILs containing the Ls-MAb linkage. Significantly, the inhibitory effect of PEG upon internalization was not observed when ILs contained the PEG-MAb linkage rather than the Ls-MAb linkage. SK-BR-3 cells treated with rhodamine-labeled ILs containing the PEG-MAb linkage displayed extensive internalization regardless of PEG concentration included (2–12 mol%). These results indicated that while PEG can inhibit binding and internalization of Ls-Fab′ ILs, possibly via steric hindrance, shifting the MAb fragment attachment site to the terminus of PEG (PEG-MAb linkage) prevents this inhibition by PEG.

To further characterize immunoliposome internalization, co-incubation studies were performed using transferrin, a ligand that undergoes rapid receptor-mediated endocytosis. SK-BR-3 cells were co-incubated with ILs containing the PEG-MAb linkage, 5 mol% PEG-PE, and rhodamine-PE as a fluorescent label, and with FITC-labeled transferrin [29]. After 10 min incubation at 37°C, confocal fluorescence microscopy showed ILs at or near the cell surface accompanied by some cytoplasmic localization, while transferrin was extensively internalized. By 30 min, ILs were also observed distributed throughout the cytoplasm and extensively co-localized with transferrin in endocytic vesicles, as indicated by superimposed images of rhodamine and fluorescein fluorescence. The specificity of immunoliposome uptake in SK-BR-3 cells was confirmed by preincubation of SK-BR-3 cells with trastuzumab at 10-fold molar excess over ILs, which totally blocked uptake of anti-HER2 ILs but not of transferrin. In addition, MCF-7 cells co-incubated with ILs and transferrin showed efficient uptake of transferrin, but no detectable uptake of ILs.

For quantitative studies of immunoliposome uptake, internalization, and intracellular drug delivery, anti-HER2 ILs were loaded with pyranine (1-hydroxypyrene-3,6,8-trisulfonic acid, HPTS), a pH-sensitive fluorophore that can be readily encapsulated in liposomes [34]. Intracellular disposition was determined by measurement of the pH-dependent fluorescence of HPTS, allowing quantitation of ILs/HPTS in neutral compartments (surface bound) vs. acidic compartments (endocytosis-associated). ILs were taken up rapidly into a neutral environment, with subsequent accumulation in acidic compartments, consistent with surface binding followed by receptor-mediated endocytosis [29]. Total uptake of ILs in SK-BR-3 cells when present at saturating concentrations was 7.21±0.45 nmol phospholipid/mg cell protein, which corresponds to uptake of 23,000 ILs/cell. Immunoliposome delivery was also extremely specific: total uptake in non-HER2-overexpressing MCF-7 cells was <0.01 nmol phospholipid/mg cell protein, indicating a selectivity of at least 700-fold for HER2-overexpressing vs. non-overexpressing target cells. This also indicated that the selectivity of anti-HER2 ILs was not simply proportional to receptor density, as the uptake differential of almost three orders of magnitude surpasses the two order-of-magnitude differential in HER2 receptor density between these two cell lines. Also, the uptake of anti-HER2 ILs was 600-fold higher than the uptake of non-targeted control liposomes in SK-BR-3 cells, but equivalent in MCF-7 cells. Consistent with the previous binding results, ILs containing MAb fragments conjugated at the liposome surface (Ls-MAb linkage) in conjunction with high concentrations of PEG-PE (2–10 mol%) displayed a reduction in cell binding by up to 100-fold and in endocytosis by two-fold. However, when MAb fragments were instead conjugated to the termini of PEG-PE (PEG-MAb linkage), binding and endocytosis were independent of total PEG-PE content. Binding and endocytosis varied depending upon the density of MAb fragments on the immunoliposome. Increasing MAb fragment density correlated with increased binding and internalization, reaching a plateau at ~40 Fab′/ILs for binding and ~10 Fab′/ILs for internalization.

To further study the intracellular disposition of anti-HER2 ILs and their contents, electron microscopy was performed using anti-HER2 ILs containing colloidal gold particles [28]. SK-BR-3 cells treated with anti-HER2 ILs showed gold-loaded ILs at the cell surface and intracellularly in coated pits, coated vesicles, endosomes, multivesicular bodies, and lysosomes. This intracellular distribution was consistent with internalization occurring via the coated pit pathway. In addition, gold particles were noted free within the cytoplasm, not associated with a membrane-bound organelle, likely due to escape of the encapsulated gold particles from the endosomal pathway.

Taken together, these studies demonstrated the ability of anti-HER2 ILs to selectively internalize in target cells, resulting in intracellular drug delivery. This strategy can provide a critical advantage to the therapeutic action of many anticancer agents.

2.4. Doxorubicin-loaded anti-HER2 ILs

Targeted delivery of doxorubicin (dox) by anti-HER2 ILs may represent a particularly advantageous combination for the treatment of HER2-overexpressing breast cancers. Some preclinical and clinical studies have suggested that HER2-overexpressing breast cancers may be relatively resistant to hormonal therapy and certain types of chemotherapy, such as cyclophosphamide- and taxane-based regimens (reviewed in Ref. [35]). On the other hand, evidence from two large randomized clinical trials indicates that HER2-overexpression is associated with particular benefit from and a steep dose–response relationship to anthracycline-based chemotherapy [36–38]. In addition, dox toxicity to both myocardium and hematopoietic cells may be much reduced by anti-HER2 immunoliposome delivery, as HER2 expression is extremely low in these cell types [6], and drug delivery by anti-HER2 immunoliposomes is dependent upon receptor density [29]. Another consideration is that clinical studies of trastuzumab have demonstrated significant cardiotoxicity with this MAb as a single agent [18], and prohibitive cardiotoxicity when used in combination with dox chemotherapy [19]. Although the mechanism has not been elucidated, this cardiotoxicity appears to be due to the antiproliferative effects of trastuzumab on HER2 signaling, which plays a central role in myocardial development and maintenance despite low antigen levels [39]. If so, then ILs should circumvent this clinical problem. Trastuzumab's antiproliferative activity requires high steady-state levels of MAb, while ILs containing trastuzumab-Fab′ do not produce sustained levels of the Fab′ when administered intermittently for drug delivery; consistent with this, ILs containing trastuzumab-Fab′ but no drug failed to inhibit HER2-dependent growth in vivo (see below). Furthermore, anti-HER2 ILs can be constructed with alternative MAb fragments (e.g., scFv C6.5 or scFv F5) that show no antiproliferative activity or other trastuzumab-like signaling effects [52]. For these reasons, anti-HER2 immunoliposome-dox may represent a particularly apt strategy to deliver dox to HER2-overexpressing cancer cells, resulting in less cardiotoxicity than dox chemotherapy alone or the combination of dox plus trastuzumab.

Anti-HER2 ILs containing encapsulated dox were prepared using the remote loading method [41]. This procedure results in high efficiency encapsulation of dox, with 10,000–15,000 dox molecules per liposome particle. Since anti-HER2 ILs can accumulate in vitro to 23,000 liposomes per target cell [29], the amount of dox that can be delivered via ILs is potentially 10^8 dox per cell. Given this enormously productive delivery, we hypothesized that immunoliposome delivery of dox to HER2-overexpressing target cells may even approach the efficiency by which free dox, a small (M_r 544), amphipathic molecule, readily diffuses through the cell membrane in vitro. In contrast, in cells that do not overexpress HER2, ILs delivery would be much less cytotoxic than free dox, and a large selective differential would be achieved. As predicted, treatment of SK-BR-3 cells for 1 h with dox-loaded anti-HER2 immunoliposomes (anti-HER2 ILs-dox) yielded dose-dependent cytotoxicity comparable to that of free dox, indicating that immunoliposome delivery of dox was as efficient as the rapid diffusion of free dox into cells in vitro [28]. Anti-HER2 IL-dox was 10- to 30-fold more cytotoxic than dox-loaded control ILs bearing irrelevant Fab′, which affected cell growth only at very high concentrations, likely due to drug leakage. The specificity of this strategy was further confirmed by anti-HER2 ILs-dox treatment of WI-38

cells, a non-malignant lung fibroblast cell line that expresses minimal levels of HER2. While free dox produced significant dose-dependent cytotoxicity against WI-38 cells, anti-HER2 ILs-dox demonstrated 20-fold less cytotoxicity.

Based on these promising results, further studies focused on the development of anti-HER2 ILs-dox.

2.5. Pharmacokinetics of anti-HER2 ILs-dox

Pharmacokinetic (PK) studies of dox-loaded anti-HER2 ILs were performed in healthy adult rats. Dox-loaded anti-HER2 ILs were prepared with 0–12 mol% PEG and with MAb fragments linked either to the liposome surface (Ls-MAb linkage) or to the termini of PEG chains (PEG-MAb linkage). After a single i.v. dose, all anti-HER2 ILs-dox constructs showed a biphasic elimination profile for dox, with terminal $t_{1/2}$ of 12–14 h and mean residence times (MRT) up to 24 h [42]. In contrast, dox levels were undetectable beyond 5 min following the administration of the equivalent dose of free dox, demonstrating a marked pharmacokinetic advantage for anti-HER2 ILs-encapsulated dox. These PK results were virtually identical to previous results with sterically stabilized liposomal dox, and greatly superior to those of 'conventional' liposomes [43].

Notably, all anti-HER2 ILs-dox constructs were long-circulating, including anti-HER2 ILs-dox lacking PEG. Thus, immunoliposomes of this composition (i.e., small unilamellar vesicles, solid bilayer, neutral charge) did not require a polymeric coating to achieve long circulation. However, the addition of PEG to these ILs, while not necessary for long circulation, was associated with further prolongation of MRT.

Direct comparison of anti-HER2 ILs vs. liposomes prepared identically except for omission of MAb fragments showed indistinguishable PK. The conjugation of MAb fragments in ILs therefore did not measurably alter clearance in normal rats.

2.6. Stability of anti-HER2 ILs-dox in vivo

The stability of dox-loaded anti-HER2 ILs in circulation was evaluated in two-component PK studies, in which dox and MAb levels were simultaneously assayed to evaluate possible drug leakage or dissociation of MAb from liposomes. For these studies, plasma PK of dox (assayed by spectrofluorometry) and of anti-HER2 Fab′ fragments (assayed by ELISA) were co-determined from the same plasma samples following single i.v. injection of dox-loaded anti-HER2 ILs. Both dox and Fab′ components showed concordant plasma PK following administration of anti-HER2 ILs, with terminal $t_{1/2} = 10$ h for both. In comparison, free dox was undetectable beyond 5 min (see above), while free trastuzumab-Fab′ has a terminal $t_{1/2}$ of 1–2 h. These results indicated that anti-HER2 ILs-dox greatly prolonged the circulation of both Fab′ and dox components, and suggested that anti-HER2 ILs remained intact in circulation, with negligible dissociation or drug leakage.

2.7. Multiple dose pharmacokinetics of anti-HER2 ILs

A major limitation of many immunoconjugate strategies has been the immunogenicity of the construct, as either the MAb component, effector/cytotoxic component, or linker can elicit a host immune response, thus preventing repeated administration and potentially contributing to immune complex-related toxicities. In order to circumvent this problem, anti-HER2 ILs were constructed using starting components of minimal immunogenicity: non-immunogenic sterically stabilized liposomes and MAb fragments of either humanized or human origin. For example, trastuzumab, a fully humanized MAb, has been shown to be associated with very low levels of circulating human anti-human antibodies (HAHA) [17]. MAb fragments (Fab′ or scFv) rather than intact IgG were used to further reduce the potential for immune recognition of Fc sequences. To confirm that repeat administration of anti-HER2 ILs does not lead to accelerated clearance, multiple dose PK studies were performed in normal rats. Radiolabeled anti-HER2 ILs (PEG-MAb linkage) were administered once a week for three doses, using the same dose and schedule as in the therapy studies (see below). Anti-HER2 ILs showed equivalent plasma PK in rats following the third and final dose as in naïve rats without prior immunoliposome treatment [40]. Furthermore, anti-HER2 ILs and sterically stabilized liposomal dox (prepared identically except

for omission of Fab′ fragments) also showed indistinguishable plasma PK results. Thus, the presence of Fab′ fragments on ILs did not result in accelerated clearance over a multiple dose regimen, even when humanized antibody sequences were introduced into an immunocompetent rodent host. These results suggest that the construct design for anti-HER2 ILs succeeded in minimizing immunogenicity, at least over the duration of a therapeutic course.

2.8. Development of animal models

We hypothesized that tumor-targeted delivery using anti-HER2 ILs containing dox would increase the therapeutic index of dox chemotherapy. In order to rigorously test this hypothesis, we performed a series of therapy studies in four different animal models. Highly tumorigenic sublines of HER2-overexpressing human breast cancer cell lines were derived by in vivo selection in nude mice. These included two xenograft lines independently derived from the BT-474 cell line (~10^6 HER2 receptors/cell; '3+' IHC score). The first BT-474 model (BT-474/MSKCC) was developed by our collaborators (J. Baselga and J. Mendelsohn, Memorial Sloan-Kettering Medical Center, NY, USA). We later developed a separate model, BT-474/SF, using an independently derived tumorigenic subline. We also developed two additional models: MCF7/HER2 (MCF7 cells stably transfected with HER2 [44], ~10^6 receptors/cell; '3+' IHC score), and MDA-MB-453 (~10^5 receptors/cell, '2+' IHC score). For each model, tumor cells were implanted subcutaneously in the back or flank of nude mice, either with or without matrigel. Estradiol pellets were also implanted to increase tumorigenicity. In each study, treatment was initiated after tumors had become fully established and were at least 200 mm^3 in volume. Therapy studies were also performed with extremely large tumors (up to 1000 mm^3).

2.9. Antitumor efficacy of anti-HER2 ILs-dox

The antitumor efficacy of anti-HER2 immunoliposome-dox constructs was extensively evaluated in these four HER2-overexpressing breast cancer xenograft models. Anti-HER2 ILs were prepared with 0–7 mol% PEG and with either the Ls-MAb or PEG-MAb linkage. Anti-HER2 ILs-dox was administered i.v. at a total dox dose of 15–22.5 mg/kg, divided over three doses once a week. Other treatment conditions included: saline; free dox, administered by the same schedule and at its maximum tolerated dose (MTD) in these mice (7.5 mg/kg; see below); and sterically stabilized liposomal dox, prepared identically to anti-HER2 ILs-dox but without MAb conjugation, and administered at the same dose and schedule. Additional treatment conditions included 'empty' anti-HER2 ILs (no dox), which were administered by the same schedule and lipid dose as dox-loaded ILs; and free rhuMAb HER2, administered at its optimal dose and schedule (0.3 mg/kg i.p. twice weekly) as described [45]. Additional controls included dox-loaded ILs containing an irrelevant Fab′ fragment (rhuMAb H52) or inactivated scFv C6.5. Both of these irrelevant or inactivated immunoliposomes yielded comparable results as non-targeted (no MAb fragment) liposomal dox, and thus the latter was used as the non-targeted control liposomal dox for most of the therapy studies.

All of the anti-HER2 ILs-dox constructs tested produced marked antitumor effects, including tumor growth inhibition, tumor regressions, and cures of mice, and showed superior activity to all other treatment conditions. For example, initial studies of anti-HER2 ILs-dox containing 1 mol% PEG and Ls-MAb linkage demonstrated suppression of tumor growth that persisted after treatment; tumor inhibition was clearly superior to that associated with free dox, which was only marginally better than saline in this model, or liposomal dox. The increase in efficacy of anti-HER2 ILs vs. liposomal dox was highly significant at $P < 0.0001$.

In direct comparisons, the improved immunoliposome construct design containing 6 mol% PEG and the PEG-MAb linkage produced superior antitumor activity vs. ILs containing 1 mol% PEG, Ls-MAb linkage. This result was presumably due to the incremental pharmacokinetic advantage associated with higher PEG concentration. Subsequent studies therefore included these optimized anti-HER2 ILs containing 6 mol% PEG and PEG-Fab′ linkage. Also, subsequent studies used a matrigel-free version of the BT-474/SF tumor xenograft model. Matrigel

was omitted once tumor localization studies revealed that liposomes and anti-HER2 ILs were non-specifically trapped in matrigel deposits (R. Shalaby et al., submitted). Omission of matrigel resulted in elimination of this artifact, and allowed improved tumor penetration of liposomes and ILs.

Therapy studies using the optimized anti-HER2 ILs-dox construct in the matrigel-free BT-474/SF model further demonstrated that targeted delivery of dox was associated with enhanced efficacy. Anti-HER2 ILs-dox produced substantial tumor regressions in this model, including high rates of cured mice (defined as complete regression of tumor during the study, persisting until study termination and confirmed histopathologically at sacrifice). Anti-HER2 ILs containing either rhuMAb HER2-Fab′ or an alternative anti-HER2 antibody fragment, scFv C6.5, produced significantly superior growth inhibition than liposomal dox ($P < 0.0001$ for either anti-HER2 ILs-dox construct vs. liposomal dox). Furthermore, cure rates in this model reached 6/11 mice (55%) for rhuMAb HER2-Fab′-immunoliposomes and 5/10 mice (50%) for scFv C6.5-ILs, vs. 0/11 mice (0%) for liposomal dox. These differences were also highly significant, with $P < 0.0001$ for either anti-HER2 ILs-dox construct vs. liposomal dox.

Treatment with anti-HER2 ILs-dox containing rhuMAb HER2-Fab′ also produced superior efficacy compared with either anti-HER2 MAb (trastuzumab) or 'empty' anti-HER2 ILs lacking encapsulated dox. In fact, empty anti-HER2 ILs, administered at the same lipid dose and schedule as anti-HER2 ILs-dox, produced minimal effects on tumor growth, indicating that the therapeutic effect of anti-HER2 ILs-dox was mainly derived from targeted drug delivery and not the antiproliferative activity associated with the trastuzumab-derived Fab′ fragments. When given three times per week instead of once weekly, empty immunoliposomes containing rhuMAb HER2-Fab′ did show some inhibition of tumor growth. These results are consistent with previous studies of trastuzumab, which requires attainment of steady-state blood levels of 5–10 μg/ml for tumor inhibition in vivo [45]. The dose and intermittent administration used for anti-HER2 ILs-dox were clearly insufficient to achieve these levels.

As an additional control, anti-HER2 immunoliposome-dox was also evaluated in a *non*-HER2-overexpressing xenograft model, MCF-7, which express low or basal levels of HER2 (~10^4 receptors/cell; '1+' IHC score). Previous studies of anti-HER2 immunoliposome binding and internalization in MCF-7 cells in vitro showed undetectable uptake by confocal microscopy and quantitative fluorometry; in addition, uptake of immunoliposomes was indistinguishable from that of control liposomes lacking MAb fragments [29]. To evaluate in vivo specificity, anti-HER2 immunoliposome-dox was administered i.v. in the MCF-7 xenograft model using the same dose and schedule as in the other therapy studies. Therapeutic effects of ILs were indistinguishable from those of non-targeted (no MAb fragment) sterically stabilized liposomes. This result confirmed that anti-HER2 immunoliposomes require a threshold receptor density or activity for appreciable drug delivery in vivo as well as in vitro.

Taking all of the therapy studies together, in each of the four HER2 overexpressing animal models (BT474/MSKCC, BT474/SF, MCF7/HER2, and MDA-MB-453), treatment with anti-HER2 ILs-dox was significantly superior to all other treatment conditions. In contrast to dox, which was associated with modest tumor growth inhibition in these tumor xenograft models, anti-HER2 ILs-dox showed potent antitumor efficacy. In eight separate studies comparing anti-HER2 ILs-dox vs. liposomal dox, MAb-targeted delivery gave significantly superior therapeutic results, with P values from <0.0001 to 0.04. Furthermore, anti-HER2 ILs, but not free dox or liposomal dox, produced frequent cures in mice. The total cure rate with anti-HER2 ILs in all studies was 18/115 mice (16%), with higher rates observed using optimized anti-HER2 ILs in the non-matrigel model as described. In contrast, no cures were observed in any of the mice treated with free dox (0/46) or liposomal dox (0/78). This difference was highly significant ($P < 0.0001$).

2.10. Antitumor efficacy of anti-HER2 ILs-dox vs. combination therapy

Additional therapy studies in the BT-474/SF model were performed to compare the antitumor efficacy of anti-HER2 ILs-dox vs. combination therapies consisting of free MAb (trastuzumab) plus either free dox or liposomal dox. Trastuzumab has

been shown to significantly increase the efficacy of dox-based chemotherapy in both preclinical [45] and clinical studies [19]. Nevertheless, anti-HER2 ILs-dox was markedly superior to the combination of trastuzumab plus free dox in this model ($P < 0.0001$). Anti-HER2 ILs-dox was also compared to combination therapy with free trastuzumab plus liposomal dox, using Doxil as the liposomal dox. Anti-HER2 ILs showed significantly superior efficacy than the combination in this direct comparison ($P < 0.0001$).

2.11. Toxicity of anti-HER2 immunoliposome-dox

In addition to enhanced efficacy, encapsulation of dox in liposomes or immunoliposomes was associated with markedly reduced host toxicity as compared with free dox. In studies to determine maximum tolerated dose (MTD) in nude mice, varying doses of free dox, sterically stabilized liposomal dox, and anti-HER2 ILs-dox were administered i.v. weekly for three doses. MTD was defined as the highest tested dose level that gave less than 20% weight loss and no treatment-related deaths. The MTD of anti-HER2 ILs-dox ($\geq$22.5 mg/kg) and liposomal dox (18.8–22.5 mg/kg) were 2.5-fold greater than that of free dox (7.5 mg/kg).

2.12. Tumor distribution and intracellular localization of anti-HER2 ILs in vivo

Given the significant superiority of ILs-dox vs. non-targeted liposomal dox in all of the therapy studies, it was possible to hypothesize that this enhanced efficacy was due to increased accumulation in tumor tissue associated with immunoliposome binding. To address this possibility, we compared radiolabeled immunoliposome and liposome accumulation in HER2-overexpressing tumors following i.v. administration. Both anti-HER2 ILs and non-targeted sterically stabilized liposomes demonstrated extremely efficient localization in breast tumor xenografts, including HER2-overexpressing tumors (BT474, MCF7HER2) as well as non-overexpressing tumors (MCF7), reaching levels of 7–8% injected dose/g tumor tissue in each of these models. Hence, surprisingly, the presence of anti-HER2 MAb fragments in immunoliposomes did not confer any significant improvement in tumor tissue levels over non-targeted sterically stabilized liposomes, even in high HER2-overexpressing tumors. This result suggests the primacy of the EPR effect in determining tumor tissue accumulation of long circulating liposomes, and suggests that immunoliposome extravasation is the rate-determining step in tumor localization, rather than antibody-mediated binding. Indeed, the accumulation of ILs and liposomes were both substantially greater than that of anti-HER2 MAb 4D5 (murine version of trastuzumab) in similar xenograft models [14]. These results also demonstrated that increased immunoliposome accumulation in tumor tissue was *not* the mechanism for the therapeutic advantage of immunoliposome delivery.

Detailed studies of the tumor localization and intratumoral distribution of anti-HER2 ILs indicated a novel mechanism by which ILs produce targeted drug delivery in vivo. As discussed, anti-HER2 ILs undergo receptor-mediated endocytosis in target cells in vitro, resulting in intracellular delivery of encapsulated agents [28,29]. Using gold-labeled anti-HER2 ILs and control liposomes, we further studied the distribution of these particles within tumor tissue following i.v. administration [40]. Gold-labeled anti-HER2 ILs and sterically stabilized liposomes (prepared identically except for omission of Fab′) were administered i.v. in the BT-474/SF and MCF7/HER2 tumor xenograft models, and subsequently visualized by light microscopy following silver enhancement. Anti-HER2 ILs were observed to have distributed widely throughout tumor tissue, with no apparent impediment to penetration into the interior of large tumors or into necrotic regions. Importantly, ILs were distributed in a completely different pattern than non-targeted liposomes. ILs were predominantly located within the cytoplasm of individual tumor cells, indicating intracellular delivery. In contrast, sterically stabilized liposomes were predominantly observed in stromal areas of the tumor and within tissue macrophages, and were not observed within tumor cells. As an additional control, ILs and liposomes were also administered in the non-HER2-overexpressing MCF-7 breast cancer xenograft model. Both ILs and liposomes distributed similarly in MCF-7 tumors, showing only stromal and macrophage accumulation and no tumor cell uptake. The results of these tumor distribution studies confirmed

that the mechanism of drug delivery by ILs involves intracellular accumulation in HER2-overexpressing tumor cells, and suggested that the therapeutic advantage obtained with anti-HER2 ILs-dox derived from this novel mechanism.

2.13. Anti-HER2 ILs-dox: preclinical and clinical development

Having demonstrated proof-of-concept for anti-HER2 immunoliposome drug delivery, anti-HER2 ILs-dox (NSC 701315) has undergone further optimization of construct design and developmental studies to support clinical testing.

ILs containing a series of different anti-HER2 MAb fragments were constructed and compared to assess the effect of ligand affinity. As mentioned, ILs containing scFv C6.5 ($K_d \sim 10^{-8}$ M for the scFv) proved to be equivalent to ILs containing rhuMAb HER2 (trastuzumab)-Fab′ ($K_d \sim 10^{-9}$ M for the Fab′), despite the 10-fold difference in binding affinity for the individual ligands. Furthermore, anti-HER2 ILs containing scFv C6.5 yielded equivalent therapeutic results as ILs containing scFv ML3-9, a high affinity variant ($K_d \sim 10^{-10}$ M) derived from scFv C6.5 by affinity maturation, as well as ILs containing diabodies of the ML3-9 variant ($K_d \sim 10^{-11}$ M). These results indicated that immunoliposome efficacy was unaffected over a wide range of ligand affinities, and thus high affinity ligands were not required for optimal therapeutic activity of anti-HER2 ILs. This was presumably due to the highly multivalent character of the ILs (30–50 binding sites per immunoliposome particle), which conferred tremendous avidity that obviated the need for high affinity ligands. scFv C6.5 was initially selected as the MAb fragment for clinical development, based on the evidence that ILs containing scFv C6.5 were equivalent in efficacy to anti-HER2 ILs containing rhuMAb HER2-Fab′. An advantage for scFv C6.5-containing ILs is the avoidance of the intrinsic antiproliferative and signaling effects of trastuzumab, which could potentially increase toxicity in combination with dox delivery. scFv C6.5 was re-engineered for clinical use, including deletion of extraneous sequences (c-myc epitope tag, $(his)_6$ affinity tag) and switch to an alkaline phosphatase-based bacterial expression system. A purification protocol involving multiple ion exchange chromatography steps was developed as a scalable process.

During scale up, a limitation of scFv C6.5 that became increasingly problematic was the presence of the internal disulfide in the antigen-binding domain, which was susceptible to reduction and consequent loss of binding activity. Therefore, additional unique anti-HER2 scFv's were derived from the same phage antibody library [31]. For this purpose, a novel phage antibody selection strategy utilizing cell internalization was developed, based on the rationale that scFv's optimal for internalizing ILs should be capable of mediating efficient internalization of phage as well. In this selection procedure, scFv-expressing phage were incubated with HER2-overexpressing breast cancer cells at 37°C; after an appropriate interval to allow for internalization to occur, bacteria were subjected to stringent wash under low pH conditions to strip surface-bound but non-internalized phage. Internalized phage were then recovered following bacterial cell lysis.

One clone identified by this method, scFv F5, was determined to specifically recognize the extracellular domain of HER2 at an epitope distinct from trastuzumab or scFv C6.5. Although scFv F5 showed lower binding affinity to HER2 than scFv C6.5, scFv F5 showed significantly more internalization in a panel of HER2-overexpressing cells than scFv C6.5, either as free scFv or when conjugated in ILs. scFv F5 also contains sequences enabling affinity purification, and demonstrated superior stability during reduction and conjugation than scFv C6.5. For these reasons, anti-HER2 ILs-dox containing scFv F5 were adopted as the clinical candidate. In collaboration with the NCI Developmental Therapeutics Program (DTP), we have developed a highly efficient and scalable manufacturing process for scFv F5 using affinity purification and ion exchange.

In parallel with MAb fragment optimization, the conjugation procedure was also optimized for commercial application. In addition to the Ls-MAb and PEG-MAb conjugation strategies, a new micellar incorporation strategy involving two-step conjugation of scFv to preformed drug-loaded liposomes was developed (Fig. 1) [46]. The first step involves conjugation of MAb fragment (e.g. scFv) to modified PEG-PE linker in solution, resulting in immunoconjugates that are able to form micelles. In the

second step, the scFv-PEG-PE conjugates are incorporated into liposomes (e.g. Doxil) by controlled heating, resulting in conversion of Doxil into anti-HER2 immunoliposome-dox. Anti-HER2 ILs-dox produced by this method were equivalent in binding, internalization, and in vivo efficacy as ILs produced by our previous method of conjugation of MAb fragments to modified PEG chains on sterically stabilized liposomes (PEG-MAb linkage). This new micellar incorporation strategy greatly facilitates the manufacturing process, as existing liposomal agents such as Doxil can simply be converted to immunoliposome versions, without the need for de novo liposome production. More generally, this conjugation strategy makes it possible to envision a combinatorial approach to the development of targeted therapeutics, in which panels of MAb fragments and panels of liposomal agents provide the starting materials for an array of potential targeted therapeutics (see below).

Our final, optimized anti-HER2 ILs-dox construct therefore consists of scFv F5 conjugated via micellar incorporation into sterically stabilized liposomal doxorubicin (Fig. 2). Additional therapy studies in HER2-overexpressing breast cancer xenograft models demonstrated that this optimized construct was equivalent to our previous versions of anti-HER2 ILs-dox, including ILs containing rhuMAb HER2-Fab′ or scFv C6.5, and ILs with either MAb fragment constructed via PEG-MAb linkage. Consistent with all of the previous therapy studies, all anti-HER2 ILs-dox versions were significantly superior to non-targeted control liposomes, including matched liposomal dox lacking MAb, ILs with inactivated C6.5, and Doxil.

Plans for a Phase I clinical trial of anti-HER2 ILs-dox in advanced breast cancer are in development. The anticipated clinical trial will be conducted at UCSF in collaboration with the NCI.

2.14. Alternative small molecule drug delivery

The versatility of liposome drug encapsulation can be exploited to develop ILs containing a range of small molecule drugs. The lead construct for preclinical and clinical development is anti-HER2 ILs-dox, due to the strong rationale for this particular combination. As discussed, HER2-overexpressing breast cancers appear to be particularly sensitive to dox chemotherapy. While the combination of dox plus trastuzumab causes unacceptable cardiotoxicity, anti-HER2 ILs-dox is likely to circumvent this problem. Furthermore, due to the development of the micellar incorporation method, pre-existing liposomal agents, such as the approved agent sterically stabilized liposomal dox, can be directly linked to MAb fragments, thus expediting drug development time.

Immunoliposome delivery is also a rational strategy for other small molecule drugs that require

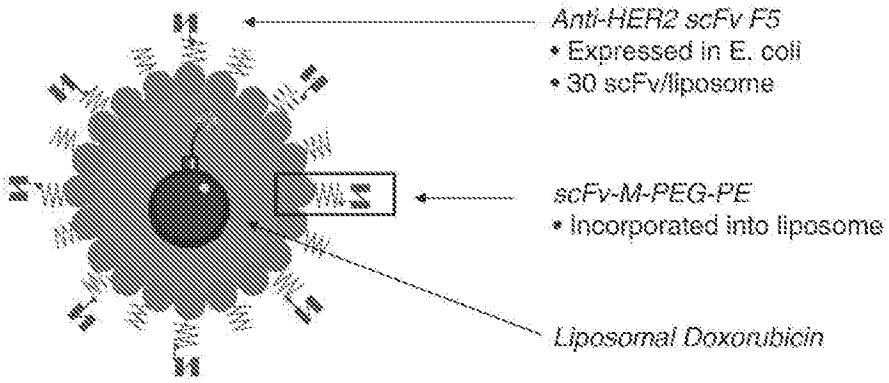

Fig. 2. Anti-HER2 immunoliposomes: optimized construct. Extensive in vitro and in vivo studies have resulted in an optimized construct design for anti-HER2 ILs, consisting of scFv F5 fragments (approx. 30 scFv/liposome) incorporated into sterically stabilized liposomal doxorubicin. scFv F5 was selected for its ability to mediate extremely efficient internalization, its high inherent stability, and its ease of manufacture. Liposomal dox was selected because of the sensitivity of HER2-overexpressing cancers to anthracyclines, the ability of ILs-dox to decrease dox and dox + trastuzumab cardiotoxicity, and the availability of approved liposomal dox for conversion to ILs-dox.

improvement in therapeutic index, which is a general limitation of all current chemotherapy agents. Immunoliposome encapsulation represents a particularly attractive strategy for drugs requiring intracellular delivery, prolonged circulation, altered biodistribution, reduced systemic toxicity, or new formulation.

Many anticancer agents have now been encapsulated in liposomes (for a review, see Ref. [47]), and these can in principle be converted to immunoliposome versions. In addition, we have developed procedures for encapsulation of several other drugs in liposomes and immunoliposomes. For example, sterically stabilized liposomes containing encapsulated vinorelbine (Navelbine®) were developed using a highly efficient remote loading technique [48]. These vinorelbine-loaded liposomes are stable after prolonged storage and show minimal drug release in serum. Liposomal vinorelbine has been used to generate anti-HER2 ILs-vinorelbine, which shows significant antitumor activity in vitro and in vivo. 17-Aminoallylgeldanamycin (AAG), a recently developed geldanamycin derivative, is currently in Phase I trials in the US and UK. 17-AAG binds HSP90, resulting in altered molecular chaperoning of cell surface receptors including HER2 and ultimately inducing their proteosomal degradation. The poorly soluble 17-AAG has been formulated into lipid-based vehicles, including anti-HER2 immunotargeted constructs. These constructs represent a strategy to deliver to HER2-overexpressing cells a unique drug with a mechanism of action particularly appropriate for this tumor cell phenotype.

2.15. Anti-HER2 cationic ILs for oligonucleotide delivery

In addition to small molecule encapsulation, studies were performed to prepare and optimize anti-HER2 cationic ILs for the delivery of oligodeoxyribonucleotides (ODN) for antisense therapy [49]. Unlike the sterically stabilized ILs described above, which are neutrally charged and have drug encapsulated within the aqueous interior space, cationic liposomes were used for efficient complexation with ODN.

Cationic liposomes were formed by mixing together one of a series of cationic and neutral lipids (DOTAP, DDAB, DOGS, and DOPE) and PEG-PE. The resulting PEGylated cationic liposomes were then complexed with 18-mer phosphorothioate ODNs, resulting in ODNs bound electrostatically to the liposome surface. rhuMAb HER2-Fab′ was then conjugated to this liposome-DNA complex via the PEG-MAb linkage. Loading of ODN was highly efficient. Cationic liposomes could contain very high ODN/lipid molar ratios, up to 0.25 mol/mol. Loading efficiency depended upon the cationic lipid used, with DOTAP providing the highest ODN loading efficiency. Conjugation of Fab′ had no effect on ODN binding.

Elimination of the positive surface charge of cationic liposomes is an important goal for therapeutic applications. Both PEG-PE and ODN loading substantially reduced the surface charge of cationic liposomes. For example, 6 mol% PEG-PE reduced the positive zeta potential of cationic liposomes from 25–30 mV to 7–10 mV; loading of ODN further decreased the zeta potential to neutral or even negative values. Freeze-fracture electron microscopy was performed to further characterize the cationic liposome- and immunoliposome-ODN complexes. Both were typically observed to be unilamellar vesicles with a smooth surface layer, with a narrowly distributed range of diameters of 100–130 nm. The addition of Fab′ had no effect on liposome structure or size.

Liposome- and immunoliposome-ODN containing PEG were highly stable against particle aggregation as well as dissociation/drug loss. PEG-containing liposome- and immunoliposome-ODN retained their particle size after >4 weeks of storage at 4°C. In contrast, cationic liposome-ODN without PEG formed large particles which further aggregated and completely precipitated within 4 days at 4°C. Serum stability studies indicated that >60% of ODN remained associated with cationic liposomes after 4 h at 37°C in 50% human plasma, and plasma proteins did not appear to associate with the complexes.

Delivery of ODN via PEGylated cationic liposomes and anti-HER2 ILs was evaluated in SK-BR-3 breast cancer cells in vitro. Uptake of free ODN was very low (16 pmol of ODN/mg cell protein). PEGylated cationic liposomes (no Fab′) produced a 4.5-fold increase in ODN uptake, while anti-HER2 ILs produced a 13-fold increase as compared to free

ODN. While immunotargeting resulted in only a moderate increase in ODN uptake vs. non-targeted liposomes, there was a pronounced difference in the intracellular distribution of ODN. Fluorescence microscopy of SK-BR-3 cells incubated with ILs-ODN revealed extensive and diffuse cytoplasmic localization of FITC-ODN, as well as a high concentration within the nucleus of every cell. In contrast, ODN delivered by non-targeted cationic liposomes were exclusively observed within cytoplasmic vesicles, with negligible nuclear uptake.

Targeted delivery via ILs greatly enhanced the biological activity of antisense ODN. Anti-HER2 ILs-ODN constructs were used to deliver antisense ODN directed against the *bcl2* oncogene (Genta, Inc.). Anti-Bcl2 antisense ODN were loaded into either anti-HER2 cationic ILs or control cationic liposomes, and incubated with BT-474 breast cancer cells, which have both high HER2 and Bcl2 expression levels. Anti-HER2 ILs-ODN produced a 46% reduction of Bcl2 protein levels in BT-474 cells [49]. In contrast, no reduction in Bcl2 was observed with cationic liposome-ODN, while free ODN produced a 20% reduction.

2.16. Anti-HER2 cationic ILs for gene therapy

We prepared cationic ILs and liposomes for complexation and delivery of plasmid DNA, large polyanions requiring a modified liposome strategy. For ODN delivery, we prepared cationic liposomes containing PEG-PE, followed by complexation with ODN, and lastly by conjugation of MAb fragments (liposome + PEG-PE, then DNA, then Fab′). For plasmid delivery, we prepared cationic liposome-plasmid DNA complexes first, followed by addition of PEG-PE for non-targeted liposomes and Fab′-PEG-PE conjugates for ILs (i.e., liposome + DNA, then Fab′-PEG-PE). Incorporation of Fab′-PEG-PE into the cationic liposome-DNA complex is another example of the micellar incorporation strategy (Fig. 1).

We evaluated various compositions of cationic liposome-DNA complexes, and optimized these constructs with respect to stability over time and stability in vivo. We developed new techniques for cationic liposome-DNA complex preparation that resulted in significantly more stable complexes than previously reported methods [50]. This improvement was achieved by condensation of plasmid DNA with polyamines, optimization of lipid composition, and inclusion of PEG-PE. The resulting PEGylated and stabilized constructs remained active after prolonged storage at 4°C (>1 month) as well as in the presence of human plasma at 37°C.

Using these stabilized cationic liposomes as a starting material, we constructed anti-HER2 cationic immunoliposomes (ILs) via conjugation of anti-HER2 Fab′ to the termini of maleimide-derivatized PEG-PE. In cationic ILs, PEG-PE served a dual purpose: it provided a specific attachment site for MAb fragments as well as minimized non-specific reactivity with non-target cells. To test this hypothesis, we evaluated the transfection efficiency of various cationic ILs and liposomes containing the firefly luciferase (*lux*) gene as a reporter, using both HER2-overexpressing (SK-BR-3) and non-overexpressing (MCF-7) cells. For example, anti-HER2 cationic ILs composed of DDAB/POPC (1:2 ratio) produced a 133-fold increase in *lux* expression in SK-BR-3 cells vs. identical PEGylated cationic liposomes without Fab′. In general, ILs prepared from a variety of cationic liposomes yielded significantly enhanced transfection activity as compared with their non-targeted cationic liposome controls, ranging from a five- to 133-fold increase. When the identical preparations were used to transfect MCF-7 cells, in each case equivalent and very low *lux* expression was obtained with or without Fab′. These results confirmed that the inclusion of PEG can substantially reduce the non-specific transfection associated with cationic liposomes; and that immunotargeting of these PEGylated cationic liposomes can then provide much enhanced efficiency as well as specificity of gene transfer. Anti-HER2 cationic ILs were used to deliver plasmids encoding anti-HER2 ribozymes to HER2-overexpressing SK-BR-3 cells in vitro, as a strategy combining HER2-targeted delivery with HER2-specific gene therapy [51]. ILs mediated 53% reduction in HER2 expression levels, as compared with no detectable change using control liposomes, and 9% reduction using non-PEGylated cationic liposomes.

Stabilized cationic liposome-DNA complexes were evaluated for efficiency and biodistribution of gene transfer in vivo. These studies were performed

in our animal models of HER2-overexpressing breast cancer. Administration of cationic liposome-*lux* constructs by single i.v. (tail vein) injection in the BT-474 breast cancer xenograft model resulted in extremely high *lux* expression in multiple tissues, particularly in lung. In general, the levels of *lux* expression achieved using these stabilized constructs was up to 1000-fold greater than in previously published reports using non-viral vectors [50]. Recently modified cationic liposomes produced a further 10-fold increase in tumor *lux* expression, with a concomitant decrease in lung expression by four orders of magnitude. Importantly, parameters associated with high in vitro transfection did not correlate with high in vivo transfection.

The most promising cationic liposome constructs, based on these in vivo studies, were converted to anti-HER2 ILs as described above. Anti-HER2 ILs-*lux* were administered via single peritumoral injection to evaluate the effect of immunotargeting on tumor cell transfection. High levels of *lux* expression were observed in tumor tissue, up to 12 pg luciferase protein/mg total protein, which was three-fold higher than transfection via non-targeted cationic liposomes.

Anti-HER2 ILs-lux were administered via single i.v. (tail vein) administration in the BT-474 breast cancer xenograft model. ILs produced a >100-fold increase in *lux* expression in tumor tissue, as compared with control liposomes (prepared identically but without MAb fragments).

2.17. Towards combinatorial therapeutics via immunoliposome delivery

The modular organization of immunoliposome constructs makes possible a combinatorial strategy for the development of new therapeutics: MAb fragments selected from a library of scFv can be coupled to an appropriate liposomal drug chosen from a repertoire of liposomal drugs (Fig. 3).

The development of rapid, efficient, and general MAb conjugation methods now makes this strategy highly feasible. In contrast to immunotoxin or drug immunoconjugate approaches, which typically require extensive re-engineering of constructs for each

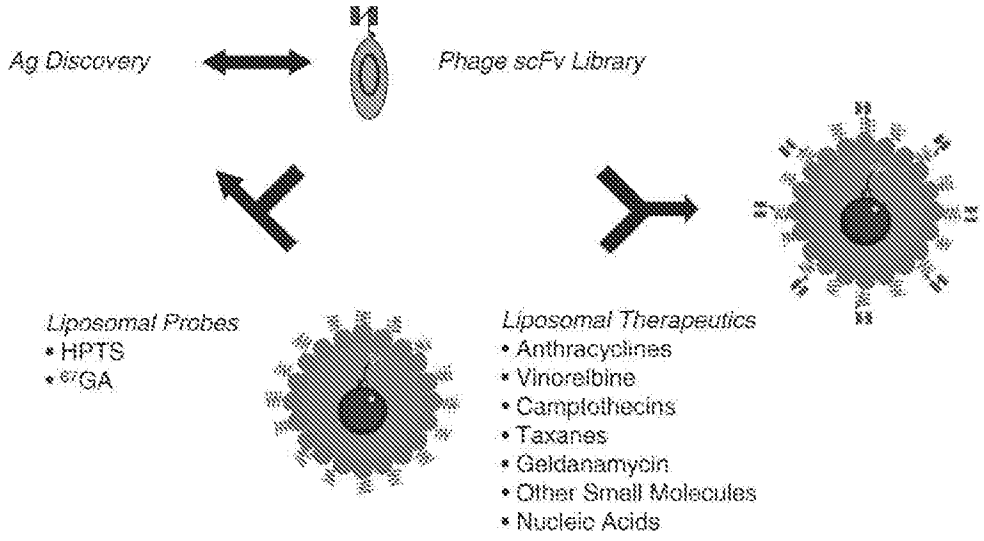

Fig. 3. Towards combinatorial therapeutics via immunoliposome delivery. The modular organization of immunoliposome constructs makes possible a combinatorial strategy for the development of new therapeutics. MAb fragments selected from a library of scFv can be coupled to an appropriate liposomal drug chosen from a repertoire of liposomal drugs. scFv can be generated against known targets (e.g. HER2) as well as novel antigens, and can be selected directly for internalization, expediting the identification of useful ligands for immunoliposome delivery. Novel antigens recognized by scFv can be characterized in conjunction with immunoliposome probes, such as HPTS for in vitro studies of binding and internalization, and radionuclides for in vivo studies of tumor localization and biodistribution.

MAb and drug/toxin component, immunoliposomes provide a general method for combining MAb fragments with liposomal agents. In principle, any MAb fragment can be easily linked to PEG-PE; this MAb module can then be stably attached via micellar incorporation into liposomal drugs. For a given MAb specificity, a range of liposomal drugs can be tested to identify the optimal combination for therapeutic application. This strategy is reflected in the generation of anti-HER2 ILs containing dox, other small molecules, oligonucleotides, and plasmid DNA as described.

Even greater combinatorial power is represented by the ability to rapidly derive MAb fragments of desired specificity from a high-diversity repertoire, such as scFv from a phage antibody library. Screening phage antibody libraries for internalizing scFv's provides a greatly expedited route to identification of ideal ligands for immunoliposomes, as with the anti-HER2 scFv F5 described. Screening can also be used to identify scFv clones of unknown specificity, which may nevertheless be useful in conjunction with immunoliposome delivery. Promising scFv clones can be further evaluated by immediate incorporation into immunoliposomes containing appropriate probes for in vitro or in vivo characterizations. In this way, immunoliposome technology can be used to assist antigen discovery efforts, while newly identified antigens can be targeted with immunoliposome therapeutics.

3. Conclusions

We have developed anti-HER2 immunoliposomes (ILs) to combine the tumor-targeting properties of certain anti-HER2 monoclonal antibodies (MAbs) with the pharmacokinetic and drug delivery properties of long circulating liposomes (Ls). We have shown that anti-HER2 ILs efficiently bind to and internalize in HER2-overexpressing cells in vitro, resulting in intracellular drug delivery. In vivo, dox-loaded ILs (ILs-dox) displayed long circulation that was identical to that of sterically stabilized liposomes. Anti-HER2 immunoliposome-dox produced marked therapeutic results in four different HER2-overexpressing tumor xenograft models, including growth inhibition, regressions, and cures. These therapy studies demonstrated that encapsulation of dox in anti-HER2 immunoliposomes greatly increased the therapeutic index of dox, both by increasing antitumor efficacy and by reducing systemic toxicity. Immunoliposome-dox was significantly superior to all other treatment conditions tested, including free dox, liposomal dox, anti-HER2 MAb (trastuzumab). When compared to non-targeted liposomal dox in a series of therapy studies involving four different HER2-overexpressing xenograft models, immunoliposome delivery produced significantly superior antitumor efficacy in each study (P values from <0.0001 to 0.04). Anti-HER2 immunoliposome-dox containing either rhuMAb HER2 (trastuzumab)-Fab', scFv C6.5, scFv F5, or variant scFv's yielded comparable therapeutic efficacy. Anti-HER2 immunoliposome-dox was also superior to combination therapies consisting of free dox + free MAb or liposomal dox + free MAb. Studies of the tumor localization of anti-HER2 ILs indicated a novel mechanism of drug delivery in vivo. Tumor tissue accumulation was unaffected by immunotargeting, reaching very high levels (7–8% injected dose/g tissue) with either ILs or non-targeted sterically stabilized liposomes. However, intratumoral distribution and particularly internalization distinguished ILs from non-targeted liposomes. Following i.v. administration, anti-HER2 ILs were observed dispersed throughout tumor tissue, and had accumulated predominantly within the cytoplasm of tumor cells. In contrast, Ls accumulated extracellularly or within macrophages. These results confirmed that ILs, unlike Ls, produced intracellular drug delivery in vitro and in vivo. This mechanism likely accounts for the significantly enhanced efficacy of anti-HER2 ILs against HER2-overexpressing tumors.

We conclude that these studies have demonstrated proof-of-concept for tumor-targeted drug delivery using anti-HER2 ILs. Anti-HER2 ILs greatly enhance the therapeutic index of dox chemotherapy, and therefore may be a potent and particularly appropriate therapy for HER2-overexpressing breast cancers. Further development of dox-loaded anti-HER2 ILs is in progress toward a Phase I clinical trial in patients with advanced breast cancer. The strategy of immunoliposome delivery may have broad utility for targeted delivery of other anticancer agents, such as those agents with narrow therapeutic

indices, pharmacokinetic limitations, or a requirement for intracellular delivery. Finally, the versatility of the immunoliposome approach is reflected in its modular organization (liposomal agent, MAb fragment), which can now be combinatorially associated using extensive repertoires of these components in conjunction with rapid and efficient conjugation methods.

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Confirmation Number:	5137
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First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Celeste A. RONEY
Attorney Docket Number	01208-0007-01US

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	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Celeste A. RONEY
	Attorney Docket Number	01208-0007-01US

	1	PARK J, et. al., "Anti-HER2 Immunoliposomes for Targeted Drug Delivery," Med Chem Res. 8(7/8):383-91 (1998).
	2	PATANKAR N, et. al., "Topophore C: A Liposomal Nanoparticle Formulation of Topotecan for Treatment of Ovarian Cancer," Invest New Drugs. 31(1):46-58 (2013). Epub 2012.
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	4	PAVAI S and YAP S, "The Clinical Significance of Elevated Levels of Serum CA19-9," Med J Malaysia. 58(5):667-72 (2003).
	5	PAVILLARD V, et al., "Determinants of the Cytotoxicity of Irinotecan in Two Human Colorectal Tumor Cell Lines," Cancer Chemother Pharmacol. 49(4):329-35 (2002).
	6	PAZ N, et al., "MM-398/PEP02, A Novel Liposomal Formulation of Irinotecan Demonstrates Stromal-Modifying Anti-Cancer Properties," Poster for abstract A63 presented at the AACR Special Conference on Pancreatic Cancer: Progress and Challenges; Jun. 18-21, 2012; Lake Tahoe, NV, 9 pages.
	7	PAZ N, et al., Abstract A63. "MM-398/PEP02, A Novel Liposomal Formulation of Irinotecan, Demonstrates Stromal-Modifying Anticancer Properties," In Proceedings of the AACR Special Conference on Pancreatic Cancer: Progress and Challenges; Jun. 18-21, 2012; Lake Tahoe, NV. Cancer Res. 2012;72(12 Suppl):Abstract nr A63, 3 printed pages.
	8	PAZ-ARES L, et al., "Efficacy and Safety of Irinotecan Liposome Injection (nal-IRI) in Patients with Small Cell Lung Cancer (SCLC)," Presentation presented at 2019 World Conference on Lung Cancer; September 7-10, 2019; Barcelona, Spain; 9 pages.
	9	PAZ-ARES L, et al., "RESILIENT part 2: An Open-Label, Randomized, Phase 3 Study of Liposomal Irinotecan Injection in Patients With Small-Cell Lung Cancer Who Have Progressed With Platinum-Based First-Line Therapy." Poster presented at the American Society of Clinical Oncology (ASCO) Annual Conference, virtual format, May 29-June 2, 2020, 7 pages.
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	11	PAZ-ARES RODRIGUEZ L, et al., Abstract OA03.03. "Initial Efficacy and Safety Results of Irinotecan Liposome Injection (NAL-IRI) in Patients With Small Cell Lung Cancer," 2019 World Conference on Lung Cancer Abstracts; September 7-10, 2019; Barcelona, Spain; pp. 220-221.

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	Examiner Name	Celeste A. RONEY
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ANTI-HER2 IMMUNOLIPOSOMES FOR TARGETED DRUG DELIVERY

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Abstract. Anti-HER2 immunoliposomes (ILs) combine the tumor-targeting properties of certain anti-HER2 monoclonal antibodies (MAb) with the pharmacokinetic and drug delivery properties of sterically stabilized liposomes (Ls). Anti-HER2 ILs efficiently bind to and internalize in HER2-overexpressing cells *in vitro,* resulting in intracellular drug delivery. Localization studies in tumor xenograft models confirm that anti-HER2 ILs, unlike liposomes, internalize in tumor cells *in vivo*. Gold-loaded ILs accumulate intracellularly in the cytoplasm of tumor cells, while liposomes lacking MAb targeting accumulate extracellularly or within macrophages. This novel mechanism of targeted, intracellular delivery may account for the significantly enhanced antitumor efficacy of anti-HER2 ILs *in vivo*. Therapy studies demonstrate that delivery of doxorubicin (dox) via anti-HER2 ILs-dox greatly increases the therapeutic index of dox, both by increasing antitumor efficacy and by reducing systemic toxicity. Anti-HER2 ILs-dox produce marked therapeutic results in multiple HER2-overexpressing tumor xenograft models, including growth inhibition, regressions, and cures. Anti-HER2 ILs-dox is significantly superior to all other relevant treatment conditions, including free dox, liposomal dox, free MAb, and combinations. In addition to dox, anti-HER2 ILs can in principle be constructed for tumor-targeted delivery of a wide variety of anticancer agents, including alternate small molecule chemotherapeutics, antisense oligonucleotides, and therapeutic genes.

Strategies for tumor-targeted drug delivery, in which drugs are efficiently and selectively introduced to tumor cells, have been intensely sought for improved cancer therapy. Current chemotherapeutic agents are generally suboptimal with respect to both efficacy and toxicity, and would clearly benefit from targeted delivery. Furthermore, advances in medicinal chemistry, such as rational drug design, combinatorial chemistry, and antisense therapy, are now yielding an unprecedented number of novel anticancer agents; many of these new molecules will require a delivery vehicle to efficiently reach their site of action.

Anti-HER2 immunoliposomes (ILs) represent a promising new technology for tumor-targeted drug delivery. Immunoliposomes have become feasible due to independent advances in the areas of liposome research and monoclonal antibody (MAb)-based therapeutics. The successful integration of these technologies has led to the develoment of immunoliposomes for tumor-targeted drug delivery (for review, see ref. (1)).

An important advance in liposomal chemotherapy has been the development of stable, long-circulating liposomes capable of enhanced tumor accumulation, such as "stealth" or "sterically stabilized" liposomes (2). Liposomal doxorubicin and liposomal daunorubicin have demonstrated improved efficacy vs. free drug in clinical trials, and have recently become commercially available. These liposomes are not directly targeted to tumor cells; they accumulate in extracellular spaces within tumor tissue, and eventually release drug for diffusion into tumor cells.

Similarly, the development of MAb for cancer therapy has finally led to clinical validation after two decades of research. A leading example has been the development of MAb directed against the $p185^{HER2}$ receptor tyrosine kinase, the product of the *HER2* (*erb*B2, *neu*) protooncogene. HER2 plays an important role in the pathogenesis of breast and other cancers (for review, see ref. (3)). HER2 is highly and stably overexpressed in a significant proportion of these cancers, and is a logical target antigen for MAb-based therapeutics. For example, muMAb4D5 and its humanized derivative, rhuMAbHER2 (trastuzumab/Herceptin™), inhibit the growth of HER2-overexpressing breast cancer cells and enhance the efficacy of certain chemotherapy drugs (4). In clinical trials in advanced breast cancer, treatment with rhuMAbHER2 has shown encouraging antitumor activity as a single agent (5, 6), and particularly in combination with chemotherapy (7).

Here we describe anti-HER2 immunoliposomes, in which long circulating liposomes are conjugated to anti-HER2 MAb fragments to generate a tumor-targeted drug carrier.

EXPERIMENTAL AND RESULTS

Construction of Anti-HER2 Immunoliposomes (ILs)

Anti-HER2 ILs incorporate multiple design elements to optimize intracellular delivery of encapsulated drug to tumor cells. These include: 1) use of MAb fragment (Fab' or scFv) rather than intact IgG to avoid accelerated clearance and immunogenicity; 2) sterically stabilized immunoliposomes for long circulation and selective tumor extravasation; 3) conjugation of MAb fragment to derivatized polyethylene glycol (PEG) linkers (see below) to facilitate immunoliposome binding and internalization; and 4) encapsulated therapeutic agents (e.g.doxorubicin, vinorelbine) for enhanced therapeutic index via targeted intracellular delivery.

Accordingly, anti-HER2 ILs were prepared as described (8, 9), as shown in Fig. 1. Anti-HER2 MAb fragments consisted of either rhuMAbHER2-Fab' (Genentech, Inc.), expressed as a recombinant protein in E. coli at high efficiency (10), or C6.5, an independently derived single-chain Fv (11). Sterically stabilized liposomes were prepared as small unilamellar liposomes consisting of hydrogenated soy phosphatidylcholine/cholesterol (HSPC/Chol, 3:2 molar ratio) and polyethylene glycol (PEG_{2000})-derivatized disteroylphosphatidylethanolamine (PEG-PE) at varying concentrations (0-12 mol%). MAb fragments were conjugated to liposomes by either of two alternative linkages. In the first of these, MAb fragment was conjugated to maleimido-phosphatylethanolamine (M-PE) at the liposome surface ("MAb-surface linkage"); in the second, to maleimide-terminated PEG-PE ("MAb-PEG linkage"). Both procedures were highly efficient, typically yielding 50-100 Fab' fragments per liposome particle.

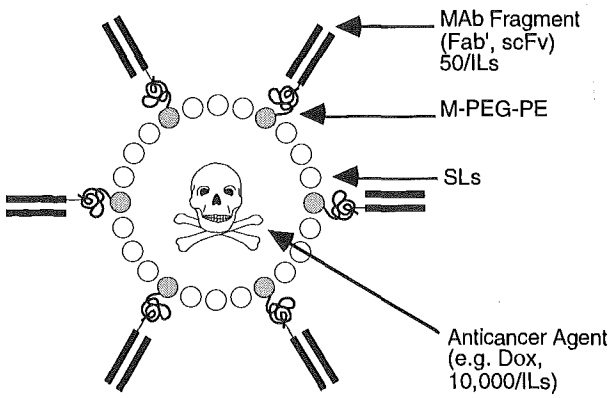

Fig. 1. Schematic representation of anti-HER2 immunoliposomes (ILs). ILs consisted of anti-HER2 MAb fragments conjugated to sterically stabilized liposomes (SLs). To avoid steric inhibition between MAb fragments and PEG, MAb fragments were conjugated to maleimide-terminated PEG-PE.

Binding and Internalization of Anti-HER2 Immunoliposomes

Quantitative studies of immunoliposome binding, internalization, and intracellular drug delivery were performed with ILs containing a pH-sensitive fluorescent probe (1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS/pyranine). These studies demonstrated rapid uptake of ILs by SK-BR-3 via receptor-mediated endocytosis (9). Total uptake of ILs in SK-BR-3 cells reached 23,000 ILs/cell, while total uptake of non-targeted control liposomes was essentially undetectable. In contrast, total uptake of ILs in non-HER2-overexpressing MCF-7 cells was 700-fold lower than in SK-BR-3 cells. ILs containing the MAb-surface linkage showed reduced binding and endocytosis due to steric inhibition by PEG, which was circumvented using ILs containing the MAb-PEG linkage.

The internalization of ILs containing colloidal gold particles was studied by electron microscopy (8). SK-BR-3 cells treated with anti-HER2 ILs showed gold-loaded ILs at the cell surface and intracellularly in coated pits, coated vesicles, endosomes, multivesicular bodies, and lysosomes, consistent with internalization occurring via the coated pit pathway.

Pharmacokinetics of Anti-HER2 Immunoliposomes

The plasma pharmacokinetics (PK) of anti-HER2 ILs containing doxorubicin (dox) have been studied in non-tumor-bearing adult rats. Dox and anti-HER2 Fab' levels obtained by sampling venous blood after single i.v. administration of ILs showed a marked prolongation of plasma half-life for both components: the terminal half-lives of dox and Fab' in ILs were $\geq$10 hours, while that for free doxorubicin at the same dose (800 μg) was less than 5 min. Importantly, ILs and sterically stabilized liposomes showed identical plasma PK, indicating no measurable effect on clearance due to the presence of MAb.

Localization of Anti-HER2 Immunoliposomes to Tumors *In Vivo*

Tumor localization studies revealed an important difference in the mechanism of delivery of anti-HER2 ILs compared with sterically stabilized liposomes. Both ILs and liposomes accumulated to very high levels in tumor xenografts with 7-8% injected dose/g tissue (12). However, examination of tumors following i.v. treatment with gold-labeled ILs or Ls demonstrated marked differences in distribution and mechanism of delivery. ILs were dispersed throughout the tumor, and, notably, were predominantly observed within the cytoplasm of tumor cells. In contrast, liposomes accumulated extracellularly or within macrophages, consistent with previous reports of the localization of sterically stabilized liposomes (13). These results confirm that anti-HER2 ILs, unlike liposomes, achieve intracellular drug delivery. This mechanism may account for the improvement in tumor response including cures seen in these animal models (see below).

Delivery of Doxorubicin Via Anti-HER2 Immunoliposomes

Anti-HER2 ILs containing dox were tested in 4 different HER2-overexpressing tumor xenograft-nude mouse models: two independently-derived strains of BT-474 (10^6 HER2 receptors/cell); MCF7/HER2, stable transfectants expressing high levels of HER2 (10^6 receptors/cell), and MDA-MB-453, a cell line expressing lower levels of HER2 (10^5 receptors/cell). In these models, experimental treatment was initiated 1-2 weeks after tumor implantation (tumor size 200-1000 mm^3). Anti-HER2 ILs produced tumor inhibition, regressions, and cures in as many as 50% of the animals (14). Tumor inhibition with immunoliposomes was significantly superior to all other treatment conditions, which included saline, free dox, free anti-HER2 MAb (rhuMAbHER2), empty anti-HER2 ILs without dox, and sterically stabilized liposomal dox. In eight separate studies comparing anti-HER2 vs. liposomal dox, ILs yielded significantly superior efficacy (p values from <0.0001 to 0.04). In addition, cure rates for immunoliposomes reached 50% (11/21) in recent studies using matrigel-free tumors, and overall 16% (18/115) in all models, vs. no cures (0/124) with free dox or liposomal dox. Anti-HER2 ILs-dox was also significantly superior to combined therapy of free dox plus free rhuMAbHER2 or liposomal dox plus rhuMAbHER2. Finally, the dose of dox that could be administered in these models was significantly augmented by encapsulation within liposomes (MTD 18-22 mg/kg, divided into three weekly injections), as compared with free dox (7.5 mg/kg).

Anti-HER2 Immunoliposomes Containing Alternate Anticancer Compounds

Due to the versatility of liposome drug encapsulation, immunoliposome delivery can be exploited in conjunction with a wide variety of cancer chemotherapeutic agents (1). For example, in addition to dox, we prepared vinorelbine-loaded anti-HER2 ILs, using a highly efficient remote loading technique; vinorelbine-loaded liposomes were stable after prolonged storage and showed minimal drug release in serum (15). The therapeutic efficacy of these ILs are currently under investigation. Other candidates for immunoliposome delivery include other anthracyclines and anthracycline prodrugs, other vinca alkaloids, methotrexate analogues, camptothecins, taxanes, cisplatin, and novel compounds. Theoretical advantages of immunoliposome delivery of such agents include enhanced efficacy due to tumor-targeted, intracellular delivery, reduced host toxicity due to altered biodistribution, and improved pharmacokinetics.

Nucleic Acid Delivery Via Immunoliposomes

In addition to providing tumor-targeted delivery of small molecules, modified immunoliposomes can be used for the intracellular delivery of nucleic acids. However, in order to efficiently deliver either oligo- or polynucleotides, ILs must be substantially modified so as to: efficiently package DNA; retain favorable pharmacologic properties (such as stability, long circulation, minimal non-specific reactivity, and reduced immunogenicity); selectively and efficiently deliver nucleic acids to tumor cells; and provide intracellular routing that allows nucleic acids to reach their target site.

For antisense oligodeoxyribonucleotide (ODN) delivery, we have constructed anti-HER2 immunoliposomes in which cationic lipids have been included for efficient complexation with nucleic acid (16). The non-specific reacitivity of these lipids was reduced by addition of a PEG coat analogous to that used in sterically stabilized liposomes and ILs. These cationic anti-HER2 ILs-ODN complexes internalized within SKBR3 cells *in vitro*, resulting in intracellular delivery and nuclear accumulation of labeled ODN. Delivery of antisense ODN directed against the *bcl-2* oncogene via cationic anti-HER2 ILs produced a significant reduction in *bcl-2* expression *in vitro*, which was greater than that associated with non-targeted cationic liposomes or free ODN.

We have also constructed similar cationic anti-HER2 ILs for the delivery of therapeutic genes for gene therapy (17). *In vitro*, these constructs efficiently transfected HER2-overexpressing cells, but not non-target cells.

DISCUSSION

Anti-HER2 ILs are as stable and long-circulating as sterically stabilized liposomes, but, unlike liposomes, bind to and internalize in target cells. As a result, anti-HER2 ILs greatly extend the therapeutic index of dox, both by increasing its antitumor efficacy and by reducing its systemic toxicity. Furthermore, anti-HER2 ILs-dox have demonstrated significantly superior efficacy vs. free dox, liposomal dox, free anti-HER2 MAb, and combination therapies in a panel of tumor xenograft models. These studies have established proof of concept that targeted delivery via anti-HER2 immunoliposomes improves antitumor efficacy.

We conclude that anti-HER2 ILs represent a promising technology for tumor-targeted intracellular drug delivery. Immunoliposome delivery has potentially broad applicability as a platform technology in conjunction with other anticancer agents. ILs can be used as a vehicle for anticancer agents requiring intracellular delivery, or may be useful in reducing adverse effects of other agents by altering biodistribution. Finally, the immunoliposome strategy in principle can be adapted for targeted drug delivery to cells expressing other antigens of interest using alternative MAb constructs.

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PRECLINICAL STUDIES

Topophore C: a liposomal nanoparticle formulation of topotecan for treatment of ovarian cancer

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Summary We have recently developed a liposomal nanoparticle (LNP) formulation of irinotecan based on loading method that involves formation of a complex between copper and the water soluble camptothecin. The loading methodology developed for irinotecan was evaluated to develop a LNP topotecan formulation (referred to herein as Topophore C) and test its activity in pre-clinical model of ovarian carcinoma. Topotecan was encapsulated into preformed liposomes containing 300 mM copper sulfate and the divalent metal ionophore A23187. Formulation optimization studies included assessments of loading efficiency, influence of temperature on drug loading and in vitro stability of the resulting formulation. In vivo assessments included drug and liposome pharmacokinetics, drug levels within plasma and the peritoneal cavity following intravenous (i.v.) administration in mice and efficacy studies on ES2 ovarian cancer model. Topotecan loading into liposomes was optimized with encapsulation efficiency of >98 % at a final drug-to-lipid (D/L) mole ratio of 0.1. Higher D/L ratios could be achieved, but the resulting formulations were less stable as judged by in vitro drug release studies. Following Topophore C administration in mice the topotecan plasma half-life and AUC were increased compared to free topotecan by 10-and 22-fold, respectively. Topophore C was 2-to 3-fold more toxic than free topotecan, however showed significantly better anti-tumor activity than free topotecan administered at doses with no observable toxic effects. Topophore C is a therapeutically interesting drug candidate and we are particularly interested in developing its use in combination with liposomal doxorubicin for treatment of platinum refractory ovarian cancer.

Keywords Liposome · Nanoparticle · Topotecan · Ovarian cancer · Copper

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Introduction

Camptothecin (CPT) was first discovered in 1958 as a potent antitumor antibiotic from the bark of the Chinese tree

Camptotheca acuminata [1]. Although this compound was interesting from a preclinical perspective its poor aqueous solubility compromised clinical development [2, 3]. Semi-synthetic and more water- soluble analogues of camptothecin have been developed to address this issue and two of these derivatives have now been approved by regulatory groups for use in patients. Camptosar® (Irinotecan hydrochloride) is used for the treatment of patients with colorectal cancer [4] and is showing some promise in other indications including lung cancer and cervical, ovarian cancer [5]. Hycamtin® (Topotecan) is approved for use in ovarian cancer patients that have relapsed following treatment with a taxane/platinum combination [4, 6, 7]. Acceptance of camptothecins as an additional class of cytotoxic agents was driven in part because of their unique mechanism of activity. Camptothecins exert their cytotoxic effect during the S-phase of the cell cycle by stabilizing the cleavable complex formed between the enzyme topoisomerase I and DNA. This process prevents re-ligation of DNA strands and ultimately leads to apoptosis due to the accumulation of DNA-topoisomerase I complexes [8–10]. As topoisomerase I inhibitors, the camptothecins act additively or synergistically with other drug classes [4, 5, 11–15], and in particular with selected topoisomerase II inhibitors such as doxorubicin [12, 13, 15, 16]. More recently, there has been a great deal of excitement about the use of combinations of poly(ADP-ribose) polymerase (PARP) inhibitors (e.g. olaparib) and topoisomerase I inhibitors such as topotecan and irinotecan [17–19], particularly in the context of patients with BRCA1 mutations [20, 21]. Further, as exposure to irinotecan can increase expression of EGFR receptor [22] camptothecins are also providing unexpected benefits in the context of chemotherapeutic regimes that include therapeutic antibodies targeting the EGFR receptor. It is therefore anticipated that the use of these camptothecins will expand over the next decade even with the introduction of more personalized therapies targeting dysregulated signaling pathways in cancer cells. Importantly, it has been recognized that the therapeutic activity of camptothecins are compromised due to a chemical characteristic. The therapeutic action of camptothecins is dependent on the integrity of the drug's α-hydroxy-lactone ring [23, 24]. This lactone ring is prone to undergo reversible hydrolysis at physiological pH, producing an inactive carboxylate derivative [24, 25]. Strategies proposed to improve the therapeutic activity of camptothecins have therefore primarily focused on preventing hydrolysis of the lactone ring. These strategies have included: i) structural modification of the compound [26–28], ii) derivatization of the lactone form [27] or iii) encapsulation into nanoparticulate carriers such as liposomes [29–36], polymer micelles [37, 38] or microemulsions [39].

Several liposomal formulations of camptothecins have been reported previously [29, 31, 40–46] with varying degree of therapeutic promise. Irinotecan and topotecan are weakly basic drugs and can be loaded into pre-formed liposomes through use of a trans-membrane pH gradient (acidic inside) [47]. The lactone ring is stabilized in its ring closed configuration when it is retained within the acidic core of the liposome. Several approaches can be used to generate a transmembrane pH gradient including: i) preparing liposomes using buffered acidic solutions [48], ii) preparing liposomes using an ammonium sulfate solution capable of generating/maintaining a pH gradient [49] or iii) preparing liposomes using solutions of monovalent or divalent metal ions coupled with the addition of an appropriate ionophore [50]. Our research team has described a loading method that involves formation of a complex between copper and irinotecan [31, 45], a method that is not necessarily dependent on formation of a pH gradient. For details regarding the method readers are requested to refer to the above mentioned references. Importantly, when using encapsulated copper in combination with a transmembrane pH gradient there were unexpected improvements in drug retention. It is not clear whether the improvements in drug retention are due to complexation of the drug to copper or, alternatively, involve a copper-membrane interaction that engenders decreases in the liposomal membrane's permeability to the camptothecin. Regardless of mechanism, the improvements in drug retention were associated with improved antitumor activity. This irinotecan formulation (referred to as Irinophore C) maintains the drug in its therapeutically active lactone conformation [31]. Since topotecan is a structurally comparable camptothecin analog, the first choice with regards to formulations was to consider whether the formulation approach developed for Irinophore C could be used to develop a LNP topotecan formulation. The objectives of this study were: i) to characterize and optimize the use of the copper/pH gradient loading methodology for topotecan; ii) to assess how a selected LNP formulation of topotecan influenced drug pharmacokinetics and distribution to the peritoneal cavity (region of ovarian carcinoma progression) following intravenous administration; and iii) to measure the therapeutic activity of the selected formulation in pseudo-orthotopic model of ovarian cancer. An optimized preparation of topotecan loaded liposomes referred to as Topophore C was identified as a suitable candidate for further development. This product candidate maintained topotecan in the lactone ring closed configuration following i.v. administration, achieved significant increases in plasma circulation half-life and AUC, delivered significantly higher amount of drug to the required site of action and was therapeutically active in an aggressive

model of ovarian cancer where tumor development was localized in the peritoneal cavity.

Materials and methods

Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) from PerkinElmer Life Sciences (Boston, MA). Hycamtin® injection (GlaxoSmithKline, Mississauga, ON, Canada; active ingredient: Topotecan-HCl; inactive ingredients: mannitol, and tartaric acid; pH of reconstituted solution: 2.5–3.5) was purchased from the pharmacy of the BC Cancer Agency (Vancouver, BC, Canada). ^{14}C-sucrose and Pico-Fluor 40 scintillation cocktail were purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Multi-use floating dialysis bags (DispoDialyzer®) were purchased from Spectrum Labs (USA). All other chemicals used were analytical or HPLC grade. The divalent cationic ionophore A23187 (calcimycin), HEPES, Sephadex G-50, cholesterol (CH) and all other chemicals (Reagent grade) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Liposome preparation

Large unilamellar vesicles (LUVs) were prepared using DSPC and CH by extrusion. Briefly, DSPC and CH were weighed, dissolved in chloroform and then mixed such that the final mole ratio of the two lipids was 55:45, respectively. A non-exchangeable and non-metabolizable lipid marker ^{3}H-CHE (5 μCi/100 μmol total lipid) was incorporated into the chloroform lipid mixture. This solution was then dried to a thin film under a gentle stream of nitrogen gas. The residual chloroform was removed by placing the lipid film under high vacuum for at least 3 h. Dried lipid films were hydrated at 65°C by mixing with 300 mM $CuSO_4$ (unbuffered, pH 3.5). Following hydration, the sample was subjected to five freeze (liquid nitrogen) and thaw (65°C) cycles. The multilamellar vesicles (MLVs) obtained were extruded 10 times through stacked polycarbonate filters of 0.1 μm and 0.08 μm pore size at 65°C using an Extruder^{EM} (Northern Lipids, Vancouver, BC, Canada). The size of the LUVs generated using this method was determined using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY). The external buffer of LUVs was exchanged with sucrose (300 mmol/L), HEPES (20 mmol/L) and EDTA (15 mmol) (SHE buffer) at pH 7.5 by running the sample through a Sephadex G-50 column equilibrated with the buffer. Liposomal lipid concentration was determined by measuring ^{3}H-CHE using liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer).

Preparation of ion gradient and optimization of topotecan loading

Liposomes with encapsulated copper sulfate (unbuffered solution, pH 3.5) were suspended in SHE buffer (pH 7.5). Subsequently, A23187 (0.5 μg per 1 mg lipid) was added to the liposomes which were then incubated at 30°C for 30 min. This mixture and a reconstituted solution of topotecan were warmed separately at 60°C for 5 min in a water bath. Immediately before addition of the drug to the liposomes a sufficient volume of 1N NaOH was added such that the final pH of the suspension was 7.0 to 7.5 after addition of topotecan (see Results). The final drug/liposome mixture was incubated in a water bath at 60°C. At specified time points, 100 μL of this mixture was placed onto 1 mL Sephadex G-50 spin columns pre-equilibrated with phosphate buffer saline (PBS, pH 7.5) to separate unencapsulated drug from liposomes. Liposomes collected in the void volumes after spinning the columns at 680×g for 3 min were analyzed for topotecan and liposomal lipid concentration. Lipid concentrations were measured using scintillation counting where ^{3}H-CHE was used as a marker for liposomal lipid. Topotecan concentrations were determined by measuring absorbance at 370 nm on a spectrophotometer (Agilent/Hewlett Packard, model: 8453, Agilent Technologies, Mississauga, ON, Canada). Briefly, an aliquot of the sample collected from the spin columns was adjusted to 100 μL followed by addition of 900 μL Triton X-100 (1 %v/v). This sample was heated in a 90°C water bath until the cloud point of the detergent was observed. Subsequently, the sample was cooled to room temperature and the absorbance was determined and compared against a topotecan standard curve in which known concentrations were treated identically to samples.

In vitro drug release

The rate of topotecan release from liposomes in vitro was determined by a dialysis method. Briefly, 300 μL of topotecan loaded liposomal suspension was mixed with 1.5 mL PBS (pH 7.5) and this mixture was placed inside disposable dialysis bags (DispoDialyzer®, SPECTRUM Laboratories, USA) with a MW cut-off of 10,000. The dialysis bag was then suspended in 1 L PBS (pH 7.5) maintained at 37°C±1° C. At specified time points, 100 μL samples were withdrawn from the dialysis bag and placed onto 1 mL Sephadex G-50 spin columns to separate liposomes from unencapsulated drug. The concentration of liposomal lipid (^{3}H-CHE) in the void volume was analyzed by scintillation counting and the topotecan concentration was determined by high

performance liquid chromatography (HPLC) using a method that was previously validated in our lab. The HPLC analysis was conducted using a Waters Alliance HPLC system equipped with a Waters Model 717 plus autosampler, a Model 600E pump, a controller and a Model 2474 Multi λ Fluorescence Detector (Waters, Milford, MA) set at an excitation wavelength of 360 nm and an emission wavelength of 425 nm. Samples were prepared by extraction with ice cold methanol. 10 μL of the diluted sample was injected onto a Waters Symmetry Shield RP C18 cartridge column (100 Å, particle size 3.5 μm; 75×4.6 mm, Waters). The mobile phase consisted of mobile phase 'A' (1 % Triethylenediamine in water, pH 6.4 adjusted with glacial acetic acid) and mobile phase 'B' (100 % acetonitrile). The sample temperature was maintained at 4°C and the column temperature was adjusted to 55°C. Each sample was run for 14 min at a flow rate of 1.0 ml/min using a gradient method, where the amount of organic phase was increased from 12 % to 40 % over 8 min. This method was able to detect ring opened carboxylate (eluted at 3 min) and ring closed lactone form (eluted at 8 min) of topotecan in a single run.

Measurement of copper concentration

Concentration of copper present inside the liposomes was determined using atomic absorption spectrometer (AA) (AANALYST 600 PerkinElmer Instruments, Woodbridge, ON). This instrument is equipped with THGA furnace with AS-800 Autosampler. Hollow cathode lamp (Cu-LUMINA. HCL) was used as a light source for copper detection. Briefly, liposomes were prepared and topotecan was loaded into pre-formed liposomes using the copper ion gradient method described above. At specified time points (5, 10, 20, 30 and 60 min) 100 μL aliquots were withdrawn from the drug loading mixture and placed onto 1 mL Sephadex G-50 spin columns equilibrated with PBS. Liposomes were collected in the void volumes after spinning the columns at 680×g for 3 min. For copper analysis an aliquot was diluted in nitric acid to achieve a final nitric acid concentration of 0.1 %. A portion of this sample was injected into the analysis chamber of the AA where it was aspirated, atomized and absorbance was determined at 325 nm. The concentration of copper from the samples was determined against a freshly prepared standard curve for copper.

Storage stability study

After selecting a LNP topotecan formulation for biological studies the stability of the product at 4°C was determined over a time frame of 2 months. This formulation, referred to as Topophore C, was monitored for a number of parameters including: i) color; ii) appearance, iii) particle size distribution as measured by Phase Analysis Light Scattering methods, iv) drug retention; v) liposome concentration as well as vi) drug-to-lipid ratio.

In vivo plasma elimination of topotecan

A single dose (5 mg/kg) of free topotecan (Hycamtin) or Topophore C was administered intravenously (iv) into female Balb/c mice (Taconic, Hudson, NY; 20–25 g). Four mice were used per time point and blood samples were collected via cardiac puncture after the mice were terminated by CO_2 asphyxiation. Blood was immediately placed into EDTA-containing microtainers (Becton Dickinson, NJ) and stored on ice until it could be centrifuged at 2500 rpm for 15 min to separate plasma from blood cells. The concentration of liposomal lipid (^{3}H-CHE) in the plasma was determined by scintillation counting and concentration of topotecan was determined by HPLC methods as described above. The drug-to-lipid ratio was estimated from these data and the plasma AUC and half-life of topotecan was determined from this data using non-compartmental pharmacokinetic model with the help of WinNonlin™ (PharSight® Corp., Mountain View, CA) software. These animal studies were completed under an animal care protocol reviewed and approved by the University of British Columbia's Animal Care Committee. The studies met current guidelines of the Canadian Council of Animal Care.

Accumulation of topotecan in the peritoneal cavity following i.v. injection

Since the pseudo-orthotopic ovarian cancer model used (see below) involve injection of the indicated tumor cell lines into the peritoneal cavity of mice, additionally since dissemination of ovarian cancer ascites is primarily in the peritoneal region studies were completed to determine the amount of topotecan that accesses this site after iv administration of free topotecan (Hycamtin) or Topophore C. ES2 tumor bearing mice (see below) were injected i.v. with a 5 mg/kg dose of topotecan. At specified time points the mice were terminated by asphyxiation with CO_2 and subsequently the animal's peritoneal cavity was washed (lavaged) with ice cold HBSS injected along the midline into the peritoneal cavity with a 27 G needle and the lavage fluid was collected and transferred to a 15 ml graduated polypropylene tube until the total volume collected was 5 mL. The peritoneal cavity was vigorously massaged after injection of lavage buffer to achieve good mixing and the fluid which was then collected with a syringe equipped with a 20 G needle. No further processing of the samples was done, thus the measured concentration of topotecan included both cell-associated and free material. The topotecan concentration was determined by HPLC as described above.

In vivo antitumor activity

The antitumor activity of Topophore C was evaluated in female mice using pseudo-orthotopic model of ovarian cancer. ES-2 cells are model of a chemorefractory clear cell carcinoma [51]. These cell lines were purchased from American Type Culture Collection, ATCC (Rockville, MD). ES-2 cells (1×10^5/500 μl) were inoculated intraperitoneally (i.p.) into female NCr-Fox1^{nu} mice (Taconic, Hudson, NY). Seven days after tumor cell inoculation free topotecan (Hycamtin) or Topophore C was administered i.v. (Q7D x 3) at the indicated drug doses. Control mice groups were injected with saline. The health status of all animals inoculated with tumor cells was monitored carefully to assess animal health as judged by recorded changes in body condition and weight as well as tumor growth and associated morbidities. If the health status was poor, as assessed by qualified animal health technicians following a standard operating procedure-sheet for animal monitoring, and when reaching specified humane endpoints the mice were terminated by CO_2 asphyxiation. Morbidity measured in efficacy studies was understood to be a reflection of both treatment related side effects and tumor progression. Improvements in "survival" of mice inoculated with tumor cells were recorded for all groups, as follows: the overall health status of the animal was used to assess morbidity and a scoring system utilized to indicate when an animal should be terminated due to that morbidity. The day of death was reported as 1 day following termination due to morbidity. Necropsies were performed on terminated animals to assess gross signs of toxicity and tumor progression. As indicated above these animal studies were conducted according to a protocol approved by Institutional Animal Care Committee (IACC) of the University of British Columbia, Canada.

Statistical analysis

Results were analyzed using ANOVA. Significant differences between groups were identified using Students-Newman-Keul's multiple comparison post hoc test (GraphPad Instat software -San Diego, CA, USA). Survival curves generated using Kaplan-Meier plot were compared for statistical significance using Log-rank (Mantel-Cox) test (GraphPad Prism software, GraphPad, CA, USA). Differences between the groups were considered significant if $p<0.05$.

Results and discussion

Topotecan loading into copper containing liposomes

Incubation of topotecan with the pre-formed liposomes prepared suspended in SHE (pH 7.5) buffer resulted in a dramatic decrease in the solution pH. If the pH of the solution was below 7.0, then the loading efficiency was very poor. Thus it was critical to have an external pH of 7.0–7.5 after topotecan addition in order to achieve optimum drug loading. The protocol developed to achieve this has been summarized in Table 1 for formulations prepared to achieve a final drug to lipid ratio of 0.1 (mol/mol). A pre-determined volume of 1N NaOH was added to the liposomes prior to topotecan addition. The volume added was such that when topotecan was mixed with the liposomes the pH of the resulting solution was 7.0–7.5. Depending on the loading conditions (lipid concentration, drug concentration, temperature) this meant that the liposomes were exposed to a pH as high as 11 for only a brief period (<10 s). When the incubation temperature was 60°C topotecan encapsulation efficiencies of >98 % could be achieved for starting drug-to-lipid ratios of 0.1 (mol/mol).

As summarized in Fig. 1A, topotecan loading was rapid (>80 % loading within 5 min) however to achieve loading efficiencies of >98 % the samples required incubation for 60 min. Particle size distribution as determined by PALS was between 95 and 110 nm (with a polydispersity index (PDI) of <0.05) prior to drug loading and the mean particle size or distribution around the mean did not change following drug loading (data not shown). Optimum topotecan loading was achieved at an incubation temperature of 60° C. The effect of temperature on drug loading rate is illustrated by the data summarized in Fig. 1B. Topotecan loading efficiency was reduced significantly when the incubation temperature was decreased to 50°C and little drug loading was noted when the samples were incubated at 40°C.

Based on an understanding that pH and temperature were critical to achieve rapid and efficient drug loading, additional

Table 1 Optimization of drug loading conditions for the LNP topotecan formulation

Initial pH of liposomes	pH after NaOH addition	Vol. of NaOH (1N) required (μL)	pH after topotecan addition	Incubation time/temp.	% topotecan loading
7.5	9.0	14	5.0	30 min/60°C	80 %
7.5	10.5	30	6.0	30 min/60°C	89 %
7.5	12.0	50	7.25	30 min/60°C	>98 %

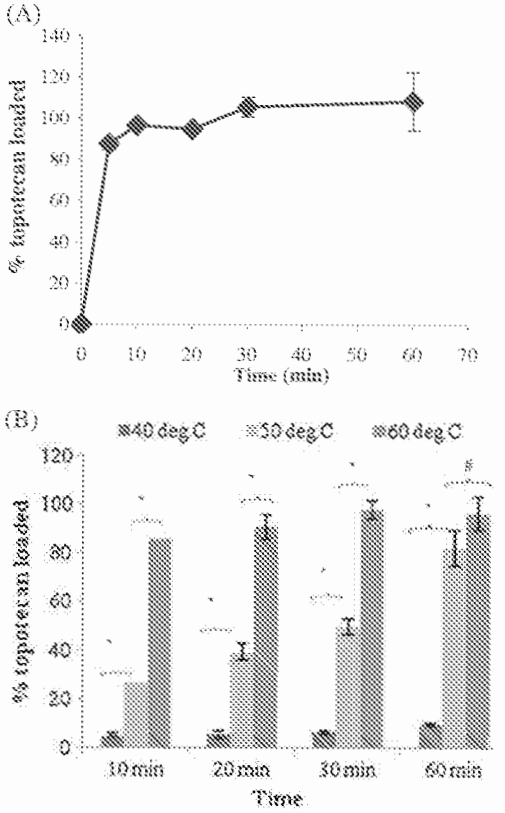

Fig. 1 $CuSO_4$ mediated topotecan encapsulation into DSPC/CH (55:45) liposomes. Liposomes were prepared with entrapped $CuSO_4$ (pH 3.5) and incubated with A23187. Topotecan was mixed with liposomal suspension to achieve a drug-to-lipid ratio of 0.1 (mol/mol) (see Table 1), this mixture was then incubated at 40°C, 50°C and 60°C for 60 min. Aliquots were removed intermittently and fractionated onto 1 mL Sephadex G-50 size exclusion column to separate unencapsulated drug. Data points represent mean±SD of values obtained from at least three experiments. *$P<0.001$, #$P<0.01$

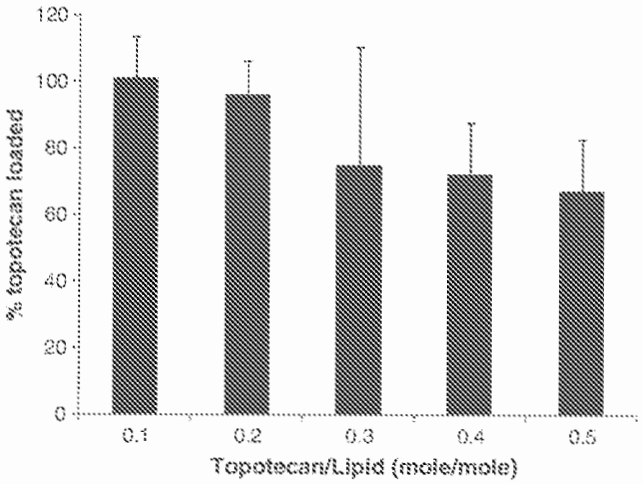

Fig. 2 Encapsulation efficiency of liposomal topotecan formulations as a function of increasing target D/L (mole/mole). Topotecan was added to liposomes prepared in 300 mM copper sulfate. The liposomes and drug were incubated separately at 60°C prior to mixing. Data points represent mean±SD of values obtained from at least three separate experiments

studies were conducted to establish the maximum drug loading capacity. This work was initiated, in part, because other LNP topotecan formulations have been described in the literature with varying drug-to-lipid ratios [29, 35, 46, 52]. Since the drug-to-lipid ratio influences lipid dose which in turn influences pharmacokinetics of injected formulations [53, 54] it was important to establish the range of formulations which could be prepared. As summarized in Fig. 2, the loading efficiency decreased as the initial drug to lipid ratio increased. When the initial drug to lipid ratio was 0.5 (mol/mol) the loading efficiency was 68 %, compared to >95 % for drug to lipid ratios of 0.2 or 0.1 (mol/mol).

To better characterize the resulting formulations, drug release was measured under the dialysis conditions described in the Methods (excess volume of PBS). This data, summarized in Fig. 3, was obtained for LNP topotecan formulations prepared at 0.1, 0.2, 0.3 and 0.4 drug to lipid ratios (mol/mol) as summarized in Fig. 3. The results demonstrated rapid release of topotecan from the liposomes when the initial drug to lipid ratio was 0.4 (~70 % drug loading). Although excellent drug retention was seen at D/L of 0.1 to 0.3 a formulation with 0.1 D/L was chosen for further studies after taking into account the dose of lipid required for maintaining the required plasma levels following in vivo administration. This formulation has been referred to herein as Topophore C.

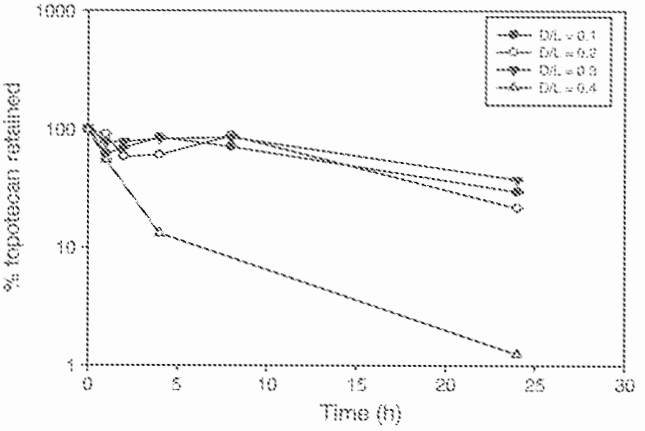

Fig. 3 In vitro drug release profile of liposomal topotecan following incubation in PBS (pH 7.4) 37°C. Briefly, liposomal suspensions were diluted appropriately with PBS buffer and placed in a dialysis bag (MWC 10000). Aliquots were removed from the dialysis bag intermittently and liposomes were separated from the free drug using spin columns prepared with Sephadex G50. Topotecan and liposomal lipids were measured using HPLC and scintillation counting respectively. Values indicate mean±SD of three individual measurements

Retention of copper following topotecan loading

It has been previously shown that the amount of retained copper after drug loading is important to achieve optimal drug retention attributes. For Irinophore C a retained copper concentration of at least 40 mM appears necessary for optimal drug retention [55]. Assuming similar mechanisms govern topotecan retention in Topophore C, it was important to determine the amount of copper retained following topotecan loading. Aliquots obtained at various time points following topotecan addition to the liposomes at 60°C were passed through a size exclusion column (see Methods) in order to separate the drug loaded liposomes from any copper that may have been released during drug loading. Subsequently the samples were analyzed for copper as described in the Methods and the results of this study have been summarized in Fig. 4. As topotecan (open circles) was encapsulated copper (filled circles) was released. In this study 90 % of the added topotecan was encapsulated within 10 min. At this time point 60 % of the encapsulated copper was released. After 60 min at 60°C, topotecan loading was >98 % and approximately 75 % of the initial liposome associated copper was released. Based on this data, the estimated copper concentration remaining in the liposomes would be approximately 75 mM.

Storage stability

Prior to initiating biological studies it was important to determine whether Topophore C could be stored at 4°C for extended time periods. In the context of the studies described here, it was known that treatment schedules to be used were based on weekly injections for at least 3 weeks, thus stability of the formulation for at least 1 month was a required. A batch of Topophore C was prepared, filter sterilized and placed into sealed 5 mL vials prior to placing the samples at 4°C. At selected time points vials were removed and the samples were analyzed for a number of parameters as specified in the Methods. This data has been summarized in Table 2. No significant changes in the formulation were noted in terms of appearance, color, particle size distribution, or drug to lipid ratio following fractionation on a size exclusion column. Topophore C was able to retain >98 % of the initially encapsulated drug for at least 2 months.

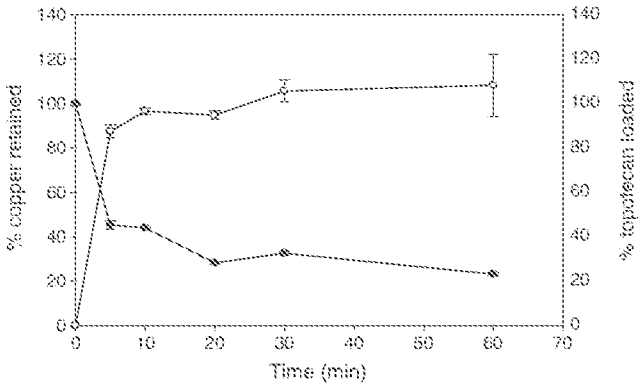

Fig. 4 Relationship between topotecan loading (open symbols) and copper release (closed symbols) from the liposomes. Liposomes were prepared with entrapped $CuSO_4$ (pH 3.5) and incubated with ionophore A23187. Topotecan was mixed with liposomal suspension to achieve a drug-to-lipid ratio of 0.1 (mol/mol), at 60°C. Aliquots were removed intermittently and fractionated onto 1 mL Sephadex G-50 size exclusion column to separate unencapsulated topotecan and released copper which were measured using UV spectrometer and atomic absorption spectroscopy respectively

In vivo pharmacokinetics following i.v. administration of Topophore C

Topotecan pharmacokinetics was evaluated in mice following administration of single i.v. bolus injection of free topotecan (Hycamtin) or Topophore C administered at a dose of 5 mg/kg drug. At selected time points blood was obtained from injected animals and the concentration of liposomal lipid and topotecan were measured in the plasma as described in the Methods. The results, summarized in Fig. 5, demonstrate that following injection of free topotecan the plasma concentration of drug decreased rapidly, with less than 0.08 % (assuming a plasma volume of 1 mL for a 22 g mouse) of the injected dose in the plasma compartment after 2 h. The concentration of topotecan was below detection limits at time points beyond 4 h. In contrast, following administration of an equivalent drug dose of Topophore C >70 % of the injected drug dose was in the plasma compartment. Topotecan levels were still measurable 24 h following administration of Topophore C, at levels comparable to those seen in plasma 2 h following administration of free drug. The plasma elimination half life

Table 2 Storage stability of Topophore C at 4°C

Time	Appearance	Size (nm)	D/L	% Drug Retained
Initial	Translucent yellow	99.4 (PDI<0.05)	0.08	>98
1 week	No change	105.8 (PDI<0.05)	0.08	>98
2 week	No change	104.8 (PDI<0.05)	0.08	>98
3 week	No change	108.1 (PDI<0.05)	0.08	>98
1 month	No change	108.1 (PDI<0.05)	0.07	>98
2 month	No change	102.9 (PDI<0.05)	0.08	>98

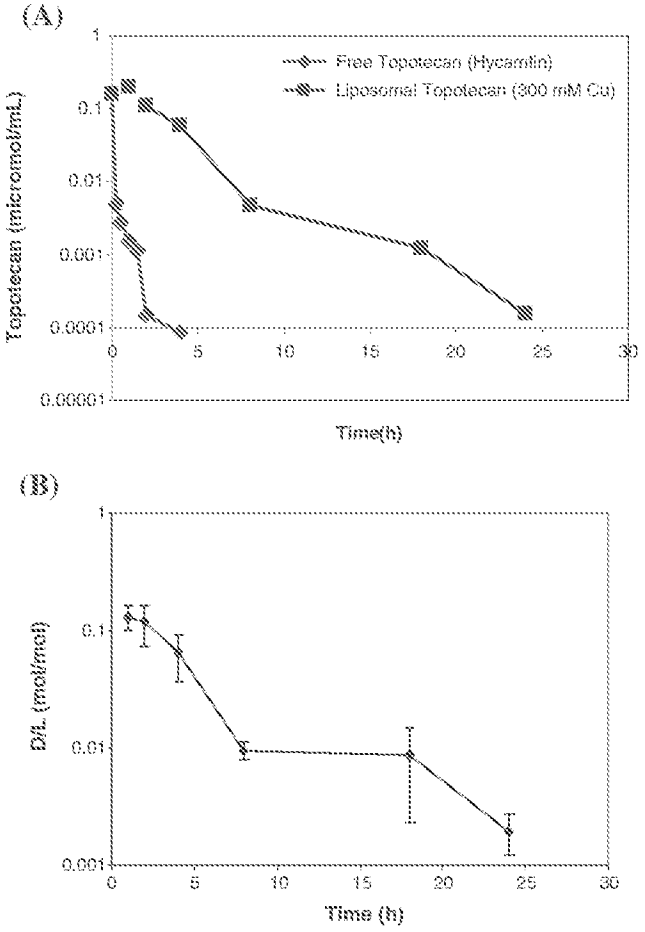

Fig. 5 Plasma elimination profiles of Topophore C compared against free topotecan (Hycamtin). Female Balb/C mice were injected intravenously with a single dose (5 mg/kg topotecan) of DSPC/Chol (55:45 mol%) liposomal topotecan formulations: (A) Concentration of topotecan remaining in plasma as a function of time. (B) Drug to lipid ratio in plasma as a function of time. Data points represent the mean±SD ($n=4$)

was 15 min for free topotecan and 2.5 h for Topophore C. The plasma $AUC_{0\text{-}24h}$ following administration of free topotecan was 14.31 μg.h.mL^{-1} compared to 317.9 μg.h.mL^{-1} following administration of Topophore C. It should be noted that at the drug dose used in these studies, the associated liposomal lipid dose was approximately 60 mg total lipid/kg. Figure 5B, represents change in drug to lipid ratio over a period of 24 h to give an idea of the rate of drug release from liposomes.

It was interesting to note that release of topotecan from liposomes and subsequent elimination from plasma was faster compared to previously studied liposomal anthracyclines or irinotecan. However, relatively faster plasma clearance of liposomal topotecan is not uncommon and other liposomal formulations of topotecan that are currently under investigation have noticed similar findings. Possible reasons may include intrinsic low $t_{1/2}$ of topotecan or higher lipid membrane permeability of topotecan which is evident from its considerable oral bioavailability (23 %). Another reason could be that, topotecan may not be able to form a gel like precipitate inside the liposomes like that of anthracyclines (doxorubicin). The HPLC data demonstrated that following 1 h of Topophore C administration >98 % of the drug measured in the plasma was in the active lactone ring closed conformation, and no detectable carboxy form was measured for as long as 18 h suggesting that the drug in the plasma compartment was primarily present in the encapsulated form. In contrast following administration of free topotecan within 1 h ~40 % of the drug measured was in the ring opened carboxy form. This data is illustrated for samples collected at the 1 h time point in Fig. 6.

Topotecan accumulation within the peritoneal cavity following i.v. administration

In advanced ovarian cancer tumor cell growth is often restricted to the peritoneal cavity and angiogenesis associated with ovarian cancer development and progression promotes peritoneal carcinomatosis and malignant ascites formation; two attributes linked to disease engendered morbidity and mortality [56, 57]. For these reasons, preclinical models of ovarian cancer often involve inoculation of tumor cells within the peritoneal cavity. Given this site of tumor progression it was reasonable to assess the extent to which topotecan distributed to the peritoneal cavity following i.v. administration of free topotecan (Hycamtin) or Topophore C. Further, studies from our lab have already demonstrated that following i.v. administration of LNP anticancer drug formulations there is significant accumulation of drug within the peritoneal cavity in the presence or absence of tumor [58]. To determine if Topophore C was able to enhance topotecan delivery to the peritoneal cavity of mice the concentration of topotecan in peritoneal fluid obtained from ES2 tumor bearing mice was measured following iv administration of either free topotecan (Hycamtin) or Topophore C (see Methods). Results of this study are summarized in Fig. 7. Approximately 96 ng/ml of topotecan was recovered from the peritoneal cavity 1 h following free topotecan administration; equivalent to about 0.5 % of the injected dose (based on the total 5 mL volume recovered from the peritoneal cavity). In comparison, delivery of drug to the peritoneal cavity was significantly lower 1 h after administration of Topophore C. This is consistent with previous results suggesting that extravasation of circulating LNPs from the blood compartment to sites of tumor growth is a slow process [59, 60]. After the 1 h time point, the amount of topotecan decreased in the peritoneal cavity of mice that received free topotecan, but increased in those animals given Topophore C.

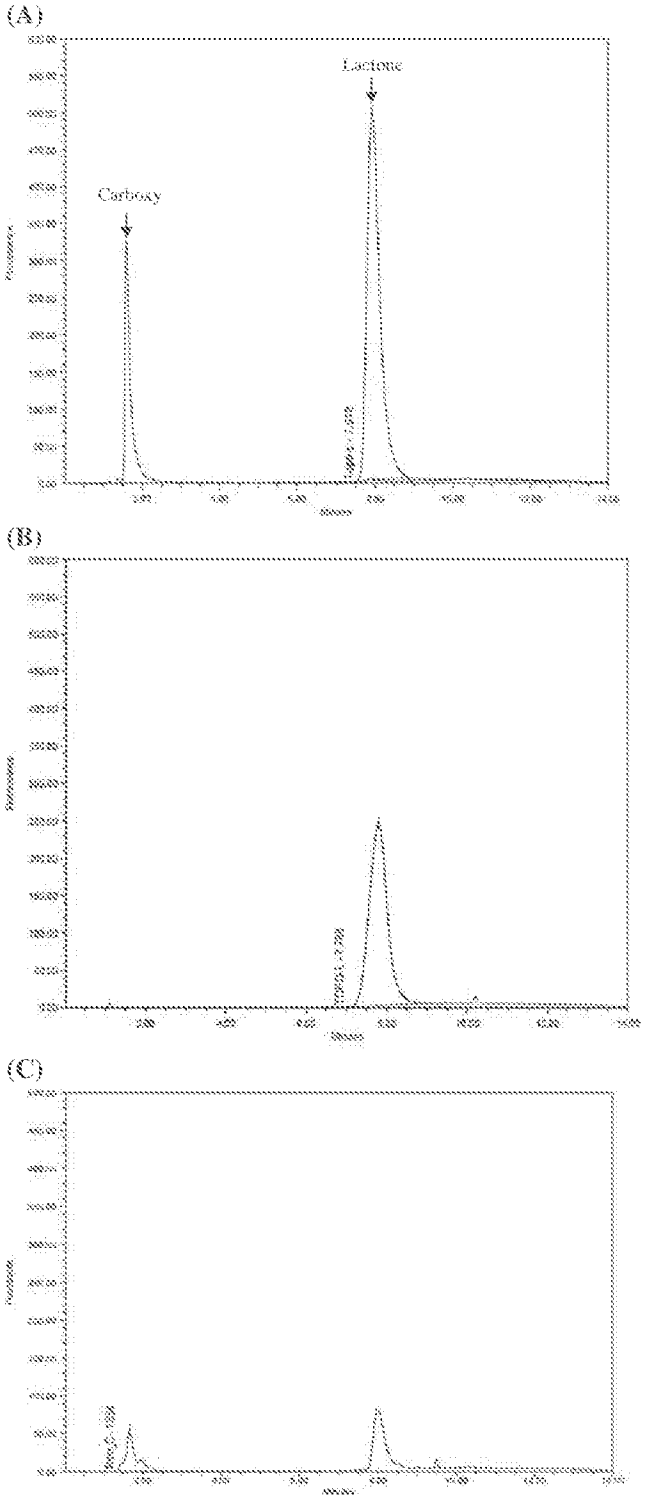

Fig. 6 HPLC chromatograms of topotecan representing (A) overlay spectra for separately injected carboxy and lactone standards, (B) 1 h mouse plasma sample following i.v. administration of Topophore C (5 mg/kg) and (C) 1 h mouse plasma sample following i.v. administration of free topotecan (5 mg/kg)

At 8 h, for example, the level of topotecan in the peritoneal cavity was not detectable following administration of free topotecan while the level obtained following administration of Topophore C were 76 ng/ml. This data clearly demonstrate that following i.v. administration of Topophore C there was significantly higher topotecan levels in the peritoneal cavity of ES2 tumor bearing mice when compared to animals treated with free topotecan (Hycamtin).

In vivo efficacy studies in model of ovarian cancer

Clear cell carcinoma is a rare subtype of ovarian cancer that is clinically distinct from serous adenocarcinoma of the ovary, and is resistant to chemotherapy [61, 62]. In these studies we used human ovarian cancer cell lines that have been classified as clear cell carcinoma (ES2 cells). As indicated in the Methods, these cells were inoculated i.p., tumor related morbidity (ES2 cells) was monitored as a function of time following treatment. Treatments were given i.v. using a Q7D x 3 schedule. Dose response curves were generated and it should be noted that free topotecan (Hycamtin) was tolerated better than Topophore C under this dosing schedule. Non-tumor related toxicity data indicated that free topotecan was better tolerated (upto 15 mg/kg (Q7D x 3) except for the decreased motor activity and dry skin. Whereas, Topophore C administered at 10 mg/kg (Q7D x 3) resulted in greater than 20 % weight loss in mice which was recovered following the last dose. This type of observation is expected with targeted therapeutics especially in cancer treatment because while more drug is delivered to the cancer cells with the help of nanotherapeutics, some rapidly proliferating healthy cells (cells of GIT and bone marrow) may also receive exposure to higher amount of drug. However, higher efficacy against cancer cells with lower dose usually outweigh such toxicity concerns as observed with Topophore C. For both free topotecan and Topophore C the toxicity observed at their maximum tolerated doses was comparable. It is understood that the therapeutic activity and toxicity of topotecan are very dose and dose schedule dependent [63], thus a comparison of therapeutic activity between topotecan and Topophore C requires a range of studies that go far beyond the scope of the current study. For this reason, efficacy data was obtained using a dose of topotecan which caused no observable effects based on gross observations (5 mg/kg given Q7D x3) and this was compared to an equivalent or lower dose of Topophore C to provide proof of concept data supporting further development of Topophore C as a product candidate for ovarian cancer or other indications where topotecan appears to have activity.

Results obtained using the ES2 ovarian cancer model are summarized in Figs. 8 and 9 in the form of median survival plot and kaplan-Meier survival plot respectively. 100 % of control animals (saline treated) were terminated due to disease progress within 29 days. Free topotecan administered at

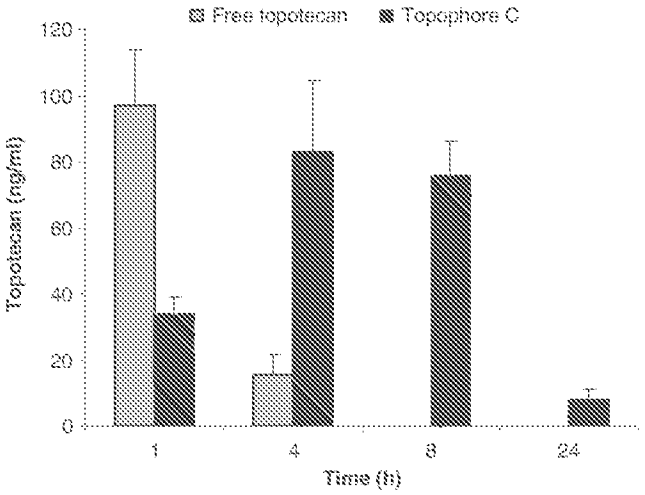

Fig. 7 Amount of topotecan recovered from the peritoneal cavity of tumor bearing mice following i.v. administration of single dose (5 mg/kg) of Hycamtin or Topophore C. Animals were terminated and then the peritoneal cavity was washed (lavaged) with buffer such that the total volume collected was 5 mL. Data points indicate mean ± SEM, $n=4$, $P<0.01$

a dose of 5 mg/kg extended the survival time of ES2 bearing mice, yet 100 % of these animals needed to be terminated due to disease progression by day 36. The median survival time (MST) for control animals was estimated to be 19 days and for animals treated with free topotecan (5 mg/kg) the MSTs was 29 days, representing a 53 % increase in median life span. The therapeutic activity of Topophore C given at 1.25, 2.5 and 5.0 mg/kg topotecan was significantly better than that observed using free topotecan given at 5 mg/kg. For example, when animals were treated at a dose of 1.25 mg/kg the MST for the treated animals was 48 days; representing a 152 % increase in MST relative to controls. This was 70 % better than that which could be achieved with the free topotecan given at a dose that was 4-fold higher. Interestingly, Topophore C did not exhibit a significant dose response curve, i.e. the MST for mice treated with 5 mg/kg of Topophore C (44 days) was not significantly different from the results obtained with 1.25 mg/kg Topophore C. The ES2 ovarian cancer model is considered to be a treatment refractory, aggressive model.

Topotecan has been recently approved by FDA to be used as a single agent in the second line treatment of recurrent ovarian cancer. It has proven to be a potent anticancer agent and has shown very good therapeutic efficacy in terms of reductions in measurable disease and improving the progression free survival time for patients with ovarian cancer [64]. Our group and many others believe that application of suitable nano-scale drug delivery technology will enhance the therapeutic effects of topotecan. Enhanced activity would be due to: i) maintaining the drug in its lactone ring closed configuration following administration, ii) enhanced delivery of therapeutically active drug to the site of tumor growth and iii) increased efficacy at lower drug doses which may be better tolerated and more suitable for use in a drug combination setting. As indicated above, other groups have pursued development of LNP topotecan formulations. In some examples low topotecan loading efficiency and rapid drug loss from the formulation limited their further development [32, 52, 65–69]. Others have reported on formulations prepared using novel methods relying on transmembrane gradients of triethylammonium salts of polyphosphate or sucrosecctasulfate to form topotecan precipitates within the liposomes [35]. These formulations exhibit extended circulation lifetimes, increased topotecan AUC, and improved therapeutic activity [35, 41]. With the help of copper based active loading method, we were able to load topotecan into liposomes at an extent that is either significantly higher or equivalent to the comparable formulations

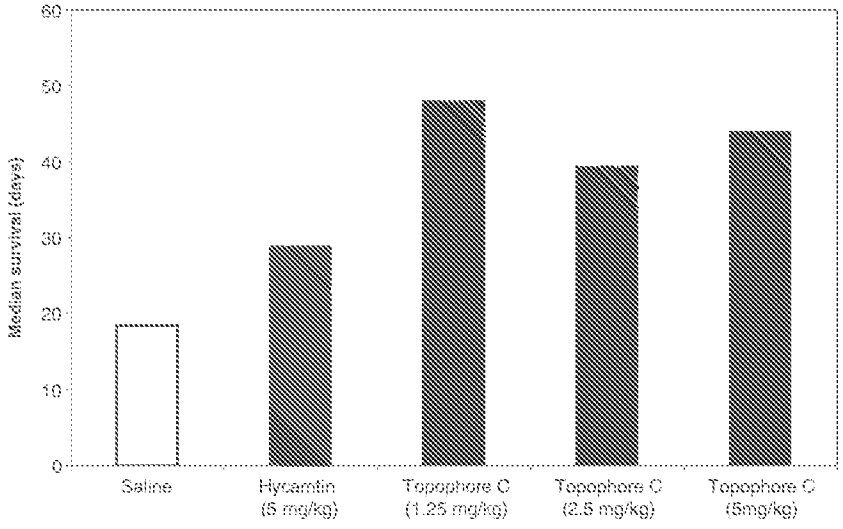

Fig. 8 Median survival plot for ES-2 ovarian tumor bearing mice following q7d x3 i.v. administration of Hycamtin (5 mg/kg) or Topophore C (1.25 mg/kg, 2.5 mg/kg and 5 mg/kg). Animals were treated 7 days after tumor cell inoculation and health status of the animals (see Methods) was assessed daily.data points represent median survival (days)

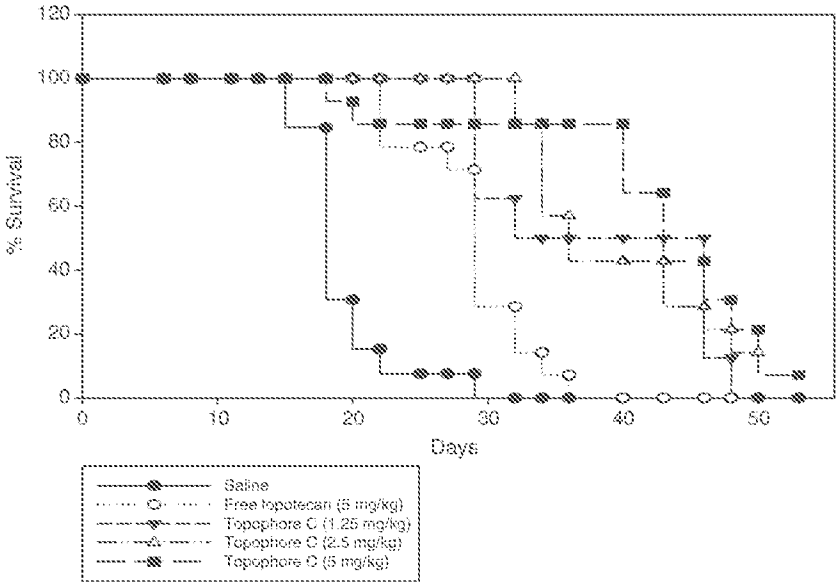

Fig. 9 Kaplan-Meier survival plot for ES-2 ovarian tumor bearing mice following q7d x3 i.v. administration of Hycamtin (5 mg/kg) or Topophore C (1.25 mg/kg, 2.5 mg/kg and 5 mg/kg). Animals were treated 7 days after tumor cell inoculation and health status of the animals (see Methods) was assessed daily. When the health status indicated that the animals needed to be terminated for humane reasons, the day of death due to tumor progression was recorded as the following day. Data points represent mean ± SD ($n \geq 8$)

by other researchers in the field. More importantly, our formulation showed excellent storage stability even after close to 100 % encapsulation efficiency. At this time it is too early to assess the benefits/limitations of these formulation methods when compared to the one described in this report. Further improvements in Topophore C e.g. in terms of longer plasma circulation time can be achieved. For example, plasma circulation time of liposomes can be improved by shielding them from opsonins and macrophages with the help of hydrophilic protective coating like PEG (stealth liposomes). However, benefits of PEG coating on liposomes are under debate due to issues like increased drug leakage, increased immunogenicity, compromised intracellular delivery etc. and therefore careful consideration should be given in balancing different factors.

In addition there needs to be a comparison made between different LNP topotecan formulations currently under development. At this point in time there does not appear to be any compelling reason to develop multiple LNP topotecan formulations, thus in the interest of patients in need of better treatment options it would be ideal if comparator studies could be completed to identify one formulation that has the best chance of providing benefits for patients. We are currently proceeding to evaluate Topophore C in combination with Doxil, a liposomal formulation of doxorubicin that is already approved for use in patients with relapsed, platinum refractory ovarian cancer.

On a final note, as a topoisomerase I inhibitor, topotecan's activity is known to be a schedule dependent. Studies by Guichard et al. which compared different administration schedules of topotecan in ovarian cancer xenografts have demonstrated that administration schedules that maintained therapeutically active topotecan concentrations in plasma over a prolong period were more therapeutically effective than those which maintained similar plasma levels for less time following administration of same total dose [63]. In light of such observations, improved cytotoxic activity observed with LNP topotecan formulation warrant dose scheduling studies with these formulations.

Conclusion

The formulation described here relies on use of encapsulated copper and a transmembrane pH gradient to achieve improvements in topotecan retention. The developed formulation was stable for at least 2 months at 4°C, exhibited a topotecan encapsulation efficiency of >98 % and altered topotecan pharmacokinetics and distribution in a manner that resulted in improved therapeutic activity as judged in an aggressive model of ovarian cancer. The encapsulated drug was maintained in its active ring closed lactone form. It will be clearly important to assess the toxicity and efficacy of Topophore C against other aggressive models of recurrent ovarian cancer and to complete a more comprehensive comparison between Topophore C and free topotecan given using optimal schedules and dosing parameters.

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Conflict of interest The authors declare that they have no conflict of interest with the financial institutions that supported this research.

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ORIGINAL PAPER

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Effects of oxaliplatin and CPT-11 on cytotoxicity and nucleic acid incorporation of the fluoropyrimidines

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Abstract *Purpose*: The addition of oxaliplatin or CPT-11 to 5-FU has become common practice in the treatment of colorectal cancer. It is not known, however, which fluoropyrimidine drug (5-FU, FUdR, or FUR) will produce superior cytotoxicity when combined with either oxaliplatin or CPT-11. The purpose of the study was to determine the effects of oxaliplatin and CPT-11 on cytotoxicity and nucleic acid incorporation of all three fluoropyrimidines. *Methods*: HT-29 cells were exposed for 2 h to IC_{10}, IC_{30}, and IC_{70} of oxaliplatin and CPT-11. Subsequently, cells were exposed for 24 h to IC_{10}, IC_{30}, and IC_{70} of 5-FU, FUdR, and FUR. Cytotoxicity was measured by the MTT assay. Nucleic acid incorporation of [^{3}H]fluoropyrimidine was then compared in the presence and absence of oxaliplatin or CPT-11 pretreatment. *Results*: Synergistic cytotoxicity was displayed when IC_{30} of oxaliplatin or CPT-11 was combined with IC_{10} and IC_{30} of the fluoropyrimidines. One fluoropyrimidine did not achieve superior cytotoxicity over the others. After pretreatment with oxaliplatin or CPT-11, cytotoxic antagonism was observed as the concentration of a fluoropyrimidine increased up to IC_{70}. The increasing cytotoxic antagonism correlated with decreases in fluoropyrimidine nucleic acid incorporation. The most significant incorporation difference existed within the 5-FU treated group. *Conclusions*: No single fluoropyrimidine is more cytotoxically effective over the others when combined with oxaliplatin or CPT-11. Correlation of cytotoxic antagonism to the inhibition of fluoropyrimidine nucleic acid incorporation implies difficulties in drug transport and/or metabolism only after oxaliplatin or CPT-11 pretreatment.

Keywords Oxaliplatin · CPT-11 · 5-FU · FUdR · FUR

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Introduction

Colorectal cancer is one of the leading causes of cancer death in the world. Palliative chemotherapy remains the only option for the majority of patients. For the last 40 years, 5-Fluorouracil (5-FU) has been the most commonly used chemotherapeutic agent in the treatment of colorectal cancer (Schmoll et al. 1999). However, the response rate to this drug has usually been under 25% and patient survival has not been significantly prolonged (Link et al. 1988). The inadequate clinical results observed with 5-FU could be attributed to its relatively slow anabolism to the active metabolites in most cells. Clinical dissatisfaction with the drug also stems from increased cases of resistance. Figure 1 illustrates the pathways through which the fluoropyrimidines are activated and incorporated into DNA and RNA. One pathway leads to the conversion to FUdR (5-fluoro-2'-deoxyuridine), which subsequently forms FdUMP. FdUMP is a potent inhibitor of the enzyme thymidylate synthase (TS). This step is essential for DNA synthesis and its inhibition is highly regarded to be responsible for the cytotoxic effects of 5-FU (Langenbach et al. 1972; Pinedo and Peters 1988; Peters et al. 1991; Shuey et al. 1995; Sanguedolce et al. 1998; Sun et al. 2002). The incorporation of 5-FU into RNA represents an alternative pathway in its metabolism. This route has also been accepted as the main explanation for 5-FU's actions (Houghton et al. 1979; Mandel et al. 1979; Kufe and Major 1981; Glazer and Lloyd 1982; Doinick and Pink 1983; Geoffroy et al. 1994).

As a result of the clinically unsatisfactory effects of 5-FU, a significant amount of interest exists to utilize

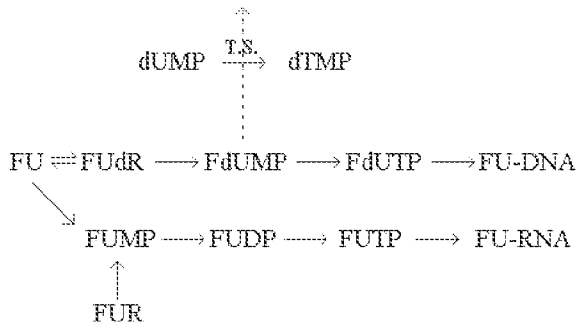

Fig. 1 Metabolic pathways for the fluoropyrimidines. FU, FUdR, and FUR are 5-fluorouracil, 5-fluoro-2'-deoxyuridine, and 5-fluorouridine, respectively. FdUMP is 5-fluoro-2'deoxyuridine-5'-monophosphate and FdUTP is 5-fluoro-2'deoxyuridine-5'-triphosphate. FUMP, FUDP, and FUTP are 5-fluorouridine-5'mono-, di-, and triphosphate, respectively. The enzyme thymidylate synthase is indicated by *T.S.* The *dotted arrow* displays the inhibitory action of FdUMP on T.S. activity

FUdR or FUR (5-Fluorouridine) as substitutes. FUdR has shown significantly superior antitumor activity in animal models compared with 5-FU (van Laar et al. 1993; van Laar et al. 1998). Arisawa et al. demonstrated higher in vitro activity of FUdR than 5-FU and reduced acquired resistance when treated for a shorter period with combination therapy (Arisawa et al. 1994). In addition, many clinical trials have shown significantly superior response rates for FUdR when compared to 5-FU (Ansfield and Curreri 1963; Buroker et al. 1976; Levin and Gordon 1993). Despite this existing evidence for the potential superiority of FUdR, 5-FU is still mainly preferred due to its assumed lower toxicity and cost. While 5-FU and FUdR are currently used in the treatment of gastrointestinal tumors, the use of FUR has been limited due to its systemic toxicity. However, Link et al. demonstrated cytotoxicity could be achieved with FUR at substantially lower doses and shorter exposure times than with 5-FU (Link et al. 1988). FUR has irreversible and non-phase-specific RNA-directed cytotoxic effects and has displayed more rapid cellular uptake and efficient conversion to nucleotides when compared to 5-FU (Wilkinson and Pitot 1973). In another study, FUR was also found to be more effective at inhibiting DNA synthesis than 5-FU (Kessel et al. 1971). The differences in metabolism, favorable pharmacokinetics, and mechanism of action of FUR have increased its use in treating superficial bladder cancers (Richie 1992; Boring et al. 1994; Song et al. 1997). As with 5-FU and FUdR, the development of resistance to FUR often occurs.

In an effort to improve response rates and survival, there has been an increased effort to find agents to potentiate the effects of the fluoropyrimidines or treat tumors that are unresponsive to the fluoropyrimidines. Oxaliplatin was recently developed for clinical use and is rapidly being utilized for the treatment of colorectal cancer. De Gramont et al. recently established the clinical superiority of a combination of oxaliplatin/5-FU/leucovorin over 5-FU/leucovorin in terms of response rate and progression-free survival (De Gramont et al. 2000). The tested drug sequence for this Phase III clinical trial was oxaliplatin/leucovorin for 2 h followed by 5-FU during 48 h, repeated every 2 weeks. Other studies have also shown superior response rates when oxaliplatin and 5-FU were combined (Levi et al. 1992; Levi et al. 1994; Levi et al. 1997; Levi et al. 1999; Giacchetti et al. 2000). Another new drug that has also recently stimulated interest is irinotecan or CPT-11. Similar to oxaliplatin, CPT-11 alone is active against colorectal cancer and in patients resistant to 5-FU (Cunningham et al. 1998; Rougier et al. 1998). It was recently shown that the combination of CPT-11/5-FU/leucovorin produced a higher response rate (Douillard et al. 2000; Saltz et al. 2000) and prolonged survival (Douillard et al. 2000) than 5-FU/leucovorin in advanced colorectal cancer patients.

Since cytotoxic and clinical success is demonstrated when 5-FU is combined with oxaliplatin and CPT-11, efforts now need to be made to explore the effects of combining FUdR or FUR with oxaliplatin and CPT-11. Researchers have often observed the potential superiority of FUdR and FUR over 5-FU. If oxaliplatin and CPT-11 act as synergistic agents with 5-FU, perhaps similar or superior results will ensue when these drugs are combined with FUdR or FUR. The goal of this study was to determine which fluoropyrimidine acts most synergistically with oxaliplatin and CPT-11. It was expected that it would be possible to establish the concentrations of each drug in a combination that displays superior cytotoxic synergy or antagonism. The MTT cytotoxic assay was utilized with HT-29 colon cancer cells to determine cytotoxicity. Because of the growing evidence for superior clinical success, it was hypothesized that the combination of oxaliplatin or CPT-11 with FUdR would display higher cytotoxic synergy than when the two drugs were combined with 5-FU or FUR. The final aim of the study was to correlate cytotoxic synergy and antagonism with fluoropyrimidine nucleic acid incorporation. After pretreatment with oxaliplatin or CPT-11, nucleic acid incorporation of varying concentrations of [^{3}H]5-FU, FUdR, and FUR was compared. The final nucleic acid incorporation potentially represents each fluoropyrimidine's ability to be transported and/or metabolized.

Materials and methods

Materials

HT-29 human colon adenocarcinoma cells were purchased from ATCC. RPMI 1640 culture medium was obtained from Gibco. LKT laboratories supplied oxaliplatin and CPT-11. [^{3}H]5-FU, [^{3}H]FUdR, and [^{3}H]FUR were purchased from Moravek Biochemicals. All other chemicals were obtained from Sigma Chemical.

Cell culture

HT-29 human colon adenocarcinoma cells were grown in monolayer cultures in RPMI 1640 media supplemented with 5% heat inactivated Fetal Bovine Serum. No antibiotics were added to the medium. The cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. The cells were trypsinized and passed once a week. Doubling time was noted to be approximately 24 h.

Individual drug cytotoxicity assay

Prior to determining cytotoxicity for combinations of oxaliplatin or CPT-11 with the fluoropyrimidine drugs, a percentage-inhibition curve and inhibitory concentrations for each of the individual drugs needed to be established. Growth inhibition was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based cytotoxicity assay (Freshney 2000). Briefly, 200 µl of the cell suspension (5×10^3 HT-29 cells) and 200 µl of RPMI 1640 growth medium were added to each well of a 96-well flat bottom microtitration plate. All plates were incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. Eight dilutions of each drug (oxaliplatin, CPT-11, 5-FU, FUdR, and FUR) were prepared in growth medium to give eight different concentrations for each drug. Each drug solution was filter sterilized. After incubation, 400 µl of growth medium was removed and 200 µl of each drug concentration or growth medium (controls) were distributed in the 96-well plates. Plates containing 5-FU, FUdR, and FUR were incubated for 24 h at 37°C. Oxaliplatin and CPT-11 were exposed to the cells in the other plates for 2 h, based on the length of time that has demonstrated clinical success (De Gramont et al. 2000; Saltz et al. 2000). Following incubation, the drugs were removed and each well was rinsed with growth medium. Wells in each plate then received 200 µl of fresh growth medium and cells were allowed to incubate for 2 days. At the end of the growth period, 50 µl of MTT (2 mg/ml) were added to each well and the cultures were incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. The medium and MTT were removed from the wells and the remaining MTT-formazan crystals were dissolved by adding 200 µl of DMSO. After adding Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH) to the wells containing DMSO, absorbance was measured with an ELISA plate reader at 570 nm (Dynex MRX Microplate Reader). Each experiment was performed using three replicated wells for each drug concentration and carried out independently three times. Results were expressed as percent cell survival. The percent cell survival for each drug concentration was calculated by the following formula: % survival = (absorbance of test wells/absorbance of control wells) × 100. A graph of % cell survival versus concentration for each drug was plotted to determine inhibitory concentrations (IC). The concentrations at which 90%, 70% and 30% of cells survived corresponded to IC_{10}, IC_{30}, and IC_{70}, respectively. These values were determined for each drug based on their respective dose response graph.

Drug combination cytotoxicity assay

The MTT cytotoxicity assay was utilized to examine cell survival after particular combinations of oxaliplatin or CPT-11 with the fluoropyrimidines. After a 24 h incubation period, HT-29 cells were exposed for 2 h to IC_{10}, IC_{30}, and IC_{70} of oxaliplatin or CPT-11 (200 µl). The plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere. The drugs were then removed and the wells were rinsed with growth medium. These cells were subsequently exposed for 24 h to IC_{10}, IC_{30}, and IC_{70} of 5-FU, FUdR, and FUR. Every possible inhibitory concentration combination between the fluoropyrimidines and oxaliplatin or CPT-11 was represented. Cytotoxicity for each individual drug alone represented the controls. After removing the drug solutions from the wells and filling them with fresh medium, MTT was added. Percent cell survival was then determined based on the MTT assay described above. Samples were examined in triplicate and the experiment was repeated three times.

Cytotoxicity values from the various combinations were subjected to the multiple drug effect analysis of Chou and Talay (Chou and Talay 1987). Using the computer program CalcuSyn (Biosoft, Cambridge, UK), the Combination Index (CI) for every combination was computed. The CI indicated synergism when smaller than 0.8, antagonism when greater than 1.20, and additive cytotoxic effects when located between 0.80 and 1.20.

Fluoropyrimidine nucleic acid incorporation after pretreatment with oxaliplatin or CPT-11

Cytotoxic synergisms and antagonisms of particular combinations of oxaliplatin or CPT-11 with 5-FU, FUdR, or FUR should be explained by differences in nucleic acid incorporation. In each of 16 75-cm^2 flasks, 3.5×10^6 HT-29 cells were seeded with 18 ml of growth medium for 2 days. Eight flasks were designated to receive oxaliplatin pretreatment and the remaining eight flasks contained cells that would not receive pretreatment (controls). After the 2-day incubation period, IC_{30} of oxaliplatin was added to each of the designated pretreatment flasks for 2 h. All flasks were then rinsed with medium and trypsinized. After pooling all of the cells from a particular group (oxaliplatin or control), cell counts were measured and the cell suspensions were centrifuged at 2,000 RPM for 5 min. Following the removal of the supernatant, the remaining cells were resuspended in transport buffer. Cells (105 µl) were distributed into 1.5-ml Eppendorf tubes and 45 µl of

tritium-labeled fluoropyrimidine were then added for 1 h. IC_{30} and IC_{70} of [^{3}H]5-FU, [^{3}H]FUdR, and [^{3}H]FUR represented the fluoropyrimidine concentrations that were added to the control and oxaliplatin pretreated cells. After the incubation period, all samples were centrifuged and washed with PBS (600 µl) for 5 min. All treated cells then received 5% cold PCA (500 µl). This step disintegrated the cell such that only nucleic acid incorporation of the tritium-labeled fluoropyrimidines remained. After waiting 30 min, samples were centrifuged at 2,000 RPM for 5 min and the supernatant was discarded. The remaining nucleic acids were dissolved in 0.5 M NaOH and later neutralized with 0.5 N HCl. Samples were transferred to vials, mixed with scintillation fluid, and counted for radioactivity. Each combination of oxaliplatin with IC_{30} and IC_{70} of all three fluoropyrimidines was examined in triplicate. The overall experiment was then repeated two separate times. Fluoropyrimidine nucleic acid incorporation was expressed as cpm/10^6 cells. Incorporation was compared between those cells receiving the IC_{30} and those receiving the IC_{70} of each of the tritium-labeled fluoropyrimidines.

The same procedure was utilized to evaluate fluoropyrimidine nucleic acid incorporation after pretreatment with IC_{30} CPT-11. Samples were examined in triplicate and the experiment was repeated twice. Fluoropyrimidine incorporation was then compared between those cells receiving the IC_{30} and those receiving the IC_{70} of each of the tritium-labeled fluoropyrimidines.

Statistical analyses

All data are presented as mean ± SD. A one-way analysis of variance (ANOVA) was utilized to determine statistical significance among groups. *t*-tests with Bonferroni correction were used for individual contrasts within each group at a $P < 0.05$.

Table 1 Drug inhibitory concentrations (IC) determined by the MTT cytotoxicity assay on HT-29 cells. Fluoropyrimidines were exposed for 24 h, while oxaliplatin and CPT-11 were exposed for 2 h. Results are averages of three separate trials

Drug	Inhibitory Concentrations (µM)		
	IC_{10}	IC_{30}	IC_{70}
5-FU	0.083	0.95	9.6
FUdR	0.000355	0.00106	0.00918
FUR	0.00088	0.00815	0.0575
Oxaliplatin	0.0068	0.114	0.776
CPT-11	0.0835	1.374	25.21

Results

Individual drug cytotoxicity

The MTT cytotoxicity assay revealed percent cell survival over a range of concentrations for oxaliplatin, CPT-11, 5-FU, FUdR, and FUR. From the dose response plots, the values for IC_{10}, IC_{30}, and IC_{70} for each of the drugs were interpolated and are displayed in Table 1.

Drug combination cytotoxicity

After determining the inhibitory concentrations for each drug, combinations of oxaliplatin or CPT-11 with 5-FU, FUdR, or FUR were examined. The IC_{10}, IC_{30}, and IC_{70} of each of the drugs were combined to determine the most synergistic and antagonistic relationships. Table 2 illustrates percent survival data for every combination of oxaliplatin or CPT-11 with a fluoropyrimidine. After a particular oxaliplatin or CPT-11 pretreatment (i.e., IC_{10}, IC_{30}, and IC_{70}), there were no survival differences seen between the fluoropyrimidines when their IC_{10}, IC_{30}, and IC_{70} data were specifically

Table 2 Percent survival of HT-29 cells after various combinations of oxaliplatin or CPT-11 with 5-FU, FUdR, and FUR (mean ± SD). Results are averages of three separate trials. (Fluoropyrimidines—24 h exposure, *Oxal* = oxaliplatin pretreatment for 2 h, *CPT* = CPT-11 pretreatment for 2 h)

Drug	Control	Percent cell survival (%)					
		Oxal IC_{10}	Oxal IC_{30}	Oxal IC_{70}	CPT IC_{10}	CPT IC_{30}	CPT IC_{70}
Control	100	92.5 ± 3.1	72.4 ± 4.3	32.3 ± 2.7	91.8 ± 2.5	73.6 ± 5.1	28.7 ± 3.9
5-FU IC_{10}	88.1 ± 2.7	89.4 ± 4.5	60.1 ± 3.2*	31.4 ± 3.9*	86.2 ± 2.8	61.7 ± 2.9*	22.9 ± 3.6*
5-FU IC_{30}	69.4 ± 3.0	67.8 ± 3.7	50.3 ± 4.6*	31.1 ± 1.2*	66.1 ± 3.6	58.5 ± 4.8*	22.1 ± 2.2*
5-FU IC_{70}	28.6 ± 3.8	42.8 ± 2.3*	41.1 ± 4.2*	31.0 ± 2.0	48.1 ± 2.6*	39.8 ± 4.1*	20.1 ± 3.4
FUdR IC_{10}	88.8 ± 4.7	87.9 ± 3.3	59.3 ± 2.6*	33.5 ± 2.9*	88.8 ± 3.7	63 ± 3.9*	23.5 ± 1.7*
FUdR IC_{30}	73.4 ± 5.1	71.2 ± 4.3	53.2 ± 3.2*	32.4 ± 3.8*	69.4 ± 3.9	57.4 ± 2.9*	21.4 ± 1.8*
FUdR IC_{70}	27.9 ± 3.5	45.3 ± 2.8*	42 ± 4.1*	29.1 ± 2.5	53.3 ± 3.0*	41 ± 3.6*	20.6 ± 2.9
FUR IC_{10}	91 ± 4.8	88.3 ± 3.7	61.1 ± 4.3*	32.2 ± 2.7*	88.5 ± 4.2	64.1 ± 5.7*	27.7 ± 3.2*
FUR IC_{30}	72.8 ± 2.9	69.7 ± 3.4	54.4 ± 3.1*	31.9 ± 3.9*	66.2 ± 4.1	54.4 ± 4.0*	24.2 ± 2.6*
FUR IC_{70}	28.8 ± 3.2	46.7 ± 3.6*	39.4 ± 2.8*	30.2 ± 3.1	50.9 ± 4.2*	43.2 ± 3.2*	21.3 ± 2.9

Utilizing Bonferroni multiple comparisons, * indicates a significant difference ($P < 0.05$) in cell survival between a particular combination and the fluoropyrimidine control alone

compared. The table also illustrates that pretreatment with IC_{10} oxaliplatin or CPT-11 did not result in synergy when combined with any fluoropyrimidine concentration (confirmed with a lack of CI < 0.8). However, cytotoxic synergy was observed when IC_{30} of oxaliplatin and CPT-11 were combined with IC_{10} and IC_{30} of any of the fluoropyrimidines. These particular combinations all resulted in CI values less than 0.8 (IC_{30} oxaliplatin/IC_{10} and IC_{30} fluoropyrimidine CI between 0.70–0.79, IC_{30} CPT-11/IC_{10} and IC_{30} fluoropyrimidine CI between 0.58–0.78). Pretreatment with IC_{70} of oxaliplatin and CPT-11 was not improved by the addition of any IC value of a fluoropyrimidine. Cell survival remained at approximately 31% after IC_{70} oxaliplatin pretreatment, regardless of the type and concentration of fluoropyrimidine that was later added. After pretreatment with IC_{70} of CPT-11, the addition of IC_{10} and IC_{30} of a fluoropyrimidine did not offer superior cytotoxic results compared to giving IC_{70} of CPT-11 alone. Surprisingly, pretreating the cells with IC_{10} and IC_{30} of both oxaliplatin and CPT-11 resulted in antagonism when the IC_{70} of 5-FU, FUdR, and FUR were subsequently added. The exposure of IC_{70} of a fluoropyrimidine alone resulted in the survival of approximately 28% of the cells. However, pretreatment with IC_{10} and IC_{30} of oxaliplatin or CPT-11 combined with IC_{70} of a fluoropyrimidine resulted in cell survival ranging from 39% to 53%. This antagonism was confirmed with CI values that were all greater than 1.2 (IC_{10} and IC_{30} of oxaliplatin/IC_{70} fluoropyrimidine CI between 1.7–2.3, IC_{10} and IC_{30} of CPT-11/IC_{70} fluoropyrimidine CI between 2.0–3.3).

The results that demonstrated unexpected antagonism prompted an exploration into possible mechanistic explanations. The difference between cytotoxicity achieved by the combination of IC_{30} oxaliplatin or CPT-11 with a fluoropyrimidine and the fluoropyrimidine alone decreased as the fluoropyrimidine IC increased (confirmed with increasing CI values). For example, a combination of IC_{30} oxaliplatin and IC_{10} 5-FU resulted in 60.1% cell survival, a 28% cytotoxic improvement when compared to IC_{10} 5-FU alone. The difference dropped to a 19% cytotoxic advantage after IC_{30} 5-FU treatment. Cytotoxic improvement did not exist once IC_{70} 5-FU was added. Instead, a 14.2% cell survival increase resulted when compared to IC_{70} 5-FU alone.

Fluoropyrimidine nucleic acid incorporation after oxaliplatin or CPT-11 pretreatment

An examination of fluoropyrimidine nucleic acid incorporation resulted in different outcomes for the control group when compared to those cells receiving pretreatment. Table 3 and Table 4 illustrate that as the fluoropyrimidine inhibitory concentration increased from IC_{30} to IC_{70} for the control cells, nucleic acid incorporation of 5-FU, FUdR, and FUR also increased. In contrast, incorporation decreased for all three fluoropyrimidines in cells receiving oxaliplatin and CPT-11 pretreatment. The most significant incorporation difference existed within the 5-FU group. The increase from IC_{30} to IC_{70} resulted in a threefold reduction of 5-FU incorporation in the oxaliplatin pretreatment group (Table 3) and a sixfold decrease in the CPT-11 pretreatment group (Table 4).

Table 3 Total nucleic acid incorporation of the fluoropyrimidines in HT-29 cells after pretreatment with oxaliplatin for 2 h (mean ± SD). Results are averages of two separate trials

Total nucleic acid incorporation (cpm/10^6 cells)		
Drug	Control	Oxaliplatin IC_{30}
5-FU IC_{30}	174 ± 21.9(a)	269 ± 29.3(b)
5-FU IC_{70}	359 ± 44.8(c)	87 ± 8.9(d)
FUdR IC_{30}	669 ± 41.9(e)	1123 ± 77.1(f)
FUdR IC_{70}	1440 ± 107.2(g)	957 ± 51.1(h)
FUR IC_{30}	2798 ± 112.1(j)	4476 ± 234.2(k)
FUR IC_{70}	5421 ± 319.3(m)	2073 ± 156.3(n)

ANOVA indicates significance with $P < 0.05$ for all fluoropyrimidine treatment groups. Bonferroni multiple comparisons show this significance between a and b, c and d, e and f, g and h, j and k, m and n

Table 4 Total nucleic acid incorporation of the fluoropyrimidines in HT-29 cells after pretreatment with CPT-11 for 2 h (mean ± SD). Results are averages of two separate trials

Total nucleic acid incorporation (cpm/10^6 cells)		
Drug	Control	CPT-11 IC_{30}
5-FU IC_{30}	131 ± 31.7	170 ± 39.2
5-FU IC_{70}	268 ± 50.6(a)	25 ± 4.9(b)
FUdR IC_{30}	627 ± 49.3(c)	969 ± 61.7(d)
FUdR IC_{70}	1236 ± 83.7(e)	825 ± 74.9(f)
FUR IC_{30}	2114 ± 102.5(g)	2854 ± 141.9(h)
FUR IC_{70}	4273 ± 271.5(j)	1821 ± 99.2(k)

ANOVA indicates significance with $P < 0.05$ for all fluoropyrimidine treatment groups. Bonferroni multiple comparisons show this significance between a and b, c and d, e and f, g and h, j and k

Discussion

The combination of oxaliplatin or CPT-11 pretreatment with 5-FU, FUdR, and FUR yielded important results seen for the first time. After oxaliplatin and CPT-11 pretreatment, one fluoropyrimidine did not produce superior cytotoxicity over the others at IC_{10}, IC_{30}, and IC_{70}. Thus, combining oxaliplatin or CPT-11 with FUdR or FUR may not result in superior clinical success when directly compared to a treatment arm consisting of oxaliplatin or CPT-11 combined with 5-FU. A comparison of the effect of oxaliplatin to CPT-11 for every fluoropyrimidine addition did not result in different cytotoxicity outcomes. Both oxaliplatin and CPT-11 are frequently entering many treatment protocols, and our study illustrates that one drug is not more cytotoxically effective over the other when combined with a fluoropyrimidine. Cytotoxic synergy was

observed when IC_{30} oxaliplatin and CPT-11 were combined with IC_{10} and IC_{30} of all three fluoropyrimidines. Since pretreatment with IC_{10} oxaliplatin and CPT-11 did not improve cytotoxicity accomplished by the fluoropyrimidines alone, it can be concluded that a particular concentration of oxaliplatin and CPT-11 must be achieved to yield efficacious results.

Despite the synergy observed at particular pretreatment and fluoropyrimidine combinations, antagonism was also observed. The difference between cytotoxicity achieved by the combination of IC_{30} oxaliplatin or CPT-11 with a fluoropyrimidine and the fluoropyrimidine alone decreased as the fluoropyrimidine IC increased. Ultimately, the addition of IC_{70} of any fluoropyrimidine to oxaliplatin or CPT-11 resulted in cytotoxic antagonism when compared to the cytotoxicity achieved by IC_{70} of the fluoropyrimidine alone. Matsuoka et al. found similar in vitro results when combining CPT-11 with 5-FU (Matsuoka et al. 1995). The investigators demonstrated that as the 5-FU concentration increased, the ratio of the cytotoxicity attained from the combination to the cytotoxicity achieved by 5-FU alone decreased. In vitro resistance results from a step-wise increase in fluoropyrimidine concentration over a specific period of time. Sobrero et al. developed cell lines resistant to FUdR by such a method (Sobrero et al. 1985). The investigators demonstrated that impaired drug transport was the mechanism of resistance and not differences in the activities of thymidylate synthase, FUdR phosphorylase, 5'-Fluorouridine kinase, 5-fluorouridine phosphorylase, and 5-Fluorouracil phosphoribosyltransferase. Another study utilizing the H-9 cell line illustrated that drug influx and nucleoside accumulation were significantly decreased in those cells resistant to FUdR and FUR compared to the sensitive cells (Agarwal et al. 2001). These investigators also did not observe differences in the expression of thymidylate synthase and multidrug-resistant protein between parental and resistant cell lines. Pretreatment with oxaliplatin and CPT-11 combined with fluoropyrimidine addition does result in cytotoxic synergy at particular fluoropyrimidine inhibitory concentrations, but antagonism appears to progressively occur as the fluoropyrimidine concentration is increased up to IC_{70}. This antagonism was then proposed to occur because of differences in cell transport or metabolism once a specific fluoropyrimidine concentration is attained. Perhaps, oxaliplatin and CPT-11 initially alter the transport or metabolism of the fluoropyrimidines and make it more difficult for the fluoropyrimidines to be later incorporated into nucleic acids at increasing concentrations.

An exploration into fluoropyrimidine influx and metabolism based on eventual nucleic acid incorporation led to many novel findings. Since a common transport inhibitor to all three fluoropyrimidines cannot be used, direct nucleic acid incorporation of the fluoropyrimidines was examined instead. Directly correlating with the cytotoxicity data, nucleic acid incorporation of the fluoropyrimidines decreased as their inhibitory concentrations increased to IC_{70} for those cells pretreated with oxaliplatin or CPT-11. As expected, nucleic acid incorporation increased when the fluoropyrimidine concentration increased to IC_{70} for the control cells that did not receive pretreatment. These results suggest that oxaliplatin or CPT-11 pretreatment does indeed inhibit fluoropyrimidine incorporation as the concentrations of the fluoropyrimidines increase. An inhibition of nucleic acid incorporation may reflect difficulties in cell transport and/or drug metabolism. The specific mechanism of action of oxaliplatin and CPT-11 could also be the cause of an inhibition of fluoropyrimidine nucleic acid incorporation. Oxaliplatin functions by forming interstrand and intrastrand cross-linking of DNA molecules. CPT-11 is a DNA topoisomerase I inhibitor. The direct effects that these agents have on DNA may make the incorporation of subsequent fluoropyrimidines more difficult as their concentrations increase. In our study, the most significant incorporation difference was seen for those cells receiving 5-FU. Since the combination of oxaliplatin or CPT-11 with 5-FU is currently being used in numerous treatment protocols, investigators must be aware of possible antagonisms if 5-FU concentration becomes exceedingly high or if it is used on a long-term basis.

Future experiments are being designed to determine what other mechanisms could be responsible for the antagonism observed at high fluoropyrimidine concentrations. Plasma membrane structure, expression of multidrug-resistance protein, expression of apoptotic markers, and measurements of catabolism all need to be compared before and after oxaliplatin or CPT-11 pretreatment. Our results indicate similar cytotoxicity for 5-FU, FUdR, and FUR when combined with oxaliplatin or CPT-11. To establish these findings clinically in terms of response rates, trials need to be conducted that involve treatment arms that directly compare 5-FU to the other fluoropyrimidines after oxaliplatin or CPT-11 pretreatment. Ultimately, our study offers insight into the possible effects of choosing alternative fluoropyrimidines to be combined with oxaliplatin or CPT-11 and the consequences associated with changing their concentrations.

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ORIGINAL ARTICLE

The Clinical Significance of Elevated Levels of Serum CA 19-9

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Summary

The tumour marker CA19-9 is a sensitive marker for pancreatic, gastric and hepatobiliary malignancies. High CA 19-9 level indicates unresectable lesions and a poor prognosis. The objective of the study was to determine the significance and implications of elevated CA 19-9 levels in the serum. A one-year retrospective review of all patients who had CA19-9 measured in our Medical Center was undertaken. 69 patients were found to have CA 19-9 level above the cut-off value (37 U/ml). Thirty-six patients had malignant and the remaining 33 had benign lesions. CA 19-9 was found to be elevated in malignancies of pancreas, colorectum, lung, liver and ovary. Benign conditions associated with elevation of CA 19-9 included disease of the hepatobiliary system, pneumonia, pleural effusion, renal failure and SLE. In two individuals, there was no obvious cause for the elevation of this marker. CA 19-9 levels were significantly lower in benign than in malignant conditions. In conclusion, elevated CA 19-9 may be found in patients with benign as well as malignant disease. Therefore, it is important (1) that elevated levels of CA 19-9 are interpreted in the light of the clinical presentation of the patient and (2) to be aware of the benign conditions that can be associated with increased levels of this marker. With these factors in mind, CA 19-9 can be used in assist in the diagnosis of pancreatic cancer and assessment of resection adequacy post-operatively.

Key Words: CA19-9, Benign conditions, Malignant conditions, Pancreatic carcinoma

Introduction

Carbohydrate antigen 19-9 (CA 19-9) was originally isolated from a human colorectal cancer cell line as a mucin like product[1]. The antigen is found in the normal epithelial cells of the gall bladder, biliary ducts, pancreas and stomach[2]. Multiple studies have shown that while elevations in serum CA 19-9 appear to be useful in the diagnosis of adenocarcinoma of the upper gastrointestinal tract and in monitoring of colonic carcinoma, its greatest sensitivity is in the detection of pancreatic adenocarcinoma[3]. Elevations in CA 19-9 level correlate with the degree of tumour differentiation as well as the extent of tumour mass[4]. In his studies, Steinberg found that CA 19-9 has over all specificity of 90% and sensitivity of 80% in detecting adenocarcinoma of the pancreas[5]. High CA 19-9 levels have been associated with unresectable lesions and a poor prognosis for patients presenting with pancreatic carcinoma[6].

Elevated CA 19-9 levels are not pathognomonic of cancer of the pancreas; it may be elevated in other malignancies as well as in benign conditions[7,8]. The objective of the study was to determine the significance and implications of elevated CA 19-9 levels in the serum.

Materials and Methods

A one-year (January 2001 to December 2001) retrospective review of all patients who had CA 19-9

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level measured was undertaken. The results were retrieved from the laboratory information system, Department of Pathology, University Malaya Medical Centre, Kuala Lumpur, and all patients who had CA 19-9 greater than the cut-off value 37U/ml were noted. The full clinical records of these patients were reviewed. Ca 19-9 was measured in the serum using a commercially available immunometric assay kit (Immulite, DPC). The upper limit of normal for CA 19-9 in our study was 37 U/ml.

Results

Of 650 patients, whose sera were analyzed for CA 19-9, 69 had their level above the cut-off value 37 U/ml. Thirty six patients (52.2%) had malignancy and the remaining 33 (47.8%) had benign disease. The mean and range, and the distribution pattern of CA 19-9 in the malignant and benign conditions are shown and in Tables I and II and Figures 1 and 2 respectively. The mean of CA 19-9 in the benign group was 83.81 U/ml whereas that in the malignant group was 1632.06 U/ml; there was a statistically significant difference between the two conditions (p value < 0.005).

The benign conditions in which CA 19-9 were found to be elevated were mostly diseases of the hepatobiliary system (16/33). Other benign conditions associated with elevation of CA 19-9 were pulmonary diseases (11/33), end stage renal failure (3/33) and polymyositis. In 2 patients, the cause of the raised CA 19-9 level was not clear; neither had evidence of any malignancy or other organic diseases. In benign conditions, the tumour marker was rarely elevated more than 200U/ml except in one patient with liver cirrhosis and another with cholecystitis. Overall, the level of this tumour marker was less than 100 U/ml in 69.7% of the benign conditions. Levels higher than this were observed only in patients with liver cirrhosis and cholecystitis.

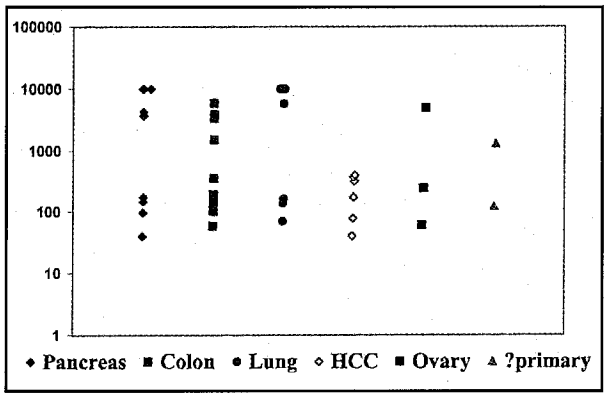
The malignant conditions with elevated CA 19-9 level were colorectal, pancreatic, hepatic, lung and ovarian carcinoma. Two patients with bony metastasis but unknown primary also had high CA 19-9 level in the serum. In the present study, this tumour marker was more than 500 U/ml in 50% of the patients with malignancies. Markedly raised levels of more than 10,000 U/ml were observed in patients with advanced stages of colorectal, pancreatic and lung carcinoma. It is notable that in a sizable proportion (22.2%) of the cases, the CA 19-9 levels were less than 100 U/ml. This included 2 patients with pancreatic cancer; both patients were post-operative cases who had testing done for assessment. With the exclusion of these 2 subjects, the majority of pancreatic cancer patients were found to have significantly elevated CA 19-9.

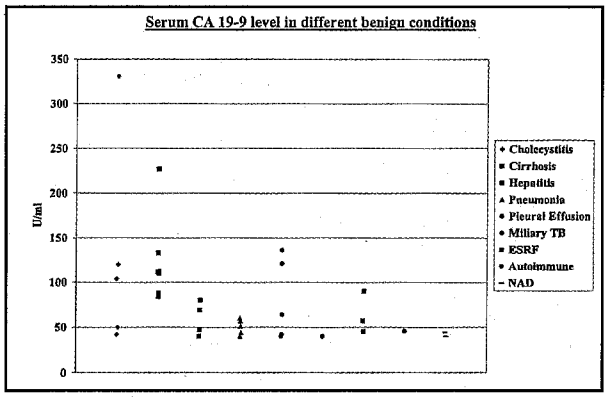
Table I: The mean and range of CA 19-9 in malignant and benign conditions

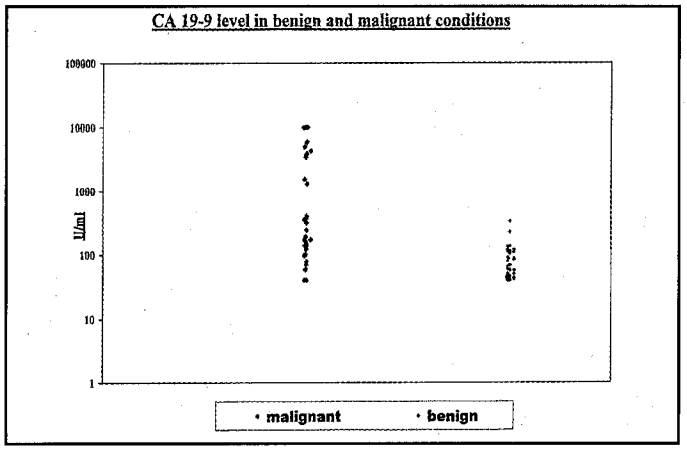
	No: of subjects	Mean	Range
Malignant Conditions			
Colorectal	10	1970.5	40 - 10000
Pancreatic	9	4274	40 - 10000
Hepatic	6	222.9	40 - 400
Lung	6	4361.7	70 - 10000
Ovary	3	1763	60 - 4986
Primary	2	709.5	122 - 1297
Benign Conditions			
Hepatitis	4	59	47 - 80
Cirrhosis	7	123.7	84 - 227
Cholecystitis	5	129.4	50 - 331
Pneumonia	5	49.6	40 - 60
Pleural effusion	5	80.6	40 - 136
Renal failure	3	64	45 - 90
Autoimmune	1	46	
Miliary Tuberculosis	1	42	
No abnormality	2	42	40 - 44

Table II: Distribution of CA19-9 assay values

	No: of subjects	37.0 -100	100.1-200	200.1-500	>500
Malignant Conditions					
Colorectal	10	1	2	1	6
Pancreatic	9	2	2	-	5
Lung	6	1	1	-	4
Hepatic	6	2	1	3	-
Ovary	3	1	1	-	1
Primary	2	1	-	-	1
Benign Conditions					
Hepatitis	4	4	-	-	-
Cirrhosis	7	2	4	1	-
Cholecystitis	5	2	2	1	-
Pneumonia	5	5	-	-	-
Pleural effusion	5	3	2	-	-
Renal failure	3	3	-	-	-
Autoimmune	1	1	-	-	-
Miliary Tuberculosis	1	1	-	-	-
No abnormality	2	2	-	-	-







Discussion

Since its discovery by Koprowski and coworkers, CA 19-9 antigen has been used widely as a tool for the investigation and management of patients with pancreatic carcinoma. CA 19-9 antigen in tissue exists primarily as an epitope present on a glycolipid, sialo-lacto-N fucopentose II ganglioside; in serum, the CA 19-9 antigen is associated with a mucin[9]. The oligosaccharide on which the CA 19-9 epitope was found is a sialylated Lewis A blood group antigen[10]. Patients who are genotypically Lewis a-b cannot synthesize the CA 19-9 antigen and thus it had been said that the maximum achievable sensitivity of this investigation in serum would be 95%[11].

The CA 19-9 is a tumour associated, but not a tumour specific antigen. It is synthesized by normal human pancreatic and biliary ductular cells, as well as by gastric, colonic, endometrial and salivary epithelia[12] and has been found in normal seminal fluid. This explains the elevated level of CA 19-9 in many malignancies.

Although CA 19-9 was found to be elevated in many different malignancies in our study, very high values were observed only in patients with advanced stages of colorectal carcinoma and in pancreatic adenocarcinoma. This finding is similar to what other workers had found in their studies[6, 13]. CA 19-9 level has been suggested as a prognostic indicator of patient survival[5]. However, we are unable to verify this in the present study, as there was no further follow up of the study subjects in most instances. We also observed markedly increased level of CA 19-9 in carcinoma of lung with metastasis. CA 19-9 had been found in epithelial tumours of the lung[14] explaining the presence of elevated levels of this antigen in lung cancer. High levels CA 19-9 were reported to be related to advanced stage adenocarcinoma of the lung[15]. Elevation of this tumour marker in hepatocellular carcinoma had been reported by other workers[16]; however, similar degrees of elevation had also been observed in patients with cirrhosis. Therefore, CA 19-9 is not considered an informative marker in the diagnosis of hepatocellular carcinoma[17]. This observation is also substantiated in our study.

In patients with primary epithelial ovarian carcinoma, CA 19-9 had been stated to have sensitivity of 55.9%[18]. However, unlike the carbohydrate antigen 125 (CA - 125), CA 19-9 shows no correlation with clinical stage[18]. In our series of patients, there was only one case of ovarian carcinoma; this patient had metastasis to the lung and a high serum CA 19-9. In the two cases of unknown primary cancer with secondaries in the bone, histopathological examination showed that the lesions were metastatic adenocarcinoma. Our observation support a previous report stating that increased levels of CA 19-9 is related to metastatic adenocarcinoma and to advanced stages of cancer of unknown primary [19].

CA 19-9 is elevated not only in hepatobiliary malignancies, but also in benign hepatobiliary disorders[20]. Of the 33 patients with benign diseases who had elevated level of CA 19-9, 16 of them had hepatobiliary disorders (48%). Cholestasis is believed to play an important role in causing raised CA 19-9 level in these patients[21]. Hence, caution is needed in interpreting elevated CA 19-9 in patients with jaundice. In fact, extraordinarily elevated CA 19-9 had been reported in patients with acute cholangitis causing diagnostic dilemma in those patients[22]. In all our cases of hepatitis, the tumour marker was less than 100 U/ml; however, we noted higher levels in patients with liver cirrhosis and cholecystitis with gallstones.

Other non-malignant conditions in which we found raised CA 19-9 included benign pulmonary diseases and end stage renal failure. In all but 2 of our cases, both of whom had benign pulmonary disease, CA 19-9 was elevated to less than 100U/ml. CA 19-9, had been shown to be expressed in mucous cells of the bronchial gland and surface of the bronchiolar surface epithelium cells in benign pulmonary disease by immunohistochemical staining[23]. This may explain the elevated level of CA 19-9 in pulmonary diseases. Increased evidence of malignancy had been reported in end stage renal failure patients. Tumours of kidney and corpus uteri are the most common forms of neoplasia seen in renal failure[24]. In our three cases of renal failure the tumour marker was above the cut-off value but less than 100 U/ml; none of them had any sign of malignancy. Tumour markers have been reported to be higher in uraemic patients compared to the normal controls[25], which may be related to the metabolic aberrations in this condition[26]. Therefore, care should also be exercised in interpreting the tumour marker level in renal failure patients.

The tumour marker was elevated in one patient with polymyositis; Shimomura et al had also reported the same findings[27]. CA 19-9 elevation may indicate severe disease or involvement of lungs due to the underlying disease process[27]. In two individuals in whom the tumour marker had been requested as a screening

procedure, CA 19-9 was found to be elevated, albeit only mildly. In these two individuals, there was no evidence of malignancy or any benign diseases. This emphasizes the fallacy of using tumour markers as a routine screen in patients in whom there are no indications.

Our data shows that CA 19-9 is elevated in both benign and malignant conditions, although the level in malignancy is significantly higher. The marker is useful as an adjunct in diagnosis of pancreatic carcinoma and in the assessment of surgical adequacy post-operatively. However, its interpretation must be made in conjunction with clinical findings and other ancillary investigations. It should not be used as a screening test for malignancies. Patients with a variety of benign conditions such as hepatobiliary diseases, pulmonary diseases and renal failure have levels above the cut-off value.

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ORIGINAL ARTICLE

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Determinants of the cytotoxicity of irinotecan in two human colorectal tumor cell lines

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Abstract *Purpose:* Irinotecan is a drug of the camptothecin family that has proven activity in advanced colon cancer, with about 20% responses in untreated as well as in 5-fluorouracil-resistant tumors. Irinotecan is considered as a prodrug which needs to be activated to SN-38 by carboxylesterases to become able to interact with its target, topoisomerase I. The work reported here intended to identify the determinants of the cytotoxicity of irinotecan in two human colorectal tumor cell lines, LoVo and HT-29, at the level of the target of the drug and at the level of the availability of the active metabolite to the target. *Results:* The cytotoxicity of irinotecan and SN-38 markedly differed in the two cell lines: irinotecan IC_{50} values were 15.8 μM for LoVo cells and 5.17 μM for HT-29 cells; SN-38 IC_{50} values were 8.25 nM for LoVo cells and 4.50 nM for HT-29 cells. Topoisomerase I expression (at the mRNA and the protein levels) and catalytic activity were similar in the two cell lines. Irinotecan induced similar amounts of cleavable complexes at its IC_{50} in both cell lines. SN-38 induced a concentration-dependent formation of cleavable complexes, which was not significantly different in the two cell lines. Expression of the carboxylesterase CES1 was higher in HT-29 than in LoVo cells. Expression of the carboxylesterase gene CES2 was comparable in the two cell lines and much higher than CES1 gene expression. Carboxylesterase activity was extremely low using *p*-nitrophenylacetate as a substrate (1.45 and 1.84 pmol/min per mg proteins) and could not even be detected using irinotecan as a substrate. Cell accumulation of irinotecan was markedly different, reaching consistently higher levels in HT-29 cells than in LoVo cells. *Conclusions:* Our results indicate that (1) the cytotoxicity of irinotecan was likely due to the drug itself and not to its metabolite SN-38, and (2) that irinotecan uptake was more predictive of its cytotoxicity than topoisomerase I availability and activity in these two cell lines.

Keywords Camptothecin · Irinotecan · Topoisomerase I · Drug activation · Colon cancer

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Introduction

In Western countries colorectal cancer represents 15% of all cancers and is the most frequently observed [2]. The majority of patients are metastatic at first presentation or later, and will require chemotherapy. Colon cancer chemotherapy has been based upon the use of 5-fluorouracil for 40 years. Recently, irinotecan (CPT-11), a derivative of camptothecin, has been shown to have efficacy against advanced colorectal cancers, both when used alone (20% responses in patients pretreated with 5-fluorouracil [6, 26]) or combined with 5-fluorouracil (near 50% responses [9]).

Irinotecan is a prodrug which needs to be transformed by carboxylesterases to its active metabolite SN-38 [19]. Camptothecin and its analogues are specific inhibitors of eukaryotic DNA topoisomerase I, a ubiquitous enzyme involved in DNA templating processes such as replication, transcription and repair [13]. These compounds can interfere with DNA-topoisomerase I cleavable complexes and stabilize them, leading to DNA damage by inducing single-strand breaks that are converted to double-strand breaks during S phase by collisions with replication forks [24].

Since the original work of Giovanella et al. [11], the level of topoisomerase I in human tumors has been considered as determining their sensitivity to camptothecin derivatives [16]. It has been shown that the amplification of the gene encoding topoisomerase I is able

to sensitize breast cancer cells to camptothecin [23]. Using another approach, Goldwasser et al. [12] have shown a relationship between the sensitivity of colon tumor cell lines and the amount of cleavable complexes formed in the presence of camptothecin. Studying colon cancer cell lines and xenografts, Jansen et al. [17] have also found topoisomerase I activity to be a determinant of irinotecan and SN-38 cytotoxicity. However, in lung cancer cells in culture, the same group have observed that the major determinant of irinotecan cytotoxicity is its conversion to SN-38 through carboxylesterase activity [29].

Concerning the involvement of carboxylesterases in irinotecan cytotoxicity, a recent study has shown that the transfection of a human liver carboxylesterase cDNA sensitizes by 17-fold the A549 human lung tumor cell line to irinotecan [21]. However, no evaluation of carboxylesterase activity has been systematically made in human tissues and tumors. It has not been established, therefore, whether the local generation of SN-38 by the tumor itself is of significance in determining irinotecan activity. Two distinct carboxylesterases have recently been characterized [15]. The first one, hCE1, isolated from macrophages, is relatively inefficient in activating irinotecan to SN-38 as compared to the second one, hCE2, isolated from the liver. The respective expression and activity of these enzymes have not yet been studied in tumors or tumor cell lines.

The importance of efflux pumps in determining drug resistance of tumor cells has been recognized for a long time. However, neither the *MDR*1 gene product, P-glycoprotein, nor the multidrug-resistance protein, MRP1, have been shown to be able to transport irinotecan or SN-38 out of tumor cells [18, 22]. Recently, it has been shown that irinotecan and SN-38, but not camptothecin itself, can be expelled by a new ABC pump, BCRP or ABCG2 [4, 30].

We studied the cellular determinants of irinotecan cytotoxicity in two colon tumor cell lines, LoVo and HT-29. We also studied the cellular determinants related to the availability of the active metabolite of irinotecan (irinotecan uptake and transformation into SN-38, carboxylesterase expression and activity, ABCG2 expression), and the cellular determinants related to the target of this drug (expression of topoisomerase I, catalytic activity, and amounts of cleavable complexes stabilized in the presence of irinotecan or SN38 in living cells). This in vitro study was undertaken as a prelude to an ex vivo study on tumor biopsies of colorectal cancers which is ongoing.

Materials and methods

Drugs and chemicals

Irinotecan and SN-38 were provided by Rhône-Poulenc Rorer (Antony, France). For growth inhibition and cell uptake studies, irinotecan was dissolved directly in culture medium from the stock solution at 20 mg/ml. SN-38 was first diluted in dimethylsulfoxide at 1 mg/ml, then in culture medium at appropriate concentrations.

Cell culture

The human colon tumor cell lines LoVo and HT-29 were obtained from the American Type Culture Collection (Rockville, Md.). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (10% for LoVo cells and 20% for HT-29 cells), an antibiotic cocktail (penicillin 1000 U/l, streptomycin 100 µg/ml) and 2 m*M* glutamine, and were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. The cell culture media and supplements originated from Seromed (Berlin, Germany).

Growth inhibition assays

Exponentially growing cells were seeded in 20 cm^2 Petri dishes with an optimal cell number for each cell line (20,000 for LoVo cells, 100,000 for HT-29 cells). They were treated 2 days later with increasing concentrations of irinotecan or SN-38 for one cell doubling time (24 h for LoVo cells, 40 h for HT-29 cells). After washing with 0.15 *M* NaCl, the cells were further grown for two doubling times in normal medium, detached from the support with trypsin-EDTA and counted in a hemocytometer (Coulter Counter ZX, Coultronics). The IC_{50} values were then estimated as the drug concentrations responsible for 50% growth inhibition as compared with cells incubated without drug.

Evaluation of cellular accumulation of irinotecan

Evaluation of the concentrations of irinotecan in cells after exposure to various extracellular concentrations (1, 5, 10, 25 µ*M*) was performed by HPLC as previously described [25]. The drug was added to the complete culture medium 16 h before incubation with cells in order to reach the lactone-carboxylate equilibrium. After a 4-h incubation, the cells layers were rinsed and the cells were recovered by scraping and pelleted. Cell extracts were obtained in methanol/acetonitrile (50/50 v/v) containing 1% HCl. Separation was carried out on a C-18 reversed-phase column (Nova-Pak, Radial Pak, Waters, Saint-Quentin-en-Yvelines, France) with a mobile phase consisting of a mixture of acetonitrile and 0.075 *M* ammonium acetate buffer, pH 6.0, containing 5 m*M* tetrabutyl ammonium phosphate (Pic-A, Waters). This mobile phase was delivered isocratically at a flow rate of 1.5 ml/min with a Spectra Systems P4000 XR pump (Thermo Quest, Les Ulis, France). Fluorometric detection was carried out with excitation and emission wavelengths set at 355 and 515 nm respectively, using the Spectra Systems FL 3000 (Thermo Quest) detector. Peaks were quantified by reference to a standard calibration curve obtained by spiking known amounts of drugs in untreated cell extracts, using PC1000 software (Thermo Quest).

Carboxylesterase activity

Carboxylesterase activity was evaluated in microsomal extracts of each cell line using two different substrates of the enzyme, irinotecan and *p*-nitrophenylacetate (PNPA), a generic substrate for carboxylesterases. Determination of carboxylesterase activity was performed according to a technique originally developed in our laboratory [14]. Human liver microsomes were used as a positive control of carboxylesterase activity. These microsomes were kindly provided by Dr C. Riché (University of Brest).

The cells (50×10^6) were mechanically detached from Petri dishes with 0.15 *M* NaCl and centrifuged at 250 *g* for 10 min. Further steps were performed on ice. Cell pellets were lysed by sonication three times for 10 s, and maintained on ice for 10 min. The cell suspensions were centrifuged two times at 7500 *g* for 30 min to eliminate nuclei and mitochondrial fractions, and the

supernatant was centrifuged at 100,000 *g* for 2 h to pellet the microsomal fraction. Microsomes were suspended in 40 m*M* Tris-HCl, pH 6.9, and kept at −80 °C until use. The amounts of protein were determined using the method of Bradford [3]. The purity of the microsomal fraction was checked by the relative enrichment in specific enzymatic activities: succinate dehydrogenase for mitochondria, sulfatase C for microsomes, phosphoglucomutase for cytosol. Standard enzymatic techniques were used for this control [1, 5, 7].

Microsomes (1 mg/ml) were incubated for 30 min with 5 µ*M* irinotecan lactone (diluted in 0.01 *M* citric acid) in 0.1 *M* Tris-HCl, pH 6.9, at 37 °C (final volume 40 µl). At the end of the reaction, 50 µl of a mixture of acetonitrile and methanol (1:1 v/v) were added to the tubes together with 10 µl 2.5 *N* HCl and 50 ng internal standard (camptothecan). After centrifugation at 10,000 *g* for 2 min to precipitate the proteins, the amount of SN-38 was determined by HPLC in 100 µl of the supernatant. A special technique allowing improved sensitivity for SN-38 quantification was used [10]. It differed from our standard method by replacing the mobile phase with a mixture of 0.1 *M* potassium buffer (pH 6.8) and acetonitrile (2:1 v/v), running isocratically at 1 ml/min, and excitation and emission wavelengths set at 228 and 540 nm, respectively. The limit of quantification was 0.005 µ*M* of SN-38 (limit of detection 0.2 ng).

For the determination of PNP formation, microsomes were first activated at 4 °C for 10 min with Triton X-100 (0.5% v/v). Incubations were performed with 100 µg microsomal proteins and 50 µ*M* PNPA in 1 ml 40 m*M* Tris-HCl, pH 6.9, at 37 °C. The kinetics of PNP formation were monitored spectrophotometrically at 405 nm for 4 min. A control without microsomal extract was used to subtract the spontaneous hydrolysis of PNPA. The results were analyzed and are expressed as the quantity of PNP formed per minute per milligram microsomal protein.

Western blotting of topoisomerase I

Exponentially growing cells (about 30×10^6 cells) were mechanically detached and centrifuged at 250 *g* for 5 min at 4 °C. All the following procedures were performed at 4 °C. Cell pellets were homogenized in a Potter homogenizer before centrifugation at 250 *g* for 5 min. The pellets were resuspended in 1 ml hypertonic solution (1 *M* NaCl) and centrifuged for 30 min at 18,000 *g* to eliminate DNA. Aliquots of the nuclear extracts of LoVo and HT-29 cells containing 100 µg proteins were loaded onto an 8% polyacrylamide gel and allowed to migrate at 40 V for 2.5 h at 4 °C in electrophoresis buffer. The proteins were then transferred to Immobilon P membrane (Millipore, Saint-Quentin-en-Yvelines, France) using an electroblotting system (Milliblot, Millipore) at 2.5 mA/cm^2 for 1 h.

The membranes were incubated at room temperature for 2 h with rabbit anti-human topoisomerase I antibody (TopoGen, Columbus, Ohio) diluted 1:2000, then for 1 h with horseradish peroxidase-labeled donkey anti-rabbit immunoglobulin as secondary antibody (Amersham-Pharmacia, Orsay, France) diluted 1:4000. Protein was detected using the peroxidase substrate Lumigen PS-3 acridan (ECL+, Amersham-Pharmacia), and visualized by autoradiography.

The chemoluminescence signals on the autoradiograms were analyzed using a video camera (Kodak DC120 Zoom Digital Camera, Rochester, N.Y.) coupled to a microcomputer, using the Kodak Digital Science 1D image analysis software. Signal intensities were recorded and expressed in arbitrary units.

Topoisomerase I catalytic activity

The catalytic activity of topoisomerase I was evaluated in 0.35 *M* nuclear extracts using its ability to relax supercoiled DNA. The nuclear extracts were prepared as described above. The substrate of the reaction was the pBSKS$^+$ phagemid (Clontech, Palo Alto, Calif.)

Serial dilutions of LoVo and HT-29 nuclear extracts were prepared so as to contain between 5 and 100 ng proteins, and incubated at 37 °C for 30 min with 1 µg pBSKS$^+$ in 20 µl reaction buffer. Samples containing 20 ng DNA were loaded onto a 1% agarose gel containing ethidium bromide (0.2 µl/ml) and submitted to electrophoresis at 80 V for 1 h. Electrophoresis allowed the separation of supercoiled DNA (substrate) and relaxed DNA (product of the reaction). A sample treated under the same conditions but without nuclear extract was used as a control.

Spot intensities were quantified by densitometry using the same camera and software as for Western blot analysis. The catalytic activity of topoisomerase I was evaluated as the amount in nanograms of DNA relaxed per nanogram protein in the nuclear extract.

Evaluation of cleavable complexes

DNA-topoisomerase I complexes were evaluated after 30-min incubations of the cells with irinotecan or SN-38 by a slot-blot method adapted from that of Subramanian et al. [27] using the Topo I Link Kit (TopoGen).

The cells ($1–10\times10^6$) were incubated for 30 min at 37 °C with different irinotecan or SN-38 concentrations, chosen as multiples of the IC_{50} values of the drugs. After removing the medium, the cells were lysed with 1 ml lysis buffer. Cell lysates were loaded at the top of a cesium chloride gradient, and centrifuged at 100,000 *g* for 16 h at 20 °C. Fractions of 200 µl were removed from the top of the gradient, and an aliquot of each fraction (10 µl) was diluted and loaded onto a 1% agarose gel containing ethidium bromide. In parallel, another aliquot of each fraction (50 µl) was diluted with an equal volume of 25 m*M* sodium phosphate buffer and loaded onto a nitrocellulose membrane (Schleicher and Schuell, Ecquevilly, France) using a slot-blot device.

Topoisomerase I was revealed in the slots with the immunoblotting technique used for the Western blots. A signal was seen in two different groups of slots, those not containing DNA (free topoisomerase I, top of the gradient) and those containing DNA (topoisomerase I-DNA complexes, bottom of the gradient). Signal intensities in the DNA-containing slots were normalized before comparing irinotecan or SN-38-treated and untreated cells. The results are expressed as the relative increase in topoisomerase I-DNA complexes, i.e. the amount of topoisomerase I-DNA complexes in treated cells as compared to that in untreated cells.

Evaluation of gene expression of topoisomerase I, carboxylesterases, and ABCG2

About 10×10^6 cells in exponential growth were recovered by scraping in 0.5 ml RNA extraction buffer and homogenized in a Dounce homogenizer. The homogenate was laid over a cesium chloride cushion and centrifuged at 150,000 *g* for 16 h at 20 °C. Pellets were then suspended in a buffer, extracted with 2.5 ml chloroform/butanol (4:1 v/v) and centrifuged for 10 min at 5000 *g*. The aqueous phase, containing RNAs, was submitted to a second extraction and purification cycle. RNAs were then pelleted with 3 *M* sodium acetate, pH 5.0, and absolute ethanol at a volume ratio of 0.1:2.

Reverse transcription was performed on 400 ng RNA in 20 µl 10 m*M* Tris-HCl buffer, pH 8.3, containing 50 m*M* KCl and 5.5 m*M* $MgCl_2$, with 0.5 m*M* dNTP mixture, 8 U RNase inhibitor, 2.5 µ*M* random hexamers and 25 U reverse transcriptase from Mule. The reaction was performed for 10 min at 25 °C followed by 30 min at 48 °C.

Real-time PCR was performed on 1- or 5-µl samples of cDNAs in 50 µl PCR buffer (TaqMan Universal Master Mix; PE Biosystems, Courtaboeuf, France) with 50, 300 or 900 n*M* oligonucleotide primers and 80 n*M* specific fluorogenic probe (all reagents from PE Biosystems). After a hot start (10 min at 95 °C), the denaturation steps were 15 s at 95 °C and the hybridization steps were 1 min at 60 °C in a thermocycler (GeneAmp 5700 Sequence

Detection System, PE Biosystems). A total of 40 cycles were performed.

The following primers and fluorogenic probes were used:

- For topoisomerase I (68 bp): *sense* 5′ tga cag ccc cgg atg aga 3′, *antisense* 5′ tgc aac agc tcg att ggc 3′, *fluorogenic probe* 5′ cat ccc agc aag atc ctt tct tat aac cgt 3′.
- For CES1 (66 bp): *sense* 5′ tgt ttt gtc tcc att ggc ca 3′, *antisense* 5′ gtg agg gcc acg cca ct 3′, *fluorogenic probe* 5′ acc tct tcc acc ggg cca ttt ctg 3′.
- For CES2 (65 bp): *sense* 5′ gtc cgc tgc gat ttg ca 3′, *antisense* 5′ ggt tcc atc cct cac acc ac 3′, *fluorogenic probe* 5′ ccc ctg agc ccc ctg aat ctt gg 3′.
- For ABCG2 (71 bp): *sense* 5′ tgc aac atg tac tgg cga aga T 3′, *antisense* 5′ tct tcc aca agc ccc agg 3′, *fluorogenic probe* 5′ ttg gta aag cag ggc atc gat ctc tca 3′.
- For GAPDH oligonucleotides and fluorogenic probe were purchased from PE Biosystems.

The fluorescence generated at each amplification cycle by liberation of the fluorochrome from the fluorogenic probe was detected by a computer-controlled cooled CCD camera (PE Biosystems). Values were then collected to determine the threshold cycle (Ct) of each reaction. GAPDH expression was used as a reference. The Ct of GAPDH was subtracted from the Ct of the gene of interest to determine a value called ΔCt. The lowest values of ΔCt correspond to the highest levels of expression of the gene of interest.

Results

Growth inhibition by irinotecan and SN-38

The IC_{50} values (mean ± SD) of irinotecan were 15.8 ± 5.1 and 5.17 ± 1.4 μ*M* for LoVo and HT-29, respectively. For SN-38, the values were 8.25 ± 1.1 and 4.50 ± 1.50 n*M* for LoVo and HT-29, respectively. This two- to threefold difference in sensitivity to both drugs prompted us to seek to identify the factors responsible.

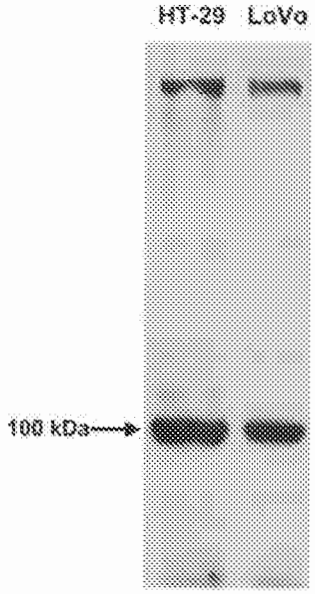

Fig. 1 Western blotting of topoisomerase I in nuclear extracts from LoVo and HT-29 cells

Evaluation of topoisomerase I expression by Western blotting and RT-PCR

Figure 1 presents the immunoblots of topoisomerase I present in the nuclear extracts from the two cell lines. Three independent nuclear extracts were analyzed. When compared by densitometry, the autoradiography signals provided by 50 μg of nuclear extracts proteins were similar, with a nonsignificant 20% higher intensity in HT-29 cells than in LoVo cells. When analyzed by real-time RT-PCR, the levels of topoisomerase I expression (average of three independent RNA preparations) were 30% higher in HT-29 cells than in LoVo cells (Table 1) but this difference did not reach significance.

Topoisomerase I catalytic activity

We performed the relaxation assays by incubating a constant amount of DNA substrate (1 μg) with increasing amounts of nuclear extracts (0 to 100 ng proteins) at 37 °C for 30 min. Three independent nuclear protein preparations were analyzed in duplicate (Fig. 2).

Table 1 Levels of gene expression of topoisomerase I, BCRP and carboxylesterases 1 and 2 as evaluated by real-time RT-PCR in cell RNA extracts. Three independent RNA extractions were performed and two PCRs were performed. The values presented are ΔCt (means ± SD) calculated as Ct(gene of interest)–Ct(GAPDH)

Gene	LoVo cells	HT-29 cells
Topoisomerase I	6.66 ± 0.71	4.48 ± 0.93
BCRP	11.0 ± 0.9	10.5 ± 1.5
CES1	16.8 ± 1.5	9.4 ± 0.8*
CES2	4.61 ± 0.79	5.53 ± 2.81

*$P < 0.01$

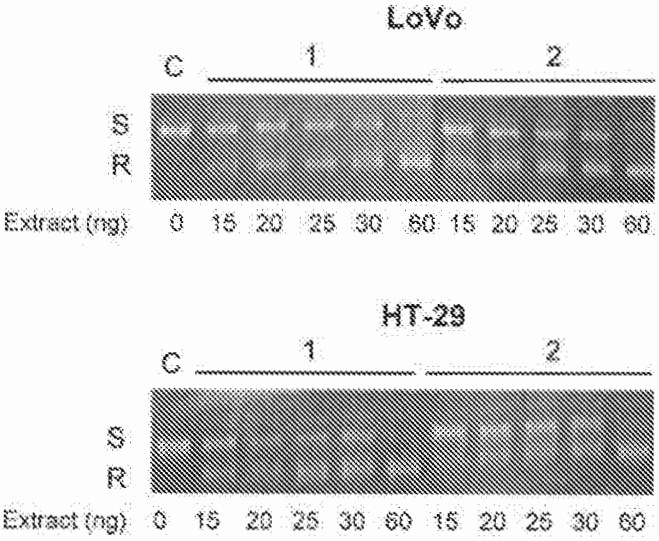

Fig. 2 Relaxation assays of pBSKS$^+$ DNA by LoVo and HT29 nuclear extracts (*C* control, *S* supercoiled DNA, *R* relaxed DNA). The amount of nuclear proteins was increased from 15 to 60 ng. For each cell line, two independent experiments are presented

Table 2 Formation of cleavable complexes in LoVo and HT-29 cells after 30-min incubations with irinotecan or SN-38. The concentrations are relative to the IC_{50} values of the drugs for each cell line. The values presented are the increases in topoisomerase I-DNA complexes relative to untreated cells, and are the means ± SD of three independent experiments (*ND* not determined)

Drug concentration	LoVo cells		HT-29 cells	
	Irinotecan	SN-38	Irinotecan	SN-38
$0.6\times IC_{50}$	ND	0.84 ± 0.22	ND	1.49 ± 0.44
$1\times IC_{50}$	1.32 ± 0.45	2.04 ± 1.41	1.53 ± 0.18	2.79 ± 1.67
$10\times IC_{50}$	ND	4.16 ± 1.04	ND	3.64 ± 1.18
$100\times IC_{50}$	ND	8.92 ± 2.00	ND	10.3 ± 1.7

Topoisomerase I catalytic activities were the same in the two cell lines: 12.7 ± 0.3 ng DNA relaxed per ng nuclear proteins in LoVo cells and 12.9 ± 0.2 in HT-29 cells (mean ± SD).

Evaluation of cleavable complexes

Table 2 presents the formation of cleavable complexes in LoVo and HT-29 cells. Irinotecan induced similar amounts of cleavable complexes at its IC_{50} value in both cell lines. SN-38 was studied over a wider range of concentrations, up to 100-fold the IC_{50} value, which was not possible for irinotecan because of the very high IC_{50} values (5–15 μ*M*). SN-38 induced a concentration-dependent formation of cleavable complexes, which was somewhat higher in the HT-29 cell line, but the difference observed did not reach significance.

Cellular accumulation of irinotecan

Cellular accumulation of irinotecan was linearly related to the dose in the LoVo and HT-29 cell lines up to 100 μ*M*. The accumulation of irinotecan was consistently twofold higher in HT-29 cells than in LoVo cells, but the kinetics of incorporation revealed a faster initial uptake in LoVo cells than in HT-29 cells (Fig. 3).

Carboxylesterase expression and activity

The expression of the CES1 gene was higher in HT-29 cells than in LoVo cells (Table 1), while the expression of the CES2 gene was quite similar in both cell lines and always much higher than the expression of the CES1 gene.

The enzymatic hydrolysis of PNPA was not significantly different in the two cell lines: 1.45 ± 0.31 and 1.84 ± 0.33 pmol/min per mg proteins in LoVo and HT-29 cells, respectively (mean ± SD). However, it was never possible to detect any formation of SN-38 with microsomal extracts of either cell line. Considering the limit of detection of SN-38 by the method used (0.2 ng), we can assume that the carboxylesterase activity transforming irinotecan was < 20 fmol/min per mg protein. In contrast, the activity in human liver microsomes was about 40 μmol/min per mg protein with PNPA and 4 pmol/min per mg protein with irinotecan as substrates.

ABCG2 expression

The expression of the ABCG2 protein was evaluated, after reverse transcription by real-time quantitative PCR using GAPDH as an internal standard of the reaction. No difference could be detected in the expression of this pump between the two cell lines (Table 1).

Discussion

LoVo cells were threefold less sensitive to irinotecan and twofold less sensitive to SN-38 than HT-29 cells. This difference can be seen as relatively minor but was quite significant and reproducible, and all the parameters studied were quantified with a reproducibility below this ratio. In the clinical setting, a two- to threefold

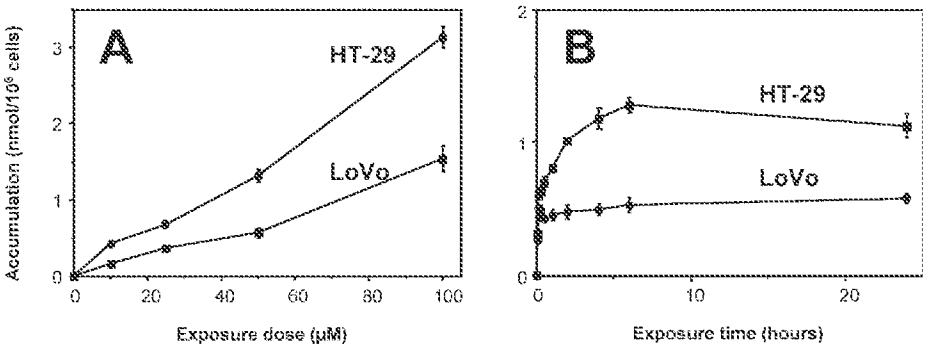

Fig. 3A, B Intracellular accumulation of irinotecan in LoVo and HT-29 cells as a function (**A**) of the external concentration of drug for an exposure time of 4 h, and (**B**) of the exposure time to the drug at a concentration of 50 μ*M*. Three independent incubations were performed and samples were analyzed twice. The *bars* represent the standard deviation of the mean values

difference in the dose administered is much larger than the difference between sensitivity and resistance to any anticancer drug, which justifies our detailed approach of the determinants of drug sensitivity. Irinotecan was about 1000-fold less cytotoxic than SN-38 in both cell lines, as already observed in vitro in cell cultures [19]. It was first remarkable that no SN-38 formation could be detected, either after incubation of the living cells with irinotecan or during the determination of carboxylesterase activity in microsomal extracts, using irinotecan as substrate. Since the limit of detection of our HPLC system was in that case especially enhanced to reach 0.2 ng SN-38, it can be concluded that the intracellular level of SN-38 formed from irinotecan during the evaluation of cytotoxicity was below the concentrations required for SN-38-induced growth inhibition [20]. Therefore, the cytotoxicity observed during irinotecan-induced growth inhibition can be attributed to irinotecan itself and not to its activated metabolic product, SN-38.

It has been shown in vitro that irinotecan can interact with topoisomerase I, leading to the same damage as SN-38, but with 1000-fold higher concentrations [8]. This means that contamination of an irinotecan preparation by SN-38 at a level as low as 0.1% could lead to the same result, which has sometimes led to the conclusion that irinotecan is totally devoid of activity. Indeed, it appears from our work that this is not true and that irinotecan can lead to cell growth inhibition in the absence of transformation to SN-38. Proof of this assertion would be given by measuring irinotecan cytotoxicity after switching off carboxylesterase activity. This is presently under study in our laboratory.

We observed that the expression of CES2 was similar in the two cell lines. This enzyme has been shown to be 50-fold more efficient in activating irinotecan than CES1 [15]. In addition, its expression in our cell lines was considerably higher than that of CES1, taking into account that the Ct scales are logarithmic. The higher expression of CES1 in HT-29 cells cannot, therefore, be considered responsible for the higher irinotecan activation, if any activation occurs in our cell lines.

No difference in topoisomerase I expression, both at the mRNA and the protein levels, could be detected between the two cell lines. As mentioned earlier, the level of topoisomerase I in a given tumor type has been related to sensitivity to topoisomerase I poisons [16, 23]. We can exclude in this study the contribution of target availability in the relative sensitivity of cell lines to irinotecan.

Cleavable complex formation was studied in the two cell lines after exposure to equitoxic concentrations of irinotecan and SN-38. This was explored in order to identify the responsibility of events occurring downstream of the drug-target interaction in mediating cytotoxicity. If the ultimate determinant of growth inhibition is the generation of cleavable complexes, then similar amounts of complexes should be obtained for similarly cytotoxic exposures. This was shown to be the case with SN-38, which induced nonsignificant differences in cleavable complex formation in the two cell lines over a wide range of concentrations. This was also the case for irinotecan after exposure at the IC_{50} value, but because of the very high IC_{50} values of irinotecan (5–15 μM), it was not possible to explore the effects of this drug at concentrations as high as could be done with SN-38. This shows that cleavable complex formation is the ultimate determinant of cytotoxicity, and that the two cell lines did not differ in their ability to undergo growth arrest upon drug-target interaction. The different p53 statuses of the two cell lines (wild-type in LoVo cells, mutated in HT-29 cells [28]) tends to indicate that this difference plays no role in the cytotoxicity of irinotecan or SN-38 in our cell lines.

The only difference we could identify between the cell lines which could account for the difference in sensitivity to irinotecan was the cellular accumulation of this drug during incubation. A twofold higher uptake of irinotecan was evident in HT-29 cells than in LoVo cells. This prompted us to study the expression of the only ABC protein that has been shown to transport drugs of the camptothecin family, the ABCG2 pump. However, a quantitative method (real-time PCR) failed to show any difference in ABCG2 expression between the two cell lines. This does not exclude the participation of other pumping systems in the regulation of intracellular accumulation of irinotecan and SN-38 in these tumor cell lines. This is presently under investigation in our laboratory. In addition, other unexplored factors, in combination with the difference in drug accumulation, may contribute to the difference observed in cytotoxicities.

Several factors studied are potential determinants of irinotecan activity and can also be studied in the clinical setting, using tumor biopsies or surgical samples. Topoisomerase I content and activity and carboxylesterase content and activity can easily be quantified in such specimens. However, cleavable complex formation or tumor drug uptake and accumulation cannot easily be determined, since this would require specific additional tumor samplings which might not be able to be carried out for ethical reasons.

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MERRIMACK PHARMACEUTICALS

MM-398/PEP02, a novel liposomal formulation of irinotecan demonstrates stromal-modifying anti-cancer properties

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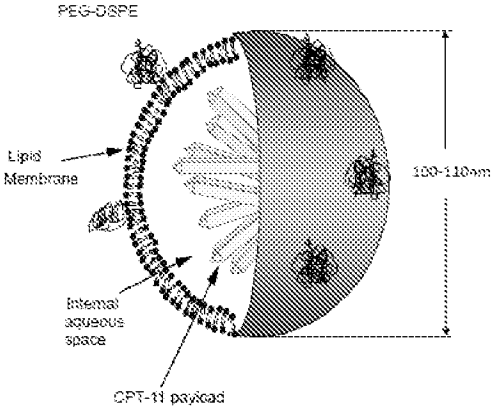
ABSTRACT

MM-398 is a stable nanoliposomal encapsulation of the pro-drug irinotecan (CPT-11) with longer plasma half-life and higher tumor deposition due to an enhanced permeability and retention effect. Pancreatic cancer has responded poorly to many therapeutics, largely because of inadequate drug penetration due to poor vascularization and the highly aggressive, hypoxic nature of the disease. We sought to better understand how MM-398, a relatively large (100nm) liposomal nanotherapeutic, could be used treat pancreatic cancer. We have tested MM-398 in several pancreatic xenograft models: BxPC3 (KRAS wild type), AsPC-1(KRAS ^{G12D}) , Panc-1 (KRAS ^{G12D}) and MiaPaCa (KRAS ^{G12C}). All models demonstrated complete tumor regression at 20 mg/kg or a human equivalent dose of 60-120 mg/m^2. At this same dose, MM-398 suppresses tumor growth in a gemcitabine insensitive AsPC-1 xenograft. MM-398 functionally blocked AsPC-1 tumor cell proliferation as measured by ki-67 staining; however, gemcitabine administered at its maximum tolerated dose did not impact proliferation. MM-398 is currently in multiple clinical trials, including a Phase 3 trial for patients with advanced gemcitabine-resistant pancreatic cancer (NAPOLI-1).

In order to further understand mechanisms driving response to MM-398, we screened and ranked several cell lines for their ability to convert irinotecan into the active metabolite, SN38. BxPC3 and HT-29 tumors ranked highest in ability to convert irinotecan to SN-38, as measured by HPLC. In a BxPC3 pancreatic orthotopic model which spontaneously metastasizes, 10 mg/kg MM-398 significantly reduced both primary and metastatic tumor load as measured by *ex vivo* biophotonic imaging of BxPC3luc cells to spleen, lung, liver, diaphragm and GI associated lymph nodes ($p<0.001$). This suggests that, in tumors with high conversion ability, MM-398 can block metastatic spread. To investigate dose-dependent effects, we explored pharmacodynamics changes and activity in the highly hypoxic HT29 model in response to five dose levels of MM-398. MM-398 reduced tumor growth in a dose-dependent manner, maximally to below starting levels.

Immunohistochemical evaluation of the levels of carbonic anhydrase IX (CAIX), a marker of HIF-1 alpha response to hypoxia demonstrated a direct correlation between decrease of tumor cell CAIX expression and tumor response to MM-398. This suggests that MM-398 is able to modulate hypoxia, which may enhance MM-398 efficacy. In this same model we have also determined that a single dose of MM-398 increases the number of blood vessels with open lumens, indicating that MM-398 acts as a stromal and vascular modifying agent.

In summary, MM-398 induces tumor regression in multiple mouse models of pancreatic cancer, including an orthotopic metastatic model. MM-398 activity may be driven in part by the ability to modify tumor microenvironment parameters, such as hypoxia and vascularization, both of which limit efficacy of chemotherapeutic agents in the treatment of pancreatic cancer. These data support the continued investigation of MM-398 in pancreatic cancer.



Systems Pharmacology of MM-398

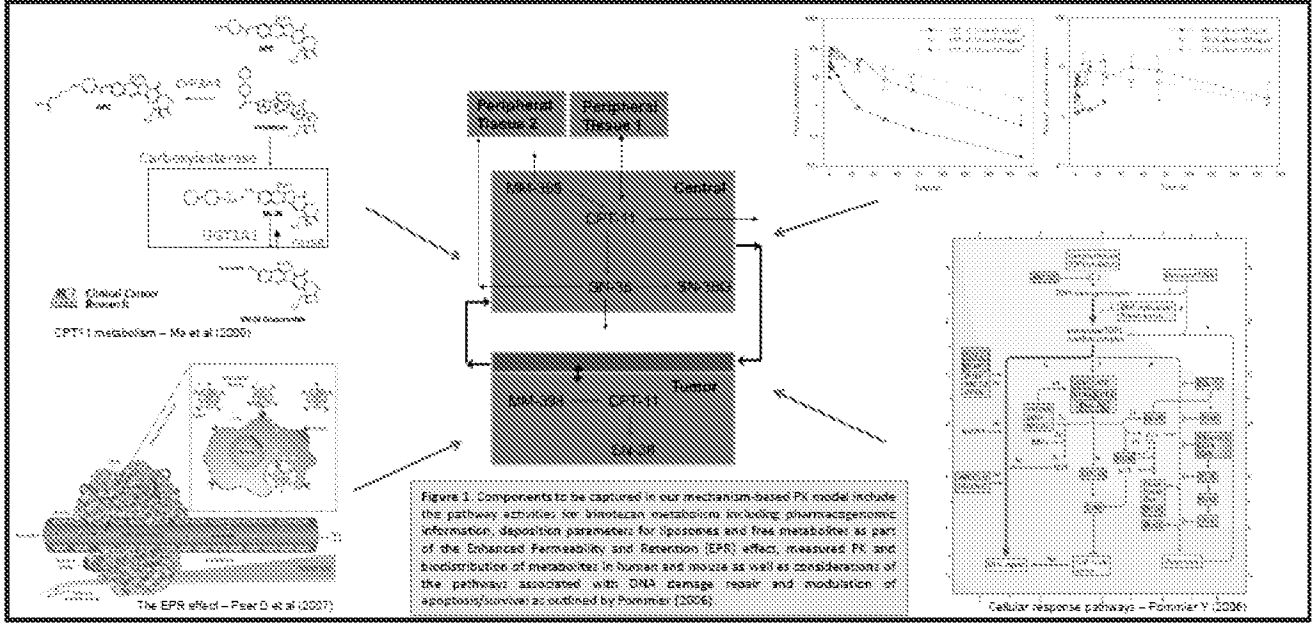
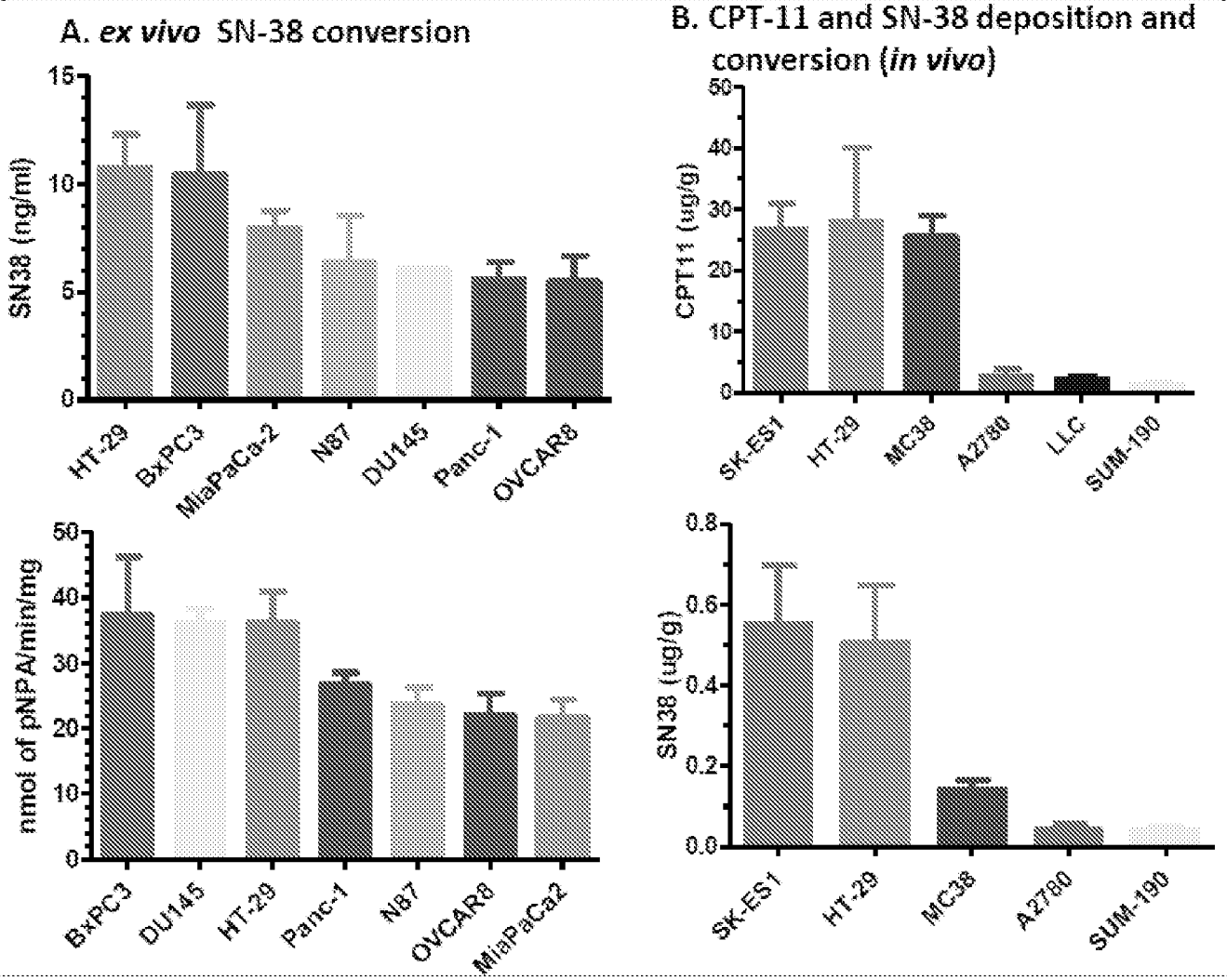
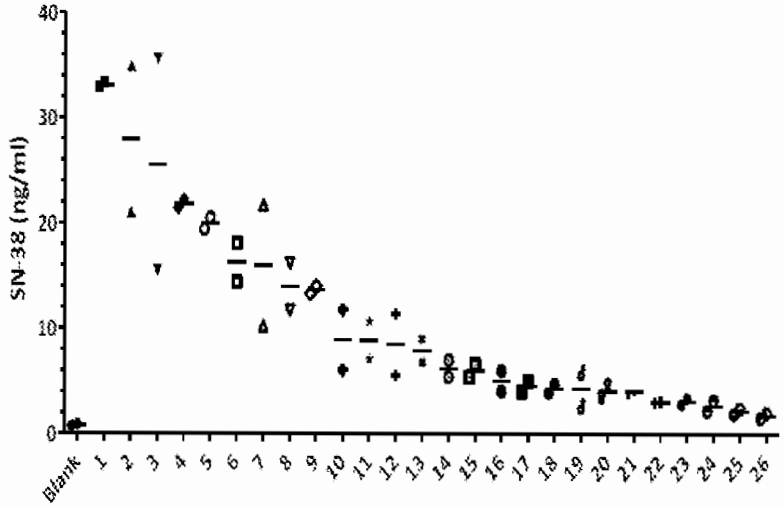


Figure 1. Components to be captured in our mechanism-based PK model include the pathway activities for irinotecan metabolism including pharmacogenomic information, deposition parameters for liposomes and free metabolites as part of the Enhanced Permeability and Retention (EPR) effect, measured PK and biodistribution of metabolites in human and mouse as well as considerations of the pathways associated with DNA damage repair and modulation of apoptosis/survival as outlined by Pommier (2006)

Pro-drug conversion activity across multiple models



C. SN-38 conversion in Pancreatic xenografts



D. Activity in the SK-ES1 xenograft

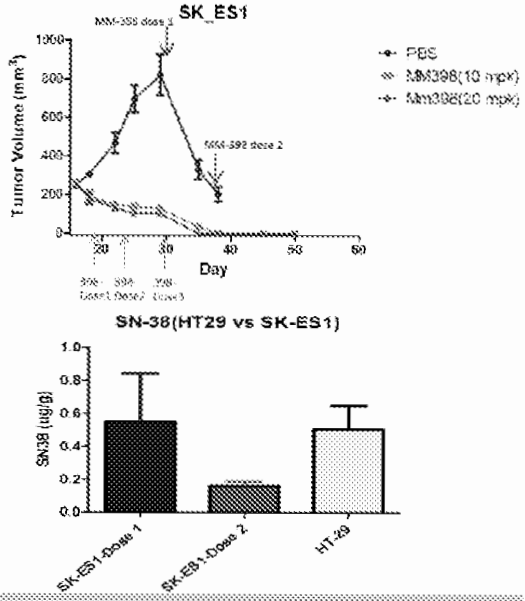
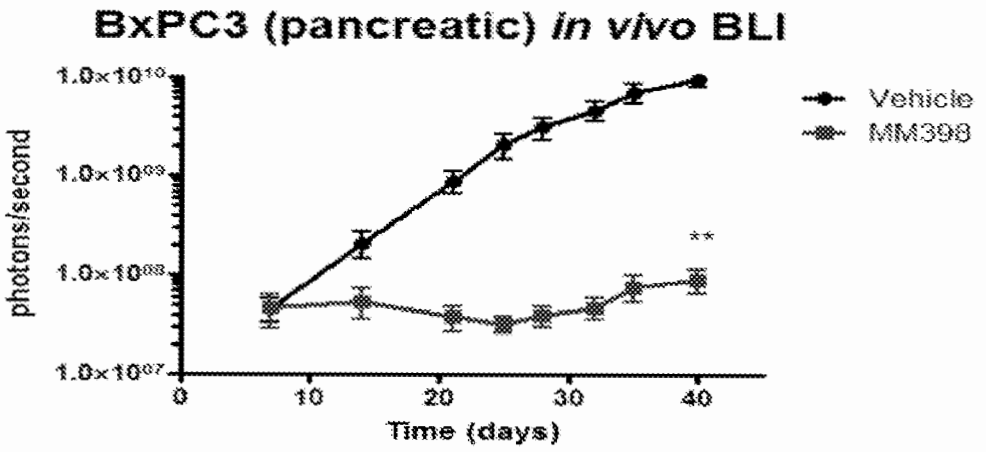


Figure 2 - Pro-drug activity ranges across multiple xenograft tumors that express Carboxylesterases: A. (Top) *ex vivo* tumor CPT-11 to SN38 conversion in the presence of exogenous CPT-11 in lysates from control untreated tumors. The values represent the conversion activity in xenograft models in which MM398 has previously demonstrated robust ativity. (Bottom) *ex vivo* tumor hydrolysis of p-nitrophenyl acetate in lysates from control untreated tumors. B. (Top) *In vivo* intratumoral deposition and conversion of CPT-11 and (Bottom) SN-38 in xenografts from mice injected with 20 mg/kg MM-398. Tumors were excised, digested and measured for CPT-11 and SN-38 levels by HPLC as compared to a standard curve. HT-29 demonstrates the highest SN-38 intratumoral levels. **C.** Results from a Conversion Activity screen in various pancreatic models. This is part of a larger multi indication screen of 100 xenograft models. D. (Top) Activity study in an SK-ES1 xenograft dosed q7d, i.v. at concentrations: 10 and 20 mpk. (Bottom) *In vivo* intratumoral deposition and conversion of SN-38 in SK-ES1 xenografts. As a reference in vivo intratumoral levels from an HT-29 xenograft post dose 1 is shown.

In Vivo Activity in High CES activity animal models

A. Activity in a pancreatic orthotopic model (spontaneous metastases)



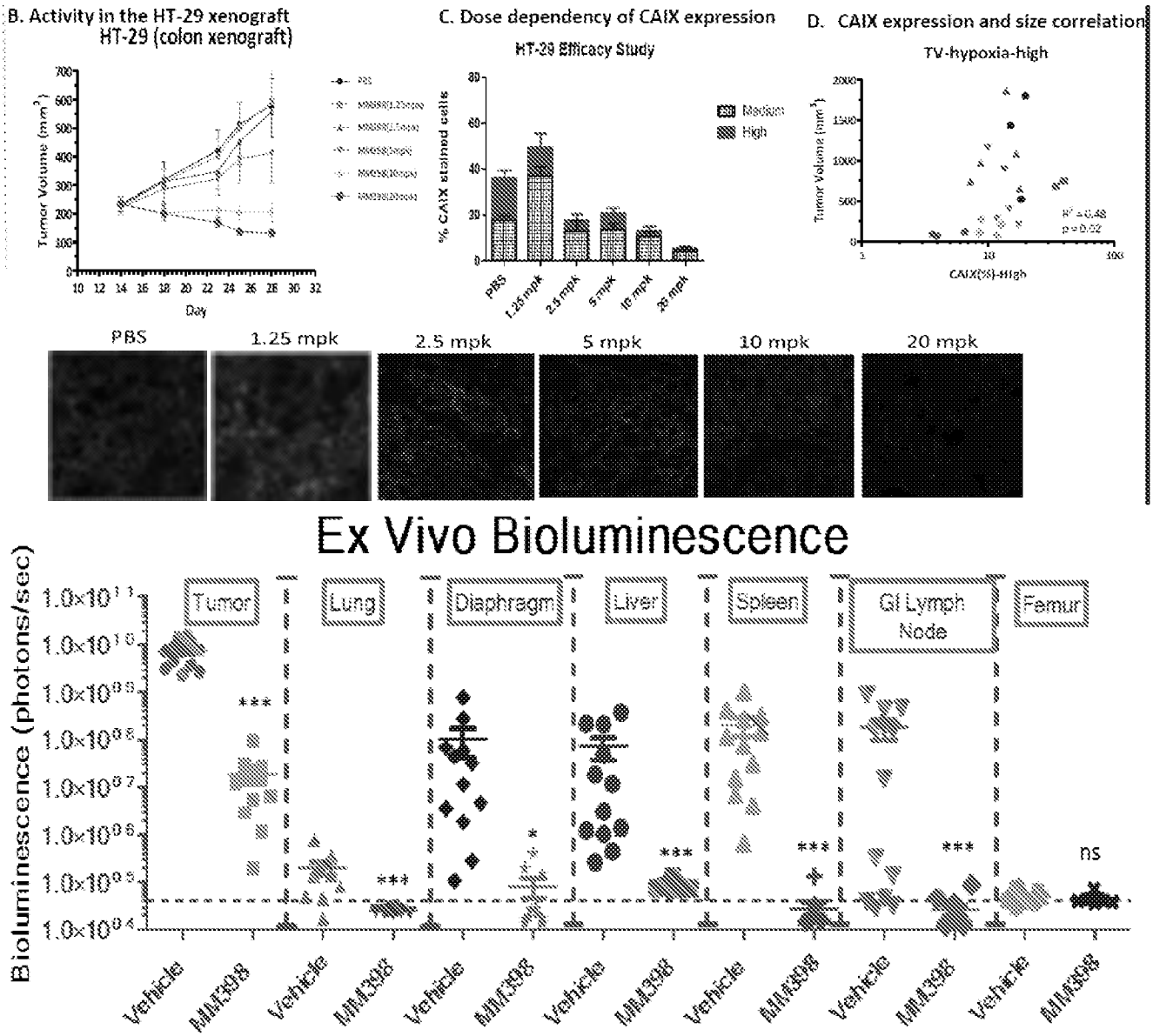
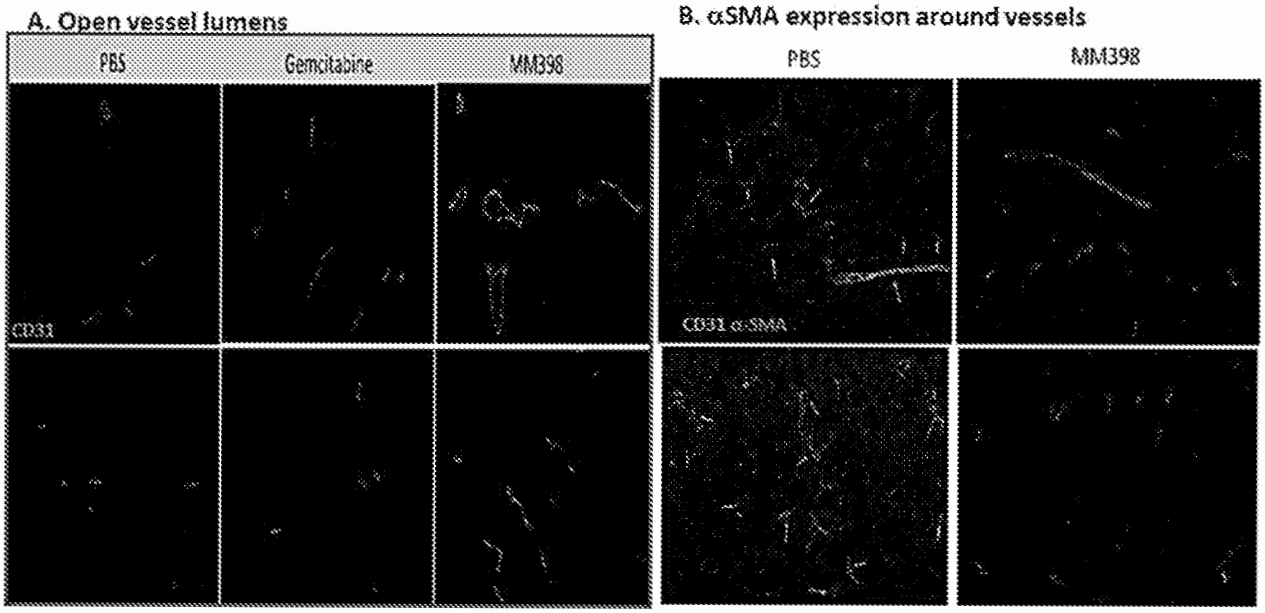
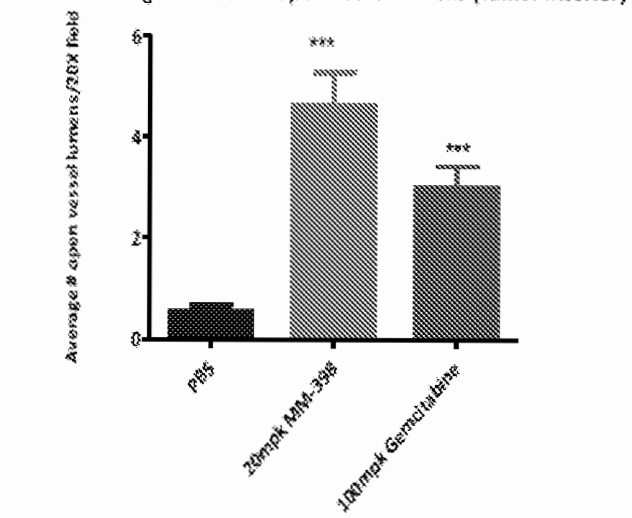


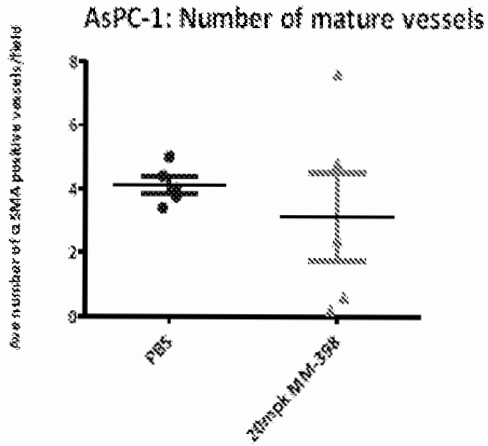
Figure 3 – *In vivo* activity in two murine models: A. BxPC3 pancreatic orthotopic model with spontaneous metastases . MM398 was administered q7d, i.v. at 10 mpk for 5 doses. (Top) *In vivo* bioluminescence (photons/seconds) measurement in the primary tumor. (Bottom) *ex vivo* bioluminescence of multiple organs at study termination. Metastatic organs were collected and imaged. **B**. Activity study in an HT-29 xenograft dosed q7d, i.v. at concentrations: 1.25, 2.5, 5, 10 and 20 mpk. **C**. Dose dependent reduction in CAIX staining as measured by whole tissue imaging and quantitation. CAIX membrane staining is averaged across five whole tumors from each study arm. CAIX marker area was normalized to a tumor mask area created over viable tissue. (Bottom) Representative images are shown. **D**. Correlation between CAIX expression and tumor volume.

MM-398 activity is associated with vascular changes



C. Vessel quantitation





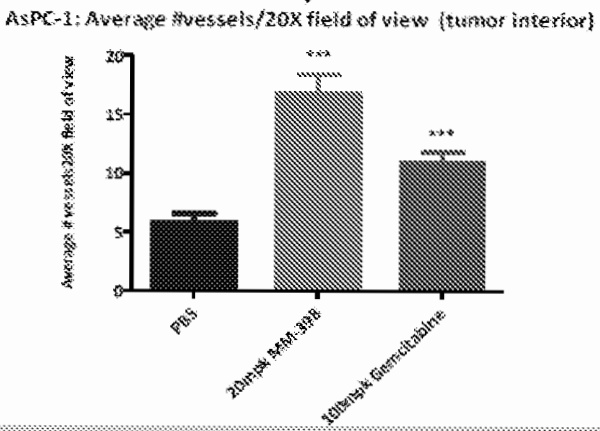
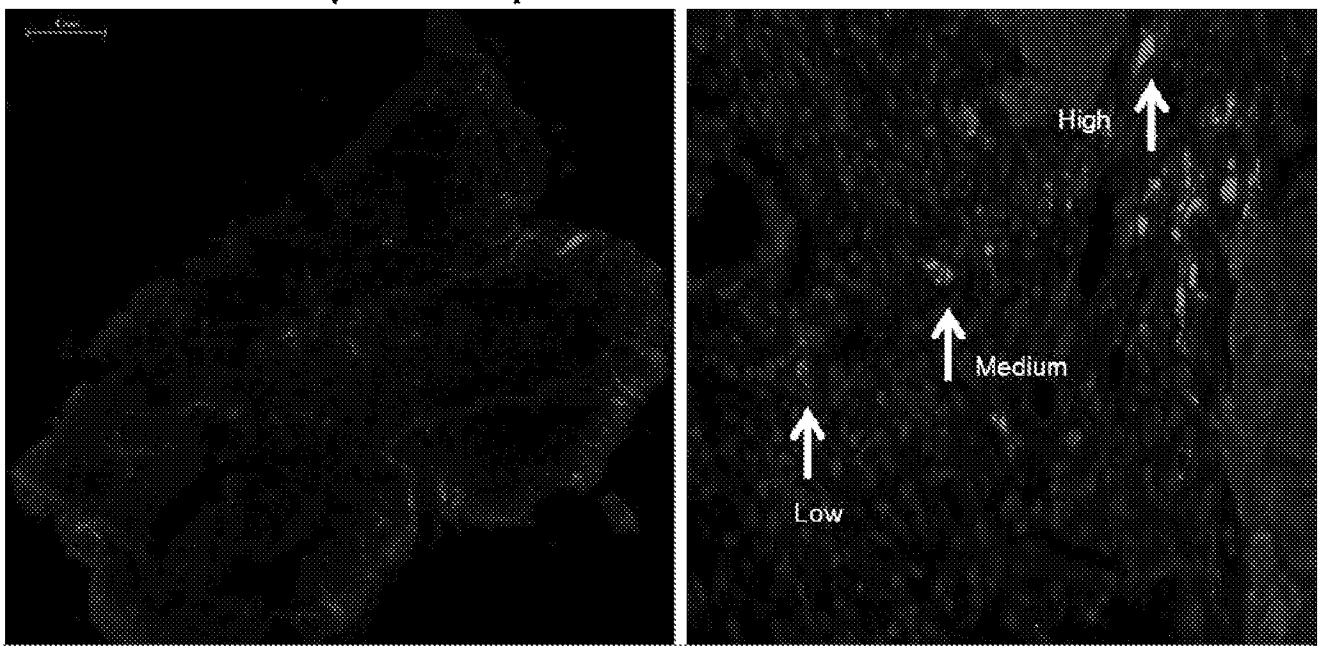
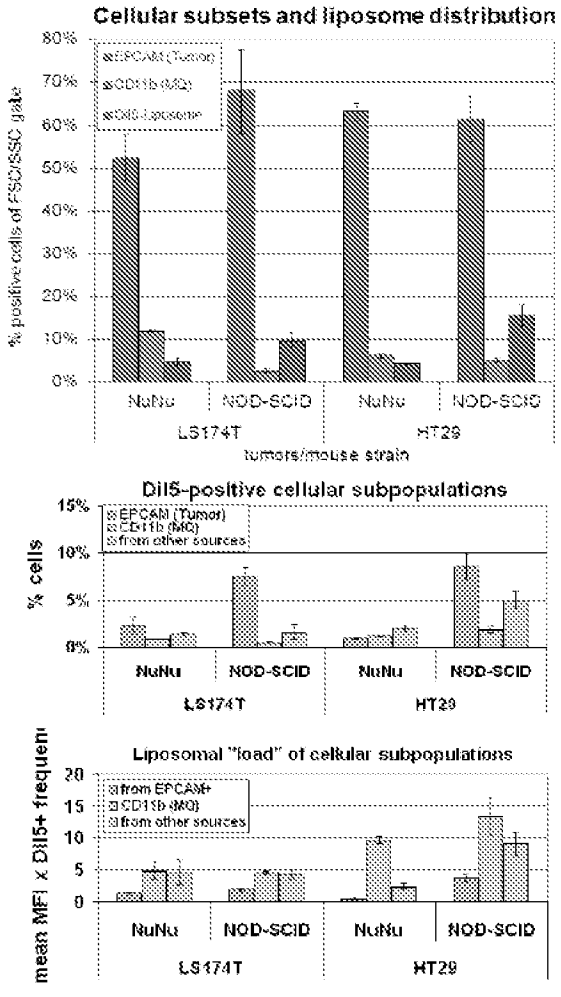


Figure 4 - Changes in vessel lumen count, microvessel density and vascular maturation: A. Tumors from Figure 4A were surveyed visually for changes in the number of open vessel lumens. **B.** Vascular maturation as determined by the dual staining of aSMA/CD31 positive vessels. **C.** Quantification (manual counting) of microvessel density, number of mature vessels, number of open vessel lumens in the AsPC-1 activity study. Magnification 20X (all images)

In Vivo Cellular and Interstitial Deposition Of Liposomes

Liposomal deposition in HT-29 tumors





Flow cytometry of HT-29 tumors after DiI5-liposome dosing

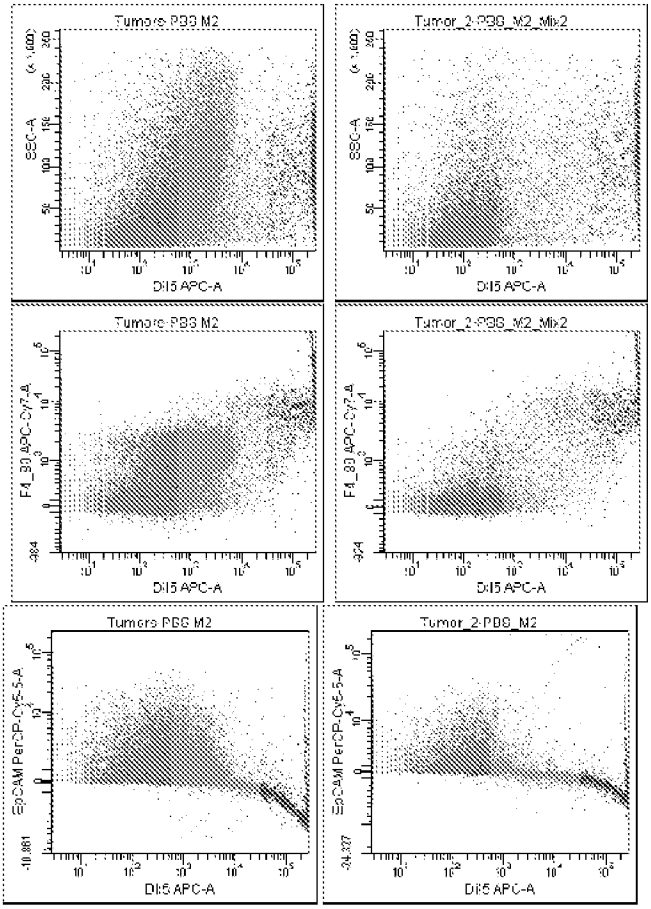


Figure 5 *In vivo* deposition of liposomes: (Left) DiI5-labeled liposomes are traced in frozen sections of HT-29 tumors 24hr after injection. The liposomes are observed in foci associated with cellular material and interstitial matrix with varying intensities. (Middle) Analysis of tumor-derived cells by flow cytometry after enzymatic and mechanical disruption demonstrates liposomal signals in both tumor and monocytic/macrophage cells. Despite lower overall frequency the CD11bpos cells capture more of the liposomes. (Right) Uptake of higher levels of liposomes in the HT-29 tumor model are mostly observed in F4/80high mature macrophages, while EpCAM-positive tumor cells display lower liposome levels. Gated population were visualized with BD FacsDiva software.

Tracking *In Vivo* Biomarkers of MM-398 Response and Drug Deposition

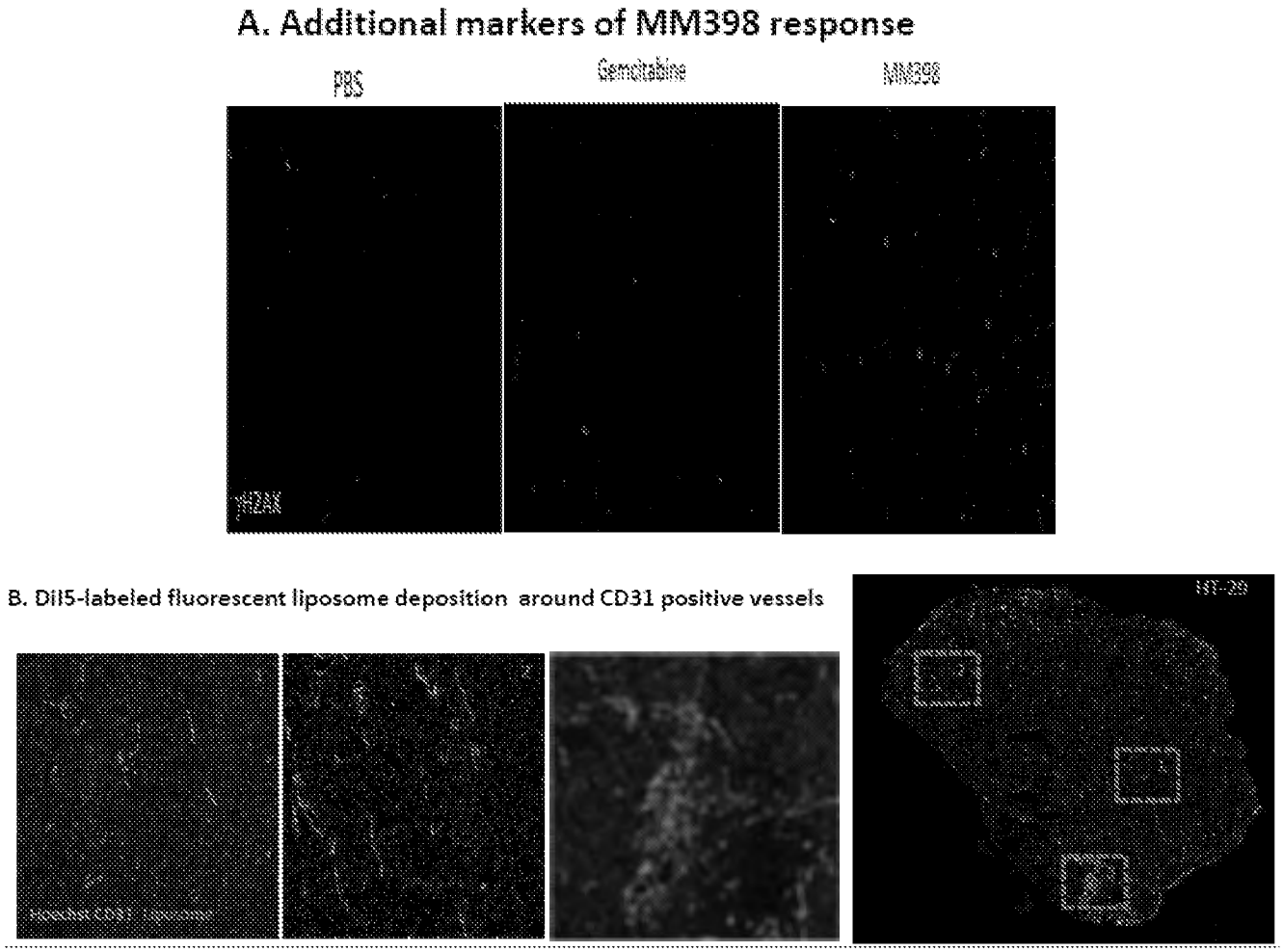


Figure 6.. A. gH2AX staining of the AsPC-1 xenograft tumors. Quantitation of the gH2AX staining is pending. **– B.** DiI5-labeled liposomes (identical to MM-398) have increased deposition in the tumor cortex of HT-29 (untreated mice) where larger vessels with open lumens are more numerous. All images were taken on a Scanscope FL whole tissue microscope and analyzed in Definiens Tissue Studio.

Current Clinical Activities

Ongoing Phase 3 monotherapy in 2nd line pancreatic cancer patients

Ongoing Phase 2 combination with 5-FU & LV in 2nd line colorectal cancer

Completed Phase 2 monotherapy in 2nd line pancreatic cancer patients

Completed Phase 2 monotherapy in 2nd line gastric cancer patients

Completed Phase 1 monotherapy in colorectal cancer full data to be presented in 2012

SUMMARY

- Intratumoral carboxylesterase 2 conversion corresponds to MM-398 activity in multiple tumor models.
- MM-398 demonstrates robust preclinical activity in an BxPC3-Luc2 pancreatic orthotopic model with spontaneous metastasis.
- In an HT-29 xenograft, (high CES2conversion model) tumor volume reduction after MM-398 treatment correlates with reduction of CAIX levels.
- MM-398 demonstrates activity in the AsPC-1 a pancreatic (KRAS mutant) gemcitabine insensitive model.
- Activity of MM-398 in AsPC-1 is associated with increased open vessel lumen density after MM-398 treatment unlike gemcitabine treated tumors.
- Activity of MM-398 in AspC-1 is associated with a trend of decreased vessel maturation and significantly increased microvessel density in the tumor interior.

Acknowledgments

We thank foremost the patients in the initial clinical trials that have yielded insight into the pharmacokinetic behavior of MM-398 and allowed continued clinical development of this drug. We thank Gabriela Garcia and Olga Burenkova for the contributions of the study on the effect of MM-398 in the BxPc3-Luc2 pancreatic orthotopic tumor model.

#A63
MM-398/PEP02, a novel liposomal formulation of irinotecan demonstrates stromal-modifying anti-cancer properties
MERRIMACK

CANCER RESEARCH

Tumor Biology

Abstract A63: MM-398/PEP02, a novel liposomal formulation of irinotecan, demonstrates stromal-modifying anticancer properties.

Nancy Paz, Peter Laivins, Ciet Niyikiza, Ulrik Nielsen, Jonathan Fitzgerald, Ashish Kalra, Milind Chalishazar, Stephan Klinz, Jaeyeon Kim, Daryl Drummond, Dmitri Kirpotin, Victor Moyo, and Eliel Bayever

DOI: 10.1158/1538-7445.PANCA2012-A63 Published July 2012

Abstract

MM-398 is a stable nanotherapeutic encapsulation of the prodrug irinotecan (CPT-11) with longer plasma half-life and higher tumor deposition due to an enhanced permeability and retention effect. Pancreatic cancer has responded poorly to many therapeutics, largely because of inadequate drug penetration due to poor vascularization and the highly aggressive, hypoxic nature of the disease. We sought to better understand how MM-398, a relatively large (100nm) liposomal nanotherapeutic, could be used treat pancreatic cancer.

We have tested MM-398 in several pancreatic xenograft models: BxPC3 (KRAS wild type), AsPC-1(KRAS G12D) , Panc-1 (KRAS G12D) and MiaPaCa (KRAS G12C). All models demonstrated complete tumor regression at 20 mg/kg or a human equivalent dose of 60-120 mg/m2. At this same dose, MM-398 suppresses tumor growth in a gemcitabine insensitive AsPC-1 xenograft. MM-398 functionally blocked AsPC-1 tumor cell proliferation as measured by ki-67 staining; however, gemcitabine administered at its maximum tolerated dose did not impact proliferation. MM-398 is currently in multiple clinical trials, including a phase 3 trial for patients with advanced gemcitabine-resistant pancreatic cancer (NAPOLI-1).

In order to further understand mechanisms driving response to MM-398, we screened and ranked several cell lines for their ability to convert irinotecan into the active metabolite, SN38. BxPC3 and HT-29 tumors ranked highest in ability to convert irinotecan to SN-38, as measured by HPLC. In a BxPC3 pancreatic orthotopic model which spontaneously metastasizes, 10 mg/kg MM-398 significantly reduced both primary and metastatic tumor load as measured by ex vivo biophotonic imaging of BxPC3luc cells to spleen, lung, liver, diaphragm and GI associated lymph nodes ($p<0.001$). This suggests that, in tumors with high conversion ability, MM-398 can block metastatic spread. To investigate dose-dependent effects, we explored pharmacodynamics changes and efficacy in the highly hypoxic HT29 model in response to 5 dose levels of MM-398. MM-398 reduced tumor growth in a dose-dependent manner, maximally to below starting levels. Immunohistochemical evaluation of carboxyl levels of carbonic anhydrase IX (CAIX), a marker of HIF-1 alpha response to hypoxia demonstrated a direct correlation between decrease of tumor cell CAIX expression and tumor response to MM-398. This suggests that MM-398 is able to modulate hypoxia, which may enhance MM-398 efficacy. In this same model we have also determined that a single dose of MM-398 increases the number of blood vessels with open lumens, indicating that MM-398 acts as a stromal and vascular modifying agent.

In summary, MM-398 induces tumor regression in multiple mouse models of pancreatic cancer, including an orthotopic metastatic model. MM-398 activity may be driven in part by the ability to modify tumor microenvironment parameters, such as hypoxia and vascularization, both of which limit efficacy of chemotherapeutic agents in the treatment of pancreatic cancer. These data support the continued investigation of MM-398 in pancreatic cancer.

Citation Format: Nancy Paz, Peter Laivins, Ciet Niyikiza, Ulrik Nielsen, Jonathan Fitzgerald, Ashish Kalra, Milind Chalishazar, Stephan Klinz, Jaeyeon Kim, Daryl Drummond, Dmitri Kirpotin, Victor Moyo, Eliel Bayever. MM-398/PEP02, a novel liposomal formulation of irinotecan, demonstrates stromal-modifying anticancer properties. [abstract]. In: Proceedings of the AACR Special Conference on Pancreatic Cancer: Progress and Challenges; Jun 18-21, 2012; Lake Tahoe, NV. Philadelphia (PA): AACR; Cancer Res 2012;72(12 Suppl):Abstract nr A63.

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IASLC | 2019 World Conference on Lung Cancer
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Conquering Thoracic Cancers Worldwide

Efficacy and Safety of Irinotecan Liposome Injection (nal-IRI) in Patients with Small Cell Lung Cancer (SCLC)

Presented by Luis G. Paz-Ares, MD, PhD

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NCT: 03088813

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Yuanbin Chen	Honorarium: BMS, Merck, Takeda, Novartis, AstraZenca, Eli-Lilly, Genentech, Guardant Health, Pfizer, Array BioPharma, Heron; Consultant: BMS, Takeda, Novartis, AstraZeneca, Genentech, Pfizer, Array BioPharma, Heron; Speaker's Bureau: BMS, Merck, Takeda, Novartis, AstraZenca, Eli-Lilly, Genentech, Guardant Health; Research/Grant Funding (Institution): BMS, AstraZeneca, Ipsen, Helsin, Guardant Health, Roche; Expert Testimony: AstraZeneca, Takeda; Clinical Trials: AstraZeneca, Ipsen, Roche, BMS
Maria Jove	Consultant: Boehringer Ingelheim; Travel & Accommodation Support: BMS, Roche, MSD
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Patricia Rich, Theresa Hayes, Vanesa Gutiérrez Calderón	Nothing to Disclose
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Bin Zhang, Yan Moore, Tiffany Wang, Natalya Nazarenko	Employment: Ipsen

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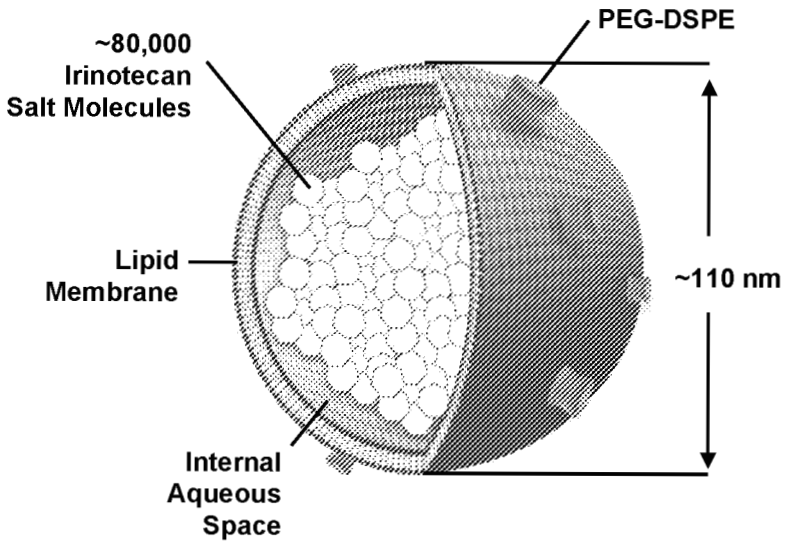
Background

- Liposomal irinotecan (nal-IRI) is a long-acting, liposomal encapsulation of irinotecan
- The half-life ($t_{½}$) of total irinotecan following administration of nal-IRI is 25.8 hours
- 95% of irinotecan remains contained within the liposome during circulation
- The ratio between total and encapsulated forms did not change from 0 to 169.5 hours post-dose
- ~5-fold higher levels of drug are found in tumors compared with plasma at 72 hours, suggesting local metabolic activation of irinotecan



Study Objectives

Primary objectives:

- To describe the safety and tolerability of nal-IRI injection monotherapy administered every 2 weeks
- To determine the recommended nal-IRI injection monotherapy dose (85 mg/m^2 or 70 mg/m^2; free-base equivalent) for future studies

Secondary objectives:

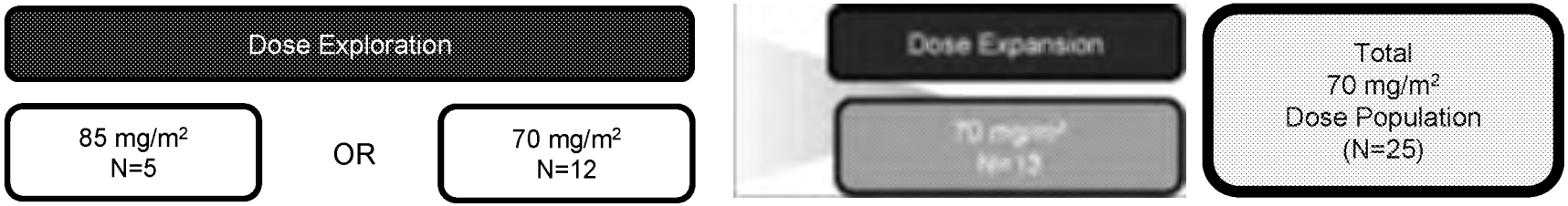
- To assess the preliminary efficacy of nal-IRI injection:
  - Objective response rate (ORR)
  - Progression-free survival (PFS)
  - Overall survival (OS)

Inclusion Criteria:

- At least 18 years of age
- ECOG Performance Status of 0 or 1
- Life expectancy ≥12 weeks
- Histopathologically or cytologically confirmed SCLC with evaluable disease per RECIST v1.1
- Progression after first-line platinum-based therapy

Study Design

This is an open-label, single-arm, safety run-in of nal-IRI administered every 2 weeks.



- A safety assessment was conducted according to a "6+6" design
- Patients were initially treated with nal-IRI injection 85 mg/m^2 every 2 weeks
  - Among the first 6 patients, if ≤2 patients experience dose-limiting toxicities (DLTs) with 85 mg/m^2 another 6 patients will be enrolled into this cohort
  - Otherwise, enrollment into the 70 mg/m^2 cohort was initiated
- Database Snapshot Date: May 8th, 2019; Database Lock: June 7th, 2019
  - At data cut-off, 7 patients continued on treatment

Study Demographics, Baseline Characteristics, and Patient Disposition

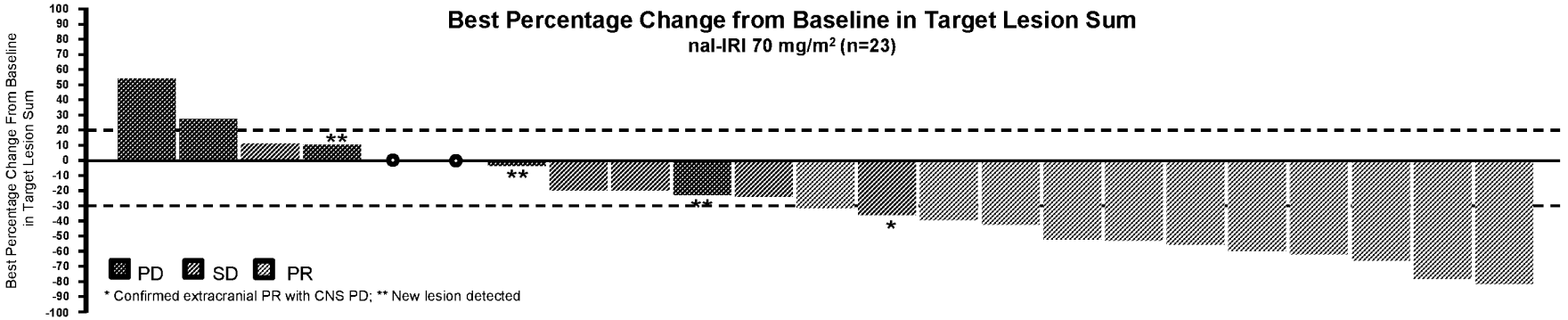
	85 mg/m² n=5	70 mg/m² n=25	Overall Doses N=30
Age (years)			
Median (range)	63.4 (59–72)	59.8 (48–73)	60.4 (48–73)
Age Group, n (%)			
<65 Years	4 (80.0)	18 (72.0)	22 (73.3)
Gender, n (%)			
Male	3 (60.0)	10 (40.0)	13 (43.3)
Race, n (%)			
White	5 (100.0)	25 (100.0)	30 (100.0)
Disease Status, n (%)%			
Locally Advanced	–	2 (8.0)	2 (6.7)
Metastatic	5 (100.0)	23 (92.0)	28 (92.3)
Baseline ECOG Performance Status, n (%)			
Fully Active (ECOG 0)	1 (20.0)	3 (12.0)	4 (13.3)
Restricted Activity (ECOG 1)	4 (80.0)	22 (88.0)	26 (86.7)
Disposition, n (%)			
Treated, n (%)	–	7 (28.0)	7 (23.3)
Discontinued, n (%)	5 (100.0)	18 (72.0)	23 (76.7)

Dose-limiting Toxicity and Treatment-related TEAEs Grade ≥3

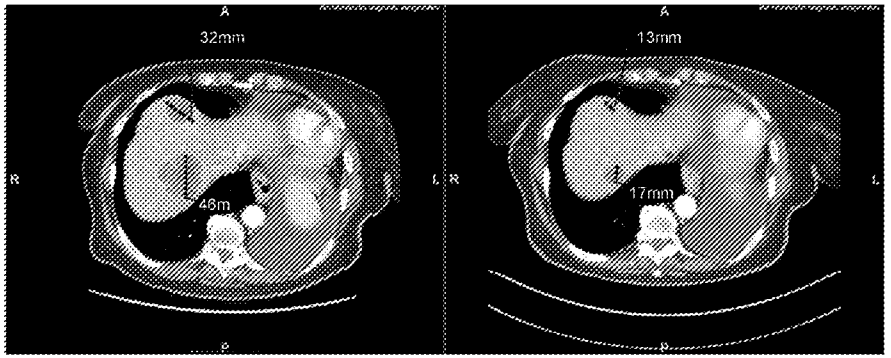
Safety & Tolerability, n (%)	85 mg/m² n=5	70 mg/m² n=25	Overall Doses N=30
Any Treatment-emergent Adverse Event (TEAE)	5 (100.0)	25 (100.0)	30 (100.0)
Grade ≥3 Related TEAEs in 70 mg/m² Treatment Arm	5 (100.0)	10 (40.0)	15 (50.0)
Neutropenia	1 (20.0)	4 (16.0)	5 (16.7)
Anemia	–	2 (8.0)	2 (6.7)
Thrombocytopenia	–	2 (8.0)	2 (6.7)
Diarrhea	3 (60.0)	5 (20.0)	8 (26.7)
Dysphagia	–	1 (4.0)	1 (3.3)
Asthenia	–	2 (8.0)	2 (6.7)
Fatigue	1 (20.0)	1 (4.0)	2 (6.7)
Abdominal Sepsis	–	2 (8.0)	2 (6.7)
Alanine Aminotransferase increased	–	1 (4.0)	1 (3.3)
Gamma-glutamyltransferase increased	–	1 (4.0)	1 (3.3)
Hypokalemia	1 (20.0)	1 (4.0)	2 (10.0)

Hematologic AEs included neutropenia (Gr3, 8%; Gr4, 8%), anemia (Gr3, 8%), febrile neutropenia (Gr3, 4%), and thrombocytopenia (Gr3, 4%; Gr4, 4%)

Clinical Response



	85 mg/m² n=5	70 mg/m² n=25	Overall Doses N=30
Best Overall Response at any time			
Complete Response (CR)	–	–	–
Partial Response (PR)	2 (40.0)	11 (44.0)	13 (43.3)
Stable Disease (SD)	1 (20.0)	7 (28.0)	8 (26.7)
Progressive Disease	1 (20.0)	5 (20.0)	6 (20.0)
Non-evaluable	1 (20.0)	2 (8.0)	3 (10.0)
Objective Response Rate (ORR)			
CR + PR	2 (40.0)	11 (44.0)	13 (43.3)



BOR (PR+SD): 72%; $DCR_{Week\ 8}$ (PR+SD): 48%

Conclusions

- The recommended dose of nal-IRI 70 mg/m^2 appeared to be well tolerated in patients with SCLC, with a reasonable safety profile
  - nal-IRI 70 mg/m^2 was assessed in the expansion phase
  - Predominantly GI toxicity was observed: Gr3 diarrhea (20%) and no Gr4 events
  - Myelosuppression was manageable: Gr3-4 neutropenia (16%) (Gr3 8%; Gr4 8%)
- Encouraging anti-tumor activity for nal-IRI 70 mg/m^2 was demonstrated in patients with SCLC, with an ORR of 44% and BOR of 72%
  - OS and PFS are still maturing
- Among 25 evaluable patients treated with nal-IRI 70 mg/m^2
  - n=11 (44%) achieved PR, with n=17 (68%) experiencing tumor shrinkage
  - 12-week disease control rate (DCR_{12wks} PR+SD) of 48%
- Future study will assess OS in two-arm randomized evaluation of nal-IRI 70 mg/m^2 and topotecan in second-line SCLC

RESILIENT part 2: an open-label, randomized, phase 3 study of liposomal irinotecan injection in patients with small-cell lung cancer who have progressed with platinum-based first-line therapy

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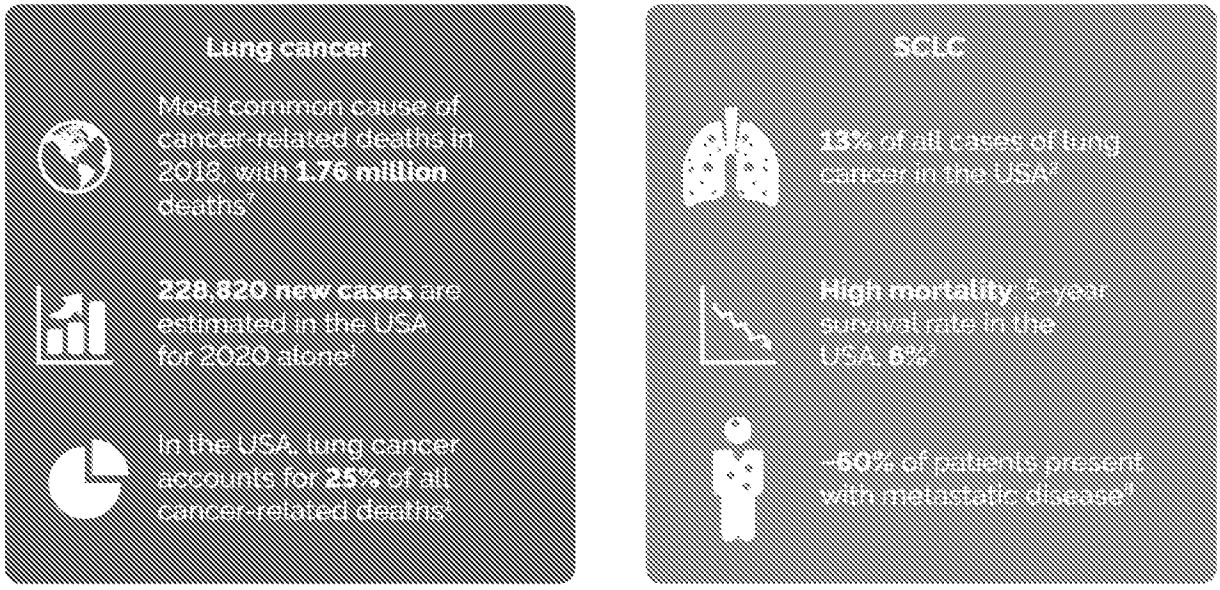
BACKGROUND

- In the USA, lung cancer accounts for approximately 25% of cancer-related deaths, with small-cell lung cancer (SCLC) occurring in approximately 13% of all lung cancer cases (**Figure 1**).[1,2]
- Many patients with SCLC develop drug resistance to established first-line (1L) therapies, and effective second-line (2L) therapies are limited.[3]
  - Currently, in the USA and Europe, the topoisomerase I inhibitor topotecan is approved as a 2L therapy to treat patients with SCLC who have relapsed.[4,5]
- Preliminary data from the first part (dose-ranging) of the two-part phase 2/3 RESILIENT study (NCT03088813) showed that liposomal irinotecan 70 mg/m^2 free base (ONIVYDE, ONIVYDE pegylated liposomal) administered intravenously every 2 weeks appeared to be well tolerated and exhibited promising antitumor activity.[6]
- Here, we present the design of the second, larger part of the RESILIENT study, which will evaluate the efficacy and safety of liposomal irinotecan versus topotecan in the same patient population as in part 1.

Figure 1. Overview of lung cancer and SCLC



SCLC, small-cell lung cancer.

STUDY DESIGN

- RESILIENT is an ongoing, two-part, phase 2/3, open-label, international study assessing the safety, tolerability and efficacy of liposomal irinotecan monotherapy in patients with SCLC who have progressed while or after receiving platinum-based 1L therapy (**Figure 2**).

Study objectives

- The primary objective for RESILIENT part 2 is to evaluate the efficacy of liposomal irinotecan in improving overall survival (OS) in patients with SCLC who have progressed with platinum-based 1L therapy (**Table 1**).
- Secondary and exploratory objectives are listed in **Table 1**.

Study population

- Consenting adults with SCLC who have progressed while or after receiving platinum-based 1L therapy will be eligible to participate in part 2 of RESILIENT.
  - **Table 2** lists key inclusion and exclusion criteria.
- Patients will be stratified according to region (North America, Asia, other) and platinum sensitivity (sensitive, resistant).

Figure 2. Study design of RESILIENT part 2

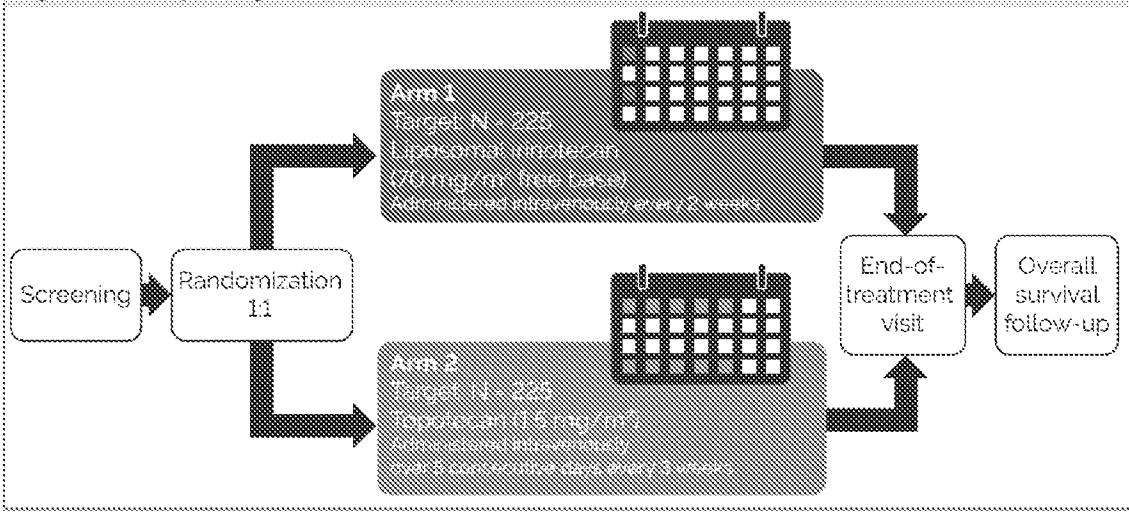


Table 1. RESILIENT part 2 study objectives

Primary objective	
OS	Time from the date of randomization to the date of death by any cause
Secondary objectives	
PFS	Time from randomization to the first documented objective disease progression as per RECIST v1.1 or death by any cause, whichever comes first
ORR	The proportion of patients with a BOR of complete or partial response as per RECIST v1.1 (or RANO criteria for CNS lesions). BOR is defined as the best response from treatment initiation to disease progression
Symptom improvement	Improvement of symptoms as measured by EORTC-QLQ-C30 and EORTC-QLQ-LC13
Safety	Severity of AEs and SAEs graded according to NCI-CTCAE v5.0. TEAEs leading to death, laboratory abnormalities, incidence of patients experiencing dose modifications (including infusion interruptions, dose omissions and dose delays) and/or premature treatment discontinuation (including reason for discontinuation)
Exploratory objectives	
PK and PD	The PKs of total irinotecan and SN-38 will be quantified using nonlinear mixed-effect modeling. Resulting estimates will be used to evaluate the association between PK and PD, and the relationship between PK exposure and associated efficacy and safety
TTF	Time from randomization to treatment discontinuation for any reason, including disease progression, treatment toxicity, patient preference or death
CNS progression	The rate of development and time to development of CNS progression and metastases will be compared
PROs	Patient-reported quality of life as reported on questionnaires such as EORTC-QLQ-C30, EORTC-QLQ-LC13 and EQ-5D-5L

AE, adverse event; BOR, best overall response; CNS, central nervous system; EORTC-QLQ-C30, European Organization for Research and Treatment of Cancer Quality of Life questionnaire – core 30; EORTC-QLQ-LC13, European Organization for Research and Treatment of Cancer Quality of Life questionnaire – lung cancer 13; EQ-5D-5L, 5-dimension EuroQol questionnaire (5 level); NCI-CTCAE v5.0, National Cancer Institute – Common Terminology Criteria for Adverse Events version 5.0; ORR, objective response rate; OS, overall survival; PD, pharmacodynamics; PFS, progression-free survival; PK, pharmacokinetics; PRO, patient-reported outcome; RANO, Response Assessment in Neuro-Oncology; RECIST v1.1, Response Evaluation Criteria in Solid Tumors version 1.1; SAE, serious adverse event; TEAE, treatment-emergent adverse event; TTF, time to treatment failure.

Table 2. Key inclusion and exclusion criteria

Key inclusion criteria
• Aged ≥ 18 years at the time at which informed consent is signed • Received one prior platinum-based therapy • Received one line of immunotherapy either as monotherapy or in combination in the first- or second-line setting • ECOG Performance Status of 0 or 1 at screening and in the 7 days before randomization • Life expectancy of > 12 weeks • Evaluable SCLC as defined by RECIST v1.1 • Adequate hematologic, hepatic and renal function • ECG without clinically significant findings
Key exclusion criteria
• Any medical condition deemed likely to interfere with the patient's ability to participate in the study or with the interpretation of results • Prior treatment with topotecan or other topoisomerase I inhibitors • Retreatment with platinum-based regimen after relapse of platinum-based 1L therapy

1L, first-line; ECG, electrocardiogram; ECOG, Eastern Cooperative Oncology Group; RECIST v1.1, Response Evaluation Criteria in Solid Tumors version 1.1; SCLC, small-cell lung cancer.

Data collection and follow-up

- Anonymized data will be collected using an electronic case-report form.
- Thirty days after permanent discontinuation of the study treatment, patients will undergo a 30-day follow-up assessment and will be observed for survival status every month until death or study end (when all patients have died or withdrawn consent, or are lost to follow-up).
- All patients will be treated until disease progression or treatment toxicity.
- Tumor assessments will be performed by computed tomography or magnetic resonance imaging every 6 weeks (± 1 week) using Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1) guidelines and Response Assessment in Neuro-Oncology (RANO) criteria for central nervous system lesions.

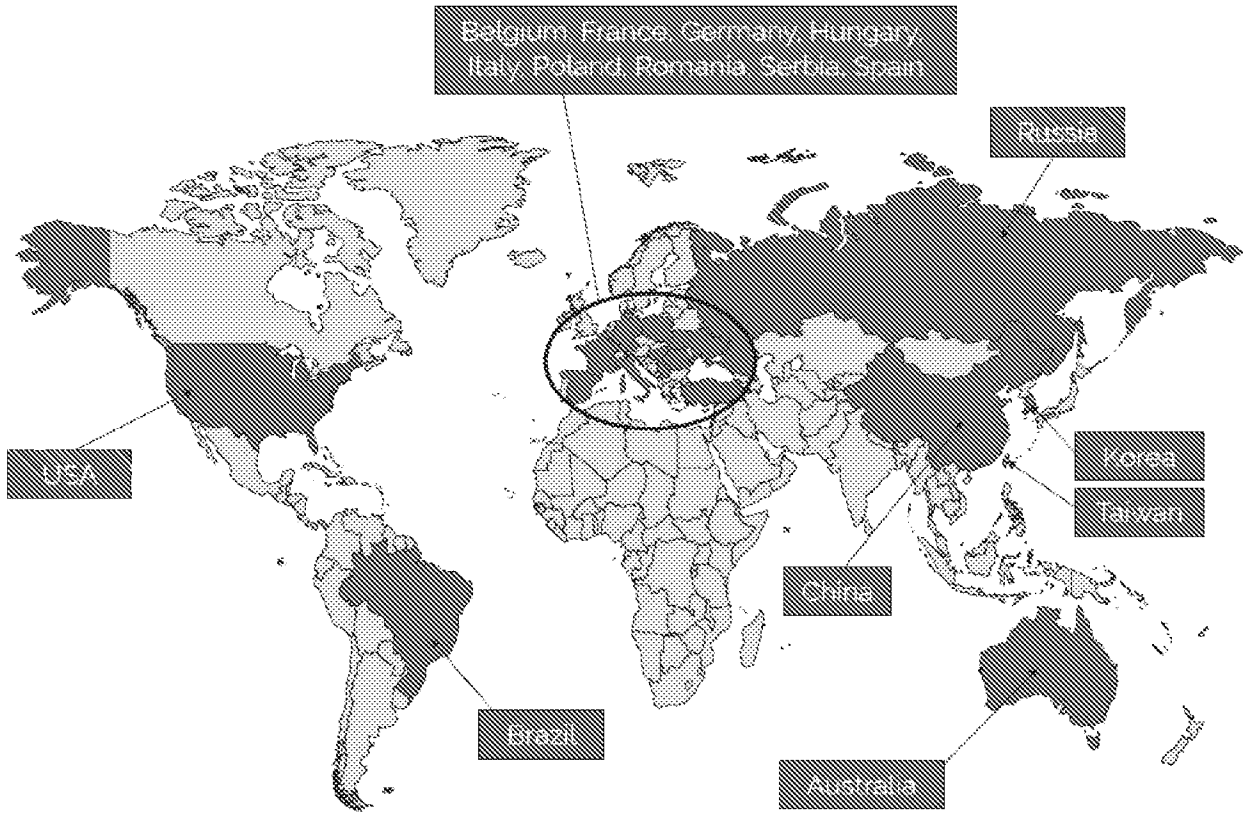
Analyses

- Efficacy analyses will be performed on the 'intent-to-treat' population.
- Safety analyses will be performed on patients who received at least one dose of any study treatment.
- OS will be assessed using Kaplan–Meier methodology, and differences between treatment arms will be assessed using a stratified log-rank test.
- Differences in progression-free survival between study arms will be evaluated using a stratified (by region and platinum sensitivity) log-rank test and displayed using Kaplan–Meier methodology. The hazard ratio will be determined using a stratified Cox proportional-hazards model.
- Differences in overall response rate between study arms will be compared using the Cochran–Mantel–Haenszel method and stratified by region and platinum sensitivity.

Recruitment update

- As of April 2020, 140 patients in total have been enrolled.
- Recruitment is ongoing at 113 centers in Europe, the USA and Asia-Pacific (**Figure 3**).

Figure 3. Distribution of patients across active countries involved in the RESILIENT study



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Author contributions

All authors have contributed to study conception/design, drafting the publication or revising it critically for scientific accuracy and important intellectual content, and final approval of the publication.

Disclosures

LPA: Adacap, Altum Sequencing, Amgen, AstraZeneca, Bayer, Blueprint Medicines, Boehringer Ingelheim, Bristol Myers Squibb, Celgene, Eli Lilly, EMA SAG, Genomica, Incyte, Ipsen, Merck Serono, MSD, Novartis, Pfizer, PharmaMar, Roche/Genentech, Sanofi, Sysmex; DS: Aeglea Biotherapeutics, Amgen, Aptitude Health, Astellas, AstraZeneca, Bayer, BIND Therapeutics, Bristol Myers Squibb, Celgene, Celldex, Clovis, Daiichi Sankyo, Dracen Pharmaceuticals, Eisai, Eli Lilly, EMD Serono, Evelo Biosciences, G1 Therapeutics, Genentech, GlaxoSmithKline, GRAIL, Ikuuda Therapeutics, Illumina, ImClone Systems, ImmunoGen, Ipsen, Janssen, MedImmune, Merck, Molecular Partners, Molecular Templates, Nektar Therapeutics, Neon, Novartis, Pfizer Pharma Mar, Roche, Seattle Genetics, Spectrum Pharmaceuticals, Takeda, Transgene, TRIPTYCH Health Partners, TRM Oncology, UT Southwestern Williams and Connolly LLP; MJ: Boehringer Ingelheim, MSD, Roche, Takeda; OJV: Abbvie, AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Eli Lilly, MSD, Pfizer, Roche/Genetech, Takeda; RBC: AstraZeneca, Bristol Myers Squibb, MSD, Roche, Takeda; AN: Boehringer Ingelheim, Oryzon Genomics, Pfizer, Roche; AD: Abbvie, Amgen, AstraZeneca, Bayer, Bristol Myers Squibb, Incuron, Ipsen, Loxo, Regeneron, Seattle Genetics, Symphogen, Takeda, Tesaro; JK: Bristol Myers Squibb, Constellation Pharma, JKMD Global Medical Solutions, Tocagen; SP: AstraZeneca, Bristol Myers Squibb, Merck, Roche.

EZ, YM, TW and JK are employees of Ipsen.

YC, PR, TH, VGC and PB have nothing to disclose.

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Presented at the Annual Meeting of the American Society of Clinical Oncology (ASCO), virtual format, May 29–June 2, 2020 (#ASCO20)

This study was sponsored by Ipsen

RESILIENT part 2: an open-label, randomized, phase 3 study of liposomal irinotecan injection in patients with small-cell lung cancer who have progressed with platinum-based first-line therapy

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BACKGROUND

STUDY DESIGN

LUNG CANCER—NON-SMALL CELL LOCAL-REGIONAL/SMALL CELL/OTHER THORACIC CANCERS

RESILIENT part II: an open-label, randomized, phase III study of liposomal irinotecan injection in patients with small-cell lung cancer who have progressed with platinum-based first-line therapy.

Luis G. Paz-Ares, David R. Spigel, Yuanbin Chen, Maria Jove, Oscar Juan-Vidal, Patricia Rich, Theresa M. Hayes, M Vanesa Gutierrez Calderon, Reyes Bernabe Caro, Alejandro Navarro, Afshin Dowlati, Bin Zhang, Yan Moore, Haofei Tiffany Wang, Jaba Kokhreidze, Santiago Ponce Aix, Paul Bunn

Hospital Universitario 12 de Octubre, Madrid, Spain; Sarah Cannon Research Institute, Nashville, TN; Cancer and Hematology Centers of Western Michigan, Grand Rapids, MI; Institut Català d'Oncologia, Barcelona, Spain; Hospital Universitario y Politécnico La Fe, Valencia, Spain; Cancer Treatment Centers of America, Atalanta, GA; South West Healthcare, Warrnambool, VIC, Australia; Hospital Regional Universitario de Málaga, Málaga, Spain; Hospital Universitario Virgen Del Rocio, Seville, Spain; Hospital Universitari Vall d'Hebron, Barcelona, Spain; University Hospitals Case Medical Center, Cleveland, OH; Ipsen Bioscience, Boston, MA; BioCancell, Cambridge, MA; Hospital Universitario 12 De Octubre, Madrid, Spain; University of Colorado, Denver, CO

Show Less

Abstract Disclosures

Abstract

TPS9081

Background: Although small cell lung cancer (SCLC) is often sensitive to established first-line therapies, many patients relapse and develop drug resistance, and second-line therapies are limited. RESILIENT (NCT03088813) is a two-part phase 2/3 study assessing the safety, tolerability, and efficacy of liposomal irinotecan monotherapy in patients with SCLC who progressed with platinum-based first-line therapy. Preliminary data from the dose-ranging part of the study (part 1) showed that liposomal irinotecan 70 mg/m^2 administered every 2 weeks was well tolerated and had promising antitumor activity (Paz-Ares *et al.* ASCO 2019; poster 318). Here, we present the design of the second, larger part of the study, which will evaluate the efficacy and safety of liposomal irinotecan versus topotecan in the same patient population. **Methods:** Part 2 of RESILIENT is a phase 3, open-label study with a planned sample size of 450. Patients are randomly allocated 1:1 to intravenous liposomal irinotecan or intravenous topotecan. Liposomal irinotecan is administered every 2 weeks at 70 mg/m^2 (free-

base equivalent) and topotecan is administered for 5 consecutive days every 3 weeks at 1.5 mg/m^2. As of January 2020, 80 patients have been enrolled in part 2 of the trial. Tumor assessments are performed using the Response Evaluation Criteria in Solid Tumors version 1.1 and the Response Assessment in Neuro-oncology criteria for CNS lesions; symptom improvement is measured using the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core 30. Safety assessments include monitoring for adverse events. The primary endpoint is overall survival (OS) and secondary endpoints are progression-free survival (PFS), objective response rate, and proportion of patients reporting symptom improvement. Patients will continue study treatment until disease progression, unacceptable toxicity or study withdrawal and will then be followed for survival until death or study end (when all patients have died, withdrawn consent or are lost to follow-up). Clinical trial information: NCT03088813.

Research Sponsor:

Ipsen

adverse events were detected. Among them, 3-4 grade adverse reactions were hypertension (8.33%), hand-foot syndrome (5.56%), hypodynamia (5.56%) and proteinuria (2.78%), which all alleviated by reducing dose and symptomatic treatment.

Table 1 Patient baseline characteristics

Age (< 65 / ≥ 65)	13/ 23	36.11%/ 63.89%
Gender (male/ female)	30/ 6	83.33%/ 16.67%
ECOG (0/ 1/ 2)	3/ 18/ 15	8.33% / 50.00%/ 41.67%
Primary lesion (Right lung/ Left lung)	25/ 11	69.44%/ 30.56%
Radiotherapy (Yes /No)	30/ 6	83.33%/ 16.67%
First line PFS (< 6 m / ≥ 6 m)	19/ 17	52.78%/ 47.22%
The number of treatment lines for apatinib		
Second line treatment	18	50.00%
Three-line treatment	15	41.67%
Four-line treatment	3	8.33%

Conclusion: Apatinib was effective for SCLC patients who failed in first-line and above chemotherapy and the adverse events were tolerable.

Keywords: apatinib, Extensive-Stage Small Cell Lung Cancer, Second-line and Later-line Therapy

OA03 SYSTEMIC THERAPIES FOR SCLC: NOVEL TARGETS AND PATIENTS' SELECTION
SUNDAY, SEPTEMBER 8 13:30–15:00

OA03.02 EFFECT OF ANLOTINIB IN ADVANCED SMALL CELL LUNG CANCER PATIENTS PREVIOUSLY RECEIVED CHEMORADIOTHERAPY: A SUBGROUP ANALYSIS IN ALTER 1202 TRIAL

Q. Wang[1], Y. Cheng[2], K. Li[3], J. Shi[4], B. Han[5], L. Wu[6], G. Chen[7], J. He[8], J. Wang[9], H. Qin[10], X.-L. Li[11]

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Background: The ALTER 1202 trial showed significant improvement in progress-free survival and well tolerant with anlotinib in advanced small cell lung cancer (SCLC) patients received at least two lines chemotherapy. Here, we reported the effect of anlotinib in patients previously received chemoradiotherapy. **Method:** The ALTER 1202 was a randomized, double-blind phase 2 trial conducted at 11 centers in China. Patients with advanced SCLC that received at least two previous lines of chemotherapy were enrolled and randomized in a 2:1 ratio to receive either anlotinib or placebo until tumor progression or unacceptable toxicity. The subgroup analysis assessed the effect of anlotinib in patients with previous concurrent, sequential and alternate chemoradiotherapy. The primary outcome was progressive-free survival (PFS). The secondary outcomes were overall survival (OS), objective response rate, disease control rate and safety. Data are reported as per the 30 June 2018, data cutoff date. This trial is registered with ClinicalTrials.gov, number NCT03059797. **Result:** Between March 30, 2017 and June 8, 2018, a total of 120 patients who met all eligibility criteria were randomly assigned to the anlotinib group (82 patients) or placebo group (38 patients). And 46 patients in anlotinib group and 22 patients in placebo group previously received chemoradiotherapy. Among them, the median PFS was 5.49 months (95% confidence interval [CI], 2.83 to 6.47) with anlotinib versus 0.69 months (95% CI, 0.66 to 0.76) with placebo (hazard ratio [HR], 0.14; 95% CI, 0.07 to 0.28; P<0.0001). Meanwhile, anlotinib significantly prolonged OS compared with placebo (9.49 months [95% CI, 7.29 to 12.68] versus 2.56 months [95% CI, 0.49 to 5.22]; HR, 0.46 [95% CI, 0.22 to 0.98]; P=0.0388) in patients previously received chemoradiotherapy. The most common adverse events were hypertension (39.13%), weight loss (39.13%), hypertriglyceridemia (36.96%) and leukopenia (30.43%). While, the most common grade 3 or worse adverse events were hypertension (15.22%), hypertriglyceridemia (10.87%), γ-glutamyl-transferase increased (8.70%). **Conclusion:** Anlotinib improved PFS and OS in advanced SCLC patients previously received chemoradiotherapy and was well tolerated.

Keywords: Anlotinib, chemoradiotherapy, advanced small cell lung cancer

OA03 SYSTEMIC THERAPIES FOR SCLC: NOVEL TARGETS AND PATIENTS' SELECTION
SUNDAY, SEPTEMBER 8 13:30–15:00

OA03.03 INITIAL EFFICACY AND SAFETY RESULTS OF IRINOTECAN LIPOSOME INJECTION (NAL-IRI) IN PATIENTS WITH SMALL CELL LUNG CANCER

L. Paz-Ares Rodríguez[1], D. Spigel[2], Y. Chen[3], M. Jove[4], O. Juan[5], P. Rich[6], T. Hayes[7], V. Gutiérrez Calderón[8], R. Bernabe[9], A. Navarro[10], A. Dowlati[11], B. Zhang[12], Y. Moore[12], T. Wang[12], N. Nazarenko[12], S. Ponce[13], P. Bunn, Jr[14]

[1]*Hospital Universitario, Madrid/Spain,* [2]*Sarah Cannon Research Institute, Nashville/United States of America,* [3]*Cancer & Hematology Centers of Western Michigan, Grand Rapids/United States of America,* [4]*Hospital Duran I Reinals, Institut Catala D'Oncologia Hospital Duran I Reinals, Barcelona/Spain,* [5]*Hospital Universitari i Politècnic La Fe, Valencia/Spain,* [6]*Cancer Treatment Centers of America, Atlanta/United States of America,* [7]*South West Healthcare, Vic/Australia,* [8]*Hospital Regional Universitario de Malaga, Malaga/Spain,* [9]*Hospital Universitario Virgen Del Rocio, Seville/Spain,* [10]*Hospital Universitari Vall D'Hebron, Barcelona/Spain,* [11]*Case Western Reserve University, Cleviland/United States of America,* [12]*Ipsen Bioscience, Boston/United States of America,* [13]*Hospital Universitario 12 de Octubre, Madrid, Spain, Madrid/Spain,* [14]*Cancer Center and Department of Medicine, University of Colorado, Denver/United States of America*

Background: SCLC accounts for ~15% of lung cancers, with 5-year survival <10%. 50-90% of patients with extensive disease respond to initial treatment; many rapidly relapse due to acquired resistance to front-line platinum-based chemotherapy. Limited treatment options are available for second-line patients. nal-IRI is a liposomal formulation of irinotecan (topoisomerase-I inhibitor), utilizing intraliposomal stabilization technology to enable high drug load and in-vivo stability. **Method:** RESILIENT (NCT03088813) is a two-part Phase 2/3 study assessing the safety, tolerability, and efficacy of monotherapy nal-IRI in SCLC patients who progressed on/after a

front-line platinum regimen. Part 1 includes dose-finding then dose-expansion. Key eligibility criteria included ECOG PS 0–1 and adequate organ function, with prior exposure to immunotherapy allowed. Eligible patients received nal-IRI 70mg/m2 or 85mg/m2 (free-base equivalent) q2w. Primary endpoints were safety and tolerability. Efficacy assessments included objective response rate (ORR), best overall response (BOR), progression-free survival (PFS), and overall survival (OS). **Result:** 30 patients were treated for >12 weeks in Part 1 (male, 43%; median age, 60.4y; platinum-resistant, 40%) with tumor assessments q6w. During dose-finding, 5 patients received nal-IRI 85mg/m2 (deemed not tolerable: dose-limiting toxicity) and 12 patients received nal-IRI 70mg/m2 (deemed tolerable: selected for dose expansion). At data cut-off** (median follow-up, 4.4mo), 25 patients had received nal-IRI 70mg/m2. Diarrhea was the most common gastrointestinal adverse events (AEs) (Gr3, 20%). Hematologic AEs included neutropenia (Gr3, 8%; Gr4, 8%), anemia (Gr3, 8%), febrile neutropenia (Gr3, 4%), thrombocytopenia (Gr3, 4%; Gr4, 4%). Preliminary efficacy identified 11 patients with partial responses (ORR 44%), BOR (PR+SD) of 72%, and 12-week disease control rate (DCR12wks PR+SD) of 48%. PFS and OS are not yet mature. **Conclusion:** Part 1 demonstrated encouraging anti-tumor activity for nal-IRI 70mg/m2 in patients with SCLC (ORR: 44%, BOR: 72%). nal-IRI 70mg/m2 was generally well tolerated. Future research is warranted to assess nal-IRI in second-line SCLC.

Table 1. Baseline Demographic, Patient Disposition, Safety & Tolerability, and Clinical Efficacy for Part 1 of the RESILIENT study

	Dose-Finding / Dose-Exploration Phase	
	Irinotecan Liposome Injection 85mg/m2 (N=5)	**Irinotecan Liposome Injection 70mg/m2 (N=25)**
Baseline Characteristics		
Gender, Male, n (%)	3 (60.0)	10 (40.0)
Age (Years, median)	62.0	59.0
Baseline ECOG		
0	1 (20.0)	3 (12.0)
1	4 (80.0)	22 (88.0)
Time Since Most Recent Progression (Weeks, median)	3.4	3.2
Disease Location, n (%)		
Locally Advanced	0	2 (8.0)
Metastatic	5 (100.0)	23 (92.0)
Disposition, n (%)		
Patient Completed Study	4 (80.0)	12 (48.0)
Patient Currently Ongoing*	-	7 (28.0)
Deaths	2 (40.0)	6 (24.0)
Disease Related	1	3
Adverse Event Not Related to Study Drug	1	1
Cardiac Arrest	1	-
Hepatic Failure	-	1
Adverse Event Related to Study Drug	0	2
Abdominal Sepsis	-	2
Patient Discontinued Treatment	5 (100.0)	18 (72.0)
Safety & Tolerability, n (%)		
Any Treatment-Emergent Adverse Event (TEAE)	5 (100.0)	25 (100.0)
Grade 3 or Higher TEAE (≥ 2 patients)	5 (100.0)	15 (60.0)
Neutropenia	1 (20.0)	4 (16.0)
Anemia	-	2 (8.0)
Thrombocytopenia	-	2 (8.0)
Diarrhea	3 (60.0)	5 (20.0)
Asthenia	-	2 (8.0)
General Physical Health Deterioration	-	2 (8.0)
Pneumonia	2 (40.0)	1 (4.0)
Abdominal Sepsis	-	2 (8.0)
Hypokalemia	1 (20.0)	2 (8.0)
Renal Failure	-	2 (8.0)
Best Overall Response		
Complete Response (CR)	-	-
Partial Response (PR)	2 (40.0)	11 (44.0)
Stable Disease	1 (20.0)	7 (28.0)
Progressive Disease	1 (20.0)	5 (20.0)
Non-evaluable	1 (20.0)	2 (8.0)
Objective Response Rate		
CR + PR	2 (40.0)	11 (44.0)
Non-responder	3 (60.0)	14 (56.0)

** Data Cut-off: May 8, 2019.
* Per RECIST v1.1 or RANO criteria.

Keywords: Irinotecan Liposome Injection, Monotherapy, small cell lung cancer

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Abstracts

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference P071520WO	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/GB2017/053293	International filing date *(day/month/year)* 01 November 2017 (01.11.2017)	Priority date *(day/month/year)* 02 November 2016 (02.11.2016)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant IPSEN BIOPHARM LTD.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☒	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☒	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 07 May 2019 (07.05.2019)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Athina Nickitas-Etienne e-mail: pct.team4@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB2017/053293	01.11.2017	02.11.2016

International Patent Classification (IPC) or both national classification and IPC
INV. A61P35/00 A61K31/4453 A61K31/4178 A61K31/513 A61K31/519 A61K9/127

Applicant
IPSEN BIOPHARM LTD

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☒ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Hornich-Paraf, E Telephone No. +49 89 2399-0

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-27
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-27
Industrial applicability (IA)	Yes:	Claims	1-27
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rules 43*bis*.1 and 70.10)

and / or

2. Non-written disclosures (Rules 43*bis*.1 and 70.9)

see form 210

SECTION V

1. *Cited documents*:

D1 MATTEO DALLA CHIESA ET AL: "Sequential chemotherapy with dose-dense docetaxel, cisplatin, folinic acid and 5-fluorouracil (TCF-dd) followed by combination of oxaliplatin, folinic acid, 5-fluorouracil and irinotecan (COFFI) in metastatic gastric cancer: results of a phase II trial", CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 67, no. 1, 5 March 2010 (2010-03-05), pages 41-48, ISSN: 1432-0843, DOI: 10.1007/S00280-010-1281-5

D2 STEFAN PEINERT ET AL: "Safety and efficacy of weekly 5-fluorouracil/ folinic acid/oxaliplatin/ irinotecan in the first-line treatment of gastrointestinal cancer", THERAPEUTIC ADVANCES IN MEDICAL ONCOLOGYENGLANDJAN 2016, vol. 2, no. 3, 1 May 2010 (2010-05-01), pages 161-174, UK ISSN: 1758-8340, DOI: 10.1177/1758834010365061

D3 Chung-Tzu Hsueh: "Nanovectors for anti-cancer drug delivery in the treatment of advanced pancreatic adenocarcinoma", 21 August 2016 (2016-08-21), Retrieved from the Internet: URL:https://www.wjgnet.com/1007-9327/full/v22/i31/7080.htm [retrieved on 2018-01-25]

D4 ANDREW KO: "Nanomedicine developments in the treatment of metastatic pancreatic cancer: focus on nanoliposomal irinotecan", INTERNATIONAL JOURNAL OF NANOMEDICINE, 1 March 2016 (2016-03-01), page 1225, AUCKLAND, NZ, ISSN: 1176-9114, DOI: 10.2147/IJN.S88084

D5 WO 2013/138371 A1

D6 WO 2013/188586 A1

D7 WO 2011/066684 A1

D8 WO 2017/034957 A1

D9 WO 2017/031442 A1

D10 WO 2017/172678 A1

Reference is made to the passages cited in the Search Report.

D1 reports on a trial investigating the combination of irinotecan, oxaliplatin, 5-fluoruracil and leucovorin (COFFI) in metastatic gastric cancer. It appears that *liposomal irinotecan* is *not* disclosed. Administration was once every two weeks.

D2 also reports on a trial on the safety and efficacy of weekly irinotecan, oxaliplatin, 5-fluoruracil and leucovorin (FUFOXIRI) in gastric cancer. Again, the document does *not* refer to *liposomal irinotecan*.

D3 reports on the usefulness of liposomal irinotecan (nal-irinotecan), 5-fluoruracil and leucovorin for the treatment of pancreas adenocarcinoma. The document also refers to a study on adding oxaliplatin to nal-IRI/LV/5-FU as 1-line treatment in metastatic pancreatic adenocarcinoma (NCT02551991). It appears that *gastric cancer* is *not* mentioned.

D4 in table 1 reports as well on the ongoing trial of nal-irinotecan with 5-fluoruracil, leucovorin and oxaliplatin in pancreatic adenocarcinoma (NCT02551991).

D5 reports on the usefulness of combination therapies i.a. including folinic acid (leucovorin), 5-fluorouracil, irinotecan and oxaliplatin (FOLFIRINOX) for the treatment of various cancers, i.a. pancreatic cancer. It is also disclosed that nanoliposomal irinotecan, MM-398 is investigated i.a. for gastric cancer.

D6 relates to the co-administration of liposomal irinotecan (MM-398) with 5-fluoruracil and leucovorin for the treatment of pancreatic cancer. Example 5 relates to phase I and II clinical studies of MM-398 in patients suffering from gastric cancer.

D7 relates to liposomes of irinotecan.

for **D8** to **D10** reference is made to **SECTION VI** of this report.

2. *Novelty* (**Art. 33(2) PCT**)

It appears that none of the cited prior art documents discloses the usefulness of liposomal irinotecan, oxaliplatin, leucovorin and 5-fluoruracil in the defined amounts for the treatment of gastric cancer. The claimed subject-matter seems therefore *novel* in the light of the available prior art.

3. *Inventive step* (**Art. 33(3) PCT**)

The usefulness of FOLFIRINOX / FOLFOXIRI / COFFI (irinotecan + oxaliplatin + leucovorin + 5-fluoruracil) for the treatment of gastric cancer is known from **D1** or **D2**.

The difference of the present application to these known regimes is obviously the use of *liposomal* irinotecan instead of the irinotecan.

It is known from the prior art that liposomal irinotecan has improved properties as to efficacy and toxicity compared to non-liposomal irinotecan (e.g. **D3**, **D4** or **D7**). Combinations of leucovorin + 5-fluoruracil with liposomal irinotecan have already been described as useful for the treatment of pancreatic adenocarcinoma (see e.g. **D3** or **D4**); in these documents reference is also made to an 'ongoing' clinical trial investigating the further addition of oxaliplatin to this combination, also for the treatment of pancreatic adenocarcinoma.

Any seen improved effect in the use of liposomal irinotecan instead of irinotecan was thus expectable.

For the skilled person it would thus have been obvious to at least investigate the effect of the use of liposomal irinotecan instead of irinotecan.

An inventive step can therefore presently not be acknowledged for the claimed subject-matter.

4. *Industrial Applicability* (**Art. 33(4) PCT**)

The patentability can be dependent upon the formulation of the claims.

Patentability, in particular novelty and inventive step, of claims 1-27 has been assessed on the basis of a purpose-limited product claim taking into account the alleged effects of the compounds used in combination.

SECTION VI

5. **D8** to **D10** were published after the priority date but before the filing date of the present application:

D8 discloses combinations of liposomal irinotecan, oxaliplatin, leucovorin and 5-fluorouracil in treating metastatic adenocarcinoma of the pancreas in a human patient.

D9 relates to liposomal irinotecan, also in combination with 5-fluoruracil and leucovorin. The usefulness for the treatment of gastric cancer is disclosed.

D10 discloses the usefulness of a combination of liposomal irinotecan, 5-fluoruracil and leucovorin for the treatment of colorectal cancer. Liposomal irinotecan was also investigated in gastric cancer.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
P071520WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB2017/053293	1 November 2017 (01-11-2017)	2 November 2016 (02-11-2016)
Applicant		
IPSEN BIOPHARM LTD		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [ ] the text is approved as submitted by the applicant
   [X] the text has been established by this Authority to read as follows:

   TREATING GASTRIC CANCER USING COMBINATION THERAPIES COMPRISING LIPOSOMAL IRINOTECAN, OXALIPLATIN, 5-FLUORURACIL (AND LEUCOVORIN)

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/053293

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61P35/00 A61K31/4453 A61K31/4178 A61K31/513 A61K31/519 A61K9/127
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MATTEO DALLA CHIESA ET AL: "Sequential chemotherapy with dose-dense docetaxel, cisplatin, folinic acid and 5-fluorouracil (TCF-dd) followed by combination of oxaliplatin, folinic acid, 5-fluorouracil and irinotecan (COFFI) in metastatic gastric cancer: results of a phase II trial", CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 67, no. 1, 5 March 2010 (2010-03-05), pages 41-48, XP019855948, ISSN: 1432-0843, DOI: 10.1007/S00280-010-1281-5 the whole document, in particular the abstract 'Patients and Methods' 'Results' 'Discussion' -/--	1-27

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 January 2018	02/02/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hornich-Paraf, E

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STEFAN PEINERT ET AL: "Safety and efficacy of weekly 5-fluorouracil/ folinic acid/oxaliplatin/irinotecan in the first-line treatment of gastrointestinal cancer", THERAPEUTIC ADVANCES IN MEDICAL ONCOLOGYENGLANDJAN 2016, vol. 2, no. 3, 1 May 2010 (2010-05-01), pages 161-174, XP055443480, UK ISSN: 1758-8340, DOI: 10.1177/1758834010365061 the whole document, in particular the abstract 'Patients and Methods' 'Results' 'Discussion'	1-27
Y	Chung-Tzu Hsueh: "Nanovectors for anti-cancer drug delivery in the treatment of advanced pancreatic adenocarcinoma", , 21 August 2016 (2016-08-21), XP055444354, Retrieved from the Internet: URL:https://www.wjgnet.com/1007-9327/full/v22/i31/7080.htm [retrieved on 2018-01-25] the whole document, in particular the abstract page 7081, column 2, paragraph 1 Table 1, last entry 'Nanoliposomal irinotecan (NAL-IRI, PEP02, MM-398)': page 7084 et seqq., in particular page 7085, column 1, paragraph 2 - page 7086, column 1, paragraph 1	1-27
Y	ANDREW KO: "Nanomedicine developments in the treatment of metastatic pancreatic cancer: focus on nanoliposomal irinotecan", INTERNATIONAL JOURNAL OF NANOMEDICINE, 1 March 2016 (2016-03-01), page 1225, XP055443469, AUCKLAND, NZ ISSN: 1176-9114, DOI: 10.2147/IJN.S88084 the whole document, in particular table 1 page 1233, column 2, paragraph 2	1-27

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1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/138371 A1 (MERRIMACK PHARMACEUTICALS INC [US]) 19 September 2013 (2013-09-19) in particular page 3, line 10 - line 15 page 5, line 11 - line 15 page 10, line 25 - page 11, line 2 page 11, line 28 - page 12, line 9 Claims, in particular claims 43-45, 72, 73, 97-99	1-27
A	WO 2013/188586 A1 (MERRIMACK PHARMACEUTICALS INC [US]) 19 December 2013 (2013-12-19) the whole document, in particular Background Summary IV. Administration VII. Treatment Protocols examples 5, 6 Claims	1-27
A	WO 2011/066684 A1 (JIANGSU HENGRUI MEDICINE CO [CN]; SHANGHAI HENGRUI PHARMACEUTICAL CO L) 9 June 2011 (2011-06-09) the whole document, in particular also the claims	1-27
X,P	WO 2017/034957 A1 (MERRIMACK PHARMACEUTICALS INC [US]) 2 March 2017 (2017-03-02) the whole document	1-27
Y,P	WO 2017/031442 A1 (MERRIMACK PHARMACEUTICALS INC [US]) 23 February 2017 (2017-02-23) in particular paragraph [0005] paragraph [0077] paragraphs [0114], [0115] example 8	1-27
Y,P	WO 2017/172678 A1 (MERRIMACK PHARMACEUTICALS INC [US]) 5 October 2017 (2017-10-05) the whole document, in particular Background Summary V. Administration VII. Treatment Protocols examples 5-7 examples 9-15 Claims	1-27

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2017/053293

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013138371 A1	19-09-2013	AU 2013201584 A1	26-09-2013
		WO 2013138371 A1	19-09-2013
WO 2013188586 A1	19-12-2013	AU 2013202947 A1	16-01-2014
		AU 2013274287 A1	29-01-2015
		CA 2875824 A1	19-12-2013
		CN 104717961 A	17-06-2015
		DK 2861210 T3	24-07-2017
		EP 2861210 A1	22-04-2015
		EP 3266456 A1	10-01-2018
		ES 2632915 T3	18-09-2017
		HK 1209627 A1	08-04-2016
		JP 2015523355 A	13-08-2015
		JP 2017149783 A	31-08-2017
		KR 20150021565 A	02-03-2015
		KR 20170104638 A	15-09-2017
		NZ 702469 A	25-08-2017
		PL 2861210 T3	29-12-2017
		PT 2861210 T	26-07-2017
		RU 2015100529 A	10-08-2016
		SI 2861210 T1	30-10-2017
		TW 201412345 A	01-04-2014
		US 2015182521 A1	02-07-2015
		US 2015328156 A1	19-11-2015
		US 2015374682 A1	31-12-2015
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		US 2016228428 A1	11-08-2016
		US 2017065578 A1	09-03-2017
		US 2017368056 A1	28-12-2017
		WO 2013188586 A1	19-12-2013
		ZA 201408804 B	28-06-2017
WO 2011066684 A1	09-06-2011	AU 2009356132 A1	21-06-2012
		BR 112012012151 A2	12-04-2016
		CA 2782911 A1	09-06-2011
		CN 102271659 A	07-12-2011
		CY 1116811 T1	15-03-2017
		DK 2508170 T3	21-09-2015
		EP 2508170 A1	10-10-2012
		ES 2547698 T3	08-10-2015
		HK 1159482 A1	17-04-2014
		HR P20150911 T1	23-10-2015
		HU E027467 T2	28-10-2016
		JP 5645954 B2	24-12-2014
		JP 2013512262 A	11-04-2013
		KR 20120089754 A	13-08-2012
		KR 20160140992 A	07-12-2016
		PT 2508170 E	16-10-2015
		RU 2012123875 A	20-01-2014
		SI 2508170 T1	31-12-2015
		SM T201500245 B	30-10-2015
		US 2012282325 A1	08-11-2012
		US 2017189392 A1	06-07-2017
		WO 2011066684 A1	09-06-2011
		ZA 201203316 B	27-11-2013
WO 2017034957 A1	02-03-2017	TW 201717933 A	01-06-2017
		US 2017049775 A1	23-02-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		WO 2017034957 A1	02-03-2017
WO 2017031442 A1	23-02-2017	TW 201713312 A	16-04-2017
		US 2017049767 A1	23-02-2017
		WO 2017031442 A1	23-02-2017
		WO 2017031445 A1	23-02-2017
WO 2017172678 A1	05-10-2017	NONE	

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB2017/053293	01.11.2017	02.11.2016

International Patent Classification (IPC) or both national classification and IPC
INV. A61P35/00 A61K31/4453 A61K31/4178 A61K31/513 A61K31/519 A61K9/127

Applicant
IPSEN BIOPHARM LTD

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☒ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Hornich-Paraf, E Telephone No. +49 89 2399-0

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-27
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-27
Industrial applicability (IA)	Yes:	Claims	1-27
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rules 43*bis*.1 and 70.10)

and / or

2. Non-written disclosures (Rules 43*bis*.1 and 70.9)

see form 210

SECTION V

1. *Cited documents*:

D1 MATTEO DALLA CHIESA ET AL: "Sequential chemotherapy with dose-dense docetaxel, cisplatin, folinic acid and 5-fluorouracil (TCF-dd) followed by combination of oxaliplatin, folinic acid, 5-fluorouracil and irinotecan (COFFI) in metastatic gastric cancer: results of a phase II trial", CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 67, no. 1, 5 March 2010 (2010-03-05), pages 41-48, ISSN: 1432-0843, DOI: 10.1007/S00280-010-1281-5

D2 STEFAN PEINERT ET AL: "Safety and efficacy of weekly 5-fluorouracil/ folinic acid/oxaliplatin/ irinotecan in the first-line treatment of gastrointestinal cancer", THERAPEUTIC ADVANCES IN MEDICAL ONCOLOGYENGLANDJAN 2016, vol. 2, no. 3, 1 May 2010 (2010-05-01), pages 161-174, UK ISSN: 1758-8340, DOI: 10.1177/1758834010365061

D3 Chung-Tzu Hsueh: "Nanovectors for anti-cancer drug delivery in the treatment of advanced pancreatic adenocarcinoma", 21 August 2016 (2016-08-21), Retrieved from the Internet: URL:https://www.wjgnet.com/1007-9327/full/v22/i31/7080.htm [retrieved on 2018-01-25]

D4 ANDREW KO: "Nanomedicine developments in the treatment of metastatic pancreatic cancer: focus on nanoliposomal irinotecan", INTERNATIONAL JOURNAL OF NANOMEDICINE, 1 March 2016 (2016-03-01), page 1225, AUCKLAND, NZ, ISSN: 1176-9114, DOI: 10.2147/IJN.S88084

D5 WO 2013/138371 A1

D6 WO 2013/188586 A1

D7 WO 2011/066684 A1

D8 WO 2017/034957 A1

D9 WO 2017/031442 A1

D10 WO 2017/172678 A1

Reference is made to the passages cited in the Search Report.

D1 reports on a trial investigating the combination of irinotecan, oxaliplatin, 5-fluoruracil and leucovorin (COFFI) in metastatic gastric cancer. It appears that *liposomal irinotecan* is *not* disclosed. Administration was once every two weeks.

D2 also reports on a trial on the safety and efficacy of weekly irinotecan, oxaliplatin, 5-fluoruracil and leucovorin (FUFOXIRI) in gastric cancer. Again, the document does *not* refer to *liposomal irinotecan*.

D3 reports on the usefulness of liposomal irinotecan (nal-irinotecan), 5-fluoruracil and leucovorin for the treatment of pancreas adenocarcinoma. The document also refers to a study on adding oxaliplatin to nal-IRI/LV/5-FU as 1-line treatment in metastatic pancreatic adenocarcinoma (NCT02551991). It appears that *gastric cancer* is *not* mentioned.

D4 in table 1 reports as well on the ongoing trial of nal-irinotecan with 5-fluoruracil, leucovorin and oxaliplatin in pancreatic adenocarcinoma (NCT02551991).

D5 reports on the usefulness of combination therapies i.a. including folinic acid (leucovorin), 5-fluorouracil, irinotecan and oxaliplatin (FOLFIRINOX) for the treatment of various cancers, i.a. pancreatic cancer. It is also disclosed that nanoliposomal irinotecan, MM-398 is investigated i.a. for gastric cancer.

D6 relates to the co-administration of liposomal irinotecan (MM-398) with 5-fluoruracil and leucovorin for the treatment of pancreatic cancer. Example 5 relates to phase I and II clinical studies of MM-398 in patients suffering from gastric cancer.

D7 relates to liposomes of irinotecan.

for **D8** to **D10** reference is made to **SECTION VI** of this report.

2. *Novelty* (**Art. 33(2) PCT**)

It appears that none of the cited prior art documents discloses the usefulness of liposomal irinotecan, oxaliplatin, leucovorin and 5-fluoruracil in the defined amounts for the treatment of gastric cancer. The claimed subject-matter seems therefore *novel* in the light of the available prior art.

3. *Inventive step* (**Art. 33(3) PCT**)

The usefulness of FOLFIRINOX / FOLFOXIRI / COFFI (irinotecan + oxaliplatin + leucovorin + 5-fluoruracil) for the treatment of gastric cancer is known from **D1** or **D2**.

The difference of the present application to these known regimes is obviously the use of *liposomal* irinotecan instead of the irinotecan.

It is known from the prior art that liposomal irinotecan has improved properties as to efficacy and toxicity compared to non-liposomal irinotecan (e.g. **D3**, **D4** or **D7**). Combinations of leucovorin + 5-fluoruracil with liposomal irinotecan have already been described as useful for the treatment of pancreatic adenocarcinoma (see e.g. **D3** or **D4**); in these documents reference is also made to an 'ongoing' clinical trial investigating the further addition of oxaliplatin to this combination, also for the treatment of pancreatic adenocarcinoma.

Any seen improved effect in the use of liposomal irinotecan instead of irinotecan was thus expectable.

For the skilled person it would thus have been obvious to at least investigate the effect of the use of liposomal irinotecan instead of irinotecan.

An inventive step can therefore presently not be acknowledged for the claimed subject-matter.

4. *Industrial Applicability* (**Art. 33(4) PCT**)

The patentability can be dependent upon the formulation of the claims.

Patentability, in particular novelty and inventive step, of claims 1-27 has been assessed on the basis of a purpose-limited product claim taking into account the alleged effects of the compounds used in combination.

SECTION VI

5. **D8** to **D10** were published after the priority date but before the filing date of the present application:

D8 discloses combinations of liposomal irinotecan, oxaliplatin, leucovorin and 5-fluorouracil in treating metastatic adenocarcinoma of the pancreas in a human patient.

D9 relates to liposomal irinotecan, also in combination with 5-fluoruracil and leucovorin. The usefulness for the treatment of gastric cancer is disclosed.

D10 discloses the usefulness of a combination of liposomal irinotecan, 5-fluoruracil and leucovorin for the treatment of colorectal cancer. Liposomal irinotecan was also investigated in gastric cancer.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
LISA A. HAILE
DLA PIPER RUDNICK GRAY CARY US LLP
4365 EXECUTIVE DRIVE, SUITE 1100
SAN DIEGO, CA 92121-2133

PATENT DOCKETING
AUG 22 2005
DLA PIPER

RECEIVED
AUG 22 2005

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference HERM1130WO	Date of Mailing (*day/month/year*) 18 AUG 2005 FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US05/15349	International filing date (*day/month/year*) 02 May 2005 (02.05.2005)
Applicant HERMES BIOSCIENCES, INC.	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 740.14.35

   For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminder

   Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90 *bis*.1 and 90 *bis*.3, respectively, before the completion of the technical preparations for international publication.

   Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Gollamudi S. Kishore, Ph.D Telephone No. 703 308 1234

Form PCT/ISA/220 (April 2002)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
LISA A. HAILE
DLA PIPER RUDNICK GRAY CARY US LLP
4365 EXECUTIVE DRIVE, SUITE 1100
SAN DIEGO, CA 92121-2133

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing (*day/month/year*) 18 AUG 2005
Applicant's or agent's file reference HERM1130WO	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US05/15349	International filing date (*day/month/year*) 02 May 2005 (02.05.2005)
Applicant HERMES BIOSCIENCES, INC.	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 740.14.35

   For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90 *bis*.1 and 90 *bis*.3, respectively, before the completion of the technical preparations for international publication.

   Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Gollamudi S. Kishore, Ph.D Telephone No. 703 308 1234

Form PCT/ISA/220 (April 2002)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference HERM1130WO	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US05/15349	International filing date (*day/month/year*) 02 May 2005 (02.05.2005)	(Earliest) Priority Date (*day/month/year*) 03 May 2004 (03.05.2004)
Applicant HERMES BIOSCIENCES, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of (4) PCT/US05/15349PCT/US05/15349 ☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the Report**
   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. ☒ **Certain claims were found unsearchable** (See Box I).
3. ☐ **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ____
   ☐ as suggested by the applicant. ☒ None of the figures
   ☐ because the applicant failed to suggest a figure.
   ☐ because this figure better characterizes the invention.

Form PCT/ISA/210 (first sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/15349

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 23-39,54-56,63-78,92-142 and 156-159
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US05/15349

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 9/127
US CL : 424/450
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,785,987 A (HOPE et al) 28 July 1998 (28.07.1998) col. 4, line 50 through col. 5, line 36, col. 9, line 26 through col. 14, line 60 and Examples.	1-7, 9-12, 40-42, 57-62, 143-148 and 153
---		---
Y		8, 13-22, 43-53, 149-152 and 154-155
X	US 6,110,491 A (KIRPOTIN) 29 August 2000 (29.08.2000) col. 6, line 18 and Examples.	40-42 and 58-62
---		---
Y		1-22, 43-51, 57, 79-91 and 149-152

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 July 2005 (27.07.2005)	18 AUG 2005
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner of Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Gollamudi S. Kishore, Ph.D Telephone No. 703 308 1234

Form PCT/ISA/210 (second sheet) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
West:
Search terms: liposome, triethylammonium salt, trimethylammonium salt, ammonium salt

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
LISA A. HAILE
DLA PIPER RUDNICK GRAY CARY US LLP
4365 EXECUTIVE DRIVE, SUITE 1100
SAN DIEGO, CA 92121-2133

FIL.
PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing (*day/month/year*)	18 AUG 2006
Applicant's or agent's file reference HERM1130WO	FOR FURTHER ACTION See paragraph 2 below

International application No.	International filing date (*day/month/year*)	Priority date (*day/month/year*)
PCT/US05/15349	02 May 2005 (02.05.2005)	03 May 2004 (03.05.2004)

International Patent Classification (IPC) or both national classification and IPC

IPC(7): A61K 9/127 and US Cl.: 424/450

Applicant

HERMES BIOSCIENCES, INC.

1. This opinion contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(*b*) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Gollamudi S. Kishore, Ph.D Telephone No. 703 308 1234

Form PCT/ISA/237 (cover sheet) (January 2004)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US05/15349

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ This opinion has been established on the basis of a translation from the original language into the following language _____, which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

☐ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☐ in written format

☐ in computer readable form

c. time of filing/furnishing

☐ contained in international application as filed.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US05/15549

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos. 23-39,54-56,63-78,92-142 and 156-159

because:

☐ the said international application, or the said claim Nos. _____ relate to the following subject matter which does not require an international preliminary examination *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 23-39,54-56,63-78,92-142 and 156-159 are so unclear that no meaningful opinion could be formed *(specify)*:

Said claims do not conform to PCT Article 6.4 (a) since these are multiple dependent claims dependent on multiple dependent claims.

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _____

☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:

the written form
- ☐ has not been furnished
- ☐ does not comply with the standard

the computer readable form
- ☐ has not been furnished
- ☐ does not comply with the standard

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See Supplemental Box for further details.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No. PCT/US05/15349

Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims 8, 13-22, 43-56, 79-91, 149-154	YES
	Claims 1-7, 9-12, 40-42, 57-62, 143-148 and 155	NO
Inventive step (IS)	Claims NONE	YES
	Claims 1-22, 40-53, 57-62, 79-91 and 143-155	NO
Industrial applicability (IA)	Claims 1-22, 40-53, 57-62, 79-91 and 143-155	YES
	Claims NONE	NO

2. Citations and explanations:

Please See Continuation Sheet

Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

V. 2. Citations and Explanations:

Claims 1-7, 9-12, 40-42, 57-62, 143-148 and 153 lack novelty under PCT Article 33(2) as being anticipated by HOPE et al (US 5,785,987).

HOPE et al disclose a method of loading liposomes with a variety of active agents such as anti-cancer agents and antibioitics using methylammonium and ethylene diammonium salts. The gradient is pH gradient. The concentration of the methylammonium salt which is encapsulated varies from 50 mM to 1M. After loading, the the ammonium salt is removed from the external medium (abstract, col. 4, line 50 through col. 5, line 36, col. 9, line 26 through col. 14, line 60, Examples). Instant method of preparation claim recites 'pre-entity' and 'entity' without reciting specific compounds. Since the compound loaded in HOPE et al is a protonatable compound and once it enters the interior of the liposome and protonated differs from the loaded compound, the unprotonated compound and protonated compound are deemed to be pre-entity and entity respectively.

Claims 40-42 and 58-62 lack novelty under PCT Article 33(2) as being anticipated by KIRPOTIN et al.

KIRPOTIN discloses liposomal compositions containing anti-neoplastic agents in the claimed ratios. The anti-neoplastic agents taught are vincristine, vinblastine and vinorelbine (col. 6, line 18 and Examples). The burden is upon applicant to show that 24 hours after the administration, the liposomes of KIRPOTIN do not retain instant amounts of the active compounds.

Claims 43-47, 52-53, 143-155 lack an inventive step under PCT Article 33(3) as being obvious over HOPE et al (US 5,785,987).

The teachings of HOPE et al have been discussed above. What is lacking in HOPE et al is the teaching of the use of prodrug. However, since the principle of loading is the same, it would have been obvious to one of ordinary skill in the art to load any drug including prodrugs with a reasonable expectation of success.

Claims 43-51, 57 and 79-91 lack an inventive step under PCT Article 33(3) as being obvious over KIRPOTIN (US 6,110,491).

The teachings of KIRPOTIN have been discussed above. what is lacking in KIRPOTIN is the teaching of the use of a prodrug. However, since the principle of loading is the same, it would have been obvious to one of ordinary skill in the art to load any drug including prodrugs with a reasonable expectation of success. Although KIRPOTIN's studies do not include rat model, in the absence of showing the criticality, it is deemed obvious to one of ordinary skill in the art to use any mammal with the expectation of obtaining at least similar results. KIRPOTIN does not express the lecithin and cholesterol in molar amounts. Assuming that they are different, it is deemed obvious to one of ordinary skill in the art to vary the amounts of the lipids to obtain the best possible results.

Claims 1-22, 48-51, 149-152 lack an inventive step under PCT Article 33(3) as being obvious over HOPE et al (US 5,785,987) in view of KIRPOTIN (US 6,110,491).

As pointed out above, HOPE et al disclose a method of loading liposomes with a variety of active agents such as anti-cancer agents and antibioitics using methylammonium and ethylene diammonium salts. What is lacking in HOPE et al is the encapsulation of anion such as a polyol with a anionic functional group such as sulfate groups.

KIRPOTIN while disclosing a method of loading of ionizable active agents using ammonium gradients teaches that ther presence of acidic compounds such as polysaccharide sulfate (chondroitin sulfate, heparin etc) increases the concentration of encapsulated compound by several fold. The loadable compounds include ionic taxanes, vinca derivatives and others (abstract, col. 5, line 9 through col. 7, line 29, col. 8, lines 45-50, and Examples).

The use of polyols with anionic functional groups in the active agent loading method taught by HOPE et al would have been

International application No.
PCT/US05/15349

Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

obvious to one of ordinary skill in the art since KIRPOTIN teaches that these anions increase the amount of the loaded compound in the liposomes. The use of taxanes as the active agents with a reasonable expectation of success would have been obvious to one of ordinary skill in the art since KIRPOTIN teaches that taxanes can be loaded using the gradient method disclosed.
Claims 1-22, 40-53, 57-62, 79-91 and 143-155 meet the criteria set out in PCT Article 33(4), and thus meet industrial applicability because the subject matter claimed can be made or used in industry.

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article," "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended ?

Under Article 19, only the claims may be amended

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Preliminary Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When ? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments ?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How ? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments ?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;
(ii) the claim is cancelled;
(iii) the claim is new;
(iv) the claim replaces one or more claims as filed;
(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under Article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing, the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see the *PCT Applicant's Guide*, Volume II.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 239669-389731	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2016/027515	International filing date *(day/month/year)* 14 April 2016 (14.04.2016)	Priority date *(day/month/year)* 14 April 2015 (14.04.2015)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant IPSEN BIOPHARM LTD.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☒	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 17 October 2017 (17.10.2017)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Agnès Wittmann-Regis e-mail: pct.team6@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2016/027515	14.04.2016	14.04.2015

International Patent Classification (IPC) or both national classification and IPC
INV. A61K9/00 A61K39/395 A61K31/4745 A61K31/513 A61K31/519 A61K45/06 A61P35/00 A61P35/02

Applicant
MERRIMACK PHARMACEUTICALS, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☒ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Fax: +31 70 340 - 3016	see form PCT/ISA/210	Taylor, Mark Telephone No. +31 70 340-0

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-62
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-62
Industrial applicability (IA)	Yes:	Claims	1-62
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rules 43*bis*.1 and 70.10)

and / or

2. Non-written disclosures (Rules 43*bis*.1 and 70.9)

see form 210

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Cited Prior Art

D1 *Cancer Medicine* 2016, **5**(4), 676

D2 *The Oncologist* 2012, **17**(12), 1486

D3 *J. Cancer Ther.* 2011, **2**(4), 470

D4 WO 2013/188586

D5 WO 2004/093795

Section V

1 Claims 1, 3-25 and 35-62 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 39.1(iv) / 67.1(iv) PCT. Patentability of such claims can be dependent upon the formulation of the claims. The EPO, for example, does not recognise as patentable claims to the use of a compound in medical treatment, but may allow claims to a product, in particular substances or compositions for use in a first or further medical treatment.

The patentability of claims 1, 3-25 and 35-62, in particular novelty and inventive step, has thus been assessed on the basis of a purpose-limited product claim taking into account the alleged effects of the compound/ composition.

2 Claims 2-6, 25 and 35 lack novelty *a priori* (Art. 33(2) PCT).

The wording of claim 2 does not include any medical indication and thus seeks protection for a sustained release injectable form of a drug which is suitable for use in combination with a second amount of the drug in an immediate release form. Sustained release formulations of drugs were already known at the date of filing, *e.g.* liposomal irinotecan (*cf.* paragraph [0008] of the application) which were suitable for use with a second amount of the drug in an immediate release form.

Thus claim 2, and its dependent claims 3-6, 25 and 35, lack novelty within the meaning of Art. 33(2) PCT.

3 The subject-matter of claims 1, 7-25, 26-34 and 36-62 would appear to meet the requirements of Art. 33(2) PCT. However, attention is drawn to the disclosures of **D1** which may become relevant for the question of novelty should the application not be entitled to its claimed priority.

4 The subject-matter of claims 1-62 does not meet the requirements of Art. 33 (2) PCT.

4.1 Not being novel, the subject-matter of claims 2-6, 25 and 35 cannot be seen as being inventive.

4.2 The subject-matter of claims 1, 7-25, 26-34 and 36-62 lacks an inventive step.

4.3 The **problem** to be solved in the present application is the provision of compositions (as well as formulations and kits) for use in the treatment of cancer, especially colorectal cancer, which treatment results in an improved therapy, particularly in the case of colorectal cancers which are resistant to current therapeutic modalities (*cf.* paragraph [0002]). In particular, 'improved' therapeutic index, pharmacokinetics or synergy are sought (*cf.* paragraph [0003]).

4.4 The **solution** provided in the application lies in the combination of a delayed-release formulation of a drug, especially liposomal Irinotecan, and a free form (i.e. having no delayed release profile) of the same drug, especially free irinotecan (*cf.* claims 1, 3, 7, 18, 34, 36 and 43). Further anti-cancer agents, Leucovorin, 5-FU and Bevacizumab may also be included (*cf.* claim 26).

4.5 The **closest prior art** document may be considered to be any of **D2-D5**.

These documents disclose combinations of delayed-release Irinotecan, Leucovorin and 5-FU (and optionally Bevacizumab) for use in the treatment of colorectal cancer. Indeed, the present applicants acknowledge in D1 that, '*The FOLFIRI regimen, combination of irinotecan with leucovorin (LV) and 5-fluorouracil (5-FU; LV/5-FU) is a standard regimen in first-line or second-line therapy of metastatic colorectal cancer (mCRC)*'.

4.6 The **difference** between the teachings of the closest prior art and the present application lie in the inclusion of a delayed release form of the drug, *e.g.* liposomal Irinotecan (MM3-98) in addition to the immediate release form of the same drug.

4.7 However, the application does not demonstrate any technical effect resulting from the combination of immediate- and delayed-release forms of the same drug. Example 1 discloses pre-clinical pharmacokinetics of MM-398 in murine models of colorectal cancer. Example 2 is a description of a, '*Phase I study of MM-398 plus irinotecan in unresectable advanced cancer*', *i.e.* a trial proposed to investigate the theory that the combination of immediate- and delayed-release forms of irinotecan has advantageous effects and solves the technical problem posed in the application. Examples of the prophetic nature of this part of the application lie, for example, at: [0151] - '*This study **will enroll** ...*'; and [0280] - '*The Investigator **will attempt** ...*'.

First, this would therefore appear to represent a scientific theory proposed to investigate the theory that the combination of immediate- and delayed-release forms of Irinotecan solve the technical problem posed in the application. Scientific theories are excluded from patentability in many jurisdiction.

Secondly, Example 2 does not demonstrate that the underlying technical problem has been solved, nor does it show make any comparison with the closest prior art, *viz.* the FOLFIRI regimen.

Thirdly, even if the results of this test were to demonstrate an advantageous or unexpected effect when compared to the FOLFIRI regimen, the use of a combination of immediate- and delayed-release forms of Irinotecan in unresectable advanced colorectal cancer does not justify the scope of protection sought, *i.e.* the combination of immediate- and delayed-release forms of any drug in the treatment of any cancer. Indeed, at a number of junctures, the application mentions synergy and an improvement of synergy. However, in order for a synergistic effect to be seen as credible proof of an inventive step, it must be demonstrated to be present over the whole scope of subject-matter for which protection is sought.

In view of the foregoing, the application lacks an inventive step (Art. 33(3) PCT).

Section VI

5 **D1** cited under Rules 64.3 and 70.10 PCT.

Section VIII

6 The claims do not meet the requirements of Art. 6 PCT.

6.1 Claims 1 and 2 mention injectable dosage forms whereas later claims (*e.g.* claim 7) do not. The subject-matter of the claims is therefore unclear as to whether this is an essential technical feature or not. Moreover, objections as to a lack of unity of invention may arise during the Regional/National phase(s).

6.2 There are too many independent claims and some of these could be made dependent upon other claims (Rule 6.4 PCT). For example, claim 18 could be made dependent upon earlier claims.

6.3 The subject-matter of claims 35, 36, 42 and 43 is unclear in view of the expression, '*improving*'. This expression is vague and indefinite, and does not provide any indication of a baseline relative to which treatment might be seen to be improved, nor is any indication provided of how this should be measured.

6.4 The subject-matter of claims 49, 50 and 53 is unclear in view of the expressions, '*analogs*' and '*derivatives*'. This expression is vague and indefinite and does not allow the skilled person to determine the scope of protection sought.

6.5 Claims 35, 36, 42 and 43 do not meet the requirements of Art. 6 PCT because the matter for which protection is sought is not clearly defined. These claims attempt to define the subject-matter in terms of the result to be achieved, which merely amounts to a statement of the underlying problem (*cf.* [0003]), without providing the technical features necessary for achieving this result.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
239669-389731		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2016/027515	14 April 2016 (14-04-2016)	14 April 2015 (14-04-2015)
Applicant		
MERRIMACK PHARMACEUTICALS, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [ ] the text is approved as submitted by the applicant
   [X] the text has been established by this Authority to read as follows:

   COMPOSITIONS FOR IMPROVING THE PHARMACOKINETICS AND THERAPEUTIC INDEX OF CANCER TREATMENT

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/027515

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/00 A61K39/395 A61K31/4745 A61K31/513 A61K31/519
A61K45/06 A61P35/00 A61P35/02
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	BENOIST CHIBAUDEL ET AL: "PEPCOL: a GERCOR randomized phase II study of nanoliposomal irinotecan PEP02 (MM-398) or irinotecan with leucovorin/5-fluorouracil as second-line therapy in metastatic colorectal cancer", CANCER MEDICINE, vol. 5, no. 4, 24 January 2016 (2016-01-24), pages 676-683, XP055280612, GB ISSN: 2045-7634, DOI: 10.1002/cam4.635 the whole document ----- -/--	1-62

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 June 2016	27/06/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Taylor, Mark

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. C. BENDELL ET AL: "Treatment Patterns and Clinical Outcomes in Patients With Metastatic Colorectal Cancer Initially Treated with FOLFOX-Bevacizumab or FOLFIRI-Bevacizumab: Results From ARIES, a Bevacizumab Observational Cohort Study", THE ONCOLOGIST, vol. 17, no. 12, 26 September 2012 (2012-09-26), pages 1486-1495, XP055280593, US ISSN: 1083-7159, DOI: 10.1634/theoncologist.2012-0190 the whole document --- XIJIAN ZHOU ET AL: "Clinical Analysis of Bevacizumab Plus FOLFIRI Regimen as Front-Line Therapy for Chinese Patients with Advanced Colorectal Cancer", JOURNAL OF CANCER THERAPY, vol. 02, no. 04, 1 January 2011 (2011-01-01), pages 470-474, XP055280602, ISSN: 2151-1934, DOI: 10.4236/jct.2011.24063 the whole document --- WO 2013/188586 A1 (MERRIMACK PHARMACEUTICALS INC [US]) 19 December 2013 (2013-12-19) abstract page 3, line 4 - page 6, line 14 page 8, line 23 - page 9, line 21 page 11, line 18 - page 12, line 5 page 12, line 6 - line 14 page 13, line 1 - page 14, line 10 examples 1-7 claims 1-27 --- WO 2004/093795 A2 (CELATOR TECHNOLOGIES INC [CA]; TARDI PAUL [CA]; HARASYM TROY [CA]; WEB) 4 November 2004 (2004-11-04) abstract paragraph [0002] paragraph [0008] examples 1-27 claims 1-24 ---	1-62 1-62 1-62 1-62

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/027515

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013188586 A1	19-12-2013	AU 2013202947 A1	16-01-2014
		AU 2013274287 A1	29-01-2015
		CA 2875824 A1	19-12-2013
		CN 104717961 A	17-06-2015
		EP 2861210 A1	22-04-2015
		HK 1209627 A1	08-04-2016
		JP 2015523355 A	13-08-2015
		KR 20150021565 A	02-03-2015
		TW 201412345 A	01-04-2014
		US 2015182521 A1	02-07-2015
		US 2015328156 A1	19-11-2015
		US 2015374682 A1	31-12-2015
		US 2016074382 A1	17-03-2016
		WO 2013188586 A1	19-12-2013
WO 2004093795 A2	04-11-2004	AU 2004231977 A1	04-11-2004
		CA 2522662 A1	04-11-2004
		EP 1615621 A2	18-01-2006
		JP 2006523713 A	19-10-2006
		US 2004022817 A1	05-02-2004
		US 2007148255 A1	28-06-2007
		US 2007298092 A1	27-12-2007
		WO 2004093795 A2	04-11-2004

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 239669-402422	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2016/057247	International filing date *(day/month/year)* 15 October 2016 (15.10.2016)	Priority date *(day/month/year)* 16 October 2015 (16.10.2015)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant IPSEN BIOPHARM LTD.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☒	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 17 April 2018 (17.04.2018)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Agnès Wittmann-Regis e-mail: pct.team6@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2016/057247	15.10.2016	16.10.2015

International Patent Classification (IPC) or both national classification and IPC
INV. A61K9/127 A61K31/4745

Applicant
MERRIMACK PHARMACEUTICALS, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Fax: +31 70 340 - 3016	see form PCT/ISA/210	van de Wetering, P Telephone No. +31 70 340-0

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-23
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-23
Industrial applicability (IA)	Yes:	Claims	1-23
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V.

1 **Prior Art**

Reference is made to the following documents:

D1 US 8 658 203 B2 (DRUMMOND ET AL) 25 February 2014, cited in the application

D2 ZHONG Z ET AL: "Analysis of cationic liposomes by reversed-phase HPLC with evaporative light-scattering detection", JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS, vol. 51, no. 4, 11 March 2010, pages 947-951, XP026813988

D3 AWA DICKO ET AL: "Intra and Inter-Molecular Interactions Dictate the Aggregation State of Irinotecan Co-Encapsulated with Floxuridine Inside Liposomes", PHARMACEUTICAL RESEARCH, vol. 25, no. 7, 5 March 2008, pages 1702-1713, XP019613128

2 **Inventive step (Article 33(3) PCT)**

The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 1-23 does not involve an inventive step in the sense of Article 33(3) PCT.

2.1 D1, which is considered to represent the most relevant state of the art, discloses (examples 11,13-15, 17, 82; table 11; column 22, lines 38-47; column 25, lines 9-21; column 26, lines 37-64): liposomal irinotecan compositions, wherein the liposomes are comprised of DSPC, cholesterol and PEG-DSPE in a molar ratio of 3:2:0.015; and wherein the active is entrapped in the form of irinotecan-sucrose octasulfate. The drug/lipid ratio input ratio was 0.15-0.55 g drug/mmol lipid of which more than 95% typically 98-100% was encapsulated. The drug concentration was adjusted to be in the range of 2.0-4.0 mg/ml. Example 82 discloses liposomes with a size of 112(+/-16) nm and in a buffer of pH 7.0.

The subject-matter of claim 1 differs from this composition in that the irinotecan moiety per ml of the composition is 4.3 mg, whereas in D1 a concentration of 2.0-4.0 mg/ml is disclosed. Furthermore D1 is silent on the

formation of lyso-PC during storage, although it is mentioned that the liposomes are very stable (example 17, column 56, lines 64-65). In view of the same excipients forming the liposome, it can be expected that the liposome stability is very similar as well.

The technical effect brought by these differences is neither shown, nor substantiated in the description.
The underlying technical problem to be solved by the present application may be regarded as how to provide an alternative stable composition.

Staring from the stable irinotecan-containing liposomes of D1, the skilled person would consider to change the concentration of the active compound, because varying the amounts of excipients and active in order to prepare pharmaceutical compositions is within the routine work of a person skilled in the art.
Hence, no inventive step can be acknowledged to the subject-matter of claim 1 (Article 33(3) PCT).

2.2 The same reasoning applies, mutatis mutandis, to the subject-matter of the corresponding independent claims 2 and 3, which therefore are also considered not inventive.

2.3 Dependent claims 4-23 do not appear to contain any additional features which, in combination with the features of any claim to which they refer, meet the requirements with respect to inventive step.
The features claimed in these dependent claims are either derivable from D1 alone (e.g. process parameters (claims 3-5, 19), liposome size (claim 6), pH value (claim 8, 17), irinotecan sucrose octasulfate (claims 14, 23), diameter (claim 15, 17), stability (claims 3, 9, 10, 18, 22)), or they concern minor modifications and routine experimentation which lie in the normal practice of the skilled person, e.g. varying the amounts of excipients and active (e.g. claims 7, 11-13, 16, 20, 21) . In the absence of any indication as to a surprising technical effect being obtained by these features, the claims concerned lack an inventive step (Article 33(3) PCT).

2.4 The claims contain a number of process features which have not been shown to lead to a surprising unexpected effect over the prior art.

2.5 In addition, it is noted that the application emphasises that the pH of the composition has a positive effect on the stability of the composition, due to the prevention of the formation of lyso-PC. However, this instability phenomenon (lysis of phospholipids and its effect on the stability of the

active) has already been recognised and solved in the prior art (e.g. by fine tuning of formulation parameters e.g. pH modification, see D2), also in connection with irinotecan, see e.g. D3.

3 **Industrial Applicability (Article 33(4) PCT)**

The subject-matter of claims 1-23 is industrially applicable in the sense of Article 33(4) PCT.

Re Item VII.

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in D2, D3 is not mentioned in the description, nor are these documents identified therein.

Re Item VIII.

4 **Clarity (Article 6 PCT)**

4.1 The present set of claims comprises three independent product claims of, at first sight, overlapping nature.

Article 6 PCT requires that the claims (i.e. the set of claims) shall define the matter for which protection is sought and that they (i.e. the set of claims) shall be clear. The present set of claims makes it impossible to understand for which subject-matter protection is sought. In particular it is quite unclear if the various different product claims relate to different embodiments (i.e. to different subject-matter) or that these claims are merely attempts to define the same subject-matter in different ways.

Now, two different options appear to exist (and no other options seem possible):
(i) If the claims define the same subject-matter then, quite clearly, the claims are not only confusing but then they also lack conciseness.
(ii) If the claims define different subject-matter, then the question of unity of invention arises. Now, lack of unity only occurs when at least two inventions can be recognized, each characterized by a special technical feature. In the present case, however, not a single invention can be recognized (see Item V

above). Thus, it appears that option (i) is the most likely.
Consequently, the set of independent claims lacks clarity and conciseness; Article 6 PCT.

4.2 Claims 3, 9, 10, 18, 22 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempt to define the subject-matter in terms of the result to be achieved, which merely amounts to a statement of the underlying problem, without providing the technical features necessary for achieving this result. In case the technical features present in the claim are known, a lack of novelty arises.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
239669-402422		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2016/057247	15 October 2016 (15-10-2016)	16 October 2015 (16-10-2015)
Applicant		
MERRIMACK PHARMACEUTICALS, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/057247

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/127 A61K31/4745
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 8 658 203 B2 (DRUMMOND DARYL C [US] ET AL) 25 February 2014 (2014-02-25) cited in the application examples 11,13-15, 17, 82; table 11 column 22, lines 38-47 column 25, lines 9-21 column 26, lines 37-64 -----	1-23
Y	ZHONG Z ET AL: "Analysis of cationic liposomes by reversed-phase HPLC with evaporative light-scattering detection", JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS, NEW YORK, NY, US, vol. 51, no. 4, 11 March 2010 (2010-03-11) , pages 947-951, XP026813988, ISSN: 0731-7085 [retrieved on 2009-10-09] page 951, left-hand column, paragraph 1 ----- -/--	1-23

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 December 2016	23/12/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van de Wetering, P

1

Form PCT/ISA/210 (second sheet) (April 2005)

International application No
PCT/US2016/057247

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AWA DICKO ET AL: "Intra and Inter-Molecular Interactions Dictate the Aggregation State of Irinotecan Co-Encapsulated with Floxuridine Inside Liposomes", PHARMACEUTICAL RESEARCH, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NL, vol. 25, no. 7, 5 March 2008 (2008-03-05), pages 1702-1713, XP019613128, ISSN: 1573-904X page 1703, left-hand column, paragraph 2 -----	1-23

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/057247

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 8658203 B2	25-02-2014	US 2007110798 A1	17-05-2007
		US 2014127136 A1	08-05-2014

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Pharmaceutical Nanotechnology

pH-dependent association of SN-38 with lipid bilayers of a novel liposomal formulation

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Abstract

The aim of this study was to determine the location of SN-38 molecules in a liposomal formulation as a function of pH. Steady-state fluorescence polarization anisotropy and gel filtration studies of blank (placebo) liposomes, liposomes containing SN-38 and SN-38 solutions (in some cases suspensions) were conducted before lyophilization and after re-hydration at different pH conditions. SN-38, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA–DPH), N-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl)trimethylammonium *p*-toluenesulfonate (TMAP–DPH) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were used as fluoroprobes in the polarization anisotropy measurements. The localization of SN-38 was governed by the degree of hydrophobicity of the drug molecules. At high pH, SN-38 is in its inactive, hydrophilic form and partitioned into the water phase of the liposome suspensions. In lyophilized LE-SN38 liposomes re-hydrated with low pH buffer, SN-38 was found at the water–lipid interface of the bilayer.

Keywords: SN-38; Liposomes; Lyophilization; Bilayer; Interaction; Fluorescence; Anisotropy

1. Introduction

SN-38 is an active metabolite of irinotecan, a derivative of camptothecin (CPT-11) that inhibits the activity of topoisomerase I (NeoPharm Inc., Waukegan, IL). SN-38 is currently being investigated for use in the treatment of metastatic colon cancer (NeoPharm Inc., Waukegan, IL). As with other camptothecin class of compounds, SN-38 undergoes pH-dependent reversible hydrolysis of the active α-hydroxy-δ-lactone ring to form an inactive carboxylate derivative in aqueous solutions (pH ≥ 7) and plasma (Burke et al., 1992; Burke and Mi, 1993 and Fig. 1). For almost all camptothecins at 37 °C, the half-life of this reversible conversion is about 16 min in PBS and about 12 min in plasma (Burke et al., 1992; Burke and Mi, 1993; Mi and Burke, 1994). Hence, to preserve the anti-tumor activity of SN-38, it is crucial to minimize its conversion to the inactive metabolite.

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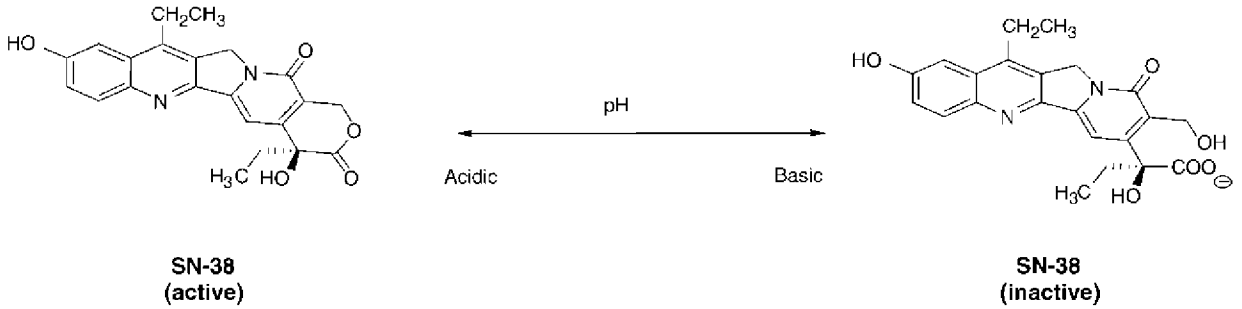

Fig. 1. pH-dependent equilibrium of SN-38 and its inactive metabolite.

SN-38 is very hydrophobic at pH < 7 (the apparent octanol/water partition coefficient ($\log P_a$) at pH 1.5 is 2.09 (unpublished data)) and hydrophilic at pH > 7 ($\log P_a = -2.49$ at pH 10.4 (unpublished data)) due to the ring-closed lactone and ring-opened carboxylate forms, respectively (Kaneda et al., 1997; Wadkins et al., 1999). Despite its hydrophobicity at low pH, SN-38 has low affinity to lipid bilayers resulting in very low drug-to-lipid entrapment in liposomal formulations (Burke et al., 1993; Wadkins et al., 1999). However, once SN-38 is entrapped within the liposomes, the pH-dependent reversible hydrolysis of the lactone ring is significantly reduced (Burke and Gao, 1994).

Recently, we reported that unilamellar liposomes of DOPC, cholesterol and cardiolipin (volume-weighted mean diameter ~150 nm) in the presence of SN-38 (LE-SN38) can be formed in high pH medium (pH > 10) (Zhang et al., 2004). At this pH, the entrapment efficiency of SN-38 is less than 10% (Zhang et al., 2004). However, upon lyophilization of the liposomes and re-hydration in acidic medium (pH < 3), SN-38 entrapment efficiency significantly increased to greater than 95%. The re-hydrated LE-SN38 liposomes were stable and showed no drug crystallization or precipitation for up to 8 h after re-hydration or after dilution in normal saline (Zhang et al., 2004).

This study aims to determine the location of SN-38 in LE-SN38 liposomes before lyophilization and after re-hydration in acidic medium by steady-state fluorescence polarization anisotropy technique using DPH derivatives and SN-38 as fluoroprobes and by gel filtration method. In aqueous medium, DPH and its derivatives partition exclusively into the lipid bilayer. The partitioning is accompanied by up to 1000-fold increase of the fluorescence of the fluoroprobes. The fluorescence polarization anisotropy of a fluoroprobe depends on the microstructure or "fluidity" of the medium (bilayer) surrounding the probe (Lentz, 1989). This makes the steady-state fluorescence anisotropy measurements one of the most sensitive methods for quantitative study of the structural order in lipid membranes in the presence or absence of drug molecules (Balasubramanian and Straubinger, 1994; Ben-Yashar and Barenholz, 1989; Bernsdorff et al., 1999; Burke et al., 1992, 1993; Lentz et al., 1976a,b; Pottel et al., 1983; Shinitzky and Barenholz, 1978). Different DPH derivatives preferentially partition and consequently probe the microstructure of different regions of the bilayer. The three fluoroprobes employed in this study, TMA–DPH, TMAP–DPH and DPH, probe the water–lipid interface, intermediate region of the bilayer and deeper region of the bilayer, respectively (Bernsdorff et al., 1999; Lentz et al., 1976a,b; Lentz, 1989). The presence of drug molecules in the lipid bilayer will affect the local order and movement of the lipid molecules. The variations in the microstructure of the lipid bilayer due to drug–lipid interactions can be detected by comparing polarization anisotropy data obtained in the presence and absence of drug molecules. The differences in polarization anisotropy between active and placebo formulations will be the most pronounced for the fluoroprobe that partitions in the same region of the bilayer where the drug molecules are located. Consequently, the polarization anisotropy measurements can reveal the approximate position of the drug molecules within the lipid bilayer.

2. Materials

SN-38 was purchased from Qventas Inc. (Newark, DE, USA). 1,2-Dioleoyl-sn-glycero-3-phosphocholine

(DOPC), cholesterol and cardiolipin were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Hydrochloric acid and sodium hydroxide were obtained from EM Science (Gibbstown, NJ, USA). Sucrose NF grade was obtained from Mallinckrodt (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA). Sodium lactate was obtained from Fisher Scientific (Fairlawn, NJ, USA). Nitrogen, NF was obtained from BOC Gases (Carol Stream, IL, USA). TMA–DPH, TMAP–DPH and DPH were purchased from Molecular Probes (Eugene, OR, USA). Sephadex G-50 beads were obtained from Sigma (St. Louis, MO, USA). All chemicals used in the fluorescence measurements were of spectroscopic grade. All chemicals were used as received.

3. Methods

3.1. SN-38 and cholesterol assays

SN-38 was quantitated according to a previously reported HPLC method (Zhang et al., 2004). Cholesterol was quantitated by an HPLC method (unpublished data). Briefly, the HPLC system consisted of an Agilent 1100 module (Wilmington, DE, USA), a quaternary pump, mobile phase degasser, auto-sampler with thermostat and a column heater compartment. Agilent software, Chemstation, was used for data acquisition and analysis. UV variable detector set at a wavelength of 205 run and Kromasil C-18 (4.6 mm × 250 mm, 5 μm) column were utilized. The mobile phase consisted of a mixture of isopropanol and acetonitrile in 75:25% v/v ratio. During the analysis, 50 μl samples were injected in duplicate into the HPLC system at mobile phase flow rate of 1 mL/min and column temperature of 40 °C.

3.2. Vesicle size measurements

Mean vesicle size was measured by dynamic light scattering technique, using Nicomp 380 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA). Prior to sample measurement, polystyrene beads of standard size were used to verify the performance of the instrument. All samples were measured in duplicate. The data were analyzed by ZW380 Application Version 1.60 software (Particle Sizing Systems, Santa Barbara, CA, USA), assuming that vesicles are spherical. The data were reported as volume-weighted Gaussian mean diameter.

3.3. Preparation of lyophilized LE-SN38 liposomes

LE-SN38 liposomes composed of DOPC: cholesterol:cardiolipin in molar ratio 50:40:10 (60 mg/mL total lipids) in 10% sucrose solution containing 2 mg/mL SN-38 at pH 10.4 were prepared by the ethanol injection method as described elsewhere (Zhang et al., 2004). Briefly, the lipid components (DOPC, cholesterol and cardiolipin) were solubilized in ethanol. The solubilized lipid mixture was diluted into an aqueous solution of SN-38 in 10% sucrose at pH 10.4. The dispersion was then extruded through polycarbonate filter membranes with pore size of 0.4, 0.2 and 0.1 μm until a mean size of 150 nm was achieved. Ethanol was removed by evaporation under vacuum. Placebo liposomes with identical lipid composition were prepared using the same procedure. The liposomes were lyophilized immediately after preparation and stored at 2–8 °C (Zhang et al., 2004).

3.4. Preparation of samples for fluorescence measurements

Pre-lyophilized liposomes (pH 10.4) and lyophilized liposomes re-hydrated with acidic buffer (10 mM sodium lactate buffer, pH 1.5) were used. These samples were further diluted with 10% sucrose (pH 10.4) and with lactate buffer (pH 1.5), respectively, to a final concentration of 0.3 mM total lipids. The equivalent concentration of SN-38 was 15.3 μM (6 μg/mL). Dynamic light scattering measurements confirmed that the liposomes were present and stable in the diluted samples for the duration of the experiments (data not shown).

Solutions/suspensions of SN-38 (15.3 μM (6 μg/mL)) in 10% sucrose (pH 10.4) and 10 mM lactate buffer (pH 1.5) were also used.

3.5. Labeling of the liposome samples with DPH derivative fluoroprobes

Fluoroprobes were not added during the preparation of the liposomes because DPH derivatives are known to partition easily into lipid bilayers (Bernsdorff

et al., 1997). Instead, the fluoroprobes were dissolved in organic solvents and added to the prepared liposomes. TMA–DPH and TMAP–DPH were dissolved in ethanol at concentration of 1 mM. DPH was dissolved in THF at concentration of 2 mM. Small volumes (between 0.4 and 0.6 μL) of the fluoroprobe stock solutions were mixed with the diluted liposomal samples from Section 3.4 in fluorometer cells. After mixing, the fluoroprobes were incubated with the liposomes at room temperature for more than 30 min. The incubation time was long enough to attain equilibrium partitioning of the probe into the lipid bilayer as confirmed by fluorescence measurements (data not shown) following an experimental procedure described previously (Lentz et al., 1976a). The final probe-to-lipid molecular ratio was 1:1500 for TMA–DPH and TMAP–DPH, and 1:1000 for DPH. Due to the high sensitivity of the instrument, these ratios were sufficient to obtain reliable anisotropy data. At the low probe-to-lipid molecular ratios used in our study, DPH derivative fluoroprobes have been shown not to disturb the overall structure of the lipid bilayer (Lentz, 1989).

3.6. Fluorescence measurements

Steady-state fluorescence anisotropy measurements were performed on ISS-PCl Photon Counting Spectrophotometer in L-shape configuration equipped with two monochromators (ISS, Champaign, IL, USA). One centimeter rectangular quartz fluorometer cells were used. Temperature was controlled within ±0.1 °C with EcoLine RE120 water bath (Lauda-Brinkmann, Germany). Prior to each measurement, the fluorescence cell was kept for at least 10 min in the spectrophotometer to allow for temperature equilibration. Samples were continuously mixed with a magnetic stirrer placed inside the cell.

Fluorescence measurements were performed with 4 or 8 nm waveband excitation and emission slits. In the case of DPH derivative fluoroprobes, the excitation and emission wavelength were set at 355 and 430 nm, respectively (Balasubramanian and Straubinger, 1994; Campbell et al., 2001). The fluorescence anisotropy was measured over a temperature range from 15 to 45 °C (above the T_m of the bilayer). The polarization anisotropy results were corrected for the intensity of the light scattered from the liposomes, as described elsewhere (Litman and Barenholz, 1982). In lactate buffer (pH 1.5), the results were also corrected for the depolarization due to light scattering (Lentz et al., 1979).

Due to its strong fluorescence, SN-38 was also used as a fluoroprobe (Burke et al., 1992; Burke et al., 1993). In this case, the polarization anisotropy was measured at 25 °C using excitation and emission wavelengths of 370 and 550 nm, respectively.

3.7. Samples for gel filtration

The following samples were analyzed: pre-lyophilized LE-SN38 liposomes, pH 9.68; pre-lyophilized LE-SN38 liposomes, pH reduced to pH 1.75; lyophilized LE-SN38 liposomes re-hydrated with lactate buffer, initial pH 1.82, after 30 min readjusted to pH 10.4; lyophilized LE-SN38 liposomes re-hydrated with lactate buffer, final pH 1.76.

3.8. Gel filtration procedure

Three mL syringes were filled with Sephadex G-50 previously hydrated with 0.9% sodium chloride. The excess amount of liquid was removed by centrifugation for 2 min at 2000 rpm (rcf of 800 × g) at 4 °C on a Centra CL3R centrifuge, rotor 243 (Thermo IEC, Needham Heights, MA, USA). To facilitate the gel filtration, some of the samples in lactate buffer (pH 1.5) were diluted two-fold with 0.9% NaCl immediately prior to centrifugation. Sample (250 μL) was placed on the top of each syringe and centrifuged. To completely elute and recover the liposomes from the column, 250 μL of 0.9% NaCl was added to each syringe and centrifuged again. The washing procedure was conducted twice. All samples were run in triplicate. The filtrates were collected, pulled together and analyzed for SN-38 and cholesterol by HPLC as described in Section 3.1.

This gel filtration procedure ensured that the liposomes along with SN-38 embedded into the bilayer or encapsulated in the water compartment of the liposomes were eluted with the filtrate. SN-38 dissolved in the water phase outside the liposomes and SN-38 aggregates were retained in the gel. The gel filtration procedure was validated with placebo liposomes and SN-38 solutions in 10% sucrose at pH > 10 and with SN-38 suspensions in lactate buffer, pH 1.5. Ninety-nine percent of the placebo liposomes was recovered with the filtrate. Ninety-eight and hundred percent of

SN-38 was retained in the gel filtration columns at high and low pH, respectively (data not shown).

4. Results and discussion

4.1. Fluorescence polarization anisotropy of SN-38

Earlier reports have shown that the camptothecin's excited-state lifetime is relatively insensitive to alterations in microenvironment, such as solvent viscosity or binding to phospholipids (Burke et al., 1993). Consequently, an increase or decrease of the rotational correlation time of the drug molecule will lead to an increase or decrease of the steady-state fluorescence anisotropy (Burke et al., 1993). Because of instrumental limitations we were not able to measure the excited-state lifetime of SN-38 and we assumed that the observations by Burke et al. (1993), would hold for SN-38 as well. This assumption allowed us to interpret the variations of the fluorescence anisotropy as an indication of changes in the microviscosity and local order surrounding the drug molecules.

The polarization anisotropy of the fluorescence of SN-38 was measured in diluted LE-SN38 formulations and SN-38 solutions at two different pH as described in the Section 3. The results of these measurements are given in Table 1. The very low values of the polarization anisotropy at pH 10.4 suggested that SN-38 was free and dissolved in the solution. No difference was observed between SN-38 solutions and LE-SN38 formulations indicating that the drug did not associated with the lipid bilayer due to its high degree of hydrophilicity at this pH condition. This observation was confirmed by gel filtration (see the Section 4.3 below) and was in agreement with previous studies that reported low encapsulation efficiency of SN-38 at high pH (Zhang et al., 2004). At low pH however, the fluorescence anisotropy in the LE-SN38 liposomes was significantly higher than in SN-38 solutions. This suggested an increased viscosity of the microenvironment of SN-38 molecules in the LE-SN38 liposomes and could be interpreted as an indication of association of SN-38 with the lipid bilayer (Burke et al., 1992; Burke et al., 1993). The association could be driven by the increased hydrophobicity of SN-38 molecules associated with the closing of the lactone ring at low pH. The change of fluorescence anisotropy from 0.020 at high pH to 0.130 at low pH observed for SN-38 solutions could be caused by self-aggregation or stacking of SN-38 molecules that is possible at low pH (Burke et al., 1993).

Table 1
Effect of the pH on the polarization anisotropy of the fluorescence of SN-38 at 25 °C

Probe	Formulation	Polarization anisotropy[a]	
		Lactate buffer, pH 1.5	Sucrose, pH 10.4
SN-38	SN-38 solutions	0.130 ± 0.007	0.020 ± 0.002
	Liposomes with SN-38	0.185 ± 0.005	0.021 ± 0.002

[a] Averaged values and standard deviations of 10 measurements.

4.2. Fluorescence polarization anisotropy of DPH derivative fluoroprobes

The effect of the pH on the polarization anisotropy of interface probe TMA–DPH in blank liposomes and in LE-SN38 at various temperatures is shown in Fig. 2. There and in the next two figures data were averaged for 10 measurements and the standard deviation is shown for each datapoint. At high pH, there was no significant difference between the polarization anisotropy measured in placebo and LE-SN38

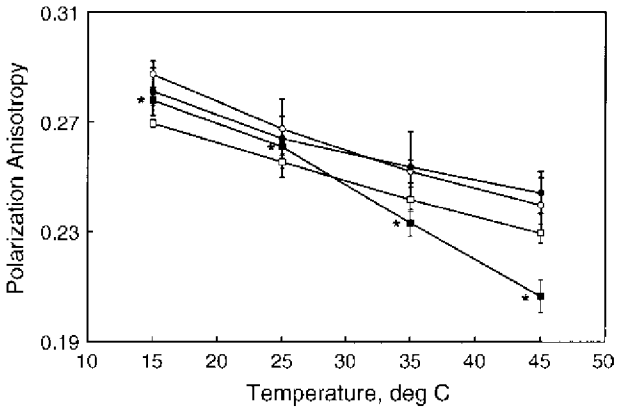

Fig. 2. Effect of the pH on the polarization anisotropy of TMA–DPH in blank liposomes (open symbols) and in liposomes with SN-38 (solid symbols): (○, ●) pH 10.4; (□, ■) lactate buffer, pH 1.5. Statistical significance in the difference of polarization anisotropy for liposomes with SN-38 compared to blank liposomes is shown on the figure: $*p < 0.001$; $n = 10$.

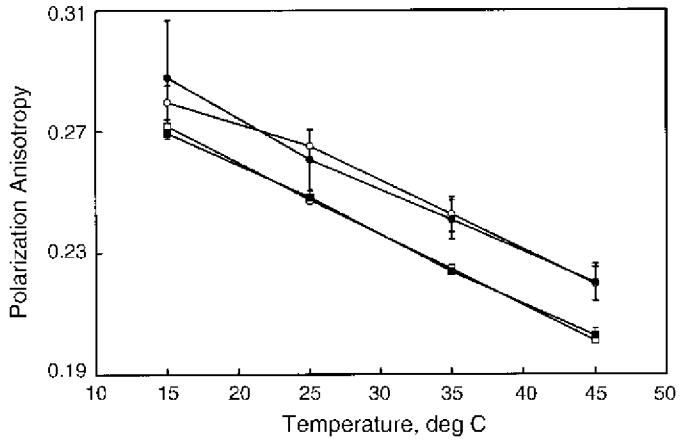

Fig. 3. Effect of the pH on the polarization anisotropy of TMAP–DPH in blank liposomes (open symbols) and in liposomes with SN-38 (solid symbols): (○, ●) pH 10.4; (□, ■) lactate buffer, pH 1.5.

liposomes suggesting that SN-38 did not associate with the lipid bilayer. This finding was supported by the polarization anisotropy results for SN-38 (see above) and by previous studies (Zhang et al., 2004). At low pH, however, there was a significant difference between the polarization anisotropy measured in placebo and LE-SN38 liposomes. The difference was more pronounced at higher temperatures (30–45 °C). The difference could be interpreted as indication that SN-38 was located near the TMA–DPH fluoroprobe. Because TMA–DPH predominantly occupies the water–lipid interface region of the bilayer our results suggested that after re-hydration with lactate buffer (pH 1.5) SN-38 was located close to the interface.

The effect of the pH on the polarization anisotropy of TMAP–DPH and DPH in blank liposomes and in LE-SN38 at various temperatures is shown in Figs. 3 and 4, respectively. There was no significant difference between the polarization anisotropy in liposomes containing or not SN-38, at high pH, confirming that SN-38 did not associate with the lipid bilayer. Furthermore, there was no significant difference at low pH, suggesting that even though SN-38 was located close to the water–lipid interface it did not penetrate into the intermediate or deeper regions of the bilayer where TMAP–DPH and DPH are located.

4.3. Gel filtration studies

From the fluorescence data at low pH it was difficult to determine if SN-38 was located inside the lipid bilayer close to water–lipid interface or formed aggregates in the water phase close to the interface. To further investigate these possibilities, we carried out gel filtration studies.

The experimental results of the gel filtration study are presented in Table 2. The table shows the amount of SN-38 and cholesterol recovered in all filtrates as a percentage of the amount initially placed on the columns. Cholesterol was used as a marker for liposome loss/recovery during the experiment. In the pre-lyophilized liposomes at high pH, 9.1% of SN-38 was associated with the liposomes. This was most probably due to passive entrapment of SN-38 inside the

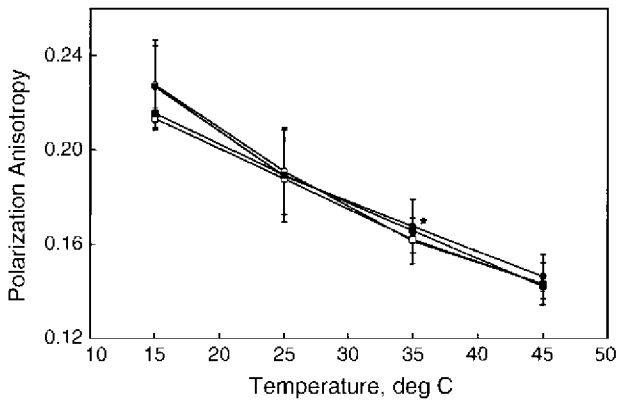

Fig. 4. Effect of the pH on the polarization anisotropy of DPH in blank liposomes (open symbols) and in liposomes with SN-38 (solid symbols): (○, ●) pH 10.4; (□, ■) lactate buffer, pH 1.5. Statistical significance of the difference of polarization anisotropy for liposomes with SN-38 compared to blank liposomes is shown on the figure: $*p < 0.001$; $n = 10$.

Table 2
Percent SN-38 associated with liposomes and percent recovery of cholesterol (as a measure of liposome recovery)

Sample	High pH (pH ≥ 10)		Low pH (pH ≤ 2)	
	SN-38 associated (%)	Cholesterol recovered (%)	SN-38 associated (%)	Cholestrol recovered (%)
Pre-lyophilized LE-SN38	9.1	99	0.8[a]	96[a]
Lyophilized LE-SN38 re-hydrated with lactate buffer	1.7[b]	102[b]	72	95

[a] Pre-lyophilized LE-SN38 liposomes, pH reduced to pH 1.75.
[b] Lyophilized LE-SN38 liposomes re-hydrated with lactate buffer, initial pH 1.82, after 30 min readjusted to pH 10.4.

liposomes during the extrusion. The result confirmed the data from the fluorescence measurements and previous findings of low encapsulation efficiency at high pH (Zhang et al., 2004). For lyophilized LE-SN38 liposomes re-hydrated with lactate buffer, 72% of SN-38 was associated with the liposomes. The remaining 28% of SN-38 was separated from the liposomes by the gel filtration procedure employed in this study.

Simple reduction of the pH in the pre-lyophilized liposomes to 1.7 did not increase the association of SN-38 with the liposomes. Instead an aggregation occurred and only 0.8% of SN-38 remained associated with the liposomes. The aggregation was apparent by the visually observed sedimentation and by the thickening of the samples. In contrast, no aggregation was observed by dynamic light scattering or by optical microscopy (data not shown) in the lyophilized liposomes re-hydrated with lactate buffer (pH 1.5).

The lyophilization by itself did not increase the association of SN-38 with the liposomes, either. Only 8.7% of SN-38 was associated with the liposomes after lyophilized LE-SN38 liposomes were re-hydrated with water and the pH was readjusted to 10.4 (cholesterol recovery 101%). The result was similar to the case of pre-lyophilized liposomes and represented the percent of SN-38 passively entrapped in the liposomes.

These results showed that the combination of lyophilization and re-hydration with an acidic buffer was the reason for association of SN-38 with the lipid bilayer. The close proximity of the drug molecules and lipid bilayers during the re-hydration allowed SN-38 to interact with the lipid bilayers. During this process, SN-38 could form micro aggregates containing several drug molecules at or very close to the water–lipid interface. SN-38 micro aggregates may adsorb at the interface which will considerably slow down further crystal growth. We speculate that the main factor facilitating the formation of such micro aggregates was the presence of the large surface area of the lipid bilayer during the re-hydration at low pH. Similar micro aggregates, called quantum dots, have already been observed on the vesicle surface in liposome suspensions (Correa and Schelly, 1998; Correa et al., 2000).

Based on the experimental results presented here we propose the following model for the localization of SN-38 in the liposomes suspensions at different pH conditions:

Case 1. High pH, pre-lyophilized SN-38 liposomes. SN-38 is soluble in water and does not associate with the lipid bilayer. Some SN-38 is passively entrapped inside the liposomes.
Case 2. Low pH, pre-lyophilized SN-38 liposomes. Upon pH reduction, SN-38 converts into its lactone hydrophobic form, its water solubility decreases and it rapidly aggregates forming bulk particles of micrometer size. These particles do not associate with the liposomes but instead sediment.
Case 3. High pH, lyophilized SN-38 liposomes. Upon re-hydration of lyophilized LE-SN38 liposomes with high pH medium, SN-38 remains soluble in water and does not associate with the bilayer. Some SN-38 remains passively entrapped inside the liposomes.
Case 4. Low pH, lyophilized SN-38 liposomes. Upon re-hydration of lyophilized LE-SN38 liposomes with low pH buffer (for example, lactate buffer), SN-38 converts into its lactone hydrophobic form. Due to the close proximity of SN-38 molecules and lipid bilayers during the re-hydration, SN-38 interacts with the lipids and forms micro aggregates close to the water–lipid interface. The micro aggregates adsorb on the liposome surface which prevents further crystal growth and aggregation.

5. Conclusions

The steady-state fluorescence polarization anisotropy and gel filtration experiments revealed that the localization of SN-38 molecules and their interactions with the lipid bilayer were governed by the hydrophobicity of the drug molecules at different pH conditions and by the preparation procedure. In the case of lyophilized SN-38 liposomes re-hydrated with low pH buffer medium (lactate buffer), the active closed-ring form of SN-38 was associated with the water–lipid interface region of the bilayer. No precipitation or sedimentation of SN-38 was observed for up to 8 h. At high pH, the inactive open-ring form of SN-38 partitioned into the water phase of the liposome suspensions. Some SN-38 was entrapped inside the aqueous core of the liposomes due to passive entrapment.

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Safety and efficacy of weekly 5-fluorouracil/folinic acid/oxaliplatin/irinotecan in the first-line treatment of gastrointestinal cancer

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Abstract:

Background: Standard chemotherapy for patients with metastatic colorectal cancer (mCRC) or gastric cancer (GC) consists of two-drug, usually fluoropyrimidine-based, combinations, with or without the addition of biological agents. Studies of triple-drug regimens combining 5-fluorouracil (5-FU)/folinic acid (FA) with both oxaliplatin and irinotecan have shown promising efficacy in studies of patients with mCRC or GC. However, improved efficacy has often been achieved at the expense of high rates of grade 3 or 4 toxicities such as neutropenia and diarrhoea, occasionally even resulting in toxic deaths.

Objective/Methods: We performed a phase II study of previously untreated patients with mCRC or GC to assess the safety and efficacy of our 5-fluorouracil/folinic acid/oxaliplatin/irinotecan (FUFOXIRI) regimen with weekly administration of irinotecan 70 mg/m^2, oxaliplatin 50 mg/m^2, FA 500 mg/m^2 and 5-FU 2000 mg/m^2 on days 1, 8, 15 and 22, repeated from day 36.

Results: A total of 22 patients were enrolled, 11 each with mCRC and GC receiving a median of four cycles per patient. The FUFOXIRI regimen was generally well tolerated with no toxic deaths, neutropenic fever or grade 4 toxicities. Most common grade 3 side effects were diarrhoea and neutropenia each affecting 24% of patients. Dose reductions due to toxicity were performed in 48% of all and 60% of patients having received at least two cycles of FUFOXIRI. The overall response rate was 46% (all partial responses), 55% and 36% for patients with mCRC and GC, respectively. Median progression-free survival for all patients, mCRC and GC patients was 9.5, 10.0 and 8.0 months, respectively. The median overall survival for all patients was 16.5, 18.0 and 15.0 months for patients with mCRC and GC, respectively.

Conclusion: These data show excellent tolerance and efficacy of the FUFOXIRI regimen in both mCRC and GC. Therefore, FUFOXIRI is a promising backbone for future studies incorporating biologic 'targeted' agents for the treatment of gastrointestinal cancers.

Keywords: chemotherapy, clinical trial, colorectal cancer, gastric cancer, gastrointestinal cancer, 5-fluorouracil, irinotecan, oxaliplatin

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Introduction

In both, metastatic colorectal cancer (mCRC) and locally advanced or metastatic gastric cancer (GC), combination chemotherapy regimens of two active treatment compounds, based on infusional 5-fluorouracil (5-FU), have been the standard of care for many years now.

In mCRC, 5-FU is commonly administered with folinic acid (FA) and with either irinotecan or oxaliplatin resulting from six phase III trials showing superior efficacy for these combinations compared with 5-FU/FA monotherapy. With the addition of either drug, reported response rates (RR) have been increased from 15–25% to 40–50% and overall survival (OS) of 10–14 months with 5-FU/FA alone was prolonged to more than 20 months if all drugs were subsequently administered [Kohne *et al.* 2005; Grothey *et al.* 2002; De Gramont *et al.* 2000; Douillard *et al.* 2000; Giacchetti *et al.* 2000; Saltz *et al.* 2000].

To further increase efficacy, novel molecularly targeted agents have been examined in randomized phase III trials, mostly in addition to chemotherapy doublets. In mCRC, the addition of bevacizumab has yielded improvement of progression-free survival (PFS) [Saltz *et al.* 2008; Hurwitz *et al.* 2004], RR and OS [Hurwitz *et al.* 2004]. The addition of cetuximab resulted in improved RR [Bokemeyer *et al.* 2009; Van Cutsem *et al.* 2009a] and PFS [Van Cutsem *et al.* 2009a], but not OS [Bokemeyer *et al.* 2009; Van Cutsem *et al.* 2009a].

Analogous to the management of mCRC, the standard treatment of GC in the Western World consists of 5-FU-based regimens, most commonly combined with cisplatin [Lutz *et al.* 2007]. Numerous studies have shown that oxaliplatin has the potential to replace cisplatin as a further standard, suggesting a favourable toxicity profile with lower rates of neutropenia, alopecia and less renal toxicity and at least equal efficacy [Al-Batran *et al.* 2008, 2004; Cunningham *et al.* 2008; De Vita *et al.* 2005; Lordick *et al.* 2005; Chao *et al.* 2004; Louvet *et al.* 2002].

Although irinotecan is less extensively evaluated in GC, phase II data have shown considerable activity when combined with 5-FU/FA [Moehler *et al.* 2005; Bouche *et al.* 2004; Pozzo *et al.* 2004; Blanke *et al.* 2001]. Furthermore, a phase III trial comparing 5-FU/cisplatin to 5-FU/irinotecan resulted in a trend for improved time to tumour progression (TTP) in favour of the irinotecan-containing schedule. Again, the experimental arm showed a better toxicity profile than 5-FU/cisplatin [Dank *et al.* 2008].

In GC, the addition of the anti-HER/2 antibody, trastuzumab, was the first targeted agent that resulted in an increased RR as well as a survival benefit when added to 5-FU or capecitabine and cisplatin [Van Cutsem *et al.* 2009b].

Another option is to combine the three most active drugs in one regimen: as 5-FU, irinotecan, and oxaliplatin show only partly overlapping toxicity profiles, a triple combination might be feasible. Moreover, these agents have different mechanisms of cytotoxicity leading to (also different) synergistic effects of combined application, as preclinically shown on colon and GC cell lines *in vitro* [Tanaka *et al.* 2005; Patel *et al.* 2004; Yeh *et al.* 2004; Fischel *et al.* 2001]. Clinically, the combination of irinotecan with oxaliplatin, *without* 5FU/FA, has been shown to be significantly inferior to 5-FU/FA and oxaliplatin (FOLFOX) regarding RR, TTP and OS [Sanoff *et al.* 2008; Goldberg *et al.* 2004].

Thus, a triple combination is expected to be more effective, mainly regarding overall RR, and extent of tumour shrinkage, which offers significant clinical benefits: a greater proportion of patients with initially inoperable metastases, mainly in mCRC with disease limited to the liver, could be converted to a resectable state and thereby potentially cured [Folprecht *et al.* 2005; Bismuth *et al.* 1996]. The rates of secondary metastatic resection in mCRC after treatment with triple-drug combinations of 5-FU/FA, irinotecan and oxaliplatin have been reported to be 15–82.4%; however, some of these results may be biased by small patient numbers and patient selection [Ychou *et al.* 2008; Ferrari *et al.* 2005; Seium *et al.* 2005; Cals *et al.* 2004; De La Cámara *et al.* 2004; Calvo *et al.* 2002; Falcone *et al.* 2002].

So far, there are two phase III studies with a direct, randomized comparison of the two-drug combination 5-FU/FA and irinotecan (FOLFIRI) with the triple-drug regimen FOLFOXIRI additionally including oxaliplatin for patients with mCRC. In both studies, the rate of secondary metastatic surgery was very similar in the FOLFIRI arms (4% and 6%, respectively) and was markedly higher with the FOLFOXIRI regimen (10% and 15%, respectively) [Falcone et al. 2007a,b; Souglakos *et al.* 2006].

In nonresectable but symptomatic patients, tumour shrinkage might offer a better relief of symptoms and/or the prevention of symptomatic disease progression: in mCRC, a recent meta-analysis has demonstrated that patients with poor performance status (ECOG PS 2) yield at least the same benefit from intensified (doublet) treatment when compared with single-agent strategies, compared with patients in good PS (0-1), with no or only few tumour symptoms [Sargent *et al.* 2009].

Moreover, trials with first-line therapy of 5-FU plus irinotecan *or* with oxaliplatin in mCRC have shown that up to 40% of the patients did not receive second-line treatment, mostly because they were not considered fit enough for further chemotherapy. With a triple combination, however, all patients will be exposed to all of the three most active agents which is, according to

the findings of a meta-analysis, a strong prognostic factor for improved OS [Grothey *et al.* 2004].

During recent years, a few phase I and II studies, mostly in patients with mCRC, have been conducted, evaluating different schedules of 5-FU/FA in combination with parallel, sequential or alternating application of irinotecan and oxaliplatin. The results regarding efficacy were quite promising with RR up to 72% [Masi *et al.* 2004], progression free survival (PFS) of 9–14 months and OS mostly well above 20 months [Falcone *et al.* 2007b, 2002; Souglakos *et al.* 2006, 2002; Abad *et al.* 2004; Cals *et al.* 2004; Masi *et al.* 2004; Calvo *et al.* 2002]; in the study of Masi *et al.* even 28.4 months [Vasile *et al.* 2009; Masi *et al.* 2004]. In cross-trial comparisons, outcome data of triple combinations in mCRC compare superior to the results obtained with dual combinations of 5-FU and either irinotecan or oxaliplatin [Kohne *et al.* 2005; Grothey *et al.* 2001; De Gramont *et al.* 2000; Douillard *et al.* 2000; Giacchetti *et al.* 2000; Saltz *et al.* 2000].

The favourable efficacy was also confirmed in a multicenter phase III trial directly comparing a three-drug combination of 5-FU/FA, oxaliplatin, and irinotecan (FOLFOXIRI) with the standard combination of FOLFIRI [Falcone *et al.* 2007a,b] while a previously reported, similar phase III trial only found a trend for improved efficacy of the triple-drug combination [Souglakos *et al.* 2006].

In GC, a triple combination of 5-FU, cisplatin and docetaxel resulted in an improved median OS when compared to the standard two-drug regimen [Ajani *et al.* 2005]. Interestingly, quality of life was maintained for a significant longer period, although high rates of grade 3/4 toxicities limit the use of this regimen in daily routine. However, the favourable efficacies observed underline the need for identification of other active triple-drug regimens.

Despite proven activity of each of the single agents 5-FU/FA, irinotecan, and oxaliplatin and several positive trials with dual combinations in GC [Al-Batran *et al.* 2008, 2004; Cunningham *et al.* 2008; Dank *et al.* 2008; De Vita *et al.* 2005; Lordick *et al.* 2005; Moehler *et al.* 2005; Bouche *et al.* 2004; Chao *et al.* 2004; Pozzo *et al.* 2004; Louvet *et al.* 2002; Blanke *et al.* 2001] data regarding triple combinations of all three drugs are still relatively scarce, but results from recent phase II studies were encouraging with RR of up to 67%, median TTP and OS of up to 9.6 and 14.8 months, respectively [Cao *et al.* 2009; Comella *et al.* 2009; Chiesa *et al.* 2007; Lee *et al.* 2007].

Toxicity, however, remains a major concern of these triple combinations with high rates particularly of grade 3 or 4 neutropenia, nausea/vomiting, diarrhoea, and considerable rates of febrile neutropenia that make routine use of these schedules difficult [Cao *et al.* 2009; Comella *et al.* 2009; Chiesa *et al.* 2007; Falcone *et al.* 2007b, 2002; Lee *et al.* 2007; Abad *et al.* 2004; Cals *et al.* 2004; Masi *et al.* 2004; Calvo *et al.* 2002; Souglakos *et al.* 2002].

Attempts have been made to attenuate toxicity by dose reduction of the individual drugs or alternating application of irinotecan and oxaliplatin, both leading to reduced dose intensity of each drug. Both strategies were able to improve tolerability, but in some trials lowered efficacy to a level also achievable with two-drug combinations [Souglakos *et al.* 2006; Aparicio *et al.* 2005; Ferrari *et al.* 2005].

The aim of this phase II study was to maintain the good tolerability and improve the efficacy of our previously reported FUFOX regimen with weekly administration of oxaliplatin (50 mg/m^2), 24-hour infusion of 5-FU/FA (2000 and 500 mg/m^2, respectively) [Lordick *et al.* 2005; Wong *et al.* 2003; Moehler *et al.* 2002; Grothey *et al.* 2001] by adding weekly administration of irinotecan. In a preceding phase I study, the maximum tolerated dose of irinotecan in conjunction with FUFOX was determined to be 70 mg/m^2 [Arnold and Grothey, unpublished data].

Patients and methods

Patient eligibility

Histologically proven mCRC or locally advanced or metastatic gastric adenocarcinoma (GC) without option for curatively intended resection; measurable disease; age of at least 18 years, no upper age limit; Karnofsky Performance Score $\geq$80%; life expectancy of at least 3 months; no prior radiotherapy or chemotherapy except for adjuvant therapy with fluoropyrimidines alone, completed at least 6 months before study entry; no further malignancy except for nonmelanoma skin cancer or *in situ* cervical carcinoma; adequate bone marrow, hepatic, and renal function (defined as white blood cell count (WBC) $\geq$ 3/nl, haemoglobin $\geq$ 6.2 mmol/l, platelets $\geq$ 100/nl;

bilirubin $\leq 1.25 \times$ upper limit of normal (ULN), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) $\leq 2.5 \times$ ULN; or bilirubin $\leq 1.5 \times$ ULN, ALT and AST $\leq 5 \times$ ULN in patients with liver metastases; serum creatinine $\leq 1.25 \times$ ULN).

Patients were excluded from the study for the following reasons: inflammatory bowel disease or chronic diarrhoea requiring treatment; total colectomy or ileostomy; bowel obstruction or subobstruction; uncontrolled metabolic disorders or active infections; uncontrolled cardiac arrhythmias; uncontrolled congestive heart failure or severe ischaemic heart disease; acute myocardial infarction within the last 6 months; history of significant neurologic or psychiatric disorders that could interfere with study treatment; pregnancy, breast feeding or lack of adequate contraception in women of childbearing potential (WOCBP) or men having unprotected sexual intercourse with WOCBP; symptomatic brain metastases; sensory neuropathy > grade 1; participation in another clinical trial within 4 weeks before initiation of treatment.

The study was conducted in accordance with the Declaration of Helsinki 1996 and Good Clinical Practice Guidelines. Patients were informed about the investigational nature of the study and provided their written informed consent before registration onto the study.

Aims of the trial and study endpoints

This was an exploratory phase II to assess the feasibility and efficacy of the FUFOXIRI regimen in patients with mCRC and GC. The aim was to accrue a total of 20 evaluable patients, 10 patients with mCRC and 10 patients with GC.

The primary endpoint of the study was PFS. Secondary endpoints were OS, safety and tolerability.

The study was offered to eligible patients reviewed in the outpatient clinics of the University Hospital Halle between March 2002 and October 2005.

Pretreatment evaluation

Patients were required to perform baseline imaging workup by CT scan of the chest, abdomen, and pelvis within 4 weeks before initiation of treatment.

A screening visit had to take place within 1 week prior to start of chemotherapy including a complete medical history, physical examination and a blood sample for differential blood cell count and routine blood biochemistry.

Treatment

Chemotherapy started with 1 hour intravenous (iv) infusion of irinotecan 70 mg/m^2/60 min, followed by oxaliplatin 50 mg/m^2 and FA 500 mg/m^2 iv over 2 hours via two different iv lines. 5-FU at a dose of 2000 mg/m^2 was administered as continuous infusion over 24 hours. All agents were given on days 1, 8, 15, 22 and were repeated from day 36.

To prevent cholinergic syndrome, 0.25 mg of atropine was injected subcutaneously prior to irinotecan administration. Antiemetic prophylaxis was performed by iv administration of 5-HT3 antagonists and 8 mg of dexamethasone.

In case of diarrhoea, patients were advised to increase oral fluid intake and start oral medication with 4 mg of loperamide followed by 2 mg every 2 hours until normalization of the stool. If diarrhoea persisted >24 hours or was accompanied by nausea and vomiting or fever >38°C, the patient was hospitalized if indicated.

Toxicity was evaluated after each course according to NCI Common Toxicity Criteria (CTC) version 3.0. Chemotherapy was postponed in the case of persisting nonhaematologic toxicity > grade 1, except for alopecia and asthenia, or in the case of haematologic toxicity > grade 2. Treatment was applied until disease progression, unacceptable toxicity or patient consent withdrawal.

Dose modifications

In the case of grade 3 or 4 diarrhoea, doses of irinotecan and 5-FU/FA were reduced by 25%. At the presence of grade 3 or 4 mucositis or hand–foot syndrome, 5-FU/FA was reduced by 25%. If patients developed sensory neuropathy ≥ grade 2, oxaliplatin was paused until resolution to grade 0 or 1.

If treatment was delayed due to leukopenia or thrombocytopenia for >1 week, doses of all, irinotecan, oxalipaltin and 5-FU/FA, were reduced by 25% each.

Patient evaluation

During treatment a complete blood cell count and white cell differential were performed every week prior to chemotherapy administration and blood biochemistry and urine analysis were done as clinically indicated, but at least every 2 weeks.

Tumour response was assessed every two cycles (10 weeks) by CT or MR imaging and evaluation according to RECIST version 1.0 [Therasse *et al.* 2000] by an independent radiologist. If a complete or partial response (CR or PR, respectively) was detected, it had to be confirmed in a subsequent scan at least 28 days later.

If there was no evidence of pulmonary or abdominal metastases, a chest X-ray or abdominal ultrasound, respectively, was performed every 6 months to test for potential new tumour manifestations.

PFS and OS were calculated as the interval between initiation of treatment and detection of progressive disease (PD) or death, respectively. Duration of response was determined as the time from first assessment of response until observation of PD.

Results

Patient characteristics

Twenty-two patients were included in this single-centre study, 11 each with mCRC and GC. Demographic data were similar in both groups (see Table 1).

The majority of the mCRC patients had metastatic disease at the time of diagnosis (7/11, 64%), two patients each with metachronous CRC metastases were initially diagnosed with stage IIB and stage III disease, all had previously been treated with adjuvant 5-FU/FA, and had relapsed with liver metastases 8, 14, 20 and 22 months after their initial diagnosis, respectively. One mCRC patient also had lung metastases at the time of relapse.

The predominant site of metastatic disease was the liver in both mCRC and GC patients (11/11 = 100% and 8/11 = 73% of patients, respectively), followed by lung metastases for mCRC patients (2/11 = 18%) and peritoneal metastases for GC patients (3/11 = 27%).

One mCRC patient had synchronous metastases at the liver, lung and bone.

Altogether, 82 cycles of FUFOXIRI were administered in this study with a median of 4 cycles per patient (range: 1–8 cycles).

Treatment toxicity

There were 21 patients evaluable for toxicity: for one patient safety data were incomplete due to loss of followup. There was no toxic death, serious adverse event (SAE), neutropenic fever or grade 4 toxicity. All patients were treated in an outpatient setting, no hospitalizations were required.

Main grade 3 toxicities were diarrhoea and neutropenia which each occurred in 5/22 = 23% of patients. An overview of the toxicity profile is shown in Table 2.

Dose delays due to toxicity were performed in 12/22 = 55% of cases; dose reductions due to treatment side effects were required in 10/22 = 46% of all and in 12/20 = 60% of patients having received at least two cycles of FUFOXIRI.

Table 1. Patient characteristics.

	All	CRC	GC
Number of patients (n)	22	11	11
Gender (M/F) [%]	64/36	82/18	46/54
Age	56 [26–68]	52 [44–64]	58 [26–68]
KPS [%]	90 [70–100]	90 [80–100]	90 [70–100]
Metastatic disease	20/22 [91]	11/11 [100]	9/11 [82]
Locally advanced disease	2/22 [9]	0	2/11 [18]
Median number of metastatic sites (range)	1 [0–3]	1 [1–3]	1 [0–2]
Liver metastases [%]	19/22 [86]	11/11 [100]	8/11 [73]
Pulmonary metastases [%]	2/22 [9]	2/11 [18]	0
Previous adjuvant chemotherapy [%]	19 [18]	4 [36]	0
Median number of cycles per patient (range)	4 [1–8]	5 [3–8]	3 [1–4]
Total cycles administered	82	45	37

CRC, colorectal cancer; GC, gastric cancer; KPS, Karnofsky performance score.

Table 2. Toxicity profile of 5-fluorouracil/folinic acid/oxaliplatin/irinotecan (FUFOXIRI).

Toxicities	Grade 1/2 (%)	Grade 3 (%)
Diarrhoea	10/22 (46)	5/22 (23)
Neutropenia	9/22 (43)	5/22 (23)
Nausea	12/22 (57)	3/22 (14)
Neurotoxicity	14/22 (67)	2/22 (10)
Anaemia	16/22 (73)	0
Fatigue	15/22 (68)	0
Hepatic	13/22 (59)	0
Alopecia	16/22 (46, all G1)	0
Thrombocytopenia	6/22 (27)	0
Stomatitis	3/22 (14)	0
Renal	5/22 (23)	0
VTE	0	0

VTE, venous thromboembolism.

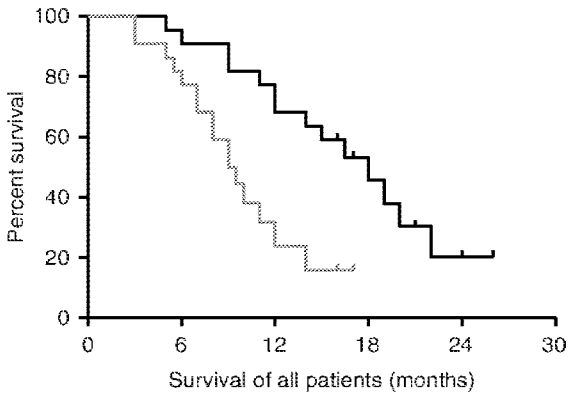

Figure 1. Overall survival (black line) and progression-free survival (red line) of all patients after 5-fluorouracil/folinic acid/oxaliplatin/irinotecan (FUFOXIRI).

Treatment efficacy

Efficacy assessment was performed in an intent-to-treat analysis. Tumour response was evaluable in 18 of the 22 patients, in 3 patients with GC and 1 patient with mCRC valid response data could not be ascertained.

The overall RR was 10/22 (46%, all PR), accounting for 6/11 (55%) and 4/11 (36%) of patients with mCRC and GC, respectively.

In each group, a further 4 patients (36%) achieved stable disease (SD) as their best response. Remarkably, no disease progression occurred during the first 10 weeks of treatment with FUFOXIRI.

Median response duration (RD) was 7.5 months in all patients with 8.0 months and 6.5 months for patients with mCRC and GC, respectively.

Median PFS was 9.5 months for all, 10.0 and 8.0 months for patients with mCRC or GC, respectively. The median OS for all patients was 16.5 months, 18.0 months and 15.0 months for patients with mCRC and GC, respectively (Figure 1).

Efficacy data are summarized in Table 3.

Subsequent treatment

None of the patients enrolled on this trial underwent secondary resection of tumour metastases.

Table 3. Efficacy of 5-fluorouracil/folinic acid/oxaliplatin/irinotecan (FUFOXIRI) in an intention-to-treat analysis.

	All (n = 22)	CRC (n = 11)	GC (n = 11)
PR [%]	46	55	36
SD [%]	36	36	36
PFS [months]	9.5	10.0	8.0
RD [months]	7.5	8.0	6.5
OS [months]	16.5	18.0	15.0

CRC, colorectal cancer; GC, gastric cancer; mo, months; OS, overall survival; PFS, progression-free survival; PR, partial response; RD, response duration; SD, stable disease.

All patients underwent subsequent systemic treatment as outlined in Table 4.

The large majority of mCRC patients received the epidermal growth factor receptor (EGFR) antibody cetuximab as well as the vascular endothelial growth factor (VEGF) antibody bevacizumab with subsequent treatment lines.

Five patients with GC were subsequently treated on an in-house phase II trial with bevacizumab, capecitabine and mitomycin-C (BECAM) [Peinert *et al.* 2006].

Discussion

The aim of this trial utilizing weekly 5-FU/FA, irinotecan and oxaliplatin in the FUFOXIRI regimen was to maintain good tolerability while obtaining comparably high efficacy in the treatment of mCRC and GC, as had been shown for other administration schedules of this triplet

Table 4. Characterisation of subsequent treatments of patients following study treatment with oxaliplatin/5-fluorouracil/folinic acid/irinotecan (FUFOXIRI).

	All (n = 22)	CRC (n = 11)	GC (n = 11)
Median no. further treatments (range)	2 (1–3)	2 (1–3)	2 (1–2)
Bevacizumab-containing [%]	14/22 (64)	9/11 (82)	5/11 (45)
Cetuximab-containing	15/22 (59)	10/11 (91)	3/11 (27)
Fluoropyrimidine-containing	21/22 (95)	10/11 (91)	11/11 (100)
Docetaxel-containing	3/22 (14)	0	3/11 (27)
Mitomycin C-containing	5/22 (23)	0	5/11 (45)

CRC, colorectal cancer; GC, gastric cancer.

combination. However, most of the previous studies achieving high RRs and durations were complicated by significant toxicity, particularly high rates of grade 3 or 4 neutropenia, diarrhoea and nausea/vomiting jeopardizing the palliative effect of efficient antitumour activity [Cao *et al.* 2009; Comella *et al.* 2009; Chiesa *et al.* 2007; Lee *et al.* 2007; Souglakos *et al.* 2006, 2002; Abad *et al.* 2004; Cals *et al.* 2004; Masi *et al.* 2004; Calvo *et al.* 2002; Falcone *et al.* 2002].

The weekly doses of the FUFOXIRI regimen administered in this study were 70 mg/m^2 of irinotecan, 50 mg/m^2 of oxaliplatin, 2000 mg/m^2 of 5-FU and 500 mg/m^2 of FA. This combination was given weekly for 4 weeks, followed by 1 week of pause.

The efficacy achieved in our small cohorts of patients was comparable to the results of previous studies with triplet combinations: the response rate for patients with mCRC was 55%, which is within the range of the previously reported RR of 23–78% with different schedules of 5-FU/FA, irinotecan and oxaliplatin [Masi *et al.* 2008, 2004; Falcone *et al.* 2007a,b, 2002; McWilliams *et al.* 2007; Souglakos *et al.* 2006, 2002; Aparicio *et al.* 2005; Ferrari *et al.* 2005; Seium *et al.* 2005; Abad *et al.* 2004; Cals *et al.* 2004; Reina *et al.* 2004; Calvo *et al.* 2002].

In terms of survival, the FUFOXIRI regimen resulted in a PFS of 10 months for the mCRC patients, which compares favourably with the results from previous studies reported in the literature (range 6.2–14 months). The OS of mCRC patients in our study was 18 months which is in line with most previous trials using triplet combinations. However, in some phase II studies, the OS was considerably longer, ranging between 24.5 and 28.5 months [Seium *et al.* 2005; Cals *et al.* 2004; Masi *et al.* 2004; Falcone *et al.* 2002]. In the two randomized phase III trials published so far, both comparing FOLFIRI with FOLFOXIRI, the OS in the triplet combination arms were 21.5 and 22.6 months, respectively, and therefore appears superior to the results of our small cohort of mCRC patients, even though at the price of increased haematologic toxicity [Falcone *et al.* 2007b; Souglakos *et al.* 2006].

For GC patients, four phase II studies have been published in recent years that report remarkable activity of triple-drug combinations of 5-FU, irinotecan and oxaliplatin with RR of 33–67%, PFS and OS of 7.3–9.6 months and 10.2–14.8 months, respectively [Cao *et al.* 2009; Comella *et al.* 2009; Chiesa *et al.* 2007; Lee *et al.* 2007]. In the treatment of GC, with the limitation of small patient numbers, our results with the FUFOXIRI regimen are in line with the reported data, revealing a RR of 36%, and PFS and OS of 8.0 and 15.0 months, respectively.

In the above-mentioned studies reporting high efficacy with triplet combination regimens that seem to be superior to FUFOXIRI at least in mCRC, irinotecan and oxaliplatin were either both administered on the same day and repeated after 2 weeks [Cao *et al.* 2009; Chiesa *et al.* 2007; Falcone *et al.* 2007a,b, 2002; Lee *et al.* 2007; Masi *et al.* 2004; Calvo *et al.* 2002] or there was a weekly schedule with alternating application of irinotecan and oxaliplatin [Aparicio *et al.* 2005; Ferrari *et al.* 2005; Seium *et al.* 2005; Cals *et al.* 2004]. In both types of regimens, the doses of the individual drugs were higher than those applied in weekly administration. This seems to be an important factor for efficacy as dose density in the weekly schedule of FUFOXIRI is comparable or even higher.

Particularly in the earlier trials with triple combinations of 5-FU/FA, irinotecan and oxaliplatin,

improvement of efficacy was at the expense of higher toxicity. Excessive rates of grade 3 or 4 neutropenia and diarrhoea in up to 86% and 45% of patients, respectively [Calvo *et al.* 2002; Falcone *et al.* 2002] and considerable rates of febrile neutropenia of up to 15% of patients have been reported [Abad *et al.* 2004; Cals *et al.* 2004; Masi *et al.* 2004; Calvo *et al.* 2002; Falcone *et al.* 2002; Souglakos *et al.* 2002]. However, the rate of severe diarrhoea in the two randomized studies comparing FOLFIRI with FOLFOXIRI (20% and 27.7%, respectively) appear comparable to that reported in the present trial (24%) [Falcone *et al.* 2007b; Souglakos *et al.* 2006].

Of note, in several of the above-mentioned trials, cases of toxic deaths were recorded [McWilliams *et al.* 2007; Reina *et al.* 2004; Calvo *et al.* 2002], and in one of these studies led to early suspension of the trial [McWilliams *et al.* 2007]. This is not acceptable in a setting where the treatment remains palliative for the majority of patients.

With weekly administration of lower doses of each drug in the FUFOXIRI regimen, we were able to completely abrogate any life-threatening toxic events: In this study, no treatment-related death, serious adverse event or grade 4 toxicity occurred.

Diarrhoea and neutropenia, two of the most common side effects of triple-drug combinations, each occurred at a rate of 24% grade 3 and none of the patients had febrile neutropenia.

The improved toxicity profile of the FUFOXIRI regimen may in part be due to the lower daily doses of each drug. In addition, 60% of all patients who received two or more cycles of FUFOXIRI experienced dose reductions during the course of their therapy.

Moreover, our patient population with a median age of 56 years was relatively young. Elderly patients >65 years of age have been shown to respond less well to FOLFOXIRI and experienced more toxicity which seems to make triple-drug combinations less suitable for this patient cohort [Vamvakas *et al.* 2009].

The strategy of weekly administration of chemotherapy in order to reduce toxicity has also been applied with the dual-drug combination of 5-FU/FA and oxaliplatin, i.e. the FUFOX regimen. As yet, there is no direct comparison of FUFOX with the standard of 2-weekly regimens, e.g. FOLFOX-4. Cross-trial comparisons, however, of grade 3 or 4 toxicities suggest that there are lower rates of neutropenia with weekly administration of 5-FU/FA and oxaliplatin without 5-FU bolus [Tournigand *et al.* 2006; Grothey *et al.* 2001; De Gramont *et al.* 2000; Buechele *et al.* 1998]. This also applies when FOLFOX or FUFOX are combined with cetuximab, a monoclonal EGFR antibody [Arnold *et al.* 2008; Tabernero *et al.* 2007].

Another way of trying to reduce the toxicity of triple-drug combinations is the alternating administration of irinotecan and oxaliplatin. As yet, there are no results from phase III studies in the treatment of mCRC available applying this strategy. There are four phase I/II studies published providing toxicity as well as survival data [Aparicio *et al.* 2005; Ferrari *et al.* 2005; Seium *et al.* 2005; Cals *et al.* 2004]. These have shown promising efficacy results that seem to be superior to those of dual-drug combinations with RR up to 78% [Seium *et al.* 2005] and median PFS and OS of 9.5–13 and 18–26.1 months, respectively. In the trial of Ferrari and colleagues, the occurrence of severe toxicities was remarkably low (8% of neutropenia being the most common), probably due to relatively low cumulative drug doses in the schedule used [Ferrari *et al.* 2005]. In the study by Cals and colleagues, however, grade 3 or 4 neutropenia occurred at a frequency of 41% of which almost 6% were complicated by fever [Cals *et al.* 2004]. An explanation for this enhanced toxicity may be the relatively high doses of weekly 5-FU used in this trial.

Not surprisingly, a similar toxicity profile was recorded when the triple-drug combination FOLFOXIRI was administered for the treatment of patients with metastatic GC. Again, (febrile) neutropenia and, in this case, nausea/vomiting and asthenia besides diarrhoea were the dose-limiting toxicities [Cao *et al.* 2009; Comella *et al.* 2009; Chiesa *et al.* 2007; Lee *et al.* 2007].

Even though for patients with GC that have distant metastases, there is no evidence for a curative treatment option [Sastre *et al.* 2006; Wagner *et al.* 2006; Wong *et al.* 2003], active chemotherapy does play an important role. First, in patients with metastatic disease, OS can be prolonged significantly [Sastre *et al.* 2006; Wagner *et al.* 2006; Wong *et al.* 2003]. Second, for patients with

initially resectable GC, Cunningham and colleagues were able to show that, in addition to prolonging PFS, perioperative chemotherapy seems to improve the chances for long-term survival and cure [Cunningham *et al.* 2006]. The treatment for this patient population was a triple-drug combination of epirubicin, cisplatin and 5-FU, one of the current standard treatment options for GC [Cunningham *et al.* 2006; Wagner *et al.* 2006]. As indicated above, the administration of oxaliplatin tends to result in improved efficacy compared with cisplatin when combined with a fluoropyrimidine and epirubicin in the treatment of advanced oesophagogastric cancer. In a large randomized phase III trial with two-by-two randomization, triple-drug combinations of epirubicin with either oxaliplatin or cisplatin and 5-FU/FA or capecitabine were compared (EOX, ECX, EOF, ECF). The EOX regimen resulted in significantly superior OS of 11.2 months compared with the other three-drug combinations while providing a similar or better toxicity profile [Cunningham *et al.* 2008].

An alternative three-drug regimen for GC is the combination of docetaxel, cisplatin and 5-FU/FA (DCF). In a large prospective randomised phase III trial with 445 patients, DCF was shown to improve RR, TTP and OS compared with CF alone. However, DCF was associated with significantly more grade 3/4 toxicities than CF, particularly the rates of neutropenia and complicated neutropenia were excessively high (82% and 29% versus 57% and 12%, respectively) [Van Cutsem *et al.* 2006]. Despite the enhanced toxicity of the triple-drug regimen, the superior antitumour activity of the DCF treatment resulted in overall clinical benefit for GC patients as compared with patients receiving CF [Ajani *et al.* 2007].

In the treatment of mCRC, the improvement of treatment efficacy is no longer restricted to modifications of the chemotherapy regimen alone. Another option to improve outcome that has already become standard of care is the addition of immunologically targeted therapies. Bevacizumab, a monoclonal antibody against VEGF, is active in combination with fluoropyrimidines, oxaliplatin and irinotecan and is included in standard first-line therapy regimens for mCRC [Hochster *et al.* 2008; Saltz *et al.* 2008; Hurwitz *et al.* 2005, 2004].

A recent report from a phase II study combining 5-FU, irinotecan, bevacizumab and oxaliplatin revealed an impressive RR of 80% translating into 6% of secondary metastatic resections, 18% amongst the patients with liver metastases only. The TTP and OS of 12 and 25 months, respectively, were similar to the results achieved with triple-drug chemotherapy alone [Santomaggio *et al.* 2009]. Another phase II study with 40 mCRC patients combined the previously reported FOLFOXIRI regimen [Falcone *et al.* 2007a,b] with bevacizumab 5mg/kg day 1 every 2 weeks. The profile of reported grade 3/4 toxicities was similar to that seen with FOLFOXIRI alone apart from the typical bevacizumab toxicities such as arterial hypertension and deep venous thromboses which occurred at 8% and 5%, respectively. Twelve percent of patients experienced grade 1 bleeding. The RR of 76% is quite promising: neither median PFS nor OS have been reported yet [Falcone *et al.* 2008].

In the neoadjuvant setting, a phase II study of capecitabine, oxaliplatin and bevacizumab for CRC patients with potentially resectable liver metastases reported a favourable RR of 73% and improved outcome for responding patients [Gruenberger *et al.* 2008].

The other biologic agent that has become a component of standard treatment for mCRC is the EGFR antibody, cetuximab. The efficacy data and particularly the response rates reported from phase II studies for the combination of cetuximab with 5-FU/FA and oxaliplatin are among the highest ever reported in the treatment of mCRC [Arnold *et al.* 2008; Tabernero *et al.* 2007]. In the study by Tabernero and colleagues, the RR of 79% enabled 23% (10 out of 43) of the patients with previously unresectable metastases to undergo curatively intended surgery. However, these favourable results could not be confirmed to the same extent in larger phase III studies of cetuximab in combination with 5-FU/FA and oxaliplatin (FOLFOX-4 ± cetuximab) or irinotecan (FOLFIRI ± cetuximab) which reported overall RR in the cetuximab arms of 46% and 46.9%, respectively. The smaller oxaliplatin-based study failed to show an improvement in PFS for the patients receiving cetuximab whereas the larger irinotecan-based trial revealed improvement of PFS but not OS for patients receiving FOLFIRI plus cetuximab. The benefit by the addition of cetuximab was confined to patients with wild-type k-ras oncogene in both studies [Bokemeyer *et al.* 2009; Van Cutsem *et al.* 2009a].

Taken together, the important role of effective chemotherapy in mCRC as well as GC is well established, and the FUFOXIRI regimen with weekly administration of 5-FU, oxaliplatin and irinotecan is one of the most promising combinations as it provides the high antitumour activity of a triple-drug regimen while avoiding excessive toxicity as observed with alternative three-drug regimens such as DCF for GC or FOLFOXIRI or XELOXIRI in the case of mCRC. This is particularly important for the design of future studies combining triple-drug regimens with biologic agents in order to define the most active treatment regimen for mCRC or GC currently available.

Conflict of interest statement

None declared.

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PEP02

Product Background

Many new formulations improve the approved drugs, such as Doxil and Abraxane, and these "me betters" have achieved significant markets over the parent drugs. PEP02 is a novel nanoparticle liposome formulation of irinotecan. Irinotecan HCl (Camptosar®) is a broad spectrum anti-cancer cytotoxic drug approved for the treatment of colorectal cancer, and the annual sales approached USD 1 billion in 2008. However, its dose limiting toxicities often result in serious side effects, such as late onset diarrhea and myelosuppression which prevent it from being explored for better anti-tumor efficacy. Regardless, irinotecan is a valuable backbone to which to deliver targeted agents, such as anti-VEGF or anti-EGFR for prolonged overall survival. PharmaEngine licensed a bench scale PEP02 from Hermes Biosciences, Inc. (South San Francisco, CA) which was acquired by Merrimack Pharmaceuticals, Inc. (Cambridge, MA).

Intellectual Properties

Patent applications for composition of matter and method of PEP02 have been filed in major countries in North America, Europe and Asia.

Chemistry and Manufacturing

Irinotecan is a water-soluble semi-synthetic derivative of camptothecin. PEP02, a liposome formulation of irinotecan, using a modified gradient loading method with sucrose octasulfate was described by Drummond et al. (Cancer Res. 66: 3271-7, 2006). The manufacturing process and product were audited and approved by a Qualified Person ("QP") in the UK. The QP declaration is required for conducting clinical studies in Europe.

Preclinical Development

A series of pharmacology, pharmacokinetics and drug metabolism, as well as toxicology and toxicokinetics studies were performed with PEP02 and CPT-11.

Pharmacology studies were conducted in xenograft mouse models of human breast, gastric, colon, cervical, brain, pancreatic, and lung cancers in comparing the efficacy of CPT-11 and PEP02 alone or in combination with 5-FU or cisplatin. PEP02 showed 3-10 fold better efficacy than CPT-11 without causing irreversible body weight loss. PEP02 in combination with cisplatin or 5-FU also showed more than additive effects than PEP02 alone.

Pharmacokinetic and plasma/tissue distribution profiles of PEP02 or ^{14}C-PEP02

in normal mice, rats and dogs, as well as in tumor bearing mice were investigated. In summary, the pharmacokinetics studies showed that the formulation of PEP02 met the characteristics of liposome drug of long circulation time, slow clearance, small volume of distribution and high tumor tissue distribution.

Toxicology and toxicokinetics of PEP02 and CPT-11 were investigated in rats and dogs either as single dose, weekly for four weeks, or every three weeks per cycle for 6 cycles. Although rats and dogs dosed with PEP02 had much higher systemic exposure of CPT-11 and SN-38 (active metabolite) than those in the CPT-11 group, the tolerability and toxicity of PEP02 were better or at least no worse than those of CPT-11. No accumulation of PEP02 in the plasma was detected upon repeat dosing.

Clinical Development

Irinotecan has shown efficacy in colorectal, gastric, lung, brain, uterine cervical and ovarian cancers. In the pre-clinical and phase I clinical studies, PEP02 demonstrated the potential in colorectal, gastric, pancreatic, brain, lung, breast, and cervical cancers. Clinical development of PEP02 will focus on the treatment for unmet needs in gastric and pancreatic cancers, and substitute irinotecan in colorectal, lung and brain cancers. We will select one or two indications to proceed to phase II or III trials in 2011.

Market forecast

Irinotecan HCl (Camptosar®) is a broad spectrum anti-cancer cytotoxic drug approved for the first and second line treatments of colorectal cancer, the annual sales approached USD1 billion in 2008. However, its dose limiting toxicities often result in serious side effects, such as late phase diarrhea and myelosuppression which prevent it from being explored for better anti-tumor efficacy. PEP02 (liposome irinotecan) target indications include gastric, pancreatic, colorectal, lung, and brain cancers. Currently, three completed phase I clinical trials showed that PEP02 has potential to become a better drug than irinotecan, in particular PEP02 can be a good backbone in combination with targeted therapies. The estimated market size is over USD1 billion for the global market.

Advantages of PEP02

Drug Itself

- Irinotecan is an efficacy-proven drug in several indications with extensive market size. Target therapies (e.g., Avastin, Erbitux) have to combine with irinotecan for better efficacy.

- PEP02 is a creative and improved liposome formulated irinotecan with patent protection.
- Stable formulation and efficient manufacture has been set up.

Plan and Strategy

- Comprehensive non-clinical data are available to support clinical development.
- A series of clinical studies covering various potential indications is in the development plan and has been preliminarily discussed and concurred by the major regulatory authority (US FDA).
- Global clinical development has been incorporated from early clinical stage.
- Collaborated with internationally recognized advisors and investigators.

Our Data

- Pharmacokinetic profile has improved with lower Cmax & higher AUC of SN-38 as compared to that of irinotecan arm.
- Efficacy
  - Better efficacy has been shown in the phase II gastric cancer study than irinotecan
  - Promising efficacy in the ongoing phase II metastatic pancreatic cancer study
  - Tumor responses were still observed in patients who have failed several prior chemotherapy regimens
- Pharmacogenetic database are built in parallel with clinical database.

Differentiation from Competitors

- PK and efficacy data are superior to other formulations of irinotecan or SN-38.
- Major competitors focus on CRC which is a relatively crowded field, but PEP02 has overall registration strategy in target tumor types with niche cancer markets (gastric, pancreatic, lung, etc.).

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Nanomedicines for Cancer Therapy: An Update of FDA Approved and Those under Various Stages of Development

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Abstract

Nanomaterials, with their size range (1-100 nm) that corresponds to basic biological materials such as DNA, vastly increased surface area (1000 m^2/g) and unique mechanical, electronic, photonic and magnetic properties are projected to have a wide range of applications from drug and gene delivery to biomedical imaging and more recently to personalized medicine. The major advantages of using nanomaterials as a carrier for anticancer agents are the possibility of targeted delivery to the tumor, tumor imaging, their ability to hold thousands of molecules of a drug and also their ability to overcome solubility, stability and resistance issues. Currently, there are several nanotechnology-enabled diagnostic and therapeutic agents undergoing clinical trials and a few are already approved by the FDA. Targeted delivery of anticancer agents is achieved by exploiting a unique characteristic of the tumor cells called "the Enhanced Permeation and Retention Effect (EPR effect). In addition to this passive targeting based mainly on size, the nanoparticle surface may be modified with a variety of ligands that would interact with specific receptors over-expressed on the surface of the tumor cells, thus imparting specificity for active targeting. Site-specific release of a drug contained in a nanoparticulate system by the application of external stimuli such as hyperthermia to a thermosensitive device is another innovative strategy for targeted delivery. This review provides an update on the FDA approved cancer nanomedicines such as Abraxane, Doxil, daunoxome, Oncaspar, DepoCyt and those in nanoplatforms that have reached an advanced stage of clinical development utilizing liposomes, lipid nanoparticles, lipoplexes, albumin nanospheres, thermosensitive devices, micelles and gold nanoparticles.

Keywords: Cancer nanomedicines; Approved products and in clinical development; Targeted delivery; Stimuli-Sensitive Release; Liposomes; Albumin-bound nanoparticles; Gold nanoparticles; RNA interference

Introduction

One of the draw backs of conventional chemotherapy for the treatment of cancer is the inability to deliver the drug in adequate quantity to the tumor site without undesirable side effects. Nanomaterials, with their size range (1-100 nm) that corresponds to basic biological materials such as DNA, vastly increased surface area (1000 m^2/g) and unique mechanical, electronic, photonic and magnetic properties are projected to have a wide range of applications from drug and gene delivery to biomedical imaging. The major advantage of using nanomaterials as a carrier for anticancer drugs is the possibility of targeted delivery to the tumor, their ability to enclose or bind thousands of molecules of a drug and deliver to the required site and to overcome solubility and stability issues.[1-4] In addition, nanoplatforms like liposomes, lipid nanoparticles, dendrimers, micelles, gold nanoparticles can offer solutions to various problems in diagnosis and treatment of cancer. Multifunctional nanoparticles (MNPs) for simultaneous diagnosis, imaging and treatment, an area referred to as Theranostics, are also being developed for personalized medicine.[5-8]

Nanoparticles (NPs) loaded with chemotherapeutic agents such as the liposomes, polymer nps and lipid nps have the ability to overcome drug resistance. Multidrug resistance (MDR) is one of the biggest challenges in cancer chemotherapy. Development of cancer drug resistance is attributed to a number of factors including inefficient drug delivery to tumor cells, partly due to inefficient targeting and the rapid removal of the drug from tumor cells by the efflux pump, P-glycoprotein (P-gp). This membrane transporter is known to be over-expressed in tumors compared to drug-sensitive parent cell lines. Approximately 50% of the anticancer drugs used clinically today are substrates of P-gp. Therefore, a plausible way to reduce MDR is to increase the efficiency of drug delivery to the tumor and reduction of MDR-based drug efflux. It has been suggested that nps may be able to circumvent P-gp-mediated resistance by partially bypassing the efflux pump as they are internalized by endocytosis.[9-13]

The size range of nanomaterials is strictly defined as 10-100 nm, although many marketed nanomedicines are in the submicron range of 100-1000 nm.[14] Many of the properties of nanomaterials are different from those of the bulk materials due to an increased surface area and quantum effects. The quantum effects coupled with surface area effects can impact on the size-dependent properties of nanomaterials, which in turn can influence their *in vivo* behavior. Nanoparticles therapeutics is especially useful for cancer and disorders of the central nervous system, because they are able to cross the blood brain barrier (BBB).[15] In addition, it is also possible to achieve a prolonged residence time and a desired drug-release pattern by controlling the size and architecture of nanoparticles.

Passive targeting

Passive targeting of drug-loaded nanocarriers and their accumulation in tumor tissue is a size-dependent process. Passive targeting occurs as a result of the pathophysiological characteristics of the tumor vessels such as a leaky vasculature and poor lymphatic drainage.

The extravasation of particulate materials in to the tumor tissue and their retention is called Enhanced Permeation and Retention (EPR) effect. However, these particles, recognized as foreign bodies, may be opsonized by the cells of the reticuloendothelial system (Mononuclear Phagocyte System, MPS), thereby reducing the availability of the drug at the required site. Well-designed nanocarriers such as those coated with polyethylene glycol, a process called pegylation, have the ability to escape capture by the MPS. Such drug delivery systems are referred to as the stealth systems.[15] Most passive- targeting nanosystems have a surface coated with PEG for biocompatibility and "stealth" purposes. A variety of pegs with varying chain length and molecular weight are being used to control the thickness of the PEG coating and the grafting efficiency. Longer chains offer greater steric influence around the nanocarrier than short chain pegs.[17] Surface modification of nanocarriers can also be achieved by using derivatives of PEG such as the block copolymers of the poloxamer type.[18] Genexol-PM, a micellar formulation formed from PEG-based block copolymers is an example of such a product available in the market (Genexol-PM was approved in 2007 in Korea and marketed in Europe).

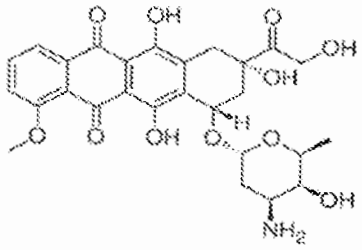
Active drug targeting

Passive targeting strategies have shown several limitations. Therefore, considerable effort is underway to maximize the accumulation of nanoparticles at the site of interest by other methods. Site- specific targeting and entry in to the tumor cell can be achieved via the over-expressed receptors of the tumor cell membrane and phagocytosis/endocytosis mechanisms. [19] The transferrin receptor,[20-23] and the folic acid receptor over-expressed in many types of cancers are targets for active drug delivery.[24,25] Transferrin is a cell membrane- associated glycoprotein involved in the cellular uptake of iron and in the regulation of cell growth. Iron uptake occurs via internalization of iron-loaded transferrin mediated by the transferrin receptor. Similarly, the folic acid receptor is also a useful target for tumor specific drug delivery. It is known that the folate receptor density increases as the grade of cancer worsens. Active targeting is also possible by targeting biomarkers specific to a tumor or by exploiting processes that are essential for tumor development such as neoangiogenesis. CA-125 is such a biomarker for ovarian cancer and is expressed in more than 85% cases of ovarian cancer. [26,27] Although the benefit of active targeting is well-known, this technology has resulted in only a few clinically validated nanoproducts to date.

Examples of Nanomedicines for Cancer Approved by FDA and Those Undergoing Clinical Trials [28] (Table 1 and Figure 1).

Doxil (Liposomal Doxorubicin)

Doxil (pegylated liposomal doxorubicin), is the first FDA approved (1995) nanodrug used to treat some types of cancers, including metastatic ovarian cancer and AIDS-related Kaposi's sarcoma. The product was formulated to improve the balance between the efficacy and toxicity of doxorubicin therapy. It contains doxorubicin (Adriamycin), a member of the anthracycline group, enclosed in an 80-90 nm size unilamellar liposome coated with PEG that allows the drug to stay in the bloodstream longer so that more of the drug reaches the cancer cell.[28-30] Doxorubicin is believed to act on cancer cells by two different mechanisms: intercalation into DNA and disruption of topoisomerase II-mediated DNA repair and generation of free radicals that result in damage to cellular membranes, DNA and proteins.[31] Doxorubicin is known to cause severe and possibly life-threatening heart problems (eg, heart failure). These problems may occur during therapy or sometimes months to years after receiving doxorubicin. In some cases, heart problems are irreversible. Myocardial damage may lead to congestive heart failure and may occur as the total cumulative dose of doxorubicin hcl approaches 550 mg/m^2. The risk may be increased in patient using certain medicines that may affect heart function or have a history of heart problems, receiving radiation treatment to the chest area, or previous therapy with other anthracyclines (eg, epirubicin) or cyclophosphamide.



Doxorubicin

The risk of developing heart problems varies depending on the dose and condition. Given as liposomes, it has fewer side effects on healthy cells than regular doxorubicin. [32-34] Liposomal doxorubicin is also called Doxil (Johnson & Johnson, USA), Caelyx (Janssen-Cilag, Europe), Evacet(Liposome company INC.,) and lipodox (Sun Pharma). Loading of Doxorubicin (DXR) in liposomes is achieved by an ammonium ion gradient method where the neutral form of DXR from a sucrose medium crosses the lipid bilayer and the protonated DXR formed inside the aqueous compartment with ammonium sulphate is retained. It is estimated that a liposome contains 10-15 thousand molecules of doxorubicin. Liposomes are sterically stabilized by PEG surface coating (Figure 1). This is achieved by Distearoyl-phosphatidyl-ethanolamine-PEG (DSPE-PEG) anchored into the phospholipid bilayers made of hydrogenated soy phosphatidylcholine and cholesterol.[35] The drug to lipid ratio is 0.125. The use of such stealth liposomes resulted in enhanced circulation time after intravenous administration (mean residence time of 4 days) and improved the pharmacokinetic profile of the drug. The area under the curve (AUC) was 900 µg.h/ml for Caelyx compared to 45 µg.h/ml for the uncoated liposomes, Myocet, and 4 µg.h/ml for doxorubicin in patients with solid tumors.[36,37]

Table 1: Examples of Nanomedicines for cancer approved by FDA and those undergoing clinical trials. (Source: FDA website, original publications, reviews and websites of pharmaceutical companies supplying/developing these drugs).

Drug product	Active ingredient	Manufacturer	Indications	FDA approved date/clinical trial status
Doxil (Caelyx)	Pegylated doxorubicin	Orthobiotech, Schering-Plough	Ovarian/breast cancer	November 1995
Abraxane	Albumin-bound Paclitaxel nanospheres.	Abraxis Bioscience, Astrazeneca	Various cancers	Jan-05
	Nab paclitaxel in combination with gemcitabine	Celgene	Metastatic pancreatic cancer	September 2013
Myocet	Liposome-encapsulated Doxorubicin	Elan Pharmaceuticals / Sopherion Therapeutics	Breast cancer	2000, Approved in Europe and Canada
DaunoXome	Liposome-encapsulated Daunorubicin	Gilead Science	HIV-related Kaposi sarcoma	Apr-96
DepoCyt	Liposomal Cytarabine	Skye Pharma, Enzon	Lymphomatous meningitis	Apr-99
Oncaspar	PEGasparaginase	Enzon	Acute Lymphocytic Leukemia	Feb-94
Onco-TCS	Liposomal Vincristine	Inex	Non-Hodgkin Lymphoma	In clinical phase I/II
LEP-ETU	Liposomal Paclitaxel	Neopharma	Ovarian/breast/lung cancers	In clinical phase I/II
Aroplatin	Liposomal Cisplatin analog	Antigenics, Inc.	Colorectal cancer	In clinical phase I/II
OSI-211	Liposomal Lurtotecan	OSI	Lung cancer/recurrent ovarian	In clinical phase II
SPI-77	Stealth Liposomal Cisplatin	Alza	Head & Neck cancer/Lung cancer	In clinical Phase III
EndoTAG-1	Paclitaxel	Medigene/SynCore Biotechnology	Breast cancer/Pancreatic cancer	In clinical phase II
Marqibo	Vincristine	Talon Therapeutics	Philadelphia chromosome-negative lymphoblastic leukemia	Aug-12
ThermoDox	Doxorubicin	Celsion Corporation	Hepatocellular carcinoma	In clinical phase III

Atragen	Liposomal all trans-retinoic acid	Aronex Pharmaceuticals	Acute promyelocytic leukemia	In clinical phase II
Lipoplatin	Liposomal Cisplatin	Regulon	Pacreatic/ Head and Neck/breast cancer	In clinical phase III
Aurimmune (CYT-6091)	TNF-α bound to colloidal Gold nanoparticles	Cytimmune Sciences	Head and Neck cancer	In clinical phase II
Auroshell	Gold nanoshells	Nanospectra Biosciences	Aurolace therapy of cancer	In clinical Phase 1
Genexol-PM	Paclitaxel-loaded polymeric micelle	Samyang	Breast cancer/small cell lung cancer	Marketed in Europe, Korea
Paclical	Paclitaxel micelles	Oasmia Pharmaceutical AB	Ovarian cancer	Phase III
Narekt -102	Irinotecan, PEGylated liposome	Nektar Therapeutics	Breast /colorectal cancer	In clinical Phase 3
NKTR-105	PEG-Docetaxel	Nektar Therapeutics	Solid tumors	Phase 1
Ontak	Diphtheria toxin and	Seragen, Inc	Cutaneous T-cell	1999

Doxorubicin hydrochloride liposome injection is currently on the FDA's drug shortage list. The U.S. Food and Drug Administration approved the first generic version of Doxil (doxorubicin hydrochloride liposome injection, February 2013) made by Sun Pharma Global FZE, a subsidiary of India's Sun Pharmaceutical Industries Ltd, to ease drug shortage. [28] Lipodox is the second generation of pegylated liposomal doxorubicin composed of distearoyl phosphatidyl choline (DSPC) and cholesterol with surface coating of PEG.[28] Lipodox has a circulation half-life of 65 hours. However, due to the long circulation time of the pegylated drug, stomatitis (inflammation of mucus lining) became the new dose-limiting toxicity. Doxil and lipodox (both pegylated) accumulate at tumor site by passive targeting mechanism. Unfortunately, both have more side effects than Myocet (non-pegylated doxorubicin). Moreover, liposomal formulations are more expensive than the non-liposomal drugs. The average per dose of doxil is approximately 10-20 fold higher compared to doxorubicin but a corresponding increase in patient survival has not been demonstrated. Another concern is the toxicity of liposomal formulations especially that of the pegylated liposomes, such as various skin reactions and hypersensitivity reactions.

Myocet

Myocet is a non-pegylated liposomal doxorubicin citrate made by Enzon Pharmaceuticals for Cephalon in Europe and for Sopherion Therapeutics in the United States and Canada. Myocet received Canadian regulatory approval on December 21, 2001 and is indicated for the first-line treatment of metastatic breast cancer in combination with cyclophosphamide. Myocet is also approved in Europe but not yet approved for use in the United States. It is currently being studied in combination with Herceptin (trastuzumab) and Taxol (paclitaxel) for treatment of HER2-positive metastatic breast cancer. Myocet has a different pharmacokinetic profile from doxorubicin, resulting in an improved therapeutic index (less cardiotoxicity and equal anticancer activity). [29]

The Myocet liposome is made up of egg phosphatidylcholine and cholesterol in a molar ratio of 55:45 and contains an equivalent of 50 mg doxorubicin hydrochloride. A pH gradient method is used to encapsulate the drug which consists of maintaining an acid pH of 4 with citric acid in the inner compartment and a pH of 7 in the outer compartment where doxorubicin hydrochloride is added. The neutral form crosses the lipid layer from the outside and the protonated form remains inside the liposome. The diameter of the liposome is about 180 nm and the drug to lipid ratio is 0.27. The product is supplied to the end-user as a three vial system. One of the vial contains a lyophilized red powder of doxorubicin hcl and another contains a suspension of the liposomes in citric acid and sodium chloride and the third vial contains a sodium carbonate buffer. The actual drug-loading occurs in the pharmacy by a specified procedure. The initial recommended dose of myocet is 60-75 mg/m^2 and cyclophosphamide (600 mg/m^2) every three weeks. The clearance of doxorubicin in breast cancer patients receiving Myocet was found to be 5-9 fold lower and the volume of distribution about 10-25 fold lower than in patients receiving conventional doxorubicin. The half-life has been reported to be between 16-50 hours, which is significantly longer than conventional doxorubicin. These findings are in agreement with the theoretical advantage of using a liposomal encapsulation for drug delivery. [30-41]

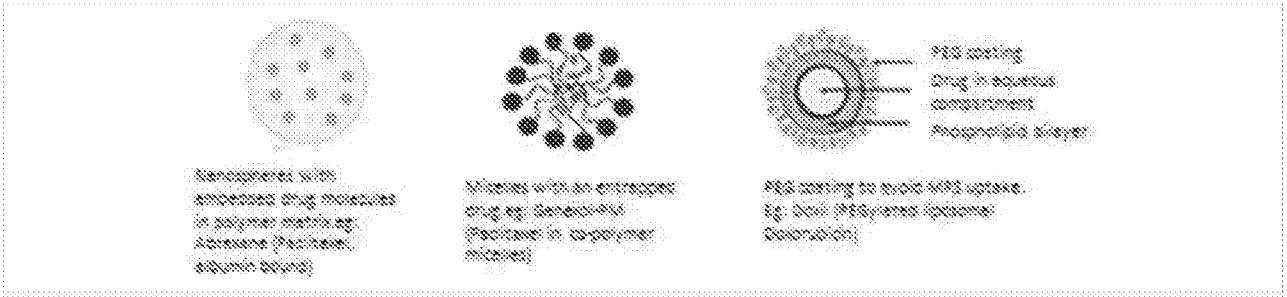


Figure 1: Nanocarriers of anticancer drugs and examples of FDA approved nanomedicines.

Although Myocet and Doxil have the same API in liposomes, they are different in terms of the lipid composition, size, loading method, different plasma and tissue distribution profile as well as different dosing and safety profiles. They are certainly not bioequivalent.

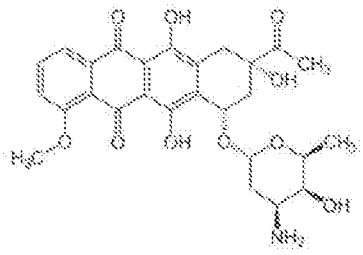
ThermoDox

A strategy leading to innovative nanoformulations focuses on nanosystems that can be triggered to release its contents on exposure to external stimuli such as heat, light, ultrasound, and magnetic fields, in order to maximize drug release at the pathological site. Thermosensitive liposomes, hydrogels, dendrimers and micelles have been studied for release of drugs at sites of elevated temperature. Generally, thermosensitive liposomes make use of lipids with transition temperature between 40-45°C. ThermoDox, a temperature-sensitive doxorubicin-pegylated liposome (Celsion, in partnership with Duke University) being developed for liver cancer, is an interesting example of stimuli-responsive nanomedicine. This new generation liposomes are being developed to release their encapsulated drug where local tissue temperatures are elevated to 39-42°C by application of radiofrequency, a technique referred to as radiofrequency ablation. The lipid components in the liposome undergo a gel to liquid transition at elevated temperatures, rendering it more permeable, thus releasing the drug. In addition, application of local hyperthermia results in the blood vessels within tumors to leak, thus increasing accumulation of liposomes in the tumor. ThermoDox is composed of a mixture of dipalmitoyl phosphatidylcholine (DPPC), monostearoyl phosphatidyl choline (MSPC) and PEG2000 DSPE (distearoyl phosphatidyl ethanolamine) in 90:10:4 molar ratios. DPPC has a transition temperature of 41.5°C, therefore, it is appropriate for temperature triggered technology. For thermodox, this technology allows concentration of the drug up to 25 times more in the treatment area than IV doxorubicin, and several fold the concentration of other liposomally encapsulated doxorubicins. [42,43] Celsion also is developing a thermosensitive liposomal Docetaxel which was evaluated in mice xenograft with lung cancer and was found to be superior in reducing the tumor volume compared to docetaxel and non-thermosensitive liposomal formulation.

DaunoXome (Liposomal Daunorubicin)

Daunoxome (Gilead sciences, now sold to Galen Pharmaceuticals)

Daunorubicin is an anthracycline antibiotic with antineoplastic activity. The anthracycline moiety is linked to an aminosugar, daunosamine via a glycosidic linkage. Daunoxome contains an aqueous solution of daunorubicin citrate encapsulated within liposomes made up of distearoylphosphatidyl choline, cholesterol and daunorubicin in a molar ratio of 10:5:1. The mean diameter is 45 nm and the total lipid to daunorubicin ratio is 18.7:1. The liposome injection has pH between 4.9 to 6 and the dispersion is red and translucent. It was approved by FDA in 1996 for HIV- associated Kaposi sarcoma. The liposomes are not rapidly cleared from the plasma by the Mononuclear Phagocyte System (MPS) and release of daunorubicin continues in a sustained manner. Preclinical studies indicate increased tissue concentrations of daunorubicin in tumor, brain, liver, spleen and intestine following daunoxome compared with free daunorubicin administration, but a reduced tissue concentration in cardiac tissue. [44] In addition, studies using radio-labelled vesicles suggest selective uptake into tumor. [45] The antitumor effects of Daunorubicin are due to intercalation into DNA and inhibition of topoisomerase II activity, resulting in decreased synthesis of both DNA and RNA. In addition, the free radical pathway that generates hydroxyl- and superoxide radicals and which peroxidizes lipids and damages cellular membranes also contributes to its antitumor activity. Pharmacokinetic studies following administration of 40 mg/m^2 in pediatric patients revealed a mono-exponential decline in all patients and the PK parameters were not dose-dependent. The total plasma clearance was 0.423 L/h/m^2, elimination half-life of 5.5 h and a volume of distribution of 3.7 L/m^2.[46,47] These differences in the volume of distribution and clearance result in higher daunorubicin exposure than with daunorubicin hcl.

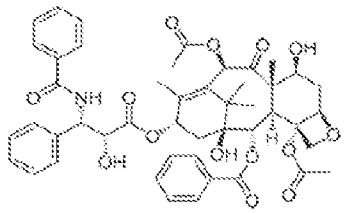


Daunorubicin

Abraxane (Albumin-bound Paclitaxel, Paclitaxel protein bound)

Paclitaxel (Taxol, Bristol-Myers Squibb) belongs to an

important class of antitumor agents called taxanes.. Paclitaxel is a semisynthetic diterpenoid taxane derivative used for the treatment of a variety of malignancies including breast, lung, non-small cell lung cancer and ovarian cancer. Taxanes are cell cycle-specific agents and bind with high affinity to microtubules resulting in inhibition of mitosis and cell death.

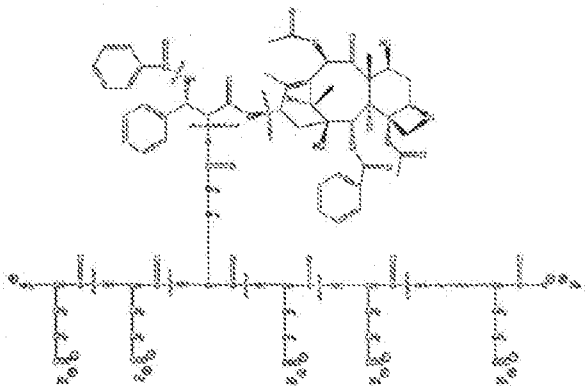


Paclitaxel

Taxanes are highly hydrophobic molecules and therefore have very low solubility in water. In order to solubilize paclitaxel, a derivative of castor oil, called polyethoxylated castor oil (cremophore EL, a non-ionic surfactant) and ethanol (50:50) is used. However, the use of the solvent-based formulation is associated with serious and dose-limiting toxicities. Cremophore itself causes hypersensitivity reactions (premedication with a steroid (oral dexamethasone) and antihistamine (diphenhydramine) was required) and is known to leach out plasticizers from standard intravenous injection tubing, necessitating special infusion set.[48] The albumin bound paclitaxel, Abraxane, however, do not contain cremophore (Figure 1). Therefore, no premedication or special tubing is required to administer abraxane. Abraxane (Abraxis Bioscience/ Celgene) was approved by the FDA in January 2005 for treatment of metastatic breast cancer and in October 2012 as a first line treatment for advanced non-small cell lung cancer in combination with carboplatin for patients who are not candidates for curative surgery or radiation therapy. Abraxane has also shown promise for treatment of advanced pancreatic cancer [49] and the US FDA has expanded approval to include advanced pancreatic cancer in 2013. Abraxane formulation uses nanotechnology to combine human albumin with paclitaxel allowing for the delivery of an insoluble drug in the form of nanospheres (130 nanometers in diameter). Albumin is an ideal carrier for drug delivery because of its preferential uptake in tumor and inflamed tissue, ready availability, biodegradability, and lack of toxicity and immunogenicity. Albumin-bound paclitaxel is the first biologic chemotherapeutic compound to exploit the gp60 receptor (albondin)-mediated pathway in endothelial cell walls of tumor microvessels to achieve enhanced intra-tumoral concentrations. Gp60 (albondin) is a 60-kda glycoprotein localised on the endothelial cell surface that binds to native albumin with a high affinity. The accumulation of albumin and albumin-bound drugs in the tumor interstitium is further facilitated by SPARC (Secreted Protein, Acidic and Rich in Cysteine) over- expressed in multiple type of tumors including breast, prostate, gastric, lung and kidney. More importantly, it is shown that increased SPARC levels in tumors' correlate with enhanced response to Abraxane.

Abraxane formulation has increased the bioavailability of paclitaxel and resulted in higher intra-tumor concentrations facilitated by albumin-receptor (gp60) mediated endothelial transcytosis.[50-52] Nanoparticle albumin bound (NAB) paclitaxel is administered as a suspension intravenously (260 mg/m^2 as infusion for 30 minutes). *Nab* paclitaxel can be reconstituted in normal saline at concentrations of 2–10 mg/ml, compared with 0.3–1.2 mg/ml for paclitaxel. It undergoes biphasic elimination (two-compartment model of disposition) with a terminal half-life of 27 hours (5.8 hours for paclitaxel). The clearance is 43% slower (15 L/h/m^2) and the mean volume of distribution is 632 L/m^2 (indicating extensive extravascular distribution). The drug exposure (AUC) was proportional to the dose in the range of 80-375 mg/m^2.

Another nanocarrier for paclitaxel is being developed by Cell therapeutics, called "Paclitaxel poliglumex (PPX)". This product is also known as Xyotax and Opaxio or CT 2103. It is a Soluble, large macromolecular conjugate of paclitaxel and poly-L-Glutamic acid and contains 37% of Paclitaxel. The polymer, poly-L glutamic acid, is biodegradable. When bound to the polymer, paclitaxel is inactive, potentially sparing healthy tissues from exposure to high level of paclitaxel and its associated toxicities. PPX, because of its large size, accumulates in tumor tissues by taking advantage of the enhanced permeability of tumor vasculature and lack of lymphatic drainage. The drug is released from the polymeric backbone by the intracellular lysosomal enzyme protease, cathepsin B, which is up-regulated in many tumor types.[53,54] Preclinical studies in animal tumor models demonstrate that PPX is more effective than standard paclitaxel and is associated with prolonged tumor exposure to active drug while minimizing systemic exposure. Phase 1 and 2 clinical studies with PPX showed encouraging outcomes compared to standard taxanes with reduced neutropenia and alopecia and allowed a more convenient administration schedule without the need for routine premedications.[55]



Polyglumex

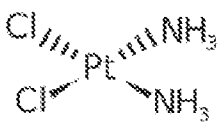
Human pharmacokinetic studies are consistent with prolonged tumor exposure to active drug and limited systemic exposure. Based on these results, three ongoing randomized phase III trials were initiated to test the efficacy of paclitaxel poliglumex in patients with advanced non-small cell lung carcinoma.[55-56]

Another agent under investigation and now in phase IV clinical trial is Genexol-PM (Samyang Biopharmaceuticals, South Korea) which consists of 20-50 nm micelles formed by the self-assembly of polyethylene glycol and polylactide polymers (Figure 1). The core of these micelles contains paclitaxel.[59] The co-polymer increases the water-solubility of paclitaxel and allows delivery of higher doses than those achievable with paclitaxel alone. Genexol-PM was launched in Korean market for breast cancer and small cell lung cancer in February 2007.

EndoTAG-1

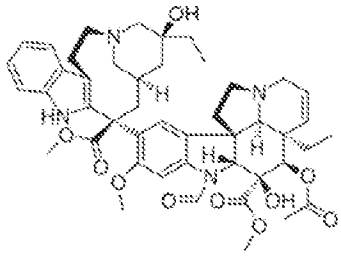
Medigene AG (a biotechnology company in Munich,Germany) obtained U.S. patent for EndoTag®-1 (formerly named lipopack) in May 2012 for the Treatment of Triple-Negative Breast cancer. Triple negative breast tumors do not have any HER2 receptors or hormone receptors for estrogen or progesterone. There are very few treatment options available for this type of cancer since the conventional antihormonal therapy or treatments targeting HER2 are not appropriate. Endotag®-1 is a cationic liposome containing paclitaxel. The liposome is formed from 1,2 dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2 dioleoyl-sn-glycero-3-phosphocholine (DOPC) and paclitaxel in a molar ratio of 50:45:5. The diameter is about 220 nm. Due to the positively charged lipids, endotag®-1 interacts with newly developing, negatively charged endothelial cells required for the growth of tumor blood vessels. The endotag®-1 targets the tumor vasculature without affecting the supply to endothelial cells of healthy tissue. By doing this, it prevent the formation of new tumor blood vessels, increases tumor vascular permeability and inhibit tumor growth.[60] A controlled phase II clinical trial for pancreatic cancer showed significantly increased survival rates in patients treated with endotag-1 and gemcitabine combination therapy. A phase II trial in triple negative breast cancer also showed a positive efficacy trend of the combination therapy. European and US authorities have granted orphan drug status for endotag-1 in the treatment of pancreatic cancer.

Lipoplatin (Liposomal cisplatin)



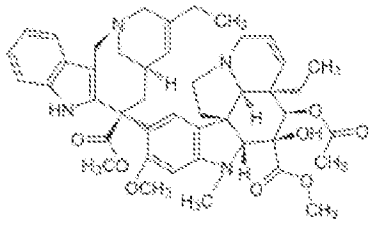
Lipoplatin is a liposomally-encapsulated (average diameter, 110 nm) drug product of the FDA-approved cytotoxic agent cisplatin. The major concern about the use of cisplatin and other platinum compounds is nephrotoxicity. Patients receiving these agents need to be hydrated to prevent renal damage. In the Lipoplatin product, cisplatin (cis-diamino dichloro-platinum) is encapsulated in a liposome shell composed of dipalmitoyl phosphatidyl glycerol, soy phosphatidyl choline, cholesterol and methoxy-polyethylene glycol-distearoyl phosphatidyl-ethanolamine lipid conjugate. The ratio of cisplatin to lipids is 8.9%:91.1% (w/w).[61,62] Lipoplatin is used against pancreatic cancer in combination with gemcitabine as first line treatment. It has shown superiority to cisplatin in combination with paclitaxel as a chemotherapy regimen in non-small cell lung cancer (NSCLC) adenocarcinomas. Lipoplatin accumulates in the cancer tissues by the extravasation of the nanoparticles through the defective vasculature of the tumor tissue during neoangiogenesis. Lipoplatin had mild hematological and gastrointestinal toxicity and did not show nephron, neuro, oto- toxicity or any other side effects characteristic of cisplatin. The half-life of total platinum in the human plasma was 60-117 hours compared to 6 hours for cisplatin.[63] Human studies have shown 40- to 200-fold higher platinum concentration compared to the adjacent normal tissue in specimens from human biopsies 20h post-infusion of the drug. The clinical development of Lipoplatin in adenocarcinomas establishes this drug as the most active platinum drug with significantly lower side effects. Lipoplatin, under the name Nanoplatin, received in 2009 the consent of European medicine agency to be tested as first line against non-squamous NSCLC mainly composed of adenocarcinomas.

OncoTCS (Vincristine)



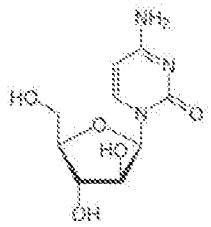
Vincristine (VCR) and Vinblastine (VLB) are alkaloids obtained from the flowering plant. *Catharanthus roseus* (Periwinkle, also called the Madagascar periwinkle). Both have powerful anticancer activity. On August 9, 2012, the Food and Drug Administration (FDA) granted accelerated approval for vincristine sulfate liposome injection (Marqibo®, made by Talon Therapeutics, Inc.) For the treatment of adult patients with Philadelphia chromosome-negative (Ph --) acute lymphoblastic leukemia (ALL). Vincristine administered as the liposomal formulation exhibits a lower clearance and higher AUC compared with conventional VCR.[64,65] INEX Pharmaceuticals is developing a liposomal formulation of vincristine (Onco TCS, vincacine, VSLI, Vincristine sulfate liposomes for injection) for the treatment of relapsed aggressive non-Hodgkin's lymphoma (NHL) and other cancers. (Inex is a Canadian biopharmaceutical company developing and commercializing proprietory drugs and drug delivery systems to improve treatment of cancer). Vincristine is being developed using INEX's proprietary drug-delivery technology platform called the "transmembrane carrier systems" (TCS). Liposomal vincristine is expected to have certain advantages over the existing standard preparation because vincristine in liposomes enables the drug to increase blood circulation time, increase the drug accumulation in the blood, increase drug accumulation in the tumor, and be released over an extended period.

Vinorelbine



Vinorelbine, a microtubule inhibitor, is a semisynthetic vinca alkaloid shown to be useful for treatment of a variety of malignancies, such as small cell lung, breast, ovarian, head and neck, cervical and kaposi sarcoma. A new formulation of vinorelbine, called Allocrest, consists of vinorelbine tartrate encapsulated in the aqueous core of a liposome (sphingomyelin-based liposomes called Optisome™). This formulation has been developed to achieve targeted delivery of the drug in high concentration in the tumor and also sustained release. In animal models, the Optisome technology resulted in prolonged plasma circulation (100-fold increased area under the concentration- time curve) and 9.5-fold enhanced cancer tissue drug penetration and accumulation as compared to that achievable with un-encapsulated, standard vinorelbine. The high intra-tumoral vinorelbine concentration is expected to improve cancer cell killing beyond the capability of standard vinorelbine. The prospect of providing enhanced anti-cancer activity without enhanced toxicity is of particular importance in the treatment of elderly cancer patients and those with marginal performance status. Nano vinorelbine (nanovnb) was found to be active against human breast cancer and lung cancer xenograft.[68]

DepoCyt (Liposomal Cytarabine)



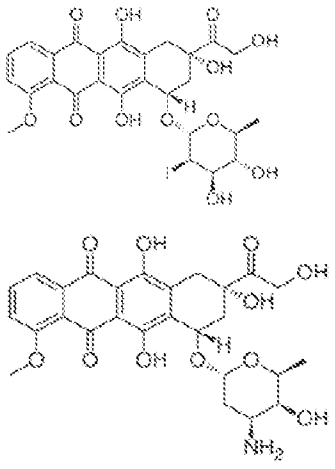
Cytarabine

DepoCyt is a sustained –release liposomal formulation of cytarabine, prepared by a unique proprietary technology called depofoam. Unlike the usual unilamellar or multilamellar liposomes, the depofoam is a multivesicular system containing hundreds of water-filled compartments separated by a lipid bilayer. This structure allows encapsulation of large quantities of drugs and ensures prolonged release.[67-69]

Cytarabine liposome injection (DepoCyt, Enzon Pharmaceuticals), was given full approval by the FDA in April 2007 for the treatment of lymphomatous meningitis, a life-threatening complication of lymphoma. It was originally approved in April 1999 under the accelerated approval regulations based on increased response rate compared to the unencapsulated drug. It is the only liposomal drug for intrathecal use and should be administered only under supervision of a qualified physician. Systemic exposure to cytarabine is negligible when the liposome is given intrathecally. Cytarabine is a cell-cycle specific antineoplastic agent affecting cells only in the s-phase of cell divison. Depofoam-encapsulation has been shown to result in a sustained-release lasting several days to weeks (Depocyt has a half-life of up to 82.4 hours compared to 3.4 hours for the unencapsulated drug) after non-vascular administration. Depocyt is well distributed throughout the cerebrospinal fluid to provide continuous tumor cells with complete exposure to cytarabine. (The routes of administration most viable for delivery of drugs via depofoam formulations include intrathecal, epidural, subcutaneous, intramuscular, intra-articular, and intraocular). Depofoam particles are distinguished structurally from unilamellar vesicles, multilamellar vesicles, and niosomes in that each particle comprises a set of closely packed non-concentric vesicles. The particles are tens of microns in diameter and have large trapped volume, thereby affording delivery of large quantities of drugs in the encapsulated form in a small volume of injection. A number of methods based on a manipulation of the lipid and aqueous composition can be used to control the rate of sustained-release from a few days to several weeks.

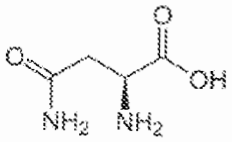
The liposome contains dioleoyl phosphatidyl choline, dipalmitoyl phosphatidyl glycerol, cholesterol and triolein. It is a long-acting, preservative-free formulation. The pharmacokinetic advantage of this formulation was that the terminal half-life was 40 times longer than that of standard cytarabine. [70] Chemical arachnoiditis, manifested primarily by nausea, vomiting, headache and fever, has been a common adverse event following administration of DepoCyt. The incidence and severity of arachnoiditis can be reduced by the co-administration of dexamethasone. Therefore, it is recommended that all patients receiving DepoCyt should be treated concurrently with dexamethasone. Cytarabine can cause fetal harm if a pregnant woman is exposed to the drug systemically. However, the concern for fetal harm following intrathecal DepoCyt administration is low because systemic exposure to cytarabine is negligible.[71]

Annamycin



Annamycin is a semisynthetic doxorubicin analog developed at the M.D Anderson Cancer Institute in Houston, Texas. Annamycin was initially investigated as an antineoplastic without the multidrug resistance related to p-glycoprotein. The p-glycoprotein is a major factor limiting the efficacy of anthracycline group of drugs.[72] As shown in the structure above, annamycin does not have the amino group in the sugar moiety as in doxorubicin. Removal of the amino group seems to reduce the cardiac toxicity without changing its antitumor properties. Annamycin intercalates into DNA and inhibits topoisomerase II. This results in the inhibition of DNA replication and repair as well as RNA and protein synthesis. Liposomal annamycin is less toxic and shows improved antitumor activity compared to annamycin and is indicated for breast cancer.[73,74]

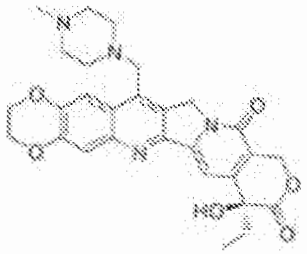
Oncaspar (Asparaginase)



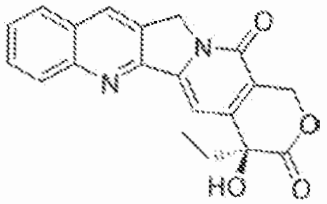
Asparagine

Leukemic cells are unable to synthesize asparagine due to lack of asparagine synthetase and are dependent on exogenous source of asparagine for survival. Asparaginase is the enzyme that depletes the amino acid asparagine. Depletion (starving the leukemic cells) of asparagine ultimately results in leukemic cell death. Normal cells are less affected because of their ability to synthesize asparagine. L-asparaginase has been an important component in the treatment of acute lymphoblastic leukemia (ALL).[75,76] Oncaspar is a modified form of the enzyme, L-asparginase. The tetrameric enzyme, derived from E.coli, is covalently conjugated with monomethoxy polyethylene glycol (mpeg). Approximately 69-82 molecules of mpeg are linked to L-Asparaginase. The molecular weight of each mpeg molecule is 5 kda. PEG asparaginase (Enzon) was approved by FDA in July, 1994 for use in acute lymphoblastic leukemia (ALL). Patients with allergy to the drug were unable to receive L-Asparaginase. The use of Oncaspar in place of L-asparaginase, markedly reduced the number of drug injections required from 21 injections of Elspar (L-asparaginase), to three injections with Oncaspar over the 20-week course of treatment. Through the process of pegylation, the half-life of L-asparaginase is significantly increased (approximately 6 days) and the L-asparaginase activity is sustained.[77] Oncaspar provides patients the full benefits of asparaginase therapy with an enhanced convenience over native L-asparaginase (non-pegylated form). Oncaspar can be administered through intramuscular (IM) injection or intravenous (IV) infusion. When utilized as a component of induction therapy for ALL, a single dose of Oncaspar achieved similar levels of asparagine depletion as 9 doses of native L-asparaginase.[77]

Camptothecins

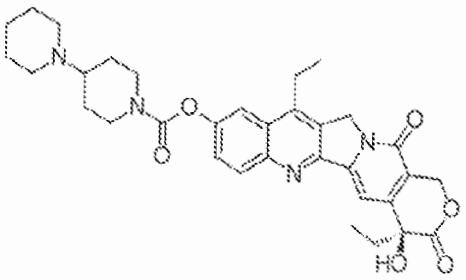


Lurtotecan



Lurtotecan is a potent semisynthetic derivative of camptothecin. This analog is water-soluble. Lurtotecan is a topoisomerase inhibitor used for epithelial ovarian cancer. Lurtotecan has been formulated into unilamellar liposomes. Pharmacokinetic studies in nude mice have demonstrated increased plasma residence time and 1500-fold increase in AUC.[78,79]

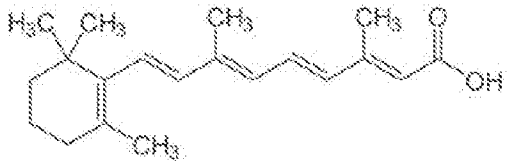
Irinotecan, another agent of this group added to fluorouracil (FU), resulted in the first major advance in many years in the treatment of colorectal cancer and the combined administration of these drugs became the standard of care.[80] Irinotecan (Camptostar,NKTR-102) is being developed by Nektar Therapeutics as a pegylated liposomal formulation for the treatment of colorectal cancer



Irinotecan (NKTR-102)

NKTR-102 is a new therapeutic option in development for advanced breast cancer. It is the first long-acting topoisomerase I inhibitor. NKTR-102 is believed to penetrate the vasculature of the tumor environment more readily than normal vasculature, increasing the concentration of active drug within tumor tissue to enhance anti-tumor activity. The unique PK profile of NKTR-102 provides continuous exposure of active drug throughout the entire chemotherapy cycle, with reduced peak exposures that can be associated with toxicities. NKTR-102 is also being evaluated

for the treatment of ovarian, glioma and lung cancers. The most significant adverse effects of irinotecan are severe diarrhea and extreme suppression of the immune system. Irinotecan received accelerated approval by the (FDA) in 1996 and full approval in 1998. During development, it was known as CPT-11.



All-trans retinoic acid

ATRA or ATRAGEN (Liposomal tretinoin, lipoatra, AR-623) is a vitamin A acid or all trans-retinoic acid. Retinoids bind to and activate retinoic acid receptors resulting in gene expressions leading to cell differentiation, decreased cell proliferation and inhibition of carcinogenesis. ATRA also inhibits telomerase, telomere shortening and eventual apoptosis of some tumor cell types. ATRA is used to treat acute promyelocytic leukemia (APL, APML) in patients who have not responded to other treatments. One of the disadvantages of oral ATRA is its very poor bioavailability. It is almost insoluble in aqueous medium with highly variable absorption from the intestine. The liposomal delivery system of ATRA alters the drug's pharmacokinetics and decreases toxicity associated with oral administration. Following (iv) injection, this formulation is able to bypass the hepatic clearance mechanism that metabolizes the oral formulation of ATRA. Additionally, in vitro studies have shown that liposomal ATRA has a greater anti-proliferative effect on neoplastic cells as compared to free ATRA. It can inhibit the proliferation of lymphoma cells in a dose-dependent manner by inducing apoptosis.[81-83] It has also been shown that the iv administration of liposomal retinoic acid in human subjects resulted in a 13-15 fold higher plasma concentration than retinoic acid.[83]

Gold Nanoparticles

One of the most studied nanotechnology-based nanoparticles is the gold nanoparticles (gnps). Gnps are potential drug carriers, photothermal agents, radiosensitizers, contrast agents and have also have shown promise for cancer therapy. There are four types of gold nanoparticles: gold colloid, gold–silica nanoshells, gold–gold sulfide nanoparticles, and gold nanorods. Each of these four particles has demonstrated success in both optical imaging and photothermal ablation of cancer. There are several methods for the preparation of gold nanoparticles. One of the methods involves reduction of chloroauric acid with sodium citrate. Varying the molar ratio of citrate to chloroauric acid results in variation of gnps sizes, from 50-150 nm. The nanoparticles are stabilized by coating with polyethylene glycol and anticancer drugs can be attached to the coated nanoparticles. Gnps exhibit unique physicochemical properties: their optical properties and ability to bind to amino and thiol groups permits surface functionalization for various biomedical applications. Tumor-specific ligands such as transferrin, folic acid, monoclonal antibodies, and tumor necrosis factor have been attached to surface of gold nanoparticles combined with a chemotherapeutic agent for specific delivery to tumor. Aurimmune (Cytimmune Sciences, Rockville, MD) is a 27nm gold nanoparticle coated with thiolated PEG and attached to recombinant human tumor necrosis factor α (TNF-α) which has the ability to accumulate in the tumor and exert its antitumor activity. TNF-α is a multifunctional cytokine known to have both cytotoxic and immunomodulatory effects. Prior to aurimmune, attempts to use TNF-α in adequate doses for its anticancer response were not successful due to dose-limiting toxicity, such as hypotension and nausea. However, no dose-limiting toxicity was observed when dose levels as high as 500-600 microgram/m^2 of TNF-α was administered to patients with solid tumors. The main side effect was only a grade 2 fever. Intracellular gnps were detectable in post-treatment tumor biopsies but not in healthy tissues. A recent study reported the amplification of biochemical action of Au-TNF by laser-induced photothermal effect in target tumor.[84] Another device that has advanced to clinical trials is a nanoparticle of silica coated with a thin layer of gold, called AuroShell (Nanospectra Bioscience Inc, Houston, Texas). Photothermal therapy of head and neck cancer is possible when the AuroShell is irradiated with near infra-red rays from a laser. Gold nanoshells are more efficient in converting the incident light in to heat than nanoparticles. This is referred to as AuroLase therapy.[84-89] The potential benefit of AuroLase therapy is the highly selective and rapid tumor destruction with minimal damage to surrounding healthy tissue. Preclinical studies have demonstrated that AuroLase Therapy is effective and causes no detectable systemic toxicity. Additionally, this therapeutic device may be used in combination with standard chemotherapy and radiation.

SiRNA Delivery

RNA interference, or gene silencing, is one of the most important discoveries in the field of biology in the last decade for which a Nobel Prize in medicine was awarded in 2006. RNA interference is a natural phenomenon which can be used to selectively turn off the genes expressed in some diseases. Molecular therapy using small interfering RNA (siRNA, synthetic RNA molecules designed to suppress production of specific proteins) has shown great therapeutic potential for diseases caused by abnormal gene over-expression or mutation. When the siRNA-based therapeutics are introduced in to the cell cytoplasm, they are guided to target protein mRNA which is then cut and destroyed, preventing subsequent production of target protein. The challenges in developing effective delivery systems include the instability of RNA in the blood and its inability to enter the cell cytoplasm. Recent developments in nanotechnology is making it possible to deliver these gene silencing agents in the true nanosize of 20-40 nm that displays favorable EPR effect and free of antigenicity.[90-92] Currently there are several phase 1 clinical trials underway using nanotechnology based delivery of siRNA including the following: CALLA 01(Calando Pharmaceuticals, a subsidiary of Arrowhead Research Corporation, a biotech company developing targeted RNAi therapeutics) using cyclodextrin nanoparticles attached to transferrin and coated with PEG for solid tumors; Atu027 (Silence Therapeutics, London, UK, now merged with Intradigm

Corporation, USA) using cationic lipids to form lipoplexes and is aimed at Protein Kinase N3(PKN3), a key factor for cancer progression and metastasis; TKM 080301 (Tekmira Pharma) targeting polo-kinase of solid tumors using lipid nanoparticles (LNP Technology).[92] Various *in vivo* delivery strategies for siRNA, including chemical modification, conjugation, lipid-based techniques, polymer-based nanosystems and physical methods, have been reviewed.[93]

Conclusion

Nanomedicines at present are in an early stage of development. They have the potential, however, to overcome the limitations of conventional cancer chemotherapy by their ability to selectively target the cancer cells without doing too much damage to healthy tissue. Properly designed nanoparticles have the ability to accumulate in tumors either by passive or active targeting and enhance the cytotoxic effects of antitumor agents. Several anticancer drugs in Nano formulations have been evaluated and a few are already approved for clinical use and others are undergoing phase 2/3 clinical trials. Although the nanomedicines have numerous advantages compared to conventional chemotherapy, there are concerns about their potential for toxicity to patients and to the environment in addition to the high cost of production and premature drug release. On the other hand, nanotechnology offers the opportunity to reformulate the discontinued drugs because of poor bioavailability, lack of selectivity to desired target or extreme toxicity. It is also known that drug loaded nanoparticles evade the efflux mechanism, maintain a high concentration within tumor cells and therefore avoid development of resistance.

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RESILIENT part 1: pharmacokinetics of second-line liposomal irinotecan in patients with small cell lung cancer

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BACKGROUND

- Liposomal irinotecan (ONIVYDE®, ONIVYDE pegylated liposomal) encapsulates the topoisomerase 1 inhibitor irinotecan in a lipid-bilayer vesicle, leading to prolonged circulation compared with the non-liposomal form.[1]
- Liposomal irinotecan is being investigated as a second-line (2L) treatment for patients with small cell lung cancer (SCLC) in the ongoing RESILIENT study (NCT03088813).

OBJECTIVES

- To describe the population pharmacokinetics (PK) of liposomal irinotecan in patients with SCLC, using data from part 1 of the RESILIENT study.

METHODS

Study design and population

- Part 1 of RESILIENT was an open-label, single-arm study comprising dose-exploration and dose-expansion phases.
- Adults (aged ≥ 18 years) were eligible for inclusion if they had SCLC that had progressed with first-line platinum-based therapy, had an Eastern Cooperative Oncology Group Performance Status of 0 or 1, and had adequate organ function; prior exposure to immunotherapy was permitted.

Treatment regimen and PK sampling

- Liposomal irinotecan (70 or 85 mg/m^2 free base) was administered intravenously over 90 minutes every 2 weeks in a 6-week cycle.
  - Patients were treated for a minimum of three cycles or until progressive disease or unacceptable toxicity.
- Seven PK samples were scheduled per patient.
  - Cycle 1: day 1 (pre dose, at the end of the liposomal irinotecan infusion and then 2 h and 24 h post infusion); day 8 (any time of day); day 15 (pre dose).
  - Cycle 2: day 22 (any time of day).

Population PK analyses

- PK parameters for total irinotecan and its active metabolite, SN-38, after liposomal irinotecan administration, were estimated using non-linear mixed-effects modelling.
  - A population PK model was used that had been developed previously using data from seven studies of liposomal irinotecan, including patients with various tumour types (N = 440).[2]
  - Here, the model was updated to include patients with SCLC using data from RESILIENT part 1.
- Assessment of model adequacy was based on the uncertainty of parameter estimates and advanced evaluation methods (e.g. a visual predictive check).
  - Interindividual variability was examined using potential covariates including patient demographics and UGT1A1*28 genotype status.
  - Derived PK parameters were computed by dose from individually predicted PK profiles.

RESULTS

Study population

- As of 2 December 2019, a total of 30 patients had received liposomal irinotecan (70 mg/m^2, n = 25; 85 mg/m^2, n = 5), four of whom had a UGT1A1*28*7/*7 homozygous genotype.

Population PK modelling

- Dose-normalized plasma concentration–time profiles of total irinotecan and SN-38 are shown in Figure 1.
- The PK of total irinotecan is described by a two-compartment model with first-order elimination, and SN-38 is formed directly by a first-order constant from the central compartment of liposomal irinotecan or after using a transit compartment (Figure 2).[2]
- Derived PK parameters for total irinotecan and SN-38 are summarized in Table 1.
- Parameter estimates based on the updated model are shown in Table 2.
- UGT1A1*28*7/*7 homozygous status did not have a significant statistical impact on SN-38 clearance.
  - Among patients receiving liposomal irinotecan 70 mg/m^2, the median average SN-38 concentration at steady state was 1.44 ng/mL and 1.46 ng/mL in patients with UGT1A1*28*7/*7 homozygous (n = 3) and non-homozygous (n = 22) genotypes, respectively.
- Model evaluation was satisfactory for total irinotecan and SN-38 (Figure 3).

Figure 1. Dose-normalized plasma concentration–time profiles of (A) total irinotecan and (B) SN-38

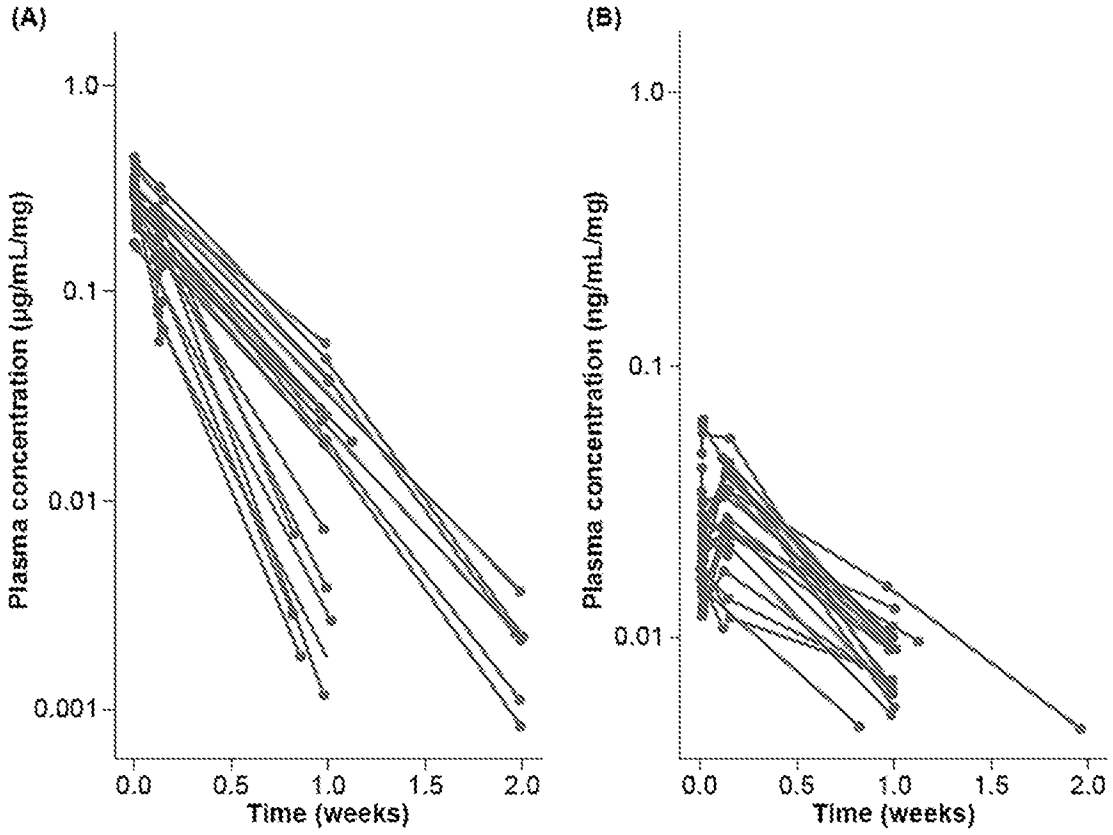
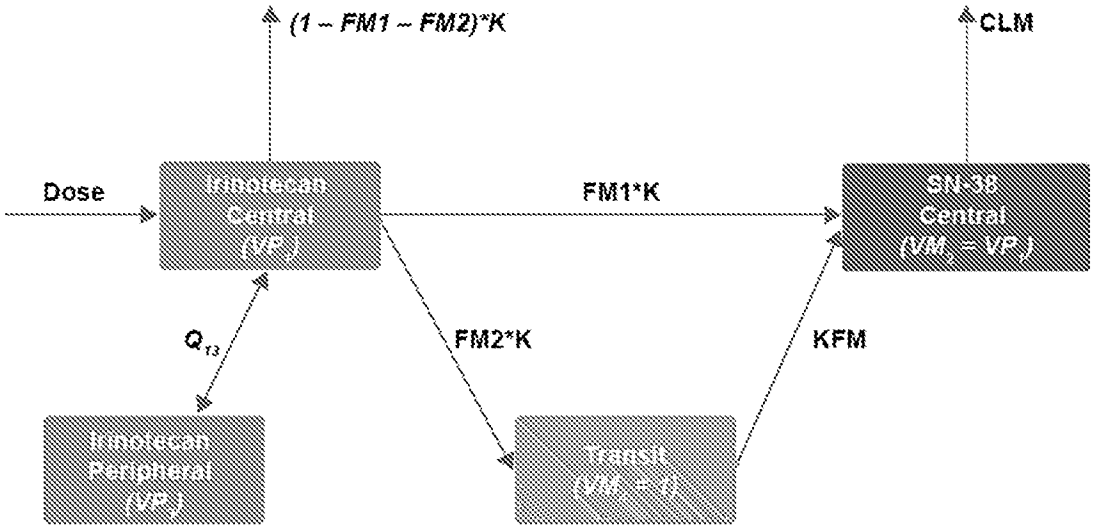
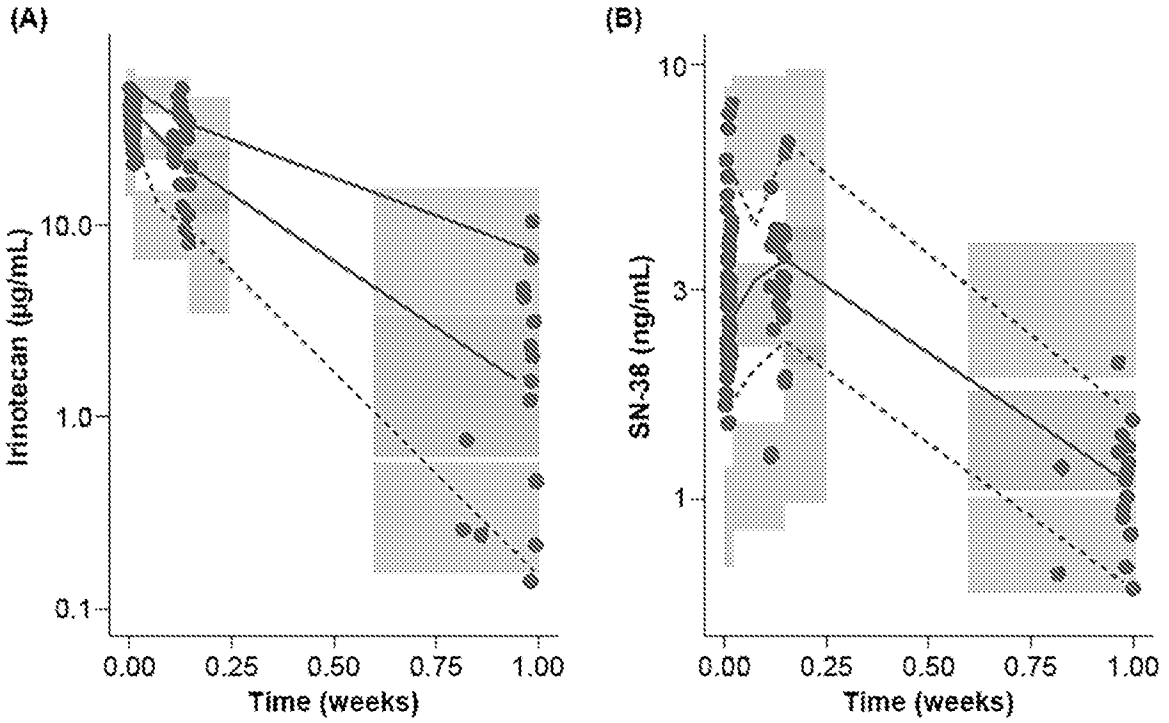


Figure 2. Population PK model representation[2]



CLM, SN-38 clearance; K, irinotecan total clearance/irinotecan central volume; FM1, fraction of parent metabolized via first-order process; FM1*K, fraction of total clearance to SN-38 (first-order); FM2, fraction of parent metabolized via transit; FM2*K, fraction of total clearance to SN-38 (transit); *(1 – FM1 – FM2)*K*, fraction of total clearance not transformed to SN-38; KFM, rate of transformation after delay; PK, pharmacokinetics; Q_{13}, intercompartmental clearance; VP_1, irinotecan central volume; VP_3, irinotecan peripheral volume; VM_1, SN-38 central volume (fixed to VP_1); VM_2, transit compartment.

Figure 3. Prediction-corrected visual predictive checks over time of (A) total irinotecan and (B) SN-38[a]



[a]Presented on a semi-log scale. Solid lines represent the observed median concentrations; dashed lines represent the 90th percentiles of the observation, pink areas represent the CIs of the prediction intervals; green areas represent the CIs of the 5th and 95th prediction intervals.
CI, confidence interval.

Table 1. Derived PK parameters for (A) total irinotecan and (B) SN-38 after administration of liposomal irinotecan (70 mg/m^2 or 85 mg/m^2 free base)

(A)	$C_{max,ss}$ (µg/mL)	$C_{min,ss}$ (µg/mL)	$C_{min,1}$ (µg/mL)	$t_{1/2}$ (days)	AUC_{ss} (µg·day/mL)
70 mg/m^2	n = 25	n = 25	n = 25	n = 24[a]	n = 25
Mean (SD)	5.16 (2.46)	35.2 (5.41)	0.232 (0.323)	5.74 (0.6)	72.3 (4.93)
CV (%)	47.7	15.4	139	73.2	47.7
Geometric mean	4.59	34.8	0.0619	5	64.3
Geometric CV (%)	54.2	15.7	717	50.4	54.3
Median (range)	3.92 (1.49–10.2)	35.9 (25.6–46.8)	0.0421 (0.001–1.43)	4.64 (2.7–23.6)	55 (20.9–144)
85 mg/m^2	n = 5	n = 5	n = 5	n = 5	n = 5
Mean (SD)	5.51 (2.13)	40.8 (5.89)	0.18 (0.224)	4.72 (0.197)	77.1 (4.26)
CV (%)	38.7	14.4	125	29.2	38.7
Geometric mean	5.17	40.4	0.0614	4.56	72.3
Geometric CV (%)	42.4	14.1	597	29.6	42.4
Median (range)	5.45 (3.12–8.2)	39.5 (33.9–50)	0.0759 (0.00889–0.529)	4.42 (3.39–6.52)	76.3 (43.6–115)

(B)	$C_{avg,ss}$ (ng/mL)	$C_{max,ss}$ (ng/mL)	$C_{min,ss}$ (ng/mL)	AUC_{ss} (ng*day/mL)
70 mg/m²	n = 25	n = 25	n = 25	n = 25
Mean (SD)	1.53 (0.522)	3.56 (1.17)	0.221 (0.144)	21.4 (1.04)
CV (%)	34.2	32.8	65	34.1
Geometric mean	1.45	3.4	0.187	20.3
Geometric CV (%)	32.6	32.2	63	32.6
Median (range)	1.44 (0.875–2.94)	3.45 (1.93–7.25)	0.169 (0.0549–0.687)	20.2 (12.2–41.1)
85 mg/m²	n = 5	n = 5	n = 5	n = 5
Mean (SD)	2.05 (0.325)	4.38 (0.45)	0.35 (0.141)	28.8 (0.646)
CV (%)	15.8	10.3	40.3	15.7
Geometric mean	2.03	4.36	0.331	28.5
Geometric CV (%)	15.2	10	37.3	15.1
Median (range)	1.96 (1.72–2.58)	4.33 (3.91–5.09)	0.288 (0.232–0.586)	27.5 (24.2–36.1)

[a]One patient had an unreliably estimated $t_{1/2}$.
AUC, area under the plasma concentration–time curve; C_{avg}, average plasma concentration; C_{max}, maximum plasma concentration; C_{min}, minimum plasma concentration; CV, coefficient of variation; PK, pharmacokinetics; SD, standard deviation; ss, steady state; $t_{1/2}$, half-life.

Table 2. Estimated PK parameters from the updated population PK model[a]

Parameters	Point estimate	RSE (%)
Irinotecan total clearance (L/week)	17.9	5.14
Asian race	× 1.204[b]	44.6
Manufacturing site	× 1.515[b]	27.9
Gender	× 0.799[b]	23.5
Irinotecan central volume (L)	4.09	2.23
BSA	× (BSA/1.71)^0.573	17.9
Manufacturing site	× 0.872[b]	29.4
Gender	× 0.886[b]	22.9
Fraction of delayed irinotecan total rate of elimination	0.629	23.4
Manufacturing site	× 1.376[b]	41
Fraction of direct irinotecan total rate of elimination	0.152	22.4
Irinotecan intercompartmental clearance (L/week)	1.35	28.6
Irinotecan peripheral volume (L)	0.421	22.6
SN-38 total clearance (L/week)	19.800	12.8
Bilirubin	× (BIL/0.41)^–0.266	17.5
Creatinine clearance	× (CRCL/85.04)^0.25	28.7
Gender	× 0.802[b]	20.3
Rate of transformation after delay (1/week)	2	5.1

Between-subject variability		
Irinotecan total clearance	0.545 (CV, 85.2%)	11
Irinotecan central volume	0.066 (CV, 26.1%)	27.5
Fraction of delayed irinotecan total rate of elimination	0.188 (CV, 45.4%)	26.4
Fraction of direct irinotecan total rate of elimination	0.928 (CV, 124%)	10.9
SN-38 total clearance	0.126 (CV, 36.6%)	13.6
Rate of transformation after delay	0.135 (CV, 38%)	29.1
Correlation between irinotecan total clearance and fraction of direct transformation	–0.558 (corr, –0.785)	12
Correlation between irinotecan total clearance and central volume	0.117 (corr, 0.617)	17.8
Correlation between irinotecan central volume and fraction of direct transformation	–0.103 (corr, –0.416)	24.4
Residual error		
Proportional error on irinotecan	0.243 (CV, 24.3%)	6.25
Proportional error on SN-38	0.291 (CV, 29.1%)	5.23
Correlation between irinotecan and SN-38 errors	0.323	26.4

[a]Model based on data from 440 patients with various tumour types in seven studies,[2] with the addition of data from 30 patients with SCLC in the RESILIENT study. [b]Values obtained as (1 ÷ x), where x is the point estimate.
BIL, bilirubin; BSA, body surface area; CRCL, creatinine clearance; corr, correlation; CV, coefficient of variation; PK, pharmacokinetics; RSE, relative standard error; SCLC, small cell lung cancer.

CONCLUSIONS

- The PK of liposomal irinotecan and SN-38 in patients with 2L SCLC is well described by the population PK model.
  - Data from part 2 of the RESILIENT study will be added to the current data set to enrich the PK characterization.
- Findings suggest that UGT1A1*28 status does not have a significant impact on SN-38 clearance in patients with 2L SCLC; however, few patients had the UGT1A1*28*7/*7 homozygous genotype.

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To download the poster or for **audio commentary**, please scan the Quick Response (QR) code.

Author contributions All authors have contributed to study conception/design, drafting the publication or revising it critically for scientific accuracy and important intellectual content, and final approval of the publication.

Conflicts of interest SP – consultant/advisor: Bristol Myers Squibb, Merck, Roche; speakers' bureau: Bristol Myers Squibb, Merck, Roche; travel/accommodation/expenses: AstraZeneca, Merck, Roche. KB – employment: Ipsen. DRS – consultant/advisor (all to institution): Artiste Health, AstraZeneca, Bayer, Bristol Myers Squibb, Celgene, Dracen Pharmaceuticals, EMD Serono, Evelo Geosciences, Genentech, GlaxoSmithKline, Ikaika Therapeutics, Illumina, Merck, Molecular Templates, Nektar Therapeutics, Novartis, Pfizer, PharmaMar, Roche, Seattle Genetics, Takeda, Triphych Health Partners, TRM Oncology, Williams & Connolly; travel/accommodation/expenses: Amgen, AstraZeneca, Celgene, Daiichi Sankyo, Bristol Myers Squibb, Genentech/Roche, GlaxoSmithKline, Janssen, Merck, Novartis, Seattle Genetics, Spectrum Pharmaceuticals, Takeda; research funding (all to institution): Aeglea BioTherapeutics, Astellas, AstraZeneca, Bind Therapeutics, Bristol Myers Squibb, Celgene, Celldex Therapeutics, Clovis Oncology, Daiichi Sankyo, Eisai, Eli Lilly, EMD Serono, G1 Therapeutics, Genentech, GRAIL, InhibRx Systems, ImmunoGen, Ipsen, Janssen, MedImmune, Merck, Molecular Partners, Nektar Therapeutics, Neon Therapeutics, Novartis, Takeda, Transgene, UT Southwestern. YC – honoraria: AstraZeneca, Bristol Myers Squibb, Genentech, Guardant Health, Lilly, Merck, Novartis, Takeda; consultant/advisor: Array BioPharma, AstraZeneca, Bristol Myers Squibb, Genentech, Heron Therapeutics, Lilly, Novartis, Pfizer, Takeda; speakers' bureau: AstraZeneca, Bristol Myers Squibb, Genentech, Guardant Health, Lilly, Merck, Novartis, Takeda; research funding: AstraZeneca, Bristol Myers Squibb, Guardant Health, Helsinn, Ipsen, Roche; expert testimony: AstraZeneca, Takeda. MJ – honoraria: Boehringer Ingelheim; travel/accommodation/expenses: Merck Sharp & Dohme, Roche, Takeda. OJ-V – consultant/advisor: AbbVie, AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Eli Lilly, Merck Sharp & Dohme, Pfizer, Roche/Genentech, Takeda. PR – nothing to disclose. TH – nothing to disclose. VGC – nothing to disclose. RBC – consultant/advisor: AstraZeneca, Bristol Myers Squibb, Merck Sharp & Dohme, Roche, Takeda. AN – consultant/advisor: AstraZeneca, Bristol Myers Squibb, Merck Sharp & Dohme, Roche, Takeda. AD – consultant/advisor: AbbVie, AstraZeneca, Bristol Myers Squibb, Seattle Genetics, Takeda; research funding: AbbVie, Amgen, Bayer, Incyte, Ipsen, Loxo, Regeneron, Seattle Genetics, Symphogen, Taxeda, Tesaro. DZ – employment, stock/other ownership and patents/royalties/other intellectual property: Ipsen. YM – employment, leadership, stock/other ownership and travel/accommodation/expenses: Ipsen. JK – employment: Ipsen; consultant/advisor: Bristol Myers Squibb, Constellation Pharmaceuticals. AP-D – employment: Ipsen. LP-A – leadership: Altum Sequencing; European Medicines Agency Scientific Advisory Groups, Genentech S.A.U.; honoraria: Advanced Accelerator Applications, Amgen, AstraZeneca, Bayer, Blueprint Medicines, Bristol Myers Squibb, Boehringer Ingelheim, Celgene, Incyte, Ipsen, Lilly, Merck Serono, Merck Sharp & Dohme, Novartis, Pfizer, PharmaMar, Roche/Genentech, Sanofi, Servier, Sysmex; research funding: AstraZeneca, Bristol Myers Squibb; travel/accommodation/expenses: AstraZeneca, Bristol Myers Squibb, Merck Sharp & Dohme, Pfizer, Roche, Takeda; other relationships: Amgen, Ipsen, Merck, Novartis, Pfizer, Roche, Sanofi, Servier. PB – nothing to disclose.

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RESILIENT part 1: pharmacokinetics of second-line liposomal irinotecan in patients with small cell lung cancer

1793P

[author block]

BACKGROUND

- Liposomal irinotecan (ONIVYDE®, OVIYDE pegylated liposomal) encapsulates the topoisomerase 1 inhibitor irinotecan in a lipid bilayer vesicle, leading to prolonged circulation compared with the non-liposomal form.[1]
- Liposomal irinotecan is being investigated as a second-line (2L) treatment for patients with small cell lung cancer (SCLC) in the ongoing RESILIENT study (NCT03088813).

OBJECTIVES

- To describe the population pharmacokinetics (PK) of liposomal irinotecan in patients with SCLC, using data from part 1 of the RESILIENT study.

METHODS

Study design and population

- Part 1 of RESILIENT was an open-label, single-arm study comprising dose-escalation and dose-expansion phases.
- Adults (aged ≥ 18 years) were eligible for inclusion if they had SCLC that had progressed with first-line platinum-based therapy and an Eastern Cooperative Oncology Group Performance Status of 0 or 1, and had adequate organ function; prior exposure to immunotherapy was permitted.

Treatment regimen and PK sampling

- Liposomal irinotecan (70 or 65 mg/m² free base) was administered intravenously over 90 minutes every 2 weeks in 4/6-week cycles.
  - Patients were treated for a minimum of three cycles or until progressive disease or unacceptable toxicity.
- Serial PK samples were scheduled per patient.
  - Cycle 1: day 1 (pre dose, at the end of the liposomal irinotecan infusion and then 2 h and 24 h post infusion), day 8 (any time of dose), day 15 (pre dose).
  - Cycle 2: day 22 (any time of dose).

Population PK analyses

- PK parameters for total irinotecan and its active metabolite, SN-38, after liposomal irinotecan administration, were estimated using non-linear mixed-effects modeling.
  - A population PK model was used that had been developed previously using data from seven studies of liposomal irinotecan, including patients with various tumor types (N = 445).[2]
  - Here, the model was updated to include patients with SCLC using data from RESILIENT part 1.
- Assessment of model adequacy was based on the uncertainty of parameter estimates and simulation methods (e.g. a visual predictive check).
  - Internal robustness was examined using patients' covariates including patient demographics and UGT1A1*28 genotype status.
  - Derived PK parameters were computed by dose from individually predicted PK profiles.

RESULTS

Study population

- As of 2 December 2019, a total of 56 patients had received liposomal irinotecan (70 mg/m², n = 29; 65 mg/m², n = 5), four of whom had a UGT1A1*28/*28 homozygous genotype.

Population PK modelling

- Dose-normalized plasma concentration-time profiles of total irinotecan and SN-38 are shown in Figure 1.
- The PK of total irinotecan is described by a two-compartment model with first-order elimination, and SN-38 is formed directly by a first-order constant from the central compartment of liposomal irinotecan or after using a transit compartment (Figure 2).[3]
- Derived PK parameters for total irinotecan and SN-38 are summarized in Table 4.
- Parameter estimates based on the updated model are shown in Table 5.
- UGT1A1*28/*28 homozygous status did not have a significant statistical impact on SN-38 clearance.
  - Among patients receiving liposomal irinotecan 70 mg/m², the median average SN-38 concentration at steady state was 1.44 ng/mL and 1.46 ng/mL in patients with UGT1A1*28/*28 homozygous (n = 3) and non-homozygous (n = 22) genotypes, respectively.
- Model evaluation was satisfactory for total irinotecan and SN-38 (Figure 3).

Table 1. Derived PK parameters for (A) total irinotecan and (B) SN-38 after administration of liposomal irinotecan (70 mg/m² or 65 mg/m² free base)

	Cycle 1 Day 1	Cycle 1 Day 8	Cycle 1 Day 15	Cycle 2 Day 22	Cycle 1 Day 1 to Cycle 2 Day 22
70 mg/m²	N = 29	N = 29	N = 29	N = 29	N = 29
Mean (SD)	3.09 (0.46)	30.2 (5.47)	0.422 (0.222)	0.74 (0.59)	72.3 (9.39)
CV (%)	47.2	18.4	126	79.2	47.1
Geometric mean	4.55	54.8	0.339	5	54.9
Geometric CV (%)	44.2	19.7	71.6	55.4	54.9
CV (%)					13.0
Median (range)	3.02 (2.40–5.01)	30.9 (20.6–46.8)	0.343 (0.107–1.01)	4.84 (2.31–23.6)	68 (47.5–88)
65 mg/m²	N = 5	N = 5	N = 5	N = 5	N = 5
Mean (SD)	8.91 (2.13)	40.5 (5.68)	0.58 (0.256)	9.72 (0.197)	77.1 (9.26)
CV (%)	24.7	14.1	128	28.2	12.0
Geometric mean	8.11	40.4	0.504	4.55	72.3
Geometric CV (%)	49.4	14.7	107	58.6	42.4
CV (%)					12.0
Median (range)	9.45 (6.13–9.72)	39.5 (32.9–46)	0.5799 (0.3–0.99)	4.42 (2.39–9.52)	76.2 (63.4–116)

	Cycle 1 Day 1	Cycle 1 Day 8	Cycle 1 Day 15	Cycle 2 Day 22
70 mg/m²	N = 29	N = 29	N = 29	N = 29
Mean (SD)	152 (25.52)	239 (51.77)	0.228 (0.144)	25.8 (21.04)
CV (%)	16.7	21.6	63	81.7
Geometric mean	149	234	0.197	20.5
Geometric CV (%)	16.5	22.1	63	82.8
CV (%)				81.7
Median (range)	144 (124–194)	245 (151–320)	0.1999 (0.0667–0.611)	20.2 (3.42–71.7)
65 mg/m²	N = 5	N = 5	N = 5	N = 5
Mean (SD)	128 (9.525)	4.36 (0.49)	0.25 (0.141)	28.5 (12.46)
CV (%)	15.5	15.3	82.3	15.7
Geometric mean	127	4.26	0.209	26.5
Geometric CV (%)	16.2	13	77.2	19.1
CV (%)				43.7
Median (range)	136 (113–139)	4.33 (3.84–4.98)	0.299 (0.0537–0.399)	25.6 (20.2–36.1)

Data presented as mean (standard deviation), unless otherwise noted.
AUC, area under the plasma concentration-time curve; C_{max}, maximum plasma concentration; $C_{max,dose}$, maximum plasma concentration normalized by dose; CL, clearance; CV, coefficient of variation; h, hour(s); min, minute(s); SD, standard deviation; $t_{1/2}$, terminal plasma half-life; t_{max}, time to maximum plasma concentration; Vss, volume of distribution at steady state; wk, week(s).

Table 2. Estimated PK parameters from the updated population PK model*

	Mean	CV (%)
Irinotecan total clearance (L/wk/kg)	12.6	3.56
Action site	4.1 [33%]	44.8
Non-functioning site	3.8 [31%]	33.9
Gender	1.27 [10%]	23.5
Irinotecan central volume (L)	2.23	3.72
BSA	1.69 [76%]	17.5
Non-functioning site	1.28 [57%]	23.8
Gender	1.13 [50%]	19.4
Fraction of delayed irinotecan total rate of administration	0.626	5.4
Non-functioning site	0.772	4.1
Fraction of direct conversion total rate of conversion	0.455	11.9
Irinotecan intercompartmental clearance (L/wk/kg)	1.16	38.5
Irinotecan peripheral volume (L)	0.821	38.5
SN-38 total clearance (L/wk/kg)	19.05	19.2
SN-38	1.26 [6.6%]	17.5
Creatinine clearance	1.0003 [0.0003–1.7]	16.7
Gender	0.9 [20%]	20.2
Rate of transformation after delay (1/week)	3	9.1
Between-occasion variability		
Irinotecan total clearance	0.565 [CV, 55.2%]	31
Irinotecan central volume	0.249 [CV, 25.1%]	12.2
Fraction of delayed irinotecan total rate of conversion	0.199 [CV, 20.4%]	36.4
SN-38 clearance		
Fraction of direct conversion total rate of elimination	0.205 [CV, 53.9%]	13.9
SN-38 total clearance	0.106 [CV, 35.3%]	15.8
Rate of transformation after delay	0.153 [CV, 39%]	20.4
Correlation between irinotecan total clearance and fraction of direct conversion	−0.559 [CV, 45.7%]	12
Correlation between irinotecan total clearance and central volume	0.337 [CV, 53.6%]	15.6
Correlation between irinotecan central volume and fraction of direct conversion	−0.325 [CV, 42.9%]	24.3
Residual error		
Proportional error on irinotecan	0.043 [CV, 21.3%]	5.28
Proportional error on SN-38	0.291 [CV, 25.5%]	5.23
Correlation between irinotecan and SN-38 errors	0.721	20.4

*Model based on data from 445 patients with various tumor types (a mixed dataset with the addition of data from 29 patients with SCLC in the RESILIENT study; "mean" is the median for CV; n, observations for a PK estimate; BS, bilirubin; BSA, body surface area; CRCL, creatinine clearance; CV, coefficient of variation; FR, fraction; IOC, inter-occasion variability; WBS, white blood cell count.

Figure 1. Dose-normalized plasma concentration-time profiles of (A) total irinotecan and (B) SN-38

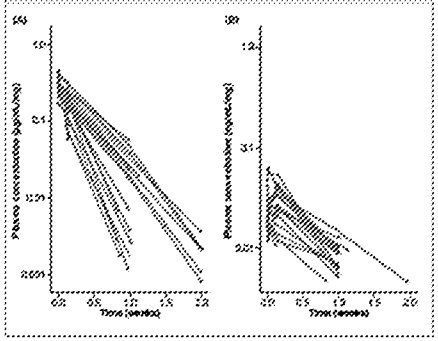
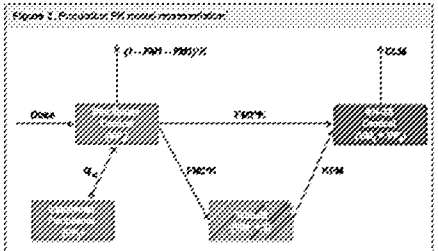
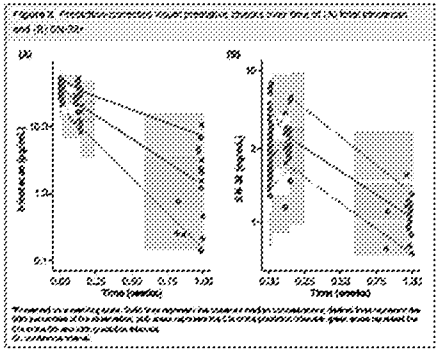


Figure 2. Population PK model representation



SN-38, SN-38 irinotecan; K, clearance and transformation rate constant; CLSN, CL/F, fraction of parent irinotecan cleared to the central; FRACTR, fraction of irinotecan converted to SN-38; KSN, CL/F, rate of conversion from the central compartment; KTR, rate of transformation from the transit; PK, pharmacokinetic; Q_{SN}, intercompartmental clearance; V_{SN}, central volume; V_{SN}, irinotecan peripheral volume; V_{SN}, SN-38 central volume; [blank] or CL/F, apparent clearance.

Figure 3. Prediction-corrected visual predictive checks over time of (A) total irinotecan and (B) SN-38



The dots represent the observed concentrations, the solid line represents the median concentration, and the area between the two dashed lines represents the 90% prediction interval. Each panel shows the observed and predicted concentrations stratified by dose level.

CONCLUSIONS

- The PK of liposomal irinotecan and SN-38 in patients with 2L SCLC is well described by the population PK model.
  - Data from part 2 of the RESILIENT study will be added to the current data set to enrich the PK characterization.
- Findings suggest that UGT1A1*28 status does not have a significant impact on SN-38 clearance in patients with 2L SCLC; however, few patients had the UGT1A1*28/*28 homozygous genotype.

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1793P **RESILIENT part 1: Pharmacokinetics of second-line (2L) liposomal irinotecan in patients with small cell lung cancer (SCLC)**

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Background: RESILIENT (NCT03088813) is a two-part phase 2/3 study of the safety, tolerability and efficacy of liposomal irinotecan monotherapy in patients with SCLC who progressed with platinum-based first-line therapy. Here we describe the pharmacokinetics (PK) of 2L liposomal irinotecan from RESILIENT part 1.

Methods: Part 1 was an open-label, single-arm study comprising dose-finding and dose-expansion phases. Patients aged ≥ 18 years, with an ECOG performance status of 0/1, received liposomal irinotecan 70 or 85 mg/m^2 free base every 2 weeks. Seven PK samples per patient were scheduled. Plasma concentration data for total irinotecan (tIRI) and SN-38 (active metabolite) were analysed using a population PK model updated for SCLC. PK parameters were estimated with non-linear mixed effects modelling. Assessment of model adequacy was based on the uncertainty of parameter estimates and advanced evaluation methods (e.g. visual predictive check). Interindividual variability was examined using potential covariates including patient demographics and UGT1A1*28 genotype status. Derived PK parameters, $C_{average}$ and C_{max}, were computed by dose from individually predicted PK profiles.

Results: As of 2 DEC 2019, 30 patients had received liposomal irinotecan (70 mg/m^2, n = 25; 85 mg/m^2, n = 5). tIRI PK is described by a two-compartment model with first-order elimination. SN-38 is formed directly by a first-order constant from the central compartment of liposomal irinotecan or following a transit compartment. UGT1A1*28 *7/*7 homozygous status (4/30 patients) was not associated with a significant impact on SN-38 clearance. Model evaluation was satisfactory for both tIRI

and SN-38. After 70 and 85 mg/m^2 respectively, median (coefficient of variation %) $C_{average}$ was 5.1 (48) and 5.5 (9) μg/mL for tiRI, and 1.6 (33) and 2.1 (16) ng/mL for SN-38, and C_{max} was 36.1 (15) and 40.7 (14) μg/mL for tiRI and 3.7 (32) and 4.4 (10) ng/mL for SN-38.

Conclusions: The PK of 2L liposomal irinotecan and SN-38 in patients with SCLC is well described by the population model. Findings suggest SN-38 clearance is not associated with UGT1A1*28 *7/*7 homozygous status. RESILIENT part 2 data will be added to the current dataset to enrich the PK characterization.

Clinical trial identification: NCT03088813.

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ORIGINAL REPORT

Phase III Southwest Oncology Group 9415/Intergroup 0153 Randomized Trial of Fluorouracil, Leucovorin, and Levamisole Versus Fluorouracil Continuous Infusion and Levamisole for Adjuvant Treatment of Stage III and High-Risk Stage II Colon Cancer

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose

Modest toxicity and possibly enhanced activity makes continuous-infusion fluorouracil (FU) an attractive alternative to FU plus leucovorin (FU/LV) for the adjuvant treatment of colorectal cancer. Intergroup trial 0153 (Southwest Oncology Group trial 9415) was developed to compare the efficacy of continuous-infusion FU (CIFU) plus levamisole to FU/LV plus levamisole in the adjuvant treatment of high-risk Dukes' B2 and C1 or C2 colon cancer.

Patients and Methods

After surgery, patients were randomly assigned to CIFU 250 mg/m^2/d for 56 days every 9 weeks for three cycles or FU 425 mg/m^2 and LV 20 mg/m^2 daily for 5 days every 28 to 35 days for six cycles. All patients received levamisole 50 mg tid for 3 days every other week. The primary end point was overall survival (OS).

Results

The study closed in December 1999 after an interim analysis demonstrated little likelihood of CIFU showing superiority to FU/LV within the stipulated hazard ratio. A total of 1,135 patients were registered. At least one grade 4 toxicity occurred in 39% of patients receiving FU/LV and 5% of patients receiving CIFU. However, almost twice as many patients receiving CIFU discontinued therapy early compared with those receiving FU/LV. The 5-year OS is 70% (95% CI, 66% to 74%) for FU/LV and 69% (95% CI, 64% to 73%) for CIFU. The corresponding 5-year disease-free survival (DFS) is 61% (95% CI, 56% to 65%) and 63% (95% CI, 59% to 68%), respectively. For all patients, 5-year OS is 83%, 74%, and 55%; 5-year DFS is 78%, 67%, and 47% for N0, N1, and N2-3, respectively.

Conclusion

CIFU had less severe toxicity but did not improve DFS or OS in comparison with bolus FU/LV.

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INTRODUCTION

Colorectal cancer is the second leading cause of cancer mortality in the United States. In 2004, it was estimated that there were approximately 146,900 new cases and 56,700 deaths from colorectal cancer.[1] Approximately 80% of patients present with cancer that is deemed resectable.[1] The 5-year survival rate after surgery alone for stage II disease is approximately 72% to 90%; for stage III disease, the 5-year survival rate disease is approximately 50% to 60%.[2-7]

Multiple cooperative group trials of adjuvant therapy, in the 10 years preceding initiation of this study, had shown consistent

benefit for fluorouracil (FU) -based treatment.[8-10] In Intergroup (INT) study 0035, the study most recently published before this trial's initiation, both disease-free and overall survival benefit were shown for FU plus levamisole compared with surgery alone for stage III disease. For patients with node-positive cancers, the progression-free survival was 44% in patients with no additional therapy, 45% for levamisole alone, and 61% in those receiving FU plus levamisole.[11] In the follow-up study, INT-0089, this FU plus levamisole regimen was compared with two different regimens of bolus FU plus leucovorin (FU/LV) and bolus FU/LV plus levamisole. While awaiting the mature results of the latter study, we chose to ask whether FU, administered by an infusion rather than bolus injection, would be superior in the adjuvant treatment of high-risk colon cancer.

FU delivered by continuous infusion has long been considered a less toxic and effective alternative to bolus FU or FU/LV for the treatment of colorectal cancer.[12-15] Continuous infusion FU, though uniquely associated with hand-foot syndrome, is less myelosuppressive than bolus therapy.[16] A meta-analysis comparing infusional and bolus therapy in the treatment of metastatic colorectal cancer determined that the response frequency for infusional therapy was higher, and that the duration of response and survival was slightly longer with infusional compared with bolus FU.[15] In addition, a prior adjuvant study had assessed the utility of continuous-infusion FU in patients with resected rectal cancer. Patients received either bolus or continuous-infusion FU concurrent with radiotherapy and were also randomly assigned to FU alone or FU with semustine before and after radiotherapy. Patients treated with continuous-infusion FU had a lower relapse rate (37% *v* 47%) and higher survival (70% *v* 60%) compared with patients treated with bolus FU. Local control was not different between the two FU regimens.[17] The results of this last study suggested that continuous-infusion FU might have increased benefit compared with bolus therapy in the eradication of micrometastases.

Because of this potential for better outcome in the setting of micrometastatic disease, the Southwest Oncology Group coordinated study INT-0153, which was developed to determine if continuous-infusion FU plus levamisole would decrease recurrence and improve overall survival compared with FU/LV plus levamisole in high-risk colon cancer. Levamisole was included in both arms because in the mid-1990s, this drug was still deemed to be a component of standard therapy.[5] The original dosing of continuous-infusion FU had been well studied in metastatic disease at 300 mg/m^2/d.[12] Because hand-foot syndrome and stomatitis were common adverse effects with such protracted treatment and because the treatment goal of this trial was to deliver 24 weeks of therapy with only two mandated breaks of 1 week each, a slightly lower dose (250 mg/m^2/d) was chosen, combined with levamisole. A pilot study of this combination was completed at the University of Southern California (Los Angeles, CA) and at Wayne State University (Detroit, MI), and acceptable tolerance was demonstrated.[18] FU/LV plus levamisole, one arm of INT-0089, was chosen as the control arm of this trial because it was expected to be at least equivalent in activity to the original FU plus levamisole schedule.

PATIENTS AND METHODS

Eligibility Criteria

Patients must have had histologic proof of adenocarcinoma of the colon and undergone complete resection of the primary tumor without gross or microscopic evidence of residual disease. All known tumor had to be resected en bloc. The entire tumor had to be above the peritoneal reflection. Surgical margins had to be more than 5 cm, although this requirement was subsequently revised to 5 cm as documented by the surgeon and/or 2.5 cm as documented by pathologic examination. Patients were eligible if they had stage II disease (TNM T3-4a, N0, M0) with total or near-total bowel obstruction or perforation, or stage III disease (TNM T1-4a, N1-3, M0). N3 defined lymph nodes along a named vascular trunk or metastasis to apical nodes as defined by the surgeon. There could be no evidence of distant metastasis as determined by the surgeon at operation and by chest radiographs. Computed tomography scan or ultrasound of the liver was encouraged but not required. Full evaluation of the colon and rectum by colonoscopy or by sigmoidoscopy with barium enema was required to exclude other synchronous, unresected primary cancers. These requirements were subsequently amended to permit intraoperative assessment of the remaining colon by the surgeon. Patients had to have evidence of adequate organ function as measured by Southwest Oncology Group performance status 0 to 2, bilirubin $\leq 2\times$ institutional upper limits of normal (IULN), AST $\leq 2\times$ IULN, alkaline phosphatase $\leq 2\times$ IULN, and serum creatinine $\leq 2\times$ IULN; white blood count more than 3,500/μL; and platelets $\geq$ institutional lower limit of normal. Patients may not have had a prior malignancy within the preceding 5 years, except for resected basal cell or squamous cell skin cancer, in situ cervical cancer, or stage I colon cancer. Patient registration had to have occurred between days 21 to 35 after definitive surgery. All patients signed informed consent forms. Institutional review board approval was required.

Treatment

Patients were randomly assigned to treatment using a dynamic balancing algorithm that stratified for T stage (T1 *v* T2 *v* T3 *v* T4), N stage (N0 *v* N1 *v* N2-3), and time from surgery to registration (days 21 to 28 *v* days 29 to 35). Treatment was to start within 4 working days of random assignment. Two treatment arms were assigned. The first arm included FU/LV plus levamisole. A cycle consisted of 5 consecutive days of FU 425 mg/m^2 intravenous push and LV 20 mg/m^2 intravenous push days 1 to 5, repeated on days 29 and 57, and then every 5 weeks for a total of six cycles. Levamisole 50 mg was delivered every 8 hours for 3 consecutive days, every 14 days, for a total of 6 months. The second arm included FU infusion and levamisole. A cycle consisted of continuous-infusion FU 250 mg/m^2/d by for three 8-week cycles. There were 1-week intervals between cycles 1 to 2 and 2 to 3.

Levamisole 50 mg was delivered every 8 hours for 3 consecutive days, every 14 days for a total of 6 months.

Venous access was established using a venous access device selected by the investigator. Because of the increased risk of thrombosis, anticoagulation was recommended. Either unfractionated heparin 10,000 U was coadministered, mixed with each week's total FU dose, or warfarin 1 to 2 mg/d orally was prescribed. Platelets and coagulation parameters were monitored weekly for 4 weeks and then monthly. A warfarin dose was chosen to be small enough not to alter PT. Heparin was to be halted if there was evidence of heparin-induced thrombocytopenia.

Dose reductions were mandated for myelosuppression, diarrhea, stomatitis, and hand-foot syndrome caused by FU. Granulocyte colony-stimulating factor could be used for persistent grade 3 to 4 neutropenia. Patients also could have dose reduction or cessation for levamisole toxicities, including fever, rash, nausea, or encephalopathy.

Treatment in both arms of the study was to be given for 6 months, with early discontinuation for unfavorable toxicity or early disease progression. After completion of chemotherapy, patients were re-evaluated by history and physical examination, hematology and chemistry panel, and chest x-ray at 9 and 12 months, and then every 6 months up to 5 years after treatment initiation. There were no mandated post-treatment carcinoembryonic antigen assessments or computed tomography scans; these were done at the discretion of the treating physician. Colonoscopy was recommended at intervals of 6 and 12 months, and then yearly or less frequently at the discretion of the investigator. Patients were observed for disease recurrence, disease-free survival, and survival.

Statistical Considerations

The primary end point for this study was overall survival. Assuming the 5-year survival probability to be 69% in the FU/LV plus levamisole arm (based on the results from INT-0035), the a priori hypothesis was that the continuous-infusion arm would be judged superior if there were a true relative increase in survival of 35% (corresponding to a 5-year survival probability of 76%). The study had an accrual goal of 1,800 eligible patients, with accrual estimated to take 6.5 years, with an additional 3.5 years of follow-up. This sample size was sufficient to detect a hazard ratio for survival of 1.35 with 95% power, using a one-sided test of significance. Disease-free survival was a secondary end point.

The study was monitored by the Southwest Oncology Group Data and Safety Monitoring Committee (DSMC). The protocol specified two formal interim analyses, timed to occur when approximately one third of the expected deaths would have occurred (expected when two thirds of the patients had been accrued), and approximately 1.5 years after the end of accrual when two thirds of the expected deaths had occurred. In 1998, the DSMC approved a modification of the accrual goal to 1,500 patients, based on slower than expected accrual and because the necessary number of events could be attained with fewer patients. This decision was based solely on accrual patterns and not on interim study results.

The three stratification factors, T stage (three levels), N stage (three levels), and time from surgery (two levels), were included as covariates in the Cox regression analysis. This model allowed for assessment of other covariates, such as race or ethnicity, sex, and age. All eligible patients were included in the analysis of survival and disease-free survival by their assigned treatment according to the intent-to-treat principle. Patients who refused treatment were not included in toxicity analyses.

RESULTS

Between December 1994 and December 1999, 1,135 patients were registered. In addition to the Southwest Oncology Group, patients were accrued from the Eastern Cooperative Oncology Group, the Cancer and Leukemia Group B, and the North Central Cancer Treatment Group.

In October 1999, the first formal interim analysis of this study was reviewed by the DSMC, which recommended early termination. This decision was based on the finding that the alternative hypothesis of a 35% improvement in survival could be rejected based on protocol-specified stopping rules.

Of the 1,135 patients, 195 were ineligible; 64 were ineligible because of insufficient prestudy documentation. The majority of the remaining ineligible patients did not have the correct disease stage, had disease below the peritoneal reflection, received inadequate prestudy surgery, or lacked pretreatment bowel assessment by sigmoidoscopy and barium enema or colonoscopy. Twenty eligible patients had major protocol violations. Fifteen patients never started protocol treatment, and one patient who was randomly assigned to the bolus FU arm received 2 days on the continuous-infusion arm instead. These patients are not included in the toxicity analysis. Of the other four major deviations, three were for patients who had incorrect doses of FU, and one patient received levamisole every other day.

Patient characteristics are listed in Table 1. There is a slightly higher percentage of males in the continuous-infusion arm than the bolus arm, although this difference is not statistically significant. The majority of patients were non-Hispanic whites. Most patients had T3 disease, and 15% were node negative. The majority of patients were registered between 29 and 35 days after surgery.

Nine hundred nineteen patients were assessable for toxicity assessment (Table 2). There were four treatment-related deaths among the eligible patients. In the bolus arm, one patient died as a result of a bowel infection, and one died as a result of sepsis. On the continuous-infusion arm, one patient developed leukemia, and one patient had a deep vein thrombosis.

Overall, at least one grade 4 toxicity occurred in 22% of the patients, with 39% in the bolus arm, and 5% in the continuous-infusion arm. The majority of the grade 4 toxicity in the bolus arm was hematologic. Febrile grade 3 to 4 neutropenia occurred in 14 patients with bolus FU/LV and was associated with two deaths. There was no grade 3 to 4 neutropenia with infusional FU. Grade 4 gastrointestinal toxicity was three-fold more common in the FU/LV arm than in the continuous-infusion arm and consisted of stomatitis and pharyngitis or diarrhea. Grade 3 to 4 thrombosis occurred in 10 patients receiving FU/LV and in 16 patients receiving continuous-infusion FU, with one

Table 1. Patient Demographics

Characteristic	FU/LV Plus Levamisole (n = 463) No. of Patients	%	Continuous Infusion FU Plus Levamisole (n = 477) No. of Patients	%
Age, years				
Median	60.6		61.3	
Minimum	27.4		19.4	
Maximum	84.2		85.0	
Sex				
Male	234	51	257	54
Female	229	49	220	46
Hispanic				
Yes	21	5	11	2
No	442	95	466	98
Race or ethnicity				
White	408	88	420	88
Black	43	9	39	8
Asian	7	2	13	3
Native American	2	0	1	0
Unknown	3	1	4	1
T stage				
T1	11	2	12	2
T2	50	11	39	8
T3	359	78	392	82
T4	43	9	34	7
N stage				
N0	66	14	78	16
N1	239	52	254	53
N2-3	158	34	145	31
No. of days from surgery to trial registration				
21-28	180	39	191	40
29-35	283	61	286	60
Performance status				
0-1	448	97	463	97
2	15	3	14	3

Abbreviations: FU, fluorouracil; LV, leucovorin.

Table 2. No. of Patients With Toxicity ≥ Grade 3

Selected Toxicities	FU/LV Plus Levamisole (n = 459) Grade 3	4	5	Continuous Infusion FU/LV (n = 460) Grade 3	4	5
Cardiovascular	17	3	0	24	2	1
Dermatologic	11	0	0	50	0	0
Flu-like symptoms	33	1	0	13	0	0
Gastrointestinal	135	44	0	68	12	0
Stomatitis or pharyngitis	70	14	0	34	3	0
Diarrhea	78	21	0	30	6	0
Vomiting	16	8	0	5	0	0
Hemorrhage	1	0	0	1	0	0
Infection	28	3	2	17	1	0
Infection with grade 3 to 4 neutropenia	10	2	2	0	0	0
Liver	4	2	0	1	0	0
Lung	3	1	0	2	2	0
Myelosuppression						
Neutropenia or granulocytopenia	110	145	0	2	2	0
Thrombocytopenia	3	1	0	1	0	0
Neurologic	12	1	0	12	1	0
Pain	14	3	0	21	1	0
Thrombosis or clotting	10	0	0	15	1	1
Maximum grade any toxicity	162	180	2	154	22	2

Abbreviations: FU, fluorouracil; LV, leucovorin.

additional patient dying as a result of complications of a clot. Thrombosis was usually related to the catheter for patients receiving continuous-infusion FU. Grade 2 hand-foot syndrome occurred in 35% and grade 3 hand-foot syndrome occurred in 7% of patients receiving continuous-infusion FU compared with 3% and less than 1% with bolus FU/LV.

Seventy-one percent of patients completed protocol treatment as planned. Fifteen patients refused any protocol treatment after random assignment, and an additional 15 patients refused to continue after beginning treatment. Despite the greater number of grade 4 toxicities experienced on the bolus arm, twice as many patients (106 *v* 64) discontinued treatment early because of adverse effects in the continuous-infusion arm compared with the bolus arm. On the basis of comments noted on the data flow sheets, many patients receiving continuous-infusion FU complained, not necessarily about high-grade toxicities, but about the logistics of pump therapy, pump malfunctions, clotting episodes, neck pain that seemed to be related to the catheter, and chronic hand-foot syndrome.

Median follow-up time was 6.52 years. The overall 5-year survival was 70% (95% CI, 66% to 74%) for the FU/LV plus levamisole arm, and 69% (95% CI, 64% to 73%) for the continuous-infusion FU plus levamisole arm (Fig 1). The corresponding 5-year disease-free survival was 61% (95% CI, 56% to 65%) and 63% (95% CI, 59% to 68%), respectively (Fig 2). The estimated hazard ratio for survival based on the Cox model was 1.16 (95% CI, 0.93 to

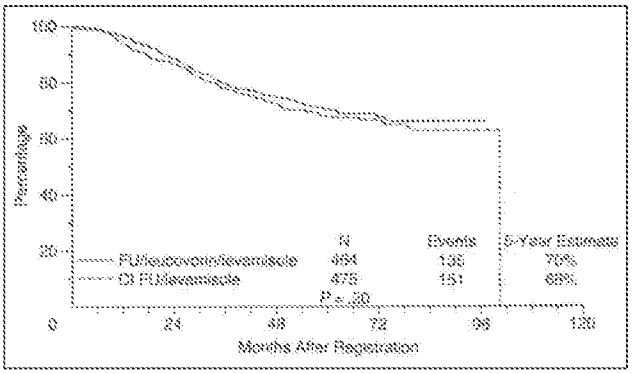

Fig 1. Overall survival, by treatment. FU, fluorouracil; CI, continuous infusion.

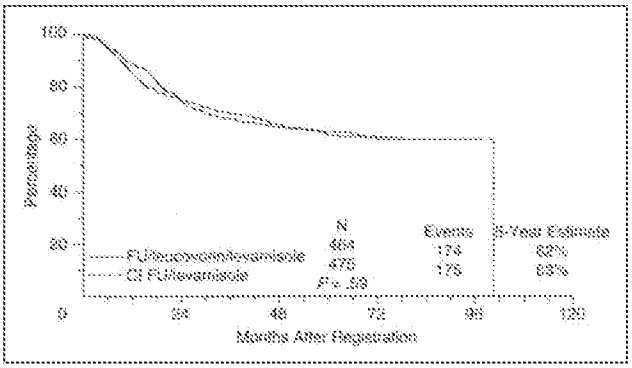

Fig 2. Disease-free survival, by treatment. FU, fluorouracil; CI, continuous infusion.

1.44; $P = .18$) for continuous-infusion FU plus levamisole compared with FU/LV plus levamisole. The estimated hazard ratio for disease-free survival was 1.05 (95% CI, 0.86 to 1.3; $P = .65$).

We also assessed potential treatment interactions by race and sex. We were unable to detect any differences in survival between males and females, nor between races or ethnicities, nor any treatment interactions with these variables.

Given the lack of differences between the treatment arms, we explored the impact of nodal involvement and of time to treatment initiation on the outcome of patients enrolled onto this trial. The estimate of 5-year disease-free survival was 78%, 67%, and 47% for N0, N1, and N2-3 patients, respectively. Five-year overall survival was 83%, 74%, and 55%, respectively (Fig 3). There were no differences in disease-free survival and overall survival based on time of treatment registration between those who registered at days 21 to 28 versus days 29 to 35.

DISCUSSION

This adjuvant study was based on the observation that continuous-infusion FU by was less toxic and had a somewhat higher level of activity than FU/LV in patients with metastatic disease. It was hypothesized that this higher level of response in metastatic disease might be amplified in the adjuvant setting.

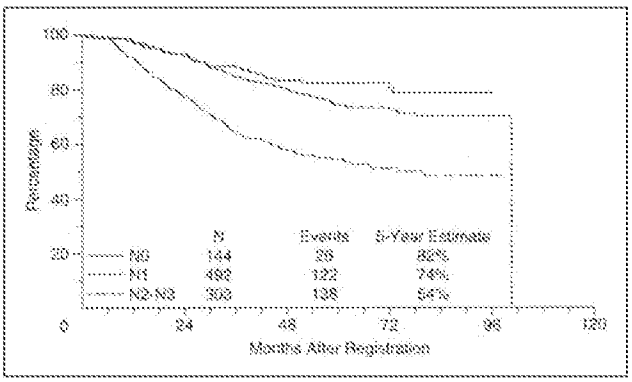

Fig 3. Overall survival by N stage.

The study suffered from a high ineligibility rate (17.2%). Patients were excluded for a number of reasons. Colonoscopy or sigmoidoscopy with barium enema was mandated to exclude synchronous colon or rectal primary tumors. Adequate surgical margins were similarly mandated. No part of the tumor could be below the peritoneal reflection. When these criteria were not fulfilled or could not be documented, registered patients were deemed ineligible. There were no differences in the results of this study in an analysis that included all patients, regardless of eligibility. However, this high ineligibility rate should prompt greater awareness of the surgical guidelines for the management of colorectal cancer patients and the need for documentation in clinical trials.[19]

No overall benefit was demonstrated for continuous infusion in this adjuvant treatment. The dose chosen for the continuous infusion was 250 mg/m^2/d rather than the standard 300 mg/m^2/d to avoid toxicity-related interruption of therapy. This lower dose may have compromised maximum benefit. However, compliance was difficult, even at this dose, because hand-foot syndrome occurred frequently, as did thrombotic episodes related to catheters. Ultimately, twice as many patients withdrew from the continuous-infusion arm as from the FU/LV plus levamisole arm.

Saini et al[20] compared short-course continuous-infusion FU to 6 months of bolus therapy, with a somewhat different outcome. Patients with stages II and III colon or rectal cancer were randomly assigned to either six cycles of FU 425 mg/m^2 and LV 20 mg/m^2 days 1 through 5 every 4 weeks for 6 months, or to continuous-infusion FU 300 mg/m^2/d for 12 weeks. Patients with rectal cancer could also receive radiation therapy, given concurrently with bolus therapy or sequentially, after the 12-week infusional regimen was completed. Patients in the infusion arm received an additional 5 weeks of FU 200 mg/m^2/d concurrent with the radiotherapy. Among the 692 eligible patients, 68% had colon cancer and 31% had rectal cancer; 42% had stage II and 57% had stage III cancers. With a median follow-up of 19.8 months, the overall survival was not statistically different. The projected 3-year relapse-free survival favored continuous-infusion FU (80% *v* 69%, respectively; log-rank $P = .023$). The subsets of stage II patients and those with rectal cancer had lower relapse rates after treatment with infusional FU compared with bolus-treated patients, although this difference was only statistically significant for patients with rectal cancer. The study by Saini et al[20] included a wider variety of patients than in our study and demonstrated benefit in patient subtypes specifically excluded from our study. However, it is noteworthy that

benefit from FU infusion was achieved with only 12 weeks of therapy.

We chose to include levamisole in both arms of our study because at the time of this study's initiation, levamisole in combination with FU was a standard of care.[11] Ultimately, INT-0089 demonstrated that FU/LV plus levamisole was not superior to FU/LV alone.[21] The QUASAR trial in the United Kingdom also compared the treatments of FU with either low- or high-dose LV and with or without levamisole in the adjuvant treatment of colorectal cancer. Again, no benefit was seen for the addition of levamisole. We can now conclude that levamisole does not add benefit to FU/LV regimens.[22]

The results of our study also emphasize the poor survival of patients with four or more positive nodes. These poor results are consistent with the extensive review by Greene et al[23] of 50,000 patients. Among patients treated from 1987 through 1993, the 5-year survival for patients with four or more positive nodes with surgery alone was approximately 22% and improved to only 33% with adjuvant therapy. Similarly, data from Gill et al[7] demonstrate 5-year disease-free survivals of 9% to 28% for patients with five or more positive nodes without additional treatment and only 5-year disease-free survivals of 21% to 44% with adjuvant therapy. This clearly is a population requiring special attention. Trials of more intensive chemotherapy or chemoradiotherapy might be considered.[24] Perhaps more rigorous staging, for example with positron emission tomography scanning, should be used in this high-risk group in which, if macrometastases were demonstrated, stage and treatment would be affected.[25]

Data based on the control arms of previous adjuvant studies would suggest that approximately 75% to 90% of stage II and approximately 50% of stage III patients will not experience disease recurrence after appropriate surgical resection and therefore do not require adjuvant therapy. Conversely, 15% to 20% of stage II and 30% of stage III patients develop progressive disease despite having received currently available adjuvant therapy.[2-5] Thus, only an estimated one third or fewer of patients treated postoperatively benefit from adjuvant FU or FU/LV treatments.[3,7,26] Intensive effort has been directed toward identified subsets with higher and lower risks, beyond TNM status. We await testing and confirmation of newer methodology with genetic profiling to help identify those at risk and those likely to benefit from adjuvant therapy.[27,28]

FU/LV has been used for adjuvant therapy for at least a decade, but it still is effective only for a portion of the currently defined at-risk population. Where are the next improvements in drug treatment? Andre et al[29] have demonstrated in a study of 905 patients with stage II and III colon cancer that LV as a 2-hour infusion plus FU 400 mg/m^2 bolus and 600 mg/m^2 22-hour continuous infusion on days 1 and 2 every 2 weeks (LVFU2) was less toxic than bolus FU/LV given daily for 5 days each month, with similar disease-free and overall survival. However, the small size of the study precludes any definitive conclusion about the relative survival benefit. The oral thymidylate synthase inhibitors are additional alternatives. Capecitabine is already approved for use in colorectal metastatic disease when fluoropyrimidine single-agent therapy is chosen.[30,31] The X-ACT adjuvant study randomly assigned 1,987 resected stage III patients to 24 weeks of FU/LV daily for 5 days each month or to capecitabine 1,250 mg/m^2 bid days 1 through 14 every 21 days. Preliminary data suggest that capecitabine appears to be equivalent with regard to disease-free survival, with a median follow-up of 3.8 years.[32] In National Surgical Adjuvant Breast and Bowel Project C-06, oral uracil plus tegafur UFT and LV taken daily for 28 of every 35 days for five cycles was compared with three cycles of FU/LV weekly for 6 of 8 weeks as the treatment of stages II and III carcinoma of the colon. Oral uracil plus tegafur and LV achieved equivalent disease-free and overall survival compared with FU/LV.[33] The two newer chemotherapy drugs, oxaliplatin and irinotecan, have undergone their initial assessments. Irinotecan in combination with bolus FU/LV did not increase the duration of failure-free survival or overall survival compared with FU/LV alone in stage III colon cancer, and was more toxic.[34] In contrast, the MOSAIC trial, comparing 12 cycles of LVFU2 to LVFU2 with the addition of oxaliplatin 85 mg/m^2 (FOLFOX) accrued 2,248 stage II and III patients between October 1998 and January 2001. With short follow-up, the 3-year disease-free survival for stage II patients was 84% for LVFU2 and 87% for FOLFOX, whereas for stage III patients, the 3-year disease-free survival was 65% and 72%, respectively.[35] Thus, the preliminary results of the MOSAIC trial suggest an incremental benefit in disease-free survival with the addition of oxaliplatin.

We look forward to the assessment in the adjuvant setting of the newly available monoclonal antibodies, cetuximab and bevacizumab; both have demonstrated activity in the treatment of metastatic colorectal cancer.[36-38] The North Central Cancer Treatment Group is now comparing an adjuvant trial of irinotecan plus FU infusion and LV to FOLFOX to sequential FOLFOX followed by irinotecan plus FU infusion and LV. All patients are also randomly assigned to no biologic treatment or cetuximab. The National Surgical Adjuvant Breast and Bowel Project is studying FOLFOX, with and without bevacizumab, for the adjuvant treatment of stages II and III colon cancer.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Irinophore C: A Liposome Formulation of Irinotecan with Substantially Improved Therapeutic Efficacy against a Panel of Human Xenograft Tumors

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Abstract

Purpose: To assess the pharmacokinetics, tumor drug accumulation, and therapeutic activity of Irinophore C, a novel liposomal formulation of irinotecan (CPT-11).

Experimental Design: The plasma lactone/carboxy levels of CPT-11 and SN-38 were determined in mice after a single i.v. dose of irinotecan (Camptosar), or Irinophore C, and the plasma $t_{1/2}$, plasma area under the curve, plasma C_{max}, and plasma clearance were calculated. Further, plasma and tumor drug levels were also measured in tumor-bearing mice following Irinophore C treatment. The efficacy of Irinophore C was compared with that of Camptosar in five s.c. human tumor xenografts using single-dose treatment (LS 180), a total of three doses administered at 4-day intervals (H460), or a total of three doses administered at 7-day intervals (Capan-1, PC-3, and HT-29).

Results: Compared with Camptosar, Irinophore C mediated an 8-fold increase in $t_{1/2}$, a 100-fold increase in C_{max}, a 1,000-fold increase in area under the curve, and a 1,000-fold decrease in clearance for the active lactone form of CPT-11. Further, the plasma and tumor SN-38 lactone levels were consistent for at least 48 h post-Irinophore C injection. Camptosar treatment (40 mg/kg) mediated a delay in the time required for tumors to increase to four times their pretreatment size compared with controls (T-C). T-Cs ranged from 2 days (LS 180 model) to 18 days (PC-3 model). Irinophore C (40 mg/kg) engendered T-Cs ranging from 14 days (LS 180 model) to 87 days (Capan-1 model).

Conclusion: Innophore C improved CPT-11/SN-38 pharmacokinetics, promoted tumor drug accumulation, and increased therapeutic efficacy in a panel of five distinct human tumor xenografts.

Camptothecins mediate their anticancer activity by stabilizing the cleavable complex formed between topoisomerase I and DNA during the S phase of the cell cycle (1); however, this mechanism is dependent on the α-hydroxy-δ-lactone ring of camptothecins (2), which is problematic because physiologic conditions promote the reversible hydrolysis of the lactone ring to yield the inactive carboxylate form (3). Resultantly, research has focused on strategies to stabilize the lactone ring structure, including the use of drug carrier technologies such as hydrogels (4), polymer conjugates (5), microspheres (6), and lipid-based systems (7–12).

The water-soluble camptothecin derivative irinotecan (CPT-11) is an attractive candidate for formulation in a nanocarrier because it has proven clinical activity against colorectal (13) and small-cell lung cancers (14) and may be active in other cancer indications (15). CPT-11 has a complicated pharmacologic profile and is extensively metabolized *in vivo* to yield a number of derivatives including the potent metabolite SN-38 (16). As with the parent drug, the cytotoxic activity of SN-38 is also dependent on the maintenance of the lactone ring (16).

The physicochemical characteristics of CPT-11 make it amenable to efficient encapsulation in pharmaceutically viable liposome systems. It is already established that CPT-11 can be actively loaded into liposomes via a transmembrane pH gradient (11). The weakly basic drug is added to the outside of liposomes suspended in buffer at pH 7.4, and at this pH a substantial proportion of the drug exists in the neutral form, which can easily permeate the liposomal membrane. On contact with the acidic environment of the aqueous core, CPT-11 is ionized and consequently trapped inside the liposome (17–19). A further benefit of this technology is that the encapsulated CPT-11 exists predominately as the active lactone, which improves therapeutic efficacy (10, 11, 20, 21).

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For many anticancer drugs, particularly those that are cell cycle specific, improved drug retention is associated with increases in drug exposure at sites of disease (15, 16). We have recently described a formulation technology that involves the use of entrapped copper ions and a transmembrane pH gradient (acidic inside), which seems to result in significantly improved CPT-11 retention in the liposomes following systemic administration (20). Drugs such as CPT-11 that have protonizable amine groups have been shown to be better retained by liposomes comprising acidic interior buffers (17); further, we have shown that CPT-11 can form a transition metal complex with copper (22). Consequently, following systemic administration, a liposome formulation with a stable internal acidic environment with copper ions mediated substantial improvements in CPT-11 retention, which translated to improved therapeutic activity in a model of colorectal cancer, when compared with carrier systems using pH gradients or copper ion gradients alone. We have named this liposome formulation Irinophore C.

The series of studies described herein investigated the influence of Irinophore C on the pharmacokinetics and tumor accumulation of both the lactone and carboxylate forms of CPT-11 and SN-38. The results will show that Irinophore C mediated a 1,000-fold increase in the plasma CPT-11 lactone area under the curve (AUC) compared with Camptosar and maintained continuous plasma levels of SN-38 lactone for at least 24 h after i.v. administration. Further, CPT-11 lactone and SN-38 lactone accumulated in tumors following Irinophore C treatment. Collectively, the pharmacokinetic improvements and tumor accumulation resulted in substantial gains in therapeutic efficacy in five different xenograft models of human cancer.

Materials and Methods

Liposome preparation. 1,2-Distearoyl-*sn*-glycero-phosphocholine and cholesterol (DSPC/Chol; 55:45 mol%; Avanti Polar Lipids) large unilamellar vesicles were prepared as previously described (23). Briefly, lipids were dissolved in chloroform at the required molar ratio, labeled with the nonexchangeable, nonmetabolizable lipid marker ^{3}H-CHE (Perkin-Elmer Life Sciences), and dried to a thin film under a stream of nitrogen gas. Subsequently, the lipid was placed in a high vacuum for ≥3 h to remove any residual solvent. The lipid films were hydrated at 65°C by mixing with 300 mmol/L copper sulfate solution before five cycles of freeze-and-thaw (5 min each, freezing in liquid nitrogen and thawing at 65°C). The multilamellar vesicle suspensions were then extruded 10 times through stacked polycarbonate filters of 0.08- and 0.1-μm pore sizes at 65°C (Extruder, Northern Lipids). The resultant large unilamellar vesicles typically possessed mean vesicular diameters in the range of 110 ± 30 nm as determined using Phase Analysis Light Scattering methods (ZetaPALS, Brookhaven Instruments Corp.). The large unilamellar vesicle external buffer was exchanged using Sephadex G-50 size exclusion chromatography with SHE buffer pH 7.5 (300 mmol/L sucrose, 20 mmol/L HEPES, 15 mmol/L EDTA).

Accumulation of irinotecan into preformed DSPC/Chol liposomes. The divalent metal ionophore A23187 [calcimycin; Sigma; 1 mg/mL (1.9 mmol/L) solution in 100% ethanol] was preincubated with liposomes (0.5 μg/mg lipid) at 60°C for 30 min. Subsequently, irinotecan hydrochloride trihydrate (Camptosar, Pharmacia; BC Cancer Agency Pharmacy) was added to liposomes (~50 mmol/L) at 50°C at a drug-to-lipid ratio of 0.2:1 (mol/mol). Drug uptake was determined after 60-min incubation by separating encapsulated drug from free drug using a Sephadex G-50 column equilibrated with PBS buffer. The excluded fractions, containing the liposomes, were analyzed to determine drug-to-lipid ratios. Lipid concentrations were measured by liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer). Irinotecan concentration was determined by measuring absorbance at 370 nm. Briefly, a portion of the sample collected from the column was adjusted to a final volume of 100 μL with PBS. Subsequently, 900 μL of Triton X-100 1% were added and the samples were heated in a water bath at >90°C until the cloud point of the detergent was observed. The samples were then cooled to room temperature and the absorbance was determined against a freshly prepared irinotecan standard curve (Hewlett Packard UV-Vis spectrophotometer, model 8453). For efficacy studies, Irinophore C was concentrated using Amicon Ultra-15 centrifugal Filter Tubes (3,000 × *g* for 30 min; Millipore) to achieve the desired dose (mg/kg) administered in a volume of 200 μL. The concentrations of lipid and irinotecan present in the final samples were confirmed as described above.

Pharmacokinetic studies. Female BALB/c mice (Taconic; 20-25 g; four per time point) were injected i.v. with a single dose of Camptosar (50 mg/kg irinotecan) or Irinophore C (50 mg/kg irinotecan; 225 mg/kg lipid) and the plasma concentrations of liposomal lipid (^{3}H scintillation counting as described above to measure associated ^{3}H-CHE) and irinotecan were determined over time (11). In addition, female RAG-2M mice ($n = 4$) with established s.c. human non–small-cell lung cancer cell line (NSCLC) H460 tumors (see "Efficacy studies") were given a single i.v. dose of Irinophore C (20 mg/kg irinotecan; 180 mg/kg lipid) and the plasma levels of drug and lipid were determined as above. High-performance liquid chromatography separation of irinotecan (CPT-11) and SN-38 lactone and carboxylate forms was done using a 250 × 4.6 mm Symmetryshield RP18 5-μm column and Symmetryshield RP18 guard column (Waters). Gradient elution was used with mobile phase A composed of 75 mmol/L ammonium acetate and 7.5 mmol/L tetrabutylammonium bromide adjusted to pH 6.4 with glacial acetic acid (Fisher Scientific) and mobile phase B was acetonitrile. Gradient profile was as follows: time, 0 min: 78% A:22% B; time, 10 min: 64% A:36% B; time, 16 min: 78% A:22% B; time, 20 min: 78% A:22% B. A 10-μL sample was injected onto the column (column temperature, 40°C) and eluted at a flow rate of 1 mL/min. CPT-11 and SN-38 forms were detected using a Waters 2475 multi-wavelength fluorescence detector (Waters) set with time program events of $\lambda_{ex} = 370$ nm; $\lambda_{em} = 425$ nm between times 0 and 12.5 min for CPT-11 forms, and $\lambda_{ex} = 370$ nm; $\lambda_{em} = 535$ nm between times 12.5 and 20 min for SN-38 forms. Before injection, all samples were maintained at 4°C to reduce conversion between lactone and carboxylate forms. Standard curves of CPT-11 and SN-38 lactone form were prepared by serial dilutions in a 2:1:1 sodium acetate (100 mmol/L)/methanol/acetonitrile pH 4.0 buffer. For the carboxylate form of CPT-11 and SN-38, serial dilutions were prepared in a 2:1:1 sodium borate (100 mmol/L)/methanol/acetonitrile pH 9.0 buffer. The limit of quantitation for CPT-11 and SN-38 lactone and carboxylate forms was 10 ng/mL. Irinotecan pharmacokinetic parameters were calculated using the noncompartmental analysis function of WinNonLin software version 5.0.1 (Pharsight).

Cell culture. All cell lines were purchased from the American Type Culture Collection and were cultured in the appropriate base media (StemCell) with fetal bovine serum (FBS; Cansera) for up to 10 passages. After 10 passages, new cells were expanded from a frozen stock stored in liquid nitrogen. The human NSCLC H460 was cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, and 10% FBS. The human colorectal adenocarcinoma cell line LS 180 was cultured in Eagle's MEM with 2 mmol/L L-glutamine and Eagle's balanced salt solution, 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and 10% FBS. The human colorectal adenocarcinoma cell line HT-29 was cultured in modified McCoy's 5a medium with

1.5 mmol/L L-glutamine and 2.2 g/L sodium bicarbonate and 10% FBS. The prostate adenocarcinoma cell line PC-3 was cultured in Ham's F12K medium supplemented with 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, and 10% FBS. Capan-1 pancreatic adenocarcinoma cells were cultured in Iscove's modified Dulbecco's medium with 4 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, and 20% FBS.

Tumor drug accumulation studies. RAG2-M mice (129S6/SvEvTac-*Rag2*^{tm1Fwa}, Taconic; 20-25 g; three per group) were inoculated s.c. into the center of the lower back with 2×10^6 H460 cells (50 μL). Tumor growth was monitored every Monday, Wednesday, and Friday with calipers and the measured dimensions (in millimeters) were converted to tumor weight (in milligrams) using the following equation: length × (width^2) / 2. When the tumors reached ~200 mg, mice were treated with a single dose of Irinophore C (20 mg/kg irinotecan). At the indicated time points, mice were euthanized and plasma and tumors were harvested. Plasma was processed as described above. Tumors were weighed and a 25% homogenate solution was prepared in ice-cold water. CPT-11 and metabolites were extracted by mixing 250 μL of homogenate with 750 μL of ice-cold acetonitrile/methanol (1:1) solution and centrifuging at 14,000 × *g* for 15 min to precipitate proteins. The supernatant was recovered and a 10-μL aliquot was injected into high-performance liquid chromatography column to quantify the levels of both conformations of CPT-11 and SN-38 using the method outlined above.

Efficacy studies. RAG2-M mice (20-25 g; eight per group) were inoculated s.c. into the center of the lower back with (*a*) 1×10^6 LS 180 cells (50 μL); (*b*) 5×10^6 HT-29 cells (50 μL); (*c*) 2×10^6 H460 cells (50 μL); (*d*) 5×10^6 PC-3 cells (100 μL); or (*e*) 2×10^6 Capan-1 cells (50 μL). Once the tumors were established and had reached a mean size of ~25 mg (LS 180), 200 mg (HT-29), 50 mg (H460), 60 mg (PC-3), or 100 mg (Capan-1), mice were treated with Camptosar at doses of 40 or 60 mg/kg (H460 only) or with Irinophore C at doses of 20, 30, or 40 mg/kg, or 60 mg/kg (H460 only), according to the following i.v. dosing schedules: LS 180, single dose; HT-29, each dose administered every 7 days; for a total of 3 doses; H460, each dose administered every 4 days for a total of 3 doses; PC-3, each dose administered every 7 days for a total of 3 doses; Capan-1, each dose administered every 7 days for a total of 3 doses. Tumor growth was monitored with calipers using a schedule governed by the growth characteristics of the tumor model and the measured dimensions (in millimeters) were converted to tumor weight (in milligrams) as described above.

Animal husbandry. Animal studies were conducted in accordance with the Canadian Council on Animal Care Guide, and the University of British Columbia animal care committee approved the animal care protocols. Mice were housed under standard conditions with enrichment and had access to food and water *ad libitum*. All animals were observed at least twice per day for morbidity, more if deemed necessary during the pretreatment and treatment periods. Signs of ill health were based on body weight loss, change in appetite, and behavioral changes such as altered gait, lethargy, and gross manifestations of stress. Animals were terminated (CO_2 asphyxiation) if signs of severe toxicity or tumor-related illness were observed, and a necropsy was done to assess other signs of toxicity.

Statistical analysis. *In vivo* efficacy data were analyzed using SPSS 13.0 (SPSS, Inc.). The time taken for s.c. tumors to increase 4-fold in relative size [relative tumor growth increase (TGI) = 4] was analyzed using Kaplan-Meier curves for survival analysis. This analysis allows modeling to the time event data (i.e., TGI = 4) in the presence of censored cases (i.e., animals who never reached this end point because of, e.g., tumor ulceration). Treatment and control groups were compared using a log-rank test. Comparisons of the maximum mean percent body weight losses (%BWL) induced following treatments/controls were compared using one-way ANOVA followed by planned contrasts and the Gabriel, Hochberg's GT2, and Games-Howell post hoc tests. The data were normally distributed as determined with the Kolmogorov-Smirnov test. There was no significant difference in variances between groups (Levene's test, $P > 0.05$).

Results

A comparison of the pharmacokinetic profiles for Camptosar and Irinophore C. BALB/c mice were injected with a single dose of 50 mg/kg irinotecan (CPT-11), administered as Camptosar or Irinophore C, and the plasma levels of CPT-11 and SN-38, both as lactone and carboxylate conformations, were determined and the results have been summarized in Fig. 1A to D. Camptosar is prepared in acidic solution that favors the active lactone form of CPT-11, and this is reflected in its pharmacokinetic profile (Fig. 1A). The maximum recorded plasma CPT-11 lactone concentration was 10 μg/mL at 5 min postinjection, decreasing by 3 orders of magnitude to 0.01 μg/mL after 8 h (Fig. 1A). In contrast, there was an initial increase in the plasma concentration of CPT-11 carboxylate from 1 μg/mL at the 5-min time point to a maximum of 5 μg/mL after 30 min as the CPT-11 lactone reached equilibrium with the carboxylate conformation under physiologic conditions (Fig. 1A). The elimination profiles of the two forms (lactone and carboxylate) of CPT-11 following Camptosar administration were similar as reflected by their calculated pharmacokinetic parameters (Table 1).

The plasma concentrations of the two forms of CPT-11 after a single dose of Irinophore C are shown in Fig. 1B. At 1 h postinjection, the CPT-11 lactone concentration was 1,071 μg/mL, and this decreased to 100 μg/mL after 24 h. These levels represent a 10- to 100-fold increase over the maximum recorded concentration achieved following injection of an identical dose of Camptosar. The carrier-mediated increase in CPT-11 levels is highlighted by the pharmacokinetic parameters shown in Table 1 and is best emphasized by the 1,000-fold increase in plasma CPT-11 lactone AUC levels afforded by Irinophore C.

It is assumed that the majority of CPT-11 detected in the plasma following Irinophore C administration is associated with the drug carrier. The pharmacokinetic parameters calculated for Irinophore C CPT-11 carboxylate form supported this belief. For example, the plasma half-life of CPT-11 in the carboxylate form increased from 1 to 9 h, the plasma AUC increased from 9.1 to 55 h μg/mL, and the plasma clearance decreased from 5,475 to 765 mL/kg/h, when compared with results obtained following administration of Camptosar (Table 1). The small proportion of CPT-11 in the carboxylate form following administration of Irinophore C is consistent with the high-performance liquid chromatography and thin liquid chromatography analyses of the formulated product, which exhibits a small amount of CPT-11 in the carboxylate form before injection.

CPT-11 is metabolized by carboxylesterase to yield SN-38, which has been reported to be 100- to 1,000-fold more potent *in vitro* than CPT-11 (24). In addition, SN-38 also exists in equilibrium between the lactone and carboxylate forms; therefore, the relative plasma levels of the two forms were quantified following Camptosar and Irinophore C i.v. administration. SN-38 lactone and carboxylate forms were both detectable in the plasma 5 min after injection of Camptosar (Fig. 1C). Equilibrium was achieved by the 30-min time point, and the plasma elimination profiles of the two forms were comparable for the remainder of the experiment, with the SN-38 lactone/SN-38 carboxylate ratio consistent at 2:1 (w/w). In

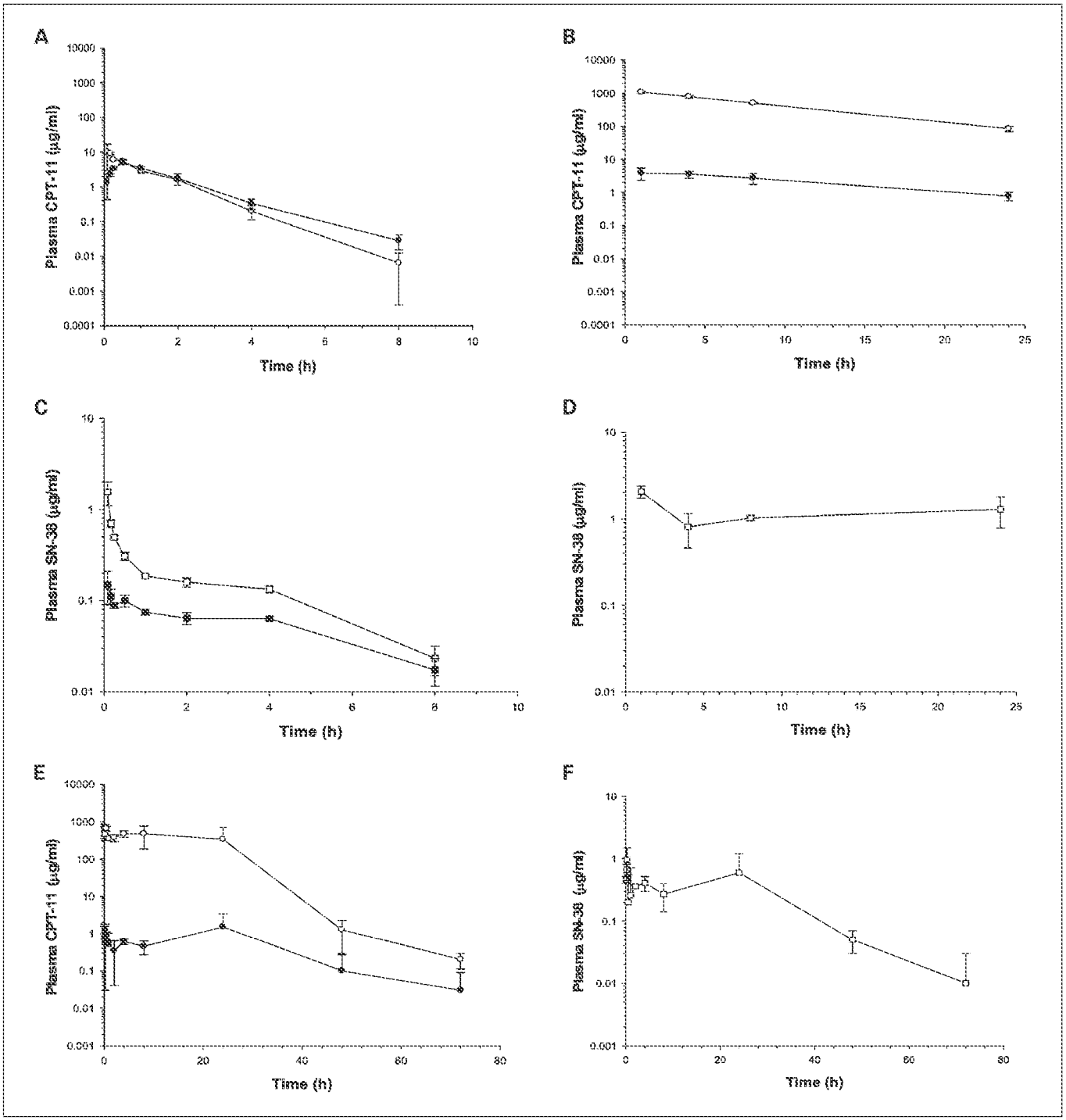

Fig. 1. Plasma elimination profiles for CPT-11 and SN-38 species following a single i.v. dose of Camptosar or Irinophore C. Female BALB/c mice were injected i.v. with a single dose of Camptosar (60 mg/kg irinotecan (CPT-11)) or Irinophore C (60 mg/kg CPT-11; 225 mg/kg lipid) and the relative plasma concentrations of CPT-11 lactone (○), CPT-11 carboxylate (●), SN-38 lactone (□) and SN-38 carboxylate (■) were determined at the indicated time points as described in Materials and Methods. *A*, Camptosar-Plasma CPT-11 (µg/mL) as a function of time. *B*, Irinophore C-Plasma CPT-11 (µg/mL) as a function of time. *C*, Camptosar-Plasma SN-38 (µg/mL) as a function of time. *D*, Irinophore C-Plasma SN-38 (µg/mL) as a function of time. Points, mean ($n = 4$); bars, SD. In addition, RAG-2M mice bearing established s.c. human NSCLC H460 xenografts were treated with a single i.v. dose of Irinophore C (20 mg/kg irinotecan; 180 mg/kg lipid) and the plasma concentrations of both forms of CPT-11 and SN-38 were determined. The symbols are as before. *E*, Irinophore C-Plasma CPT-11 (µg/mL) as a function of time. *F*, Irinophore C-Plasma SN-38 (µg/mL) as a function of time. Points, mean ($n = 4$); bars, SD.

contrast, only SN-38 lactone was detectable in the plasma following Irinophore C administration (Fig. 1D). An SN-38 lactone plasma concentration of 2 µg/mL was recorded 1 h postinjection. This level decreased to 1 µg/mL after 4 h and remained constant for the duration of the experiment achieving continuous plasma SN-38 levels over 24 h, which were greater than those observed 10 min after Camptosar administration.

Table 1. The calculated CPT-11 pharmacokinetic parameters following a single i.v. dose of Camptosar or Irinophore C

Treatment	Irinotecan (CPT-11) parameters*							
	$t_{1/2}$ (h)		C_{max} (μg/mL)		AUC (h μg/mL)		Cl (mL/h/kg)	
	Lactone	Carboxy	Lactone	Carboxy	Lactone	Carboxy	Lactone	Carboxy
Camptosar†	0.8	1.0	9.9	5.1	10	9	4,877	5,475
Irinophore C†	6.2	9.0	1,071.3	3.9	11,226	55	4	765
Irinophore C‡	6.1	8.5	654.0	1.5	14,214	41	1.4	481

Abbreviation: Cl, clearance.
*Pharmacokinetic parameters were estimated using noncompartmental analysis (WinNonLin software version 5.0.1).
†BALB/c mice were given a single i.v. bolus dose equivalent to 50 mg/kg irinotecan (CPT-11).
‡Human NSCLC H460 tumor-bearing RAG-2M mice were given a single i.v. bolus dose equivalent to 20 mg/kg irinotecan (CPT-11).

The plasma elimination profiles for both forms of CPT-11 (Fig. 1E) and SN-38 (Fig. 1F) were also quantified following a single dose of Irinophore C 20 mg/kg injected i.v. via the tail vein of RAG-2M mice bearing established s.c. human NSCLC H460 xenografts. The duration of this study was extended to 72 h postinjection and the relative levels of CPT-11 lactone and carboxy for the first 24 h are comparable to that seen in non–tumor-bearing BALB/c mice treated with a higher dose of 50 mg/kg Irinophore C (compare Fig. 1E-B). At 48 h postinjection, the CPT-11 lactone levels had decreased by 2 orders of magnitude (Fig. 1E); in contrast, the concentration of CPT-11 carboxy had remained reasonably constant. Resultantly, the ratio of CPT-11 lactone/CPT-11 carboxy changed from ~1,000:1 over the first 24 h to 10:1 by 72 h. The pharmacokinetic parameters associated with Irinophore C administration to tumor-bearing mice are shown in Table 1 and are comparable to those for BALB/c mice, with a plasma half-life for CPT-11 lactone of 6 h in both models.

The plasma SN-38 levels in the H460 xenograft model following Irinophore C injection (20 mg/kg) are illustrated in Fig. 1F. Consistent with the plasma profile in BALB/c mice (Fig. 1D), SN-38 lactone levels remained steady over 24 h, albeit at concentrations approximately half of that seen with a higher dose of Irinophore C (50 mg/kg), before decreasing by 2 orders of magnitude by 72 h; further, there were no detectable levels of SN-38 carboxy (Fig. 1F). Although SN-38 is known to be highly protein bound (>90%) and the lactone form has a higher affinity thereby forcing equilibrium for unbound plasma SN-38 toward the lactone form (16), it is unclear why SN-38 carboxy was not detected after Irinophore C administration (limit of detection, 10 ng/mL; ref. 25). It may be that SN-38 can be generated when the CPT-11 is still associated with the liposome, thereby facilitating the maintenance of the lactone form (26).

The concentration of CPT-11 and SN-38 in H460 NSCLC tumors following treatment with Irinophore C. Enhanced permeability and retention is a putative mechanism by which nanoparticle drug carrier systems can exert their antitumor effects (27). Particles in the nanometer size range that are normally too large to extravasate from blood vessels are able to passively accumulate in tumor tissue via leaky capillaries associated with the disease. Figure 2 shows the concentrations of CPT-11 and SN-38 present in human NSCLC H460 xenografts grown on the back of RAG-2M mice following a single i.v. dose of Irinophore C (20 mg/kg irinotecan). The drug concentrations have been corrected to account for the blood volume associated with the tumor (28) and, therefore, represent the drug present in the tissue.

The concentration CPT-11 lactone (*black columns*) was the greatest of the species detected over the duration of the experiment, peaking at ~12 μg/g of tumor tissue after 24 h before decreasing to ~5 μg/g of tumor tissue at 48 h. CPT-11 carboxy levels followed the same trend as CPT-11 lactone, albeit at markedly lower levels with a peak of 1 μg/g of tumor tissue detected after 24 h. Because the tumor levels of SN-38 lactone have been corrected for circulating plasma levels, the concentrations shown in Fig. 2 represent local concentrations of SN-38 (29). SN-38 lactone was detectable 1 h postinjection, but levels increased to ~1 μg/g of tumor tissue after 4 h and remained constant for the duration of the experiment, mirroring what was observed in the plasma (see Fig. 1F). SN-38 carboxy was not detected using the experimental methods described in this article.

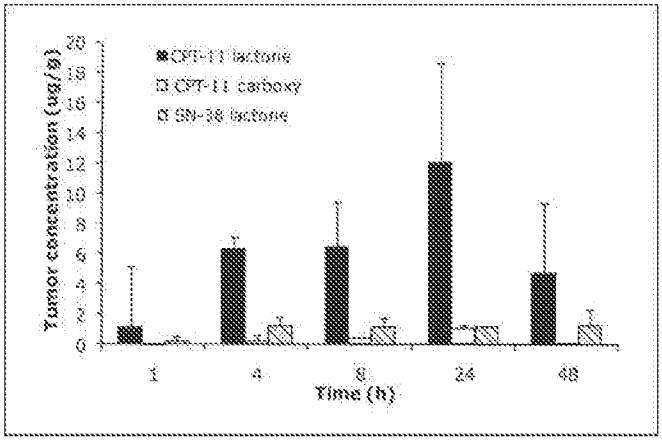

Fig. 2. CPT-11 and SN-38 tumor levels in NSCLC H460 xenografts following Irinophore C treatment. RAG2-M mice bearing established s.c. human NSCLC H460 tumors were treated with a single dose of Irinophore C (irinotecan dose of 20 mg/kg) and the tumor lactone/carboxy levels of CPT-11 and SN-38 were measured as described in Materials and Methods. Columns, mean tumor levels (μg/g) collected from three animals, corrected for drug levels associated with the plasma compartment; bars, SD.

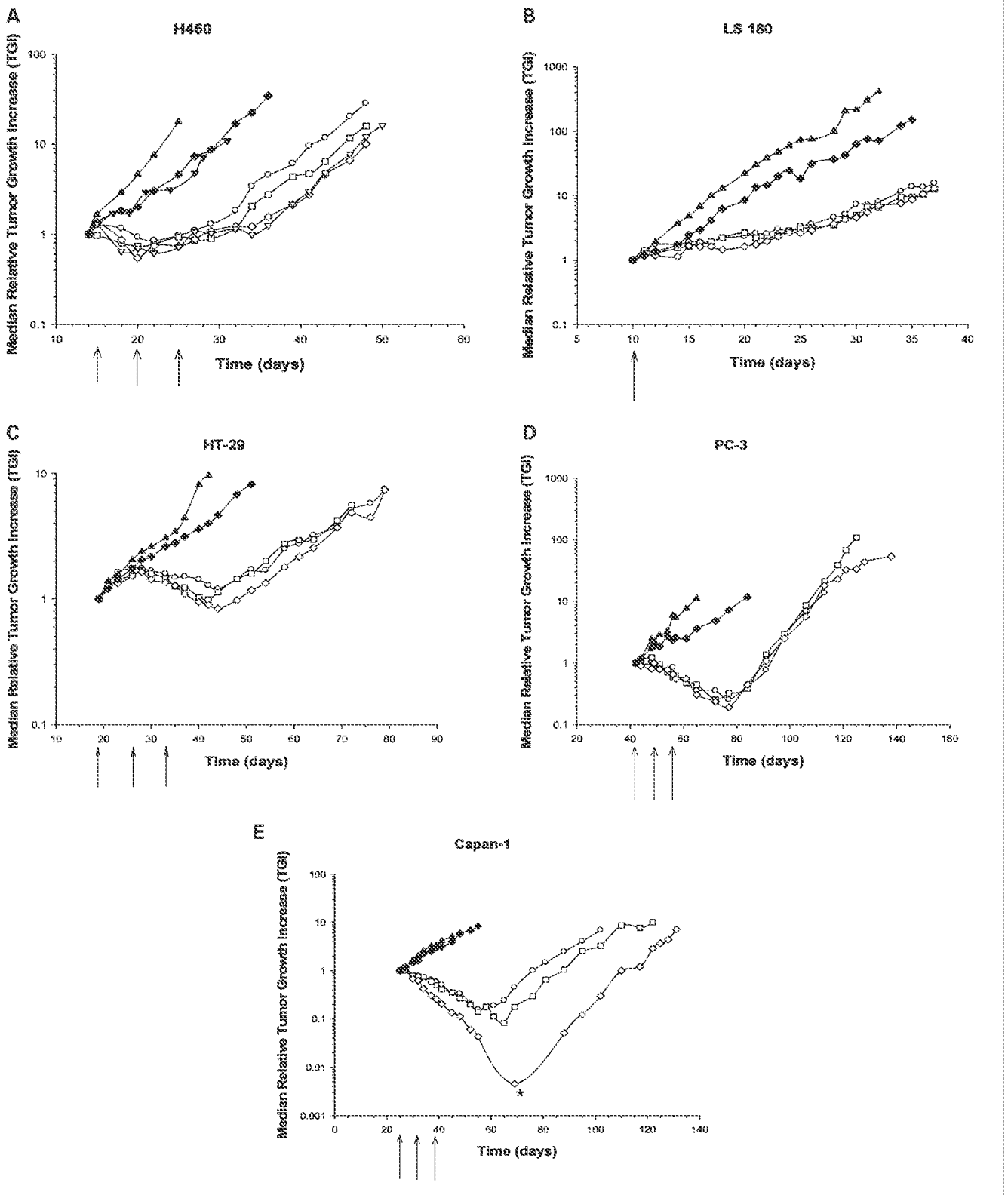

Fig. 3. The therapeutic effectiveness of Camptosar and Irinophore C measured against a panel of human tumor xenografts. RAG2-M mice bearing established s.c. human tumor xenografts were treated with saline (▲), Camptosar 40 mg/kg (◆), Camptosar 60 mg/kg (▼, H460 only), Irinophore C 20 mg/kg (○), Irinophore C 30 mg/kg (□), Irinophore C 40 mg/kg (◇), and Irinophore C 60 mg/kg (▽, H460 only). Tumor growth is represented by the median relative TGI and is expressed as a function of time. The number of doses and dosing schedule for each tumor model, as described in Materials and Methods, are indicated by the arrows. Each data point represents $n = 8$, except for Capan-1 where * indicates that this data point and subsequent data points for this treatment represent $n = 7$ because there was one durable cure. *A*, H460 NSCLC; *B*, LS180 colorectal adenocarcinoma; *C*, HT-29 colorectal adenocarcinoma; *D*, PC-3 prostate carcinoma; *E*, Capan-1 pancreatic carcinoma.

The therapeutic efficacy of Camptosar and Irinophore C against a panel of five human tumor xenografts. Following i.v. administration of Irinophore C, there was a substantial change in both CPT-11 and SN-38 pharmacokinetics, represented by significant increases in AUC for the lactone forms of these drugs, and the pharmacokinetics suggested that SN-38 levels were maintained over extended time periods; further, CPT-11 lactone and SN-38 lactone were shown to be present in the tumor. The next series of studies aimed to determine whether the altered pharmacokinetic profile of Irinophore C was associated with improvements in therapeutic activity. Camptosar and Irinophore C were used to treat RAG2-M mice bearing established s.c. human tumor xenografts. The results from five different tumor models are summarized in Fig. 3, where tumor growth is expressed as the median relative TGI as a function of time (days).

A dose of Camptosar (40 or 60 mg/kg) or Irinophore C (20, 30, 40, or 60 mg/kg) was administered every 4 days for a total of three doses to treat mice with human H460 NSCLC xenografts (Fig. 3A). Camptosar did not result in a significant delay in TGI when compared with the saline-treated controls. Significant improvements (relative to controls and the Camptosar treated animals) were seen following treatment with Irinophore C. This is emphasized by the T-C, which represents the median difference in days for treated tumors (T) compared with control tumors (C) to reach a TGI equal to 4 (Table 2). The calculated T-C for the highest Camptosar dose tested (60 mg/kg) was 8 days, significantly lower than the lowest dose of Irinophore C (20 mg/kg), which mediated a T-C of 15 days. The mean %BWL was recorded and used as a marker for toxicity (Table 2). The %BWL associated with Irinophore C 20 mg/kg was 8.4%, which was lower than all treatment groups. There was a trend toward greater %BWL at the highest doses, particularly with Irinophore C 60 mg/kg (%BWL = 18.5%; Table 2), with no significant improvements in therapeutic activity (Fig. 3A; Table 2). For this reason, further efficacy experiments focused on doses equivalent to a maximum of 40 mg/kg CPT-11.

Figure 3B shows the tumor growth curves for human LS 180 adenocarcinoma xenografts following single-dose treatment with Camptosar or Irinophore C. Consistent with the results seen with the H460 model, all doses of Irinophore C (20, 30, and 40 mg/kg) mediated a greater delay in TGI compared with Camptosar 40 mg/kg, which translated to an ~7-fold increase in T-C if the 40 mg/kg dose data are compared (Table 2). Notably, there was no significant difference in the T-C values between the Irinophore C doses; further, the tumor growth curves were comparable (Fig. 3B). Interestingly, an ~5% BWL was recorded for all treatment groups indicative of Irinophore C having a greater therapeutic index than Camptosar. It was also evident that the body weights had predominately recovered by ~7 days after injection (data not shown); therefore, for the other efficacy models, the dosing interval was increased from 4 days (used to treat H460 tumors) to 7 days.

A treatment schedule of three doses, with each dose administered every 7 days, was sufficient for all doses of Irinophore C to induce tumor regression in a human HT-29 colorectal carcinoma model (Fig. 3C). In contrast, Camptosar 40 mg/kg delayed TGI compared with saline control but did not mediate a regression in tumor size. Resultantly, Irinophore C mediated a 10-fold increase in T-C (32 days for Irinophore C 40 mg/kg compared with 3 days for Camptosar 40 mg/kg; Table 2). Interestingly, there was no significant difference in T-C recorded for the three doses of Irinophore C (Table 2), and this was supported by similar tumor growth curves (Fig. 3C). Analysis of the associated %BWL indicated that administering the treatments at 7-day intervals resulted in similar %BWL observed for single-dose treatment (Table 2; cf. LS 180) and did not result in a cumulative dosing effect that was apparent for H460 (Table 2).

The results for the treatment of RAG2-M mice with established PC-3 prostate carcinoma xenografts followed a pattern similar to that observed for the HT-29 model (i.e., all doses of Irinophore C induced tumor regression whereas Camptosar only delayed tumor growth; Fig. 3D). Further, there were no notable differences in the therapeutic activity of the three Irinophore C doses (Table 2; Fig. 3D), and the %BWL associated with the different treatments were comparable and

Table 2. The therapeutic effectiveness of Camptosar and Irinophore C against a panel of five human cancer xenografts

Treatment	H460		LS 180		HT-29		PC-3		Capan-1	
	T-C	%BWL	T-C	%BWL	T-C	%BWL	T-C	%BWL	T-C	%BWL
Irinophore C (20 mg/kg)	15*	8.4 (3.8)	12†	5.0 (2.2)	30†	5.2 (3.6)	53†	6.3 (2.6)	58†	7.9 (4.3)
Irinophore C (30 mg/kg)	19*‡	14.7 (4.3)	15†	4.8 (6.5)	24†	7.4 (3.0)	53†	6.2 (2.1)	69†‡	4.1 (2.4)
Irinophore C (40 mg/kg)	23*‡	15.0 (1.9)	14†	5.4 (2.8)	32†	5.8 (2.7)	53†	6.7 (3.2)	87†§	6.2 (1.9)
Camptosar (40 mg/kg)	5	12.1 (3.4)	2	4.9 (2.9)	3	7.0 (2.2)	18	9.6 (5.0)	6	4.9 (2.5)
Irinophore C (60 mg/kg)	24*§	18.5 (3.6)								
Camptosar (60 mg/kg)	8	13.9 (3.9)								

NOTE: Treatment schedules are described in Materials and Methods section. T-C represents the median difference in days for treated tumors (T) compared with control tumors (C) to reach a TGI = 4. %BWL represents the maximum mean body weight loss associated with the indicated treatment. The SD is included in parentheses.

*Significantly different from Camptosar 40 mg/kg and Camptosar 60 mg/kg ($P < 0.005$).

†Significantly different from Camptosar 40 mg/kg ($P < 0.005$).

‡Significantly different from Irinophore C 20 mg/kg ($P < 0.05$).

§Significantly different from Irinophore C 20 mg/kg ($P = 0.001$) and Irinophore C 30 mg/kg ($P < 0.05$).

similar to that recorded for the single-dose treatment of LS 180 (Table 2).

The final model tested was a Capan-1 pancreatic xenograft and the results are shown in Fig. 3E. This was the only model within the panel of human tumor xenografts where there was a marked difference in therapeutic efficacy associated with the dose of Irinophore C. Clearly, all doses of Irinophore C were substantially more active than Camptosar 40 mg/kg (Fig. 3E). Moreover, the highest dose of Irinophore C (40 mg/kg) was able to cure one mouse and also mediated a T-C (87 days) that was significantly greater than those recorded for the 20 and 30 mg/kg doses, as well as being 15-fold higher than the T-C for the equivalent dose of Camptosar (6 days; Table 2). Consistent with the previous models using the dosing schedule of three doses administered every 7 days, the %BWL were comparable to those observed following a single dose (Table 2).

Discussion

Liposomes represent the preeminent nanoscale drug delivery technology for the i.v. administration of cytotoxic drugs (30), with three anthracycline preparations currently licensed for clinical use (31–33) and many other liposomal formulations of antineoplastic drugs in preclinical or clinical trials (12, 34–37). The beneficial effects of liposomes have been attributed to decreased toxicity, increased drug stability, and improved biodistribution of the encapsulated drugs (38).

The clinical effect of CPT-11 on overall patient survival and quality of life has been significant (39) despite the physiologic instability of the active lactone forms of the drug and its potent metabolite, SN-38 (3). Consequently, CPT-11 is an attractive candidate for formulation with lipid nanocarriers. Burke and Gao (7) first showed that lipid-based carrier formulations could stabilize the lactone ring of topotecan; subsequently, the stabilization of CPT-11 lactone, following i.v. administration, by liposome encapsulation has been associated with improved therapeutic activity (10, 11, 20). Irinophore C is a novel liposome formulation of CPT-11 in which drug retention following systemic administration was greatly improved by entrapping the drug in an acidic copper ion environment (40).

Following a single i.v. bolus injection, Irinophore C mediated a substantial increase in the CPT-11 lactone plasma half-life, maximum plasma concentration (C_{max}), and plasma AUC and a substantial decrease in plasma clearance, when compared with an equivalent dose of Camptosar (Table 1). Likewise, Irinophore C altered the pharmacokinetic profile for CPT-11 carboxylate (Table 1; Fig. 1A and B). Presumably, this indicates that the majority of both forms of CPT-11 detected in the plasma are associated with the carrier system, which would explain why the high CPT-11 C_{max} levels following i.v. administration of Irinophore C did not precipitate a "cholinergic syndrome." This toxicity is attributed to CPT-11–mediated acetylcholinesterase inhibition (41) and limits the maximum single i.v. dose of Camptosar that RAG2-M mice can tolerate to 80 mg/kg. In contrast, Irinophore C is well tolerated at this dose in RAG-2M mice and has been administered to the plasma carboxylesterase–deficient murine strain aES1^{e}/J (42) at single doses up to 350 mg/kg with no immediate or long-term toxicities.[5] Drummond et al. (10) reported similar results for a liposomal irinotecan formulation in which multivalent anionic trapping agents were used to increases retention of the drug.

Protracted dosing schedules would be expected to be beneficial for S-phase active drugs like CPT-11 (15, 16). Clinical studies have shown that the maximum CPT-11 dose intensity achievable with long-term continuous infusion is actually two to three times lower than that observed with short-term infusions; however, the SN-38 plasma AUC levels were comparable (15, 16). This implies that higher CPT-11 doses may saturate the hepatic carboxylesterases that are predominately responsible for the generation of SN-38. Further, there is evidence to suggest that optimal anticancer activity is dependent on the maintenance of a threshold level of exposure to SN-38 (43). Furman et al. (44) reported that a prolonged CPT-11 dosing schedule optimized in xenograft models to maintain a minimum threshold plasma concentration of SN-38 could be mimicked in children with a comparable relationship between SN-38 levels and response. The long half-life of CPT-11 release from the carrier (44.4 h; ref. 40) could prevent saturation of the carboxylesterase enzymes in the liver, enabling Irinophore C to mediate a constant plasma SN-38 lactone level over the duration of the experiment (Fig. 1D and F). Indeed, the inherent accumulation of liposomes in the liver might act as a drug depot where the macrophage disruption of the carrier could slowly free CPT-11 for enzymatic conversion (45). If the lowest dose of Irinophore C (20 mg/kg) still maintained the plasma SN-38 levels above the critical threshold (Fig. 1F), this could explain the lack of dose-response effect observed for the majority of the xenograft models tested (Fig. 3).

The mechanism of tumor growth delay, or shrinkage, observed in the five xenograft models following Irinophore C treatment remains to be determined. Camptothecins have been reported to have antiangiogenic effects (46), and it may be that maintenance of a threshold plasma level of SN-38 by Irinophore C could mimic metronomic dosing, in which the anticancer action is primarily through disruption of the tumor vasculature. However, despite SN-38 being reported as up to 1,000 times more potent than CPT-11 *in vitro* (24), it is difficult to estimate the contribution of each compound to the overall cytotoxic effect *in vivo* (47). There have been *in vitro* survival studies using HT-29 cells (48) and H460 cells (25) that have attributed the observed cytotoxicity solely to CPT-11. Analysis of H460 tumor levels of CPT-11 (Fig. 2) showed that the lactone form predominates, suggesting either that CPT-11 is still encapsulated within the liposome or, alternatively, that the lower pH of the tumor microenvironment is limiting the conversion of bioavailable CPT-11 lactone to the carboxy form (49). SN-38 lactone levels within the tumor, after accounting for the presence of the active metabolite in the plasma, could be due to CPT-11 lactone that leaked from regionally localized liposomes, or it could be from the plasma compartment. The latter could be a result of drug release from liposomes within the plasma compartment or following localization in other

[5] Unpublished observations.

tissues such as the liver. However, it is also reasonable to speculate that tumor accumulation of liposomes via the enhanced permeability and retention effect and subsequent leakage of CPT-11 could also contribute to the anticancer effect of Irinophore C. We are currently investigating whether the activity of Irinophore C is mediated by a dual-action mechanism encompassing antivascular and direct tumor cell cytotoxic actions. Further, ongoing studies in our laboratory are also assessing the therapeutic efficacy of Irinophore C against a systemic melanoma model grown in aES1^{e}/J mice. These mice are deficient in plasma carboxylesterase and are believed to offer a better model of SN-38 metabolism in humans. It is hoped that these experiments will provide a more mechanistic basis for the improved therapeutic effects of Irinophore C.

Obviously, further clinical development of Irinophore C will also require a better understanding of the toxicity of this new formulation. The principal dose-limiting toxicity of irinotecan is delayed diarrhea, which is attributed to the high intestinal concentrations of SN-38 excreted directly via the biliary canal, or as the result of β-glucuronidase cleavage of SN-38G to SN-38 by the gut flora (50). Ongoing studies in our laboratory are investigating the effect of CPT-11 encapsulation on the principal toxicities associated with irinotecan therapy.

In conclusion, Irinophore C is a novel liposomal formulation of irinotecan, which substantially increases the plasma levels of CPT-11 lactone and mediates a prolonged plasma and tumor exposure to SN-38 lactone. This favorable pharmacokinetic profile translated into superior therapeutic activity compared with Camptosar when tested against a panel of five human cancer xenografts. These results would support the further development of Irinophore C for future clinical trials.

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Clinical Cancer Research

Irinophore C: A Liposome Formulation of Irinotecan with Substantially Improved Therapeutic Efficacy against a Panel of Human Xenograft Tumors

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Multicentre phase II study and pharmacokinetic analysis of irinotecan in chemotherapy-naïve patients with glioblastoma

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Background: To assess the antitumour activity and safety profile of irinotecan and its pharmacokinetic interactions with anticonvulsants in patients with glioblastoma multiforme.

Patients and methods: This multicentre phase II and pharmacokinetic study investigated the effects of irinotecan 350 mg/m^2 given as a 90-min infusion every 3 weeks either prior to (group A) or after relapse following radiotherapy (group B) in chemotherapy-naïve patients with glioblastoma. Preferred concomitant medication for seizure prevention was valproic acid. Pharmacokinetic analysis of irinotecan and its main metabolites (SN-38, SN-38-G, APC and NPC) was performed during cycle 1. An independent panel of experts reviewed the activity data.

Results: Fifty-two patients (25 patients in group A and 27 patients in group B) received a total of 191 cycles of irinotecan. Forty-six patients (22 patients in group A and 24 patients in group B) were evaluable and externally reviewed for activity. According to external review, one partial response (group B), seven minor responses (three in group A and four in group B), 12 disease stabilisations (seven in group A and five in group B) were observed. This resulted in an overall response rate of only 2.2% (95% confidence interval 0.2% to 6.5%). The median time to tumour progression was 9 weeks in group A and 14.4 weeks in group B. Six-month progression-free survival rates were 26% in group A and 43% in group B. Grade 3–4 toxicities (percentage of patients in groups A and B) consisted of neutropenia (12.5% and 25.9%), diarrhoea (8.3% and 7.4%), asthenia (12.5% and 7.4%) and vomiting (0% and 7.4%). The clearance of irinotecan was 12.4 and 14.4 l/h/m^2 in two patients who received no anticonvulsant. In patients receiving valproic acid, the clearance of irinotecan was 17.2 ± 4.4 l/h/m^2.

Conclusions: Irinotecan given at the dose of 350 mg/m^2 every 3 weeks has limited clinical activity as a single agent in patients with newly diagnosed and recurrent glioblastoma after radiotherapy. The toxicity profile and plasma disposition of irinotecan and SN-38 were not strongly influenced by anticonvulsant valproic acid therapy. Although the response rate of irinotecan as a single agent was limited, it remains an attractive drug for combination studies in patients with glioblastoma.

Key words: anticonvulsants, APC, irinotecan, NPC, SN-38, valproic acid

Introduction

Malignant gliomas represent ~60% of primary malignant tumours of the central nervous system [1–5]. Nitrosoureas (including BCNU and CCNU) [6–9] and more recently temozolomide [10] are alkylating agents classically used in patients with glioblastoma. However, newer active anticancer agents that have a different mechanism of action are urgently required to improve the outcome of patients with gliomas.

Irinotecan has been shown to exert antitumour activity against several human tumour types and was recently approved in combination with 5-fluorouracil (5-FU)/folinic acid as first-line chemotherapy for metastatic colorectal cancer [11]. In animal studies, irinotecan displayed marked antitumour activity against a broad panel of subcutaneous and intracranial human glioblastoma multiforme, ependymoma and medulloblastoma xenografts [12–14]. After i.v. injection, irinotecan has a very complex metabolism that mainly takes place in the liver. Irinotecan can be converted into two inactive metabolites, 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin (APC) and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin (NPC), by the CYP3A4 enzyme and into an active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by carboxylesterase enzymes in the liver [15, 16]. SN-38 is further metabolised to SN-38 glucuronide {7-ethyl-10-[3,4,5-trihydroxypyran-2-carboxylic acid]-camptothecin (the β-glucuronide conjugate of SN-38) (SN-38-G)} through conjugation by uridine diphosphate glucuronosyltransferase (UGT1A1) [17]. SN-38 has

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a low molecular weight and presents lipophilic characteristics but has shown poor penetration of central nervous fluid (CSF) in comparison with other camptothecins [18].

Based on these data, this multicentre phase II study was conducted to evaluate the antitumoral activity and toxicity profile of irinotecan administered every 3 weeks in chemotherapy-naïve patients with glioblastoma either previously treated by or not treated by radiotherapy. Supportive care in the management of patients with glioblastoma frequently includes use of corticosteroids to control cerebral oedema and oral anticonvulsants to prevent epilepsy. Concomitant medications may modify the metabolism of irinotecan and could affect the efficacy and toxicity profile of the drug. Valproic acid (2-propylpentanoic acid) is a frequently used anticonvulsant that is extensively glucuroconjugated with potential interactions with the disposition of irinotecan [19]. In this study, we investigated the pharmacokinetics and pharmacodynamics of irinotecan and its main metabolites, SN-38, SN-38-G, APC and NPC, with regard to concomitant medications (predominantly valproic acid) used during the trial.

Patients and methods

Inclusion criteria

Inclusion criteria were as follows: aged ≥18 years; histologically confirmed recurrent glioblastoma multiforme (grade 4 astrocytoma) measurable on contrast enhanced magnetic resonance imaging (MRI) performed within 2 weeks before study initiation; no previous chemotherapy; moderate MRC less than or equal to two neurological symptoms; and performance status ranging from 0 to 3. A minimum interval of 3 weeks between prior surgery or 6 weeks prior radiotherapy and enrolment must have elapsed. Other criteria were neutrophils $\geq 2 \times 10^9$/l, platelets $\geq 100 \times 10^9$/l, haemoglobin ≥10 g/dl, total bilirubin <1.25 × the institutional upper normal limit (UNL), alkaline phosphatases and ASAT ≤2.5 × UNL; prothrombin time ≥50% and creatinine ≤120 µmol/l. For patients with corticosteroids, a stable dose for 20 days was required before study entry. Exclusion criteria were past or present history of chronic diarrhoea, current uncontrolled infection, other investigational drugs, pregnancy and lactating women of child-bearing age had to take contraceptive measures. All patients gave written informed consent prior to registration for the study.

Drug administration

Aventis Pharma (Paris, France) supplied irinotecan in 5 ml vials (100 mg of active product). Irinotecan was diluted in 250 ml (0.9% NaCl) and administered as a 90 min i.v. infusion every 21 days at the dose of 350 mg/m^2 [20].

Two groups of patients were to be successively considered:

- Group A: patients with inoperable or incompletely resected newly diagnosed radiotherapy-naïve glioblastoma received irinotecan prior to radiotherapy. They were scheduled to receive three cycles of irinotecan followed by radiotherapy in case of stabilisation or tumour progression.
- Group B: patients relapsing after radiotherapy. Patients in group B were scheduled to receive up to six cycles of irinotecan, according to the investigator efficacy assessment.

Radiotherapy consisted of a conformational administration of 50–60 Grays into the tumour volume given over a period of 6–7 weeks.

Dose adjustments

Dose adjustment of irinotecan was based on the worst toxicity observed during the previous cycle. The next cycle was delayed until the neutrophil and platelet counts were ≥1500/µl and ≥100 000/µl, respectively, and treatment toxicity was fully resolved. If this exceeded a 2-week delay, treatment with irinotecan was discontinued. In cases of grade 4 thrombocytopenia (grade 3–4 neutropenia lasting >7 days) and febrile neutropenia, the dose of irinotecan was reduced to 300 mg/m^2 and, if necessary, could be subsequently reduced to 250 mg/m^2. Patients were withdrawn from the study if they required more than two dose reductions.

Concomitant medications

Boluses of methylprednisolone 80 mg with standard doses of ondansetron were given to prevent vomiting. Atropine (1–2 mg) was administered for treatment of cholinergic syndromes [21] and then, if necessary, given prophylactically at subsequent cycles. Patients began antidiarrhoeal treatment for delayed diarrhoea occurring more than 24 h after irinotecan administration [22]. The prophylactic use of loperamide was not allowed. Patients presenting with severe vomiting, blood in their faeces or severe diarrhoea after 48 h were hospitalised. Haematopoietic growth factors were not allowed.

To minimise interaction with the metabolism of irinotecan, patients taking anti-epileptic treatments were recommended to use valproic acid or carbamazepine 2 weeks prior to irinotecan. Valproic acid was given orally at a daily dose ranging from 20 to 30 mg/kg/day (<60 mg/kg/day). Concentrations of anticonvulsants were monitored during the study as necessary to maintain therapeutic levels. Chronic oral administration of corticosteroids was used as needed with a careful monitoring of doses.

Baseline and follow-up examinations

Patients underwent physical and neurological examination within 2 weeks prior to entering the study, immediately prior to the first irinotecan injection and then at least once before each subsequent drug infusion. Complete blood cell with differential and platelet counts, serum biochemistry and hepatic parameters were assessed weekly. Toxicity was graded according to National Cancer Institute-Common Toxicity Criteria.

Response determination was based on both the comparison of the baseline brain contrast-enhanced MRI or CT scan done 2 weeks prior to irinotecan with those performed every two to three infusions. Treatment was discontinued in case of tumour progression, unacceptable toxicity or patient refusal. Patients were followed-up monthly (MRI or CT scan every 2–3 months) after the completion of the study until death.

Evaluation of response to therapy

The primary end point of this study was to assess the anti-tumour activity according to the criteria of MacDonald et al. [23] and this was reviewed by a panel of independent experts. Patients were considered evaluable for efficacy if they had received at least two cycles of irinotecan. In addition to classical response parameters [23], a minor response was defined as a >25% but a <50% reduction from baseline in the size of enhancing tumour on either the CT or MRI scan. Follow-up examinations for tumour evaluation were performed using the same method as that used at baseline. A CT or MRI scan was performed every two cycles or every three cycles in patients entering groups A and B, respectively. Partial and minor responses had to be confirmed using the same technique, either a CT or MRI scan, after a period of at least 4 weeks had elapsed since the first onset of response. Tumours were considered stable if they met the criteria for tumour stabilisation for at least two cycles.

Pharmacokinetic evaluation

Pharmacokinetic analysis was performed during the first infusion. Blood specimens (2 ml heparinised tubes) were drawn 5 min before, 45 min during and at the end of the infusion, then 1 h, 6 h and 22.5 h after the end of the infusion (4°C). Plasma was harvested immediately by centrifugation at 1200 *g* for 30 min and stored at –80°C until analysis.

Total forms of CPT-11 and its metabolites (SN-38, SN-38-G, APC and NPC) were assayed using high-performance liquid chromatography with fluorescence detection as described by Rivory et al. [24], and with slight modifications [25]. Calibration standard responses were linear. Limits of quantification in plasma were 10 ng/ml for all compounds. Irinotecan concentrations were expressed in free base units for pharmacokinetic analyses. Irinotecan, SN-38, SN-38-G, APC and NPC plasma concentration data were analysed by non-compartmental methods. Peak plasma concentrations (C_{max}) were determined for irinotecan, SN-38, SN-38-G, APC and NPC from concentration–time curves. Areas under the plasma concentration–time curves (AUC_{0-24h}) were calculated using the linear trapezoidal rule from time zero to the last sampling with quantifiable drug concentrations.

Metabolite ratios were calculated as follows:

$[AUC_{0-\infty}$ (metabolite)$/AUC_{0-\infty}$ (irinotecan)$]\times 100$ for SN-38, SN-38-G and NPC;

$[AUC_{0-\text{last sampling}}$ (APC)$/AUC_{0-\text{last sampling}}$ (irinotecan)$]\times 100$ for APC.

The ratio of SN-38 glucuronidation was defined as follows:

$[AUC_{0-\text{last sampling}}$ (SN-38-G)$/AUC_{0-\text{last sampling}}$ (SN-38)$]\times 100$.

Statistical analysis

The primary end point was the objective response rate according to the expert panel. Secondary objectives were progression-free survival, duration of response and overall survival (Kaplan–Meier analysis).

The number of patients in each group was determined by Gehan test with a type error I ($\alpha = 5\%$) and a type error II ($\beta = 10\%$) to have a control rate of 20%. For the first stage, 14 patients were enrolled in each group. Regarding the disease control rate observed on these 14 patients, additional patients could be included for the second stage. In total, 25 patients per group had to be enrolled in this study.

The statistical (SAS software, ver. 6.12®) and pharmacokinetic analyses (WinNonlin; Scientific Consulting, Cary, NC, USA) used non-parametric Mann–Whitney and Kruskal–Wallis tests displayed using GraphPad-InStat 3.00® (GraphPad Software, San Diego, CA, USA). A two-sided value of $P < 0.05$ was considered significant.

Results

Patient characteristics

A total of 52 patients with confirmed glioblastoma were enrolled in this study (25 patients in group A and 27 patients in group B). All patients received at least one cycle of chemotherapy. One patient withdrew his consent immediately after the first cycle and was not evaluable for activity and toxicity. Baseline patient characteristics are summarised in Table 1. Most of the patients entering this trial had concomitant anticonvulsant therapy that included valproic acid, carbamazepine, phenytoin and/or phenobarbital in 40, seven, four and/or two patients, respectively. Forty patients had concomitant treatment with corticosteroids at study entry. Only one patient had neither anticonvulsant therapy nor treatment with corticosteroids at study entry. Other concomitant medications likely to interfere with the CYP3A4 metabolism of irinotecan were registered.

Treatment delivery

A total of 191 cycles were administered (69 cycles in group A and 122 cycles in group B). The median number of cycles were three (range 1–8) and four (range 1–10) in groups A and B, respectively. Short treatment delays (ranging from 3 to 6 days) were reported in two patients (two cycles) in group A and in seven patients (eight cycles) in group B. These delays were mainly due to the scheduling of cycles and were caused by irinotecan toxicity in only one patient (asthenia). Longer delays (≥7 days) were reported in three patients (five cycles) in group A and four patients (seven cycles) in group B. Longer delays were due to toxicity in two patients (four cycles). Cycles were given at the dose of 350 mg/m^2 in 68 cycles (98.6%) in group A and 107 cycles (87.7%) in group B. Dose reduction to 300 mg/m^2 irinotecan was performed for three patients (one in group A and two in group B) and 14 cycles. The median relative dose intensity of irinotecan was 0.98 (range 0.83–1.02) in group A and 0.97 (range 0.72–1.02) in group B.

Antitumour activity

The antitumour activity of irinotecan in patients with glioblastoma was evaluated using CT scans and MRI of the brain in 12 and 40 patients, respectively (Table 2).

According to the investigators, 49 patients were evaluable for activity. One partial response (in group A), four minor responses (two in group A and two in group B) and 16 tumour stabilisations (six in group A and 10 in group B) were observed, resulting in an overall control rate of 43% [95% confidence interval (CI) 28.5% to 57%].

According to expert panel review, 46 patients were considered evaluable for activity. Six patients (three in group A and three in group B) were not evaluable (in four cases, images from baseline were not technically comparable with images used for the documentation of response, one patient withdrew his consent before tumour evaluation and one died prematurely from a concomitant disease). After review, one partial response lasting 35 weeks was observed in group B, seven minor responses (three in group A and four in group B), 12 tumour stabilisations (seven in group A and five in group B) and 26 tumour progressions were reported. This resulted in an overall response rate of 2.2% (95% CI 0.2% to 6.5%). Minor and partial responses were observed in patients concomitantly treated with valproic acid. The median time-to-progression was 9 weeks (range 3.6–53.1; 95% CI 8.1–22.4) in group A and 14.4 weeks (range 5.5–36.8; 95% CI 9.0–21.1) in group B. The 6-month progression-free survival rate was 26% in group A and 43% in group B (Figure 1; no statistical difference was observed between groups A and B; Cox proportional hazard ratio = 1.34, $p = 0.38$). The median overall survival was 5.8 months (range 1.5–13.0; 95% CI 3.1–9.6) in group A and 6.8 months (range 3.0–14.4; 95% CI 5.5–11.5) in group B.

Safety

Fifty-one patients and 190 cycles were evaluable for safety. Overall safety data are presented in Table 3. No clinically relevant difference in toxicity was observed in the two groups of patients.

Grade 3–4 cholinergic syndrome with acute diarrhoea, abdominal pain, nausea, vomiting, hypersalivation, sweating and asthenia occurred in five patients (10%) and six cycles (3%).

Table 1. Patient characteristics

	Group A	Group B	Total
No. of patients	25	27	52
Male/female	12/13	16/11	28/24
Median age, years (range)	54 (33–75)	52 (26–67)	53 (26–75)
WHO performance status			
0	5	12	17
1	9	10	19
2	11	3	14
3	0	2	2
Neurological symptoms at study entry	20 (80%)	16 (59%)	36 (69%)
Site of lesions			
Frontal lobe	8 (32%)	9 (33%)	17 (33%)
Occipital lobe	6 (24%)	8 (30%)	14 (27%)
Parietal lobe	11 (44%)	13 (48%)	24 (46%)
Temporal lobe	1 (4%)	3 (11%)	4 (8%)
Other sites	6 (24%)	2 (7%)	8 (15%)
Unifocal site	18 (72%)	19 (70%)	37 (71%)
Multifocal sites	7 (28%)	8 (30%)	15 (29%)
Mean tumour sizes[a] at study entry, mm^2 (range)	1039 (228–6400)	1225 (143–3355)	1182 (143–6400)
Delay between diagnosis and enrolment, months (range)	0.59 (0.13–2.03)	7.74 (0.62–18.23)	1.97 (0.13–18.23)
Previous surgery			
Complete resection	1 (4%)	11 (41%)	12 (23%)
Partial resection	8 (32%)	11 (41%)	19 (36.5%)
Biopsy	16 (64%)	5 (18%)	21 (40.5%)
Mean dose of radiotherapy (range)	0	57 (50–60)	NA
Delay from the end of radiotherapy to enrolment, months (range)	NA	5.39 (1.41–48.1)	5.39 (1.41–48.1)
Concomitant anticonvulsant			
0	1	2	3
1	23	21	44
≥2	1	4	5

NA, not applicable; WHO, World Health Organization.
[a]Product of the larger diameters of the lesions.

Mild to moderate delayed diarrhoea was frequently observed. Grade 1 diarrhoea was observed in 13 patients (25.5%) and in 44 of 190 cycles (23%). Grade 2 diarrhoea was observed in 21 patients (41.2%) and 28 cycles (14.7%). Grade 3 diarrhoea was observed in four patients (7.9%) and five cycles (2.6%), requiring hospitalisation for hydration in three patients. No grade 4 diarrhoea was reported. Two patients (two cycles) experienced severe diarrhoea in group A and two patients (three cycles) in group B. Diarrhoea was efficiently treated with oral loperamide.

Two patients, a 60-year-old male and a 63-year-old female, who experienced febrile grade 2–3 diarrhoea without neutropenia were adequately treated with oral loperamide and ciprofloxacine. These primary events subsequently led, in both cases while the diarrhoea had resolved, to more severe sepsis and patient death. In one of these patients, who developed an acute respiratory distress syndrome, *Enterococcus faecalis*, *Staphylococcus faecalis* and *Staphylococcus maltophilia* were identified from blood cultures. No bacterial documentation was possible in the other patient. Pharmacokinetic data were not available for these two patients.

Severe grade 3–4 neutropenia was reported in a total of 10 patients (19.6%) and 21 cycles (11%). In group A, grade 4 neutropenia was observed in three patients (12.5%) with one of them experiencing febrile neutropenia, whilst in group B, seven patients (25.9%) presented with grade 3–4 neutropenia including three patients (11.1%) with febrile grade 3–4 neutropenia. In total, four patients had episodes of febrile grade 3–4 neutropenia (7.8% of patients and 2.1% of cycles). One patient treated in group A presented with grade 3 anaemia. No grade 3–4 thrombocytopenia was observed.

Table 2. Antitumour activity of irinotecan in patients with glioblastoma

	Group A		Group B		Total	
	Investigators	Experts	Investigators	Experts	Investigators	Experts
No. of patients	25	25	27	27	52	52
Evaluable	24	22	25	24	49	46
Non-evaluable	1	3	2	3	3	6
Withdrawal of consent			1	1	1	1
Died from other diseases	–	–	1	1	1	1
Insufficient imaging	1	3	–	1	1	4
Partial responses	1	–	–	1	1	1
Minor responses	2	3	2	4	4	7
Tumour stabilisations	6	7	10	5	16	12
Tumour control rate[a]	37.5% (9/24)	45.4% (10/22)	48% (12/25)	41.7% (10/24)	43% (21/49)	43% (20/46)
(95% CI)	(16.6–58.4%)	(23–68%)	(27–69%)	(20.4–63%)	(28.5–57%)	(28.5–58%)
Tumour progression	15	12	13	14	28	26
Six-month progression-free survival rate	26%		43%		35%	
Time to progression	9.0 weeks (95% CI 8.1–22.4)		14.4 weeks (95% CI 9.0–21.1)		–	
Median survival	5.8 months (95% CI 1.5–13.0)		6.8 months (95% CI 3.0–14.4)		–	

[a]Tumour control rate is defined as (partial and minor responses + tumour stabilisation)/number of evaluable patients.
CI, confidence interval.

Other toxicities were as follows: grade 3 asthenia was observed in three and two patients in groups A and B, respectively; grade 3–4 vomiting in two patients in group B; and grade 1–2 alopecia in six and nine patients in groups A and B, respectively.

Transient grade 3 elevations of transaminases were observed in three patients. One of them had a grade 3 elevation of ALAT when carbamazepine was substituted for valproic acid. Another patient had grade 3 elevation of ASAT while receiving concomitant treatment with valproic acid for a month. In these two patients, valproic acid and irinotecan were maintained with no recurrence of grade 3 hepatic toxicity at subsequent cycles. The last patient had a grade 3 elevation of ASAT while he was concomitantly treated with phenytoin and paracetamol. In this patient, recovery was obtained after withdrawal of phenytoin and paracetamol.

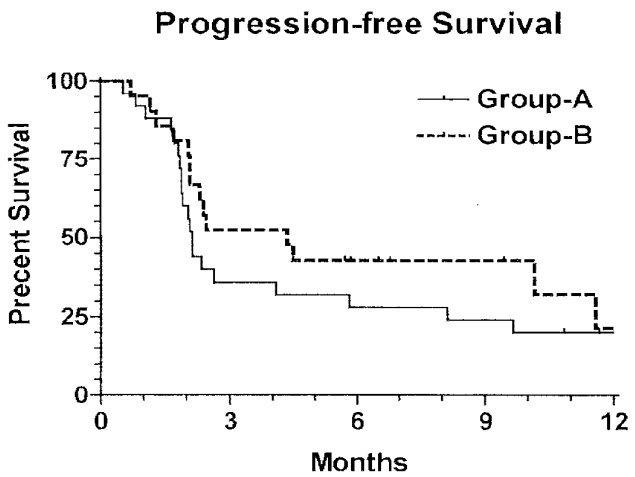

Figure 1. Progression-free survival (Kaplan–Meier analysis) of patients with glioblastoma treated with irinotecan either prior to (group A) or after radiotherapy (group B). No statistical difference was observed between groups A and B (Cox proportional hazard ratio: 1.34, $P = 0.38$).

Pharmacokinetics of irinotecan

Pharmacokinetic sampling was performed in 27 patients (14 patients in group A and 13 patients in group B). A typical pharmacokinetic profile is shown in Figure 2 and the main parameters of irinotecan and its metabolites are presented in Table 4. The mean $AUC_{0-\infty}$ of irinotecan was 18 518 ± 5323 ng·h/ml [coefficient of variation (CV): 28.7%] with a mean clearance of 17.6 ± 4.7 l/h/m^2 (CV: 26.5%). The mean $AUC_{0-\infty}$ of SN-38 was 471.6 ± 224.3 ng·h/ml (CV: 47.6%). The mean metabolic ratio of SN-38/irinotecan was 0.6 ± 0.7 % (CV: 108%). Mean $AUC_{0-\infty}$ of SN-38-G was 1243 ± 687 ng·h/ml (CV: 55.2%). The ratio of SN-38-G/irinotecan was 4.7 ± 2.0% (CV: 43%) with a glucuronidation ratio of 16.5 ± 14.3% (CV: 86.2%). Mean *AUC*s of APC and NPC were 5407 ± 2598 ng·h/ml (CV: 48%) and 533 ± 263 ng·h/ml (CV: 49%), respectively. Metabolic ratios of APC and NPC were 27.5 ± 13% (CV: 47.5%) and 1.5 ± 1% (CV: 66.6%), respectively.

Toxicity and pharmacokinetics of irinotecan according to concomitant medications

All patients were receiving concomitant medications (median number, 5; range 2–10) with several drugs. We found no influ-

Table 3. Grade 3–4 toxicity of irinotecan per patient and cycle

	Group A		Group B		Total	
	Per patient (%)	Per cycle (%)	Per patient (%)	Per cycle (%)	Per patient (%)	Per cycle (%)
No. of patients/cycle	24	68	27	122	51	190
Abdominal pain	–	–	1 (3.7)	1 (0.8)	1 (1.9)	1 (0.5)
Diarrhoea	2 (8.3)	2 (2.9)	2 (7.4)	3 (2.5)	4 (7.9)	5 (2.6)
Nausea	–	–	1 (3.7)	1 (0.8)	1 (1.9)	1 (0.5)
Vomiting	–	–	2 (7.4)	2 (1.6)	2 (3.9)	2 (1)
Asthenia	3 (12.5)	3 (4.4)	2 (7.4)	3 (2.5)	5 (9.8)	6 (3.1)
Hepatic cytolysis	2 (8.3)	2 (2.9)	1 (3.7)	1 (0.8)	3 (5.9)	3 (1.6)
Respiratory insufficiency	–	–	1 (3.7)	1 (0.8)	1 (1.9)	1 (0.5)
Skin toxicity	–	–	1 (3.7)	1 (0.8)	1 (1.9)	1 (0.5)
Infection	–	–	3 (11%)	3 (2.5%)	3 (5.9)	3 (1.6)
Neutropenia						
Grade 3	–	3 (4.4)	4 (14.8)	10 (8.2)	4 (7.8)	13 (6.8)
Grade 4	3 (12.5)	5 (7.35)	3 (11.1)	3 (2.5)	6 (11.8)	8 (4.2)
Febrile neutropenia	1 (4.2)	1 (1.5)	3 (11.1)	3 (2.5%)	4 (7.8)	4 (2.1)
Anaemia	1 (4.2)	1 (1.5)	–	–	1 (1.9)	1 (0.5)

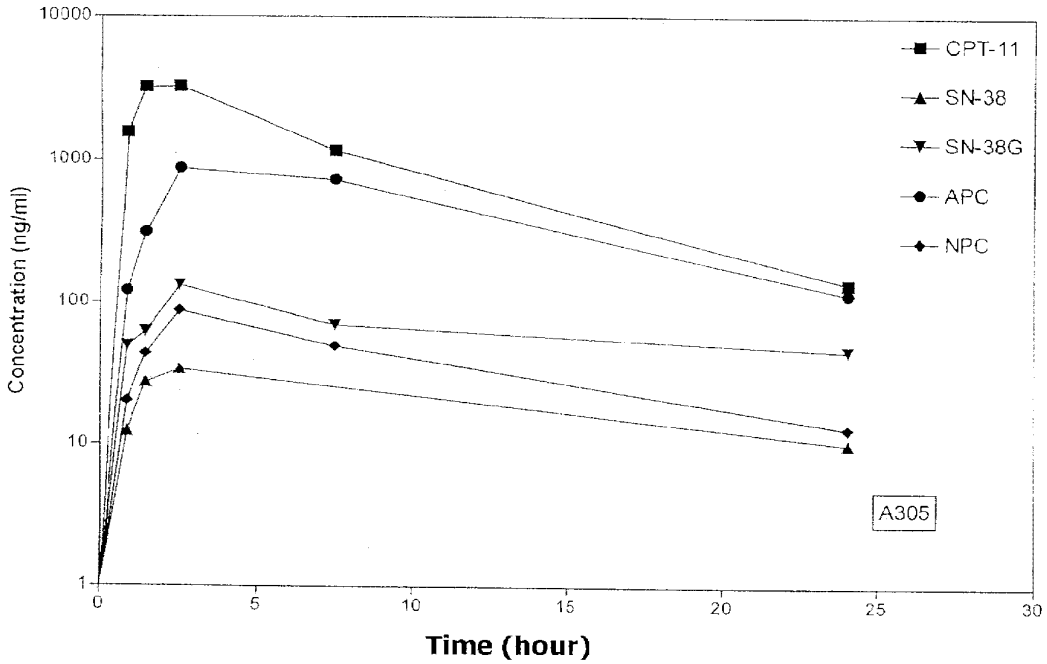

Figure 2. Typical pharmacokinetic profile of irinotecan and its main metabolites in a patient with glioblastoma. This patient received valproic acid concomitantly to irinotecan.

ence of the doses and duration of exposure to corticosteroids and omeprazole on the toxicity and the pharmacokinetic parameters of irinotecan and its metabolites (data not shown). In 40 patients treated with valproic acid for seizure prevention, grade 3–4 diarrhoea, neutropenia and asthenia were observed in nine (22.5%), 13 (32.5%) and 14 (35%) patients, respectively. Neither grade 3–4 diarrhoea, neutropenia, nor asthenia was observed in patients concomitantly treated with carbamazepine, phenytoin and/or phenobarbital.

For pharmacokinetic drug interaction analysis, three groups of patients (Figure 3) were defined: (i) patients without anti-epileptic drug (two patients); (ii) patients receiving valproic acid only (20 patients); and (iii) patients concomitantly treated with carbamazepine or phenobarbital (five patients). None of the

Table 4. Comparison of pharmacokinetic parameters of irinotecan and its metabolites in patients with glioblastoma or other tumour types

	Clinical trials									
	Malignant gliomas		Other tumour types							
	Our study	Friedman et al. [26]	Abigerges et al. [50]	Rothenberg et al. [51]	Rowinski et al. [52]	Gupta et al. [53]	Rivory et al. [54]			
Characteristics of trials										
No. of patients	27	32	7	17	31	40	5	9	3	2
Duration of infusion	90 min	90 min	30 min	90 min	90 min	90 min	30–90 min	30–90 min	30–90 min	30–90 min
Doses of irinotecan, mg/m^2	350	125	350	50–180	100–245	145	115	300–350	500	600
Irinotecan										
C_{max}, ng/ml	4416 ± 1190	1465 ± 598	7700 ± 1100	890–1970	1055–2637	1851 ± 586	1641 ± 586	7794 ± 3516	5567 ± 1992	10 138 ± 3926
$AUC_{0-\infty}$, ng·h/ml	18 518 ± 5323	4430 ± 1306	34 000 ± 4100	2790–11 750	243 776–1 267 518	11 854 ± 5294	5450 ± 1348	25 022 ± 7384	27 952 ± 6680	40 024 ± 7618
Half-life, h	4.9 ± 0.7	5.3 ± 0.86	11.2 ± 1.3	7.9 ± 2.8	5.2 (3.6–3.9)	8.8 ± 4.3	6.6 ± 1.4	6.1 ± 2.2	6.8 ± 2.4	4.7 ± 0.2
Clearance, l/h/m^2	17.6 ± 4.7	30.4 ± 8.3	11.0 ± 2.0	15.3 ± 3.5	21.12 ± 2.0	14.6 ± 6.4	19.3 ± 4.4	12.9 ± 3.9	16.3 ± 2.7	13.4 ± 2.7
SN-38										
C_{max}, ng/ml	29.3 ± 11.5	12.9 ± 5.5	56 ± 20	26.2–26.4	29.4–74.8	30 ± 12.4	31 (12–67)	47 (16–98)	59 (27–86)	118 (90–15)
$AUC_{0-\infty}$, ng·h/ml	471.6 ± 224.3	76.7 ± 50.5	451 ± 100	215–3676	98–634	372 ± 374	259 (121–431)	470 (200–402)	549 (274–1,098)	941 (666–1489)
Half-life, h	8.9 ± 4.4	8.5 ± 6.4	13.8 ± 1.4	13.0 ± 5.8	5.9 (2.8–14)	11.6 ± 8.2	13 (7.2–29)	17 (3.8–33)	11 (5.3–21)	7.1 (5.4–11)
SN-38-G										
C_{max}, ng/ml	118.3 ± 42.1	65.3 ± 25.7	–	–	–	86.9 ± 45.2	273 (125–432)	324 (96–1306)	488 (187–745)	562 (278–1022)
$AUC_{0-\infty}$, ng·h/ml	1243 ± 687	440 ± 197	–	–	–	1189 ± 1079	2670 (1079–3578)	2840 (1079–7384)	6248 (3010–11 360)	6248 (3010–11 360)
Half-life, h	8.1 ± 3.7	10.0 ± 2.9	–	–	–	10.5 ± 4.9	13 (5.7–29)	12 (4.8–26)	13 (5.6–23)	5.6 (2.8–5.3)
APC										
C_{max}, ng/ml	627.8 ± 327.8	–	–	–	–	–	617 (185–1234)	1666 (494–6170)	1481 (494–2591)	6787 (1172–11 106)
$AUC_{0-\infty}$, ng·h/ml	5407 ± 2598	–	–	–	–	–	6787 (2776–14 191)	16 042 (4319–51 211)	22 212 (6787–48 126)	66 636 (11 723–103 039)
Half-life, h	5.8 ± 0.9	–	–	–	–	–	7.6 (5.0–11)	6.7 (3.9–12)	8.2 (4.7–16)	4.9 (3.4–5.9)
NPC										
C_{max}, ng/ml	57.4 ± 26.5	–	–	–	–	–	–	–	–	–
$AUC_{0-\infty}$, ng·h/ml	553 ± 263	–	–	–	–	–	–	–	–	–
Half-life, h	5.7 ± 2.6	–	–	–	–	–	–	–	–	–

Results are expressed as mean ± standard deviations (range).

APC, 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin; NPC, 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38-G, 7-ethyl-10-[3,4,5-trihydroxy-pyran-2-carboxylic acid]-camptothecin (the β-glucuronide conjugate of SN-38).

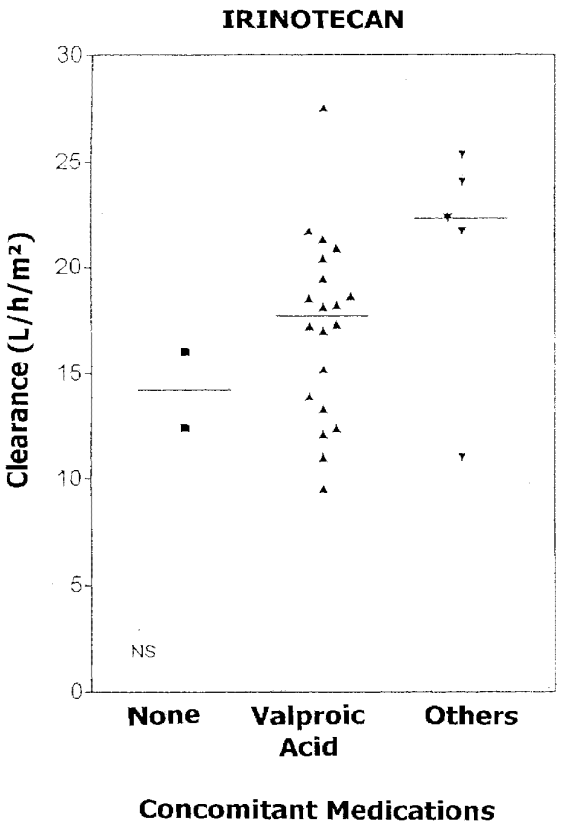

Figure 3. Clearances of irinotecan in patients with glioblastoma. Three groups of patients were defined to assess possible drug interactions: (group 1, squares) patients without anticonvulsant (two patients), (group 2, triangles) valproic acid (20 patients) and others (group 3, inverted triangles) including carbamazepine or phenobarbital (five patients).

patients analysed in the pharmacokinetic study received phenytoin. Clearances of irinotecan were 12.4 and 14.2 l/h/m^2 in two patients who received no anti-epileptic drugs, 20.9 ± 5.7 l/h/m^2 (range 11–25.3 l/h/m^2) under phenobarbital/carbamazepine and 17.2 ± 4.4 l/h/m^2 (range 9.5–27.5 l/h/m^2) under valproic acid (P >0.05). Pharmacokinetic parameters of SN-38 (as well as other metabolites) were not significantly different in patients receiving no anticonvulsants ($AUC_{SN\text{-}38}$: 131.9 ± 35 ng·h/ml) as compared with those receiving valproic acid ($AUC_{SN\text{-}38}$: 115.3 ± 136 ng·h/ml, P >0.05). Metabolic ratios of metabolites with regards to concomitant anticonvulsants are shown in Figure 4.

Discussion

In human glioma xenografts in nude mice, irinotecan displayed marked antitumour activity [12–14], which then translated into promising antitumour activity in patients with malignant gliomas [26–28]. However, clinical trials in patients with gliomas also revealed that the toxicity profile and the pharmacokinetic parameters of irinotecan were strongly modified by concomitant medications such as anticonvulsants. Seizure prevention using carbamazepine, phenytoin and phenobarbital were associated with lower exposure to SN-38; this subsequently led to the resumption of phase I/II clinical trials using increasing doses of irinotecan [26–28].

In our study, irinotecan (350 mg/m^2 every 3 weeks) displayed a good safety profile which allowed the planned dose intensity to be maintained with limited occurrences of dose reduction and treatment delays. Grade 3–4 toxicity consisted of neutropenia (19.6% per patient) and delayed diarrhoea (7.9% per patient). This toxicity profile was consistent with toxicities reported in phase III studies including patients with colorectal cancer with the same schedule (neutropenia ranged from 14% to 22% and delayed diarrhoea in 22%) [29–31]. Similarly, cholinergic syndrome and asthenia were reported in 10% and 9.8% of patients and were comparable with that previously reported in patients with colorectal cancer [30, 31]. Grade 3–4 vomiting was observed in only 3.9% of patients with gliomas and was lower than that in colon cancer patients (14%). This might be related to the antiemetic properties of chronic administration of corticosteroids combined with the effects of the antiemetic regimens used in this study. Two patients experienced severe infections of digestive origin that resulted in toxic death. In these patients, chronic administration of a high dose of corticosteroids could have led to immunosuppression.

In our study, immediate post-operative imaging was not mandatory for patients in group A. However, for patients with complete or partial resection, contrast enhanced tumour masses of >2 cm were required at study entry if these patients were to be considered measurable and therefore eligible for this study. It was considered sufficient to exclude most of the changes in post-surgical radiological enhancement, as these could sometimes be confounded with radiological responses. However, the overall response rate was lower than that reported for anticancer drugs currently used in the treatment of glioblastoma. In addition, our results seem to be lower than those reported by Friedman et al. [26], who showed eight objective responses among 48 patients with glioblastoma using a weekly administration of irinotecan. Poor prognosis factors in our patient population (large tumour sizes, multiple lesions, age, performance status and number of complete or partial resections) might have influenced the result [35–37]. For instance, the median age of patients in our study was about 10 years older than in most of the recent chemotherapy trials (median age 53 versus 44 [34], 46 [33] or 46 [26] in other clinical trials). More recently, Cloughesy et al. [38] reported the results of a phase II study where irinotecan was given at a dose of 300 mg/m^2 as a 90-min infusion every 3 weeks, which was then escalated to 350 mg/m^2 in the absence of severe haematological toxicity. In that study, two patients (14%) presented a partial response and two additional patients had tumour stabilisation with a median time-to-progression of 6 weeks. These data appeared to be comparable with those reported in our study. In our study, the median time-to-progression was 9 weeks (range 3.6–53.1; 95% CI 8.1–22.4) in group A and 14.4 weeks (range 5.5–36.8; 95% CI 9.0–21.1) in group B, and the 6-month progression-free survival rate was 26% in group A and 43% in

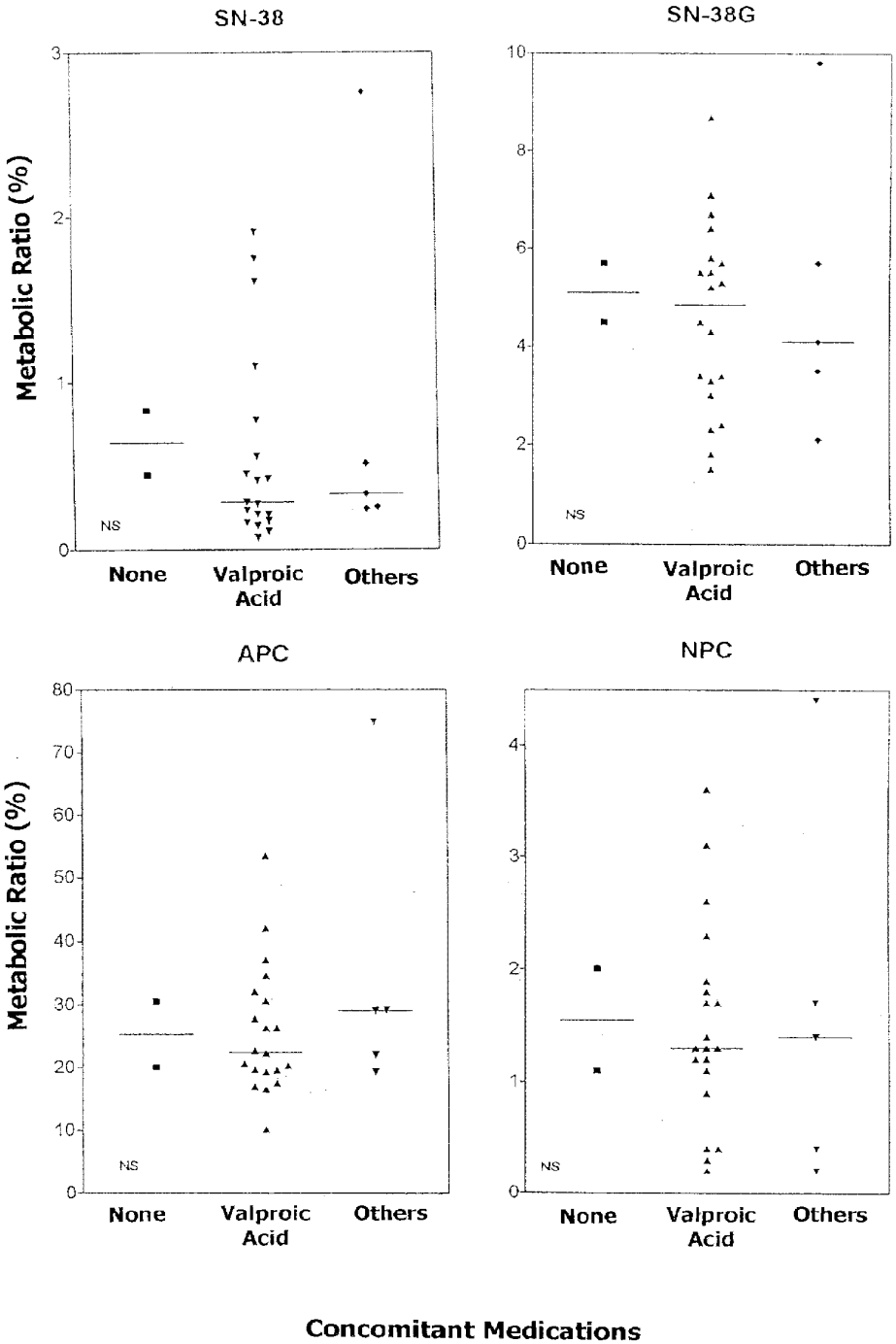

Figure 4. Metabolic ratios of irinotecan metabolites in patients with glioblastoma. Metabolite ratios of SN-38, SN-38-G, APC and NPC were calculated as follows: $[AUC_{0-\infty}$ (metabolite)$/AUC_{0-\infty}$ (irinotecan)$] \times 100$ for SN-38, SN-38-G and NPC. $[AUC_{0\text{–last sampling}}$ (APC)$/AUC_{0\text{–last sampling}}$ (irinotecan)$] \times 100$ for APC. Individual (plots) and means ratios (horizontal lines) are presented in patients taking no anticonvulsant (squares), valproic acid (triangles) and other drugs (inverted triangles) for seizure prevention (carbamazepine and phenobarbital).

group B. In this study, the 6-month control rate appears to be similar to that of nitrosourea [32] and temozolomide [33, 34].

The use of experimental agents prior to radiotherapy in newly diagnosed glioblastoma multiforme has become increasingly popular in recent years. This methodology of early screening for new compounds was used recently to optimise the chance of detecting activity that would otherwise be undetectable given the limited survival of patients relapsing after radiotherapy. How-

ever, one might also consider that participation in such a study could have negative effects on the outcome of patients by delaying radiation therapy. Grossman et al. [39] recently reported the survival of 368 patients with newly diagnosed gliohlastoma treated with investigational new drugs either prior to or following radiotherapy. In their study, no significant difference in survival was detected between the two groups. However, the authors stressed that careful monitoring of tumour progression in patients treated with new agents prior to radiotherapy is mandatory to avoid delaying salvage radiotherapy. In our study, we observed slightly higher progression-free and overall survival rates in group B patients, which could either be due to the effectiveness of radiotherapy or to selection bias that excludes patients with rapidly progressing tumours from receiving radiotherapy. In the absence of postoperative randomisation, it was impossible to compare groups A and B, although both had very similar survival outcomes.

Medications commonly used by patients with malignant gliomas (corticosteroids, phenytoin, carbamazepine and phenobarbital) affect the CYP3A4 enzyme system [26, 40–45], cause an increase in the clearance of paclitaxel [40] and irinotecan, and require the use of larger doses of chemotherapy [42–45]. In our study, the clearance of irinotecan was increased in four of five patients exposed to phenobarbital or carbamazepine, corroborating previous studies [46].

Interestingly, valproic acid is also registered for seizure prevention and does not interact strongly with the CYP3A4 enzyme. Valproic acid is conjugated in humans, reversibly inhibits hepatic UGT1A1 conjugation, both by competitive and noncompetitive mechanisms [46, 47], and interacts with drugs requiring glucuronidation [48]. In addition, valproic acid and its metabolites exert choleretic effects in animal models [49]. In Wistar rats, concomitant administration of valproic acid 200 mg/kg with irinotecan 20 mg/kg inhibited SN-38-G formation and increased $AUC_{SN\text{-}38}$ by 270% as compared with control rats receiving irinotecan alone [19]. Therefore, valproic acid was expected to result in increased SN-38 exposure and intestinal toxicity in humans. However, our study shows that valproic acid did not increase the rates of diarrhoea and neutropenia as compared with previous studies in patients treated without anticonvulsants [30, 31]. In previously published pharmacokinetic data from phase I/II studies in patients without brain tumours [50–53] (Table 4), we found that in patients without gliomas the mean clearances of irinotecan ranged from 11.0 to 21.1 l/h/m^2 (Table 4). In our study, the clearance of patients receiving valproic acid (17.2 l/h/m^2, range 9.5–27.5) appears to be similar to that observed in patients treated without anticonvulsants (14.2 l/h/m^2, range 12.4–16). In our study, the exposure to SN-38 (471.6 ± 224.3 ng·h/ml) was comparable with that previously reported by Abigerges et al. [50] and Rivory et al. [54] in phase I trials using similar doses of irinotecan. This is in contrast with the study by Friedman et al. [26] where the clearance of irinotecan in patients receiving phenytoin, carbamazepine and/or phenobarbital was 30.4 ± 8.3 l/h/m^2 and was correlated with low exposures to SN-38 and SN-38-G.

Based on our data, the glucuronidation of SN-38 did not seem to be completely inhibited in patients treated with valproic acid. Discrepancies between species might explain the absence of drug interactions between valproic acid and irinotecan in humans. In addition, we have recently shown that concomitant medications with valproic acid generate new metabolites by oxidation of the camptothecin backbone or the piperidinylpiperidine lateral chain of irinotecan [25]. Other factors that might help to explain the absence of inhibition of UGT1A1 in humans might be related to the schedule of dosage of valproic acid. In humans, valproic acid was given as a chronic daily oral administration starting several days before irinotecan, while in animal experiments a single i.v. valproic acid infusion was given immediately before the administration of irinotecan. Finally, chronic oral corticosteroids might help to counteract the effects of valproic acid by inducing UGT1A1 enzymes.

In summary, irinotecan (350 mg/m^2 every 3 weeks) has an effect that results in a median time-to-progression of 9 weeks in group A and 14.4 weeks in group B, with 6-month progression-free survival rates of 26% and 43% in groups A and B, respectively. The toxicity profile of irinotecan requires careful clinical follow-up in patients with glioblastoma. We showed that valproic acid does not increase the exposure to SN-38 and the toxicity of irinotecan in patients with glioblastoma. Although the response rate of irinotecan as a single agent was limited, it remains an attractive drug for use in combination with nitrosourea, temozolomide and radiotherapy in patients with glioblastoma.

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Original article

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A phase I/II and pharmacokinetic study of irinotecan in combination with capecitabine as first-line therapy for advanced colorectal cancer

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Purpose: The aim of this study was to determine in patients with previously untreated advanced colorectal cancer the maximum tolerated dose (MTD) and safety profile of irinotecan in combination with capecitabine, to identify a recommended dose and to determine the response rate and time to disease progression. In addition, we aimed to explore the pharmacokinetic parameters of irinotecan and capecitabine when used in different sequences of administration, with irinotecan infusion either prior to or after the first intake of capecitabine.

Patients and methods: One hundred patients were included: 43 patients were recruited into an extended phase I trial of alternating escalation in dose of both drugs where irinotecan was administered intravenously (i.v) on day 1 after first intake of capecitabine taken from days 1–14 twice daily, with cycles repeated every 3 weeks. After the determination of recommended dose a further 57 patients were treated in a phase II evaluation with the reverse sequence of drugs on day 1. Pharmacokinetic analysis was performed in patients treated at the recommended dose in two cohorts of patients in which the sequence of the first administration of each drug was reversed.

Results: The MTD of the combination was determined as irinotecan 300 mg/m^2, with capecitabine 2000 mg/m^2/day. Dose limiting toxicities were neutropenia and diarrhoea. The recommended dose is irinotecan intravenous (i.v.) 250 mg/m^2 day 1 and capecitabine 2000 mg/m^2/day days 1–14, every 3 weeks. Treatment was well tolerated, with diarrhoea the most common serious toxicity. Response rate in the phase II cohort was 42% [95% confidence interval (CI) 29% to 56%]. Median duration of response was 7.7 months (95% CI 7.5–8.9). Median time to progression was 8.3 months (95% CI 5.8–10). No significant effect on irinotecan pharmacokinetics was observed whatever the intake of capecitabine before or after irinotecan infusion. An effect of irinotecan on capecitabine and some capecitabine metabolites was observed, but irinotecan did not effect 5-fluorouracil (5-FU) pharmacokinetics.

Conclusions: Irinotecan in combination with capecitabine is a well tolerated regimen with an activity comparable to, but more convenient than, irinotecan–5-FU i.v. combinations in patients with previously untreated advanced colorectal cancer. The pharmacokinetic data suggest that the sequence of administration does not impact significantly on the metabolism of the two drugs.

Key words: capecitabine, colorectal cancer, irinotecan, phase I, phase II pharmacokinetics

Introduction

The results of systemic therapy for advanced colorectal cancer have improved significantly with the availability of cytotoxic drugs such as irinotecan and oxaliplatin, and monoclonal antibodies against growth factors and their receptors [1]. Irinotecan is a topoisomerase I inhibitor that has activity

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in 5-fluorouracil (5-FU)-pretreated advanced colorectal cancer and proven survival advantage when used as second-line monotherapy in this setting [2, 3]. Irinotecan administered in combination with 5-FU results in significant superior survival when used as first-line therapy compared with 5-FU monotherapy. Two separate trials have established this superiority when used in combination with either a bolus 5-FU regimen [4] or in combination with infusional 5-FU [5]. Recently, orally active fluoropyrimidine analogues have been introduced into routine clinical practice. Capecitabine is an oral precursor that is preferentially converted into the active compound 5-FU in malignant tissue [6] in three steps, and was shown to have improved tolerability and response rate compared with bolus 5-FU, with comparable time to progression and survival, in metastatic colorectal cancer [7, 8]. Capecitabine has gained widespread acceptance as an alternative to intravenous (i.v.) 5-FU [1]. A logical progression from these two developments is to combine irinotecan and capecitabine with the aim of maintaining the efficacy of a combination regimen and utilising the convenience of oral fluoropyrimidine therapy. We have conducted a phase I/II study of irinotecan and capecitabine in patients with advanced colorectal cancer using a 21-day cycle comprising irinotecan on day 1 with capecitabine administered twice-daily days 1–14. A clinically significant pharmacokinetic interaction between 5-FU–folinic acid (FA) and irinotecan has been reported, demonstrating a 40% reduction in the area under the curve (AUC) for the active irinotecan metabolite SN-38 when 5-FU was commenced following irinotecan compared with the reverse sequence where irinotecan was administered after completion of 5-FU infusion [9]. This interaction was associated with differences in the maximum tolerated dose (MTD) of irinotecan. Therefore, in the present study the two sequences of i.v. irinotecan, before or after capecitabine intake, were studied with a pharmacokinetic analysis of the metabolic interaction of these drugs to determine the importance of the sequence of administration of these drugs.

Patients and methods

Patients

Between May 2000 and December 2003, patients were recruited from three centres to the phase I component and subsequently from nine centres to the phase II component. The main eligibility criteria were: histologically proven metastatic adenocarcinoma of the colon or rectum with measurable disease, no prior chemotherapy for metastatic disease, age ≥18 and ≤75 years, WHO performance status 0, 1 or 2, life expectancy >3 months, adequate haematological and biochemical parameters [haemaglobin ≥10 g/dl, neutrophils $\geq 2 \times 10^9$/l, platelets $\geq 100 \times 10^9$/l, bilirubin ≤1.25× institutional upper limit of normal range (ULN), aspartate aminotransferase and alanine aminotransferase ≤5 times ULN, serum creatinine ≤1.25 ULN or creatinine clearance >50 ml/min], and written informed consent. Main exclusion criteria were: pregnant or lactating women, inadequate contraception in women with childbearing potential, prior exposure to irinotecan and/or exposure to more than one thymidine synthetase inhibitor regimen, severe concomitant conditions, inflammatory bowel disease or malabsorption. The study was approved by appropriate ethics committees covering all sites.

Study design and treatment

This study comprised a phase I and a phase II component. The phase I study was conducted as an open label, non-randomised, dose-finding study using escalation of both study drugs. Five dose levels were tested with an irinotecan dose ranging from 250 to 300 mg/m^2 in combination with capecitabine ranging from 1500 to 2500 mg/m^2/day twice daily days 1–14. The primary objective in the phase I study was to determine the MTD of irinotecan and capecitabine and to determine a recommended dose for phase II study when irinotecan is administered i.v. on day 1 combined with a 14-day oral intake of capecitabine. A secondary objective was to define the safety profile of the combination at each dose level.

The primary objective of the phase II study was to determine the response rate after treatment at the recommended dose. Secondary objectives were to determine the duration of response and time to progression, and to further evaluate the safety profile of the recommended combination dosing and schedule.

For the phase I study capecitabine was administered orally twice daily on days 1–14 every 3 weeks. Dosing was ~12 h apart (9 a.m. and 9 p.m.) and taken within 30 min after a meal. Irinotecan was administered 2 h after the first dose of capecitabine as an i.v. infusion over 30 min on day 1 repeated every 21 days (sequence A). In the phase II study, the reverse sequence of administration was used (sequence B), where irinotecan was administered over 30 min followed 2 h after end of the irinotecan infusion by the first dose of oral capecitabine (Figure 1). 5-HT$_3$ antagonists were administered within conventional institutional antiemetic protocols in routine use in participating centres. The administration of atropine sulphate 0.25 mg subcutaneously was widely adopted as prophylaxis against irinotecan-induced acute cholinergic-like syndrome.

Dose escalation proceeded according to the dose schedule (see Table 2). Three patients were entered at each dose level and dose escalation was permitted if no dose-limiting toxicity (DLT) was encountered in the first cycle. Cohorts were expanded where DLT was experienced and patients replaced if unable to comply with cycle 1, unless because of DLT. MTD was defined as being reached if the first three patients experienced a DLT or if three or more patients in an expanded cohort experienced DLT.

DLT was defined as any of the following experienced during the first cycle: grade 4 neutropenia >7 days, neutropenic fever, grade 4 thrombocytopenia, diarrhoea grade 3 or grade 2 >8 days, vomiting grade

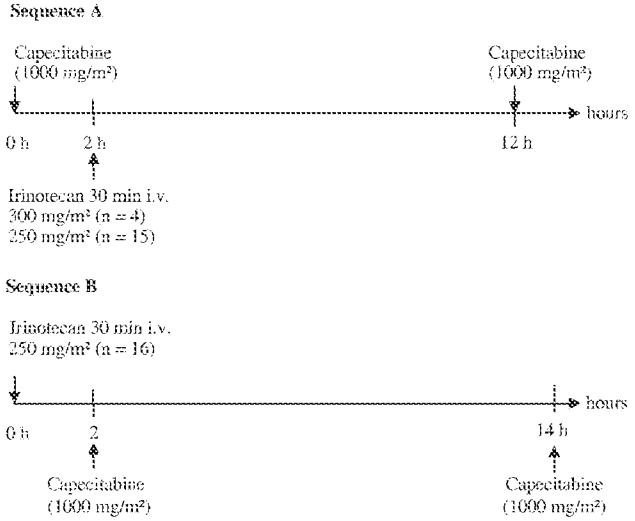

Figure 1. Sequence and timing of drug administration on day 1 of cycle 1. n indicates numbers of patients contributing to pharmacokinetic evaluation in each schedule and dose. i.v., intravenous.

4, hand–foot syndrome grade 3 for ≥2 days, any other grade ≥3 toxicity or inability to administer treatment on schedule. The recommended dose was the dose level below the MTD and a further 15 patients were to be treated at this dose level with pharmacokinetic evaluation. Within the phase II study a second cohort of 15 patients were treated with the reverse sequence (sequence B, Figure 1) with pharmacokinetic evaluation. This sequence was administered to all patients on the phase II trial.

Treatment was repeated every 21 days. Treatment was discontinued for disease progression, unacceptable toxicity, delay of >36 days in instituting the next cycle of treatment, or at investigators discretion or patient request. Patients were followed up every 3 months after completion of treatment to evaluate resolution of treatment-related toxicity and time to progression.

Toxicity was evaluated weekly during treatment. Protocol specified dose reductions and delays based on previous cycle toxicity utilising both haematological nadirs and on day of next treatment haematological/biochemical parameters. In the phase I study, the dose was reduced to the dose level below. In the phase II study, irinotecan dose was reduced by 20% and capecitabine dose by 25% depending on the degree of toxicity. Grade 3 and 4 non-haematological toxicities and grade 2 hand–foot syndrome were required to resolve to grade ≤1 prior to redosing with a reduction in one or both drugs depending on the specific toxicity.

The primary end point of the phase II part was to assess the partial plus complete response rate associated with irinotecan plus capecitabine in patients with measurable advanced colorectal cancer. The study used a single-stage Fleming design, with a response rate of ≤20% selected as not worthy of further study and a response rate ≥35% sufficiently promising for further evaluation. With type I error set at 5%, type II error set at 20% and a power of 80%, a sample size of 50 evaluable patients was required. Allowing for a 10% rate of non-evaluable patients, 56 patients were included in the phase II trial. Response was evaluated by tumour assessments every three cycles during treatment including an end of treatment assessment. WHO response criteria were used for evaluation of response in the phase I trial and RECIST criteria were used to determine response in the phase II study.

Pharmacokinetics

The pharmacokinetic component of the study was intended to determine the pharmacokinetic parameters of irinotecan and the active metabolite SN-38 and of capecitabine and relevant capecitabine metabolites 5′-deoxy-5-fluorocytidine (5′-DFCR), 5′-deoxyfluorouridine (5′-DFUR), 5-FU and α-fluoro-β-alanine (FBAL) during both sequences of administration during the extended phase I and phase II components of the study. Blood samples were drawn from an indwelling intravenous cannula and collected into heparin-coated tubes for irinotecan an into EDTA-containing tubes for capecitabine on day 1 of the first cycle as follows.

Sequence A (capecitabine preceding irinotecan). Blood samples were collected immediately prior to capecitabine intake and at 0.5, 1, 2.5, 3, 4, 5, 7 and 10 h (prior to the evening dose) after the first oral morning capecitabine dose administered.

Samples were also taken immediately prior to irinotecan infusion and at 15 and 25 min after the start of infusion, and at 15, 30, 90 min, 2.5, 4.5, 7.5, 20.5, 23 and 25.5 h after the end of the infusion of irinotecan.

Sequence B (irinotecan preceding capecitabine). Samples were taken immediately prior irinotecan infusion, at 15 and 25 min after the start of infusion and at 15, 30, 90 min and 2.5, 4, 6, 9, 23 and 28 h after the end of infusion of irinotecan. Samples were also taken immediately prior to intake of capecitabine, and at 0, 5, 1, 2, 3, 4, 5, 7 and 10 h after first oral morning dose administered. After sampling, all blood specimens were immediately put in an ice water bath (4 °C) until centrifugation at 2000 r.p.m. for 15 min at 4 °C. Then plasma samples were stored at −20 °C until analysis.

Plasma concentration of irinotecan and its metabolite SN-38 (total of lactone and carboxylate forms) were determined by validated high-performance liquid chromatography assay (HPLC) with fluorescence detection following solid phase extraction. The lower limit of quantitation was 10 μg/l for irinotecan and 2.5 μg/l SN-38 for a 50 μl sample volume.

The accuracy of the assay, defined as the present difference between the nominal and the mean measured concentrations of the quality controls, ranged from −1.4% to 4% for irinotecan and from −0.11% to 3.6% for SN-38 in plasma over the analysis period. The precision of the assay, established by the coefficient of variation of the quality controls, was lower than 5.5% for both compounds.

Concentrations of capecitabine and its metabolites 5′-DFCR, 5′-DFUR, 5-FU and FBAL were determined by HPLC with tandem mass-spectrometric detection. The lower limits of quantitation were 50 μg/l for 5′-DFUR, 11.3 μg/l for FBAL, 10 μg/l for capecitabine and 5′-DFCR, and 2 μg/l for 5-FU. The accuracy of the assay ranged from −8.3% to 4.4% for capecitabine, from −6% to 5.6% for 5′-DFCR, from −11% to 1.5% for 5′-DFUR, from −11% to 4.7% for 5-FU, and from −5.4% to 7.6% for FBAL over the analysis period. The precision of the assay was equal or lower than 4.6%, 7.9%, 8.6%, 12% and 22% for capecitabine, 5′-DFCR, 5′-DFUR, 5-FU and FBAL, respectively.

Pharmacokinetic analysis was performed by non compartmental analysis using WinNonlin software, version 3.3 (Pharsight Corp., Mountain View, CA, USA). The following parameters were determined for irinotecan, capecitabine and their metabolites: peak concentration (C_{max}), time to C_{max} (T_{max}), area under the curve from 0 to infinity (AUC) or from 0 to time (t) the last quantifiable concentration (AUC_{0-t}), half-life of the terminal phase ($t_{1/2}$), the metabolic ratio of AUC_{0-t}s of SN-38 to irinotecan and, finally, plasma clearance (CL) and volume of distribution at steady state (V_{ss}) for irinotecan only.

Statistical analysis was performed using SAS software (SAS version 8.02; SAS Institute Inc, Cary, NC, USA) with a significance level of $\alpha = 0.05$.

The effect of capecitabine on irinotecan pharmacokinetics was carried out on dose-normalised C_{max}, AUC_{0-t} or AUC of irinotecan and SN-38 and on metabolic ratio, after log-transformation using the Proc-Mixed procedure with group taken as fixed effect and with reverse sequence as reference. The 90% confidence intervals (CIs) were estimated.

The effect on irinotecan on the pharmacokinetics of capecitabine was carried out on C_{max}, AUC_{0-t} and AUC of capecitabine, 5′-DFCR, 5′-DFUR, 5-FU and FBAL, and on metabolite ratios after log-transformation using the Proc-Mixed procedure with group taken as fixed effect and with initial sequence as reference. The 90% CIs were estimated.

Results

A total of 100 patients were included. Of the 43 patients entered into the phase I study, 19 patients were treated at the recommended dose with irinotecan after capecitabine. Fifty-seven patients were treated within the phase II study. All patients were eligible and evaluable for toxicity.

Phase I study

Patient characteristics for the 43 patients evaluated in the phase I cohort are summarised in Table 1. DLTs were experienced at all dose levels and are summarised in Table 2. Dose escalation proceeded to dose level 5 (irinotecan 300 mg/m^2

Table 1. Patient characteristics

	Phase I [*n* (%)]	Phase II [*n* (%)]
Total	43 (100)	57 (100)
Median age, years (range)	62 (37–76)	62 (33–76)
Male	24 (56)	34 (60)
Female	19 (44)	23 (40)
WHO performance status[a]		
0	24 (56)	41 (72)
1	14 (33)	15 (26)
2	5 (12)	0 (0)
Primary tumor site		
Colon	13 (30)	31 (54)
Rectum	14 (33)	16 (28)
Colon rectosigmoid	16 (37)	10 (18)
Organs involved		
Median number (range)	1 (1–2)	2 (1–5)
Liver	36 (84)	41 (72)
Lung	12 (28)	21 (37)
Lymph node	6 (14)	21 (37)
Abdominal mass	0 (0)	3 (5)
Skin	0 (0)	1 (2)
Other soft tissue	0 (0)	9 (16)
Other	7 (16)	7 (12)

[a]One patient is unknown.

and capecitabine 2500 mg/m^2/day). At this dose the level grade 3 and 4 toxicity encountered in later cycles was regarded as unacceptable and cohort expansion at dose level 4 was considered appropriate. MTD was also encountered at dose level 4 (irinotecan 300 mg/m^2 and capecitabine 2000 mg/m^2/day), at which level five DLTs were experienced in the nine patients treated: neutropenia grade 4 (three patients) and diarrhoea grade 3 (one patient) and grade 4 (one patient). The recommended dose was therefore established at irinotecan 250 mg/m^2 and capecitabine 2000 mg/m^2/day. As specified per protocol, this dose level was further expanded to a total of 19 patients. This dose level was well tolerated with none of the 19 patients experiencing a haematological DLT. DLT at the recommended dose was experienced in four of 19 patients comprising one patient with grade 3–4 diarrhoea lasting 11 days resulting in grade 3 dehydration. In addition, two further patients developed grade 3 dehydration, and one patient developed grade 3 nausea and vomiting. One patient treated at dose level 1 died from complications arising from treatment-related infection without neutropenia. All treatment-related grade 3 and 4 toxicity experienced at each dose level is summarised in Tables 3 and 4 for haematological and non-haematological toxicities, respectively. At the recommended dose the most significant toxicity was diarrhoea, experienced as grade 3 or 4 in seven out of 19 patients, which was associated with grade 3 dehydration in three patients.

Efficacy in phase I cohort

Thirty-nine patients were evaluable for response. Responses were seen at levels 2, 3 and 4. Two complete responses and 11 partial responses were observed in 43 patients. Overall response rate was 30% (95% CI 17% to 46%). Overall response rate of the recommended dose level 3 was 32% (95% CI 13% to 57%). Median duration of response was 5.2 months (95% CI 4.4–17.5). Median time to progression was 7 months (95% CI 5.3–17.5).

Phase II study

Patient characteristics of the 57 patients treated within the phase II protocol are summarised in Table 1. All patients were eligible for safety and were treated with at least one cycle of chemotherapy at the recommended dose using administration sequence B (irinotecan preceding capecitabine). After evaluation of the first three cycles of the first 15 patients an acceptable safety profile was observed. Accrual was continued for additional 42 patients. A total of 314 cycles were administered

Table 2. Dose-escalation schedule and dose-limiting toxicities in phase I cohort during first cycle

Dose level	1	2	3	4	5	Total
Irinotecan (mg/m^2)/capecitabine (mg/m^2/day)	200/1500	250/1500	250/2000	300/2000	300/2500	
Patients (*n*)	3	6	19	9	6	43
Cycles (*n*)	18	45	112	68	23	266
DLTs [*n* (%)]						
Neutropenia grade 3–4	1 (33)			3 (33)	1 (17)	5 (12)
Diarrhoea grade 3–4		1 (17)	1 (5)	2 (22)		4 (9)
Nausea grade 3			1 (5)			1 (2)
Vomiting grade 3			1 (5)			1 (2)
Cardiac ischaemia grade 3		1 (17)				1 (2)
Dyspepsia grade 3					1 (17)	1 (2)
Transaminase grade 3		1 (17)				1 (2)
Dehydration grade 3			3 (15)			3 (7)

Table 3. Grade 3 and 4 haematological toxicity (worst grade per patient) in phase I cohort

Haematological toxicity	Total (n = 43)	Dose level 1: 200/1500[a] (n = 3)	Dose level 2: 250/1500 (n = 6)	Dose level 3: 250/2000 (n = 19)	Dose level 4: 300/2000 (n = 9)	Dose level 5: 300/2500 (n = 6)
Neutrophils [n (%)]						
Grade 3	3 (7)	0 (0)	0 (0)	2 (11)	0 (0)	1 (17)
Grade 4	8 (19)	1 (33)	0 (0)	2 (11)	3 (33)	2 (33)
Febrile neutropenia [n (%)]						
Grade 3	3 (7)	1 (33)	0 (0)	0 (0)	1 (11)	1 (17)
Grade 4	1 (2)	0 (0)	0 (0)	0 (0)	1 (11)	0 (0)
Platelets [n (%)]						
Grade 3	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)
Grade 4	2 (5)	0 (0)	0 (0)	1 (5)	1 (11)	0 (0)

[a]Irinotecan (mg/m^2)/capecitabine (mg/m^2/day).

(median six per patient; range 1–15). The administration of the combination was delayed in 37 cycles (12%) and the doses were reduced in five cycles (2%) for haematological toxicity and in 14 cycles (14%) for non-haematological toxicity. Treatment was generally well tolerated with a low incidence of grade 3 and 4 toxicity and no treatment-related deaths. Grade 3 neutropenia was reported as worst haematological toxicity in 12 patients and grade 4 neutropenia in five patients, occuring in 8% and 2% of cycles, respectively. There was only one episode of febrile neutropenia. Grade 4 diarrhoea was experienced by seven patients (12%), and grade 3 diarrhoea by four patients (7%), occurring in 2% and 1% of cycles, respectively. There was one episode of grade 3 dehydration. Four patients experienced grade 3 hand–foot syndrome and three patients experienced grade 3 vomiting. Thromboembolic complications were encountered in three patients. The most common all-grade toxicities per patient are summarised in Figure 2.

The relative dose-intensity for irinotecan was 99.96% (range 83% to 104%) and for capecitabine was 96.77% (17% to 111%).

Efficacy in phase II cohort

Of the 57 patients entered, 46 patients were evaluable for response. Eleven patients were not evaluable for response because they discontinued treatment before the first scheduled evaluation without definite proof of progressive disease. The reasons for withdrawal from the study as assessed by the local investigator were bowel obstruction (four patients), deterioration of performance status (three), nausea/vomiting (one), coronary vein spasms (one), cholinergic syndrome (one) and patient refusal (one).

Two complete responses and 22 partial responses were observed. Stable disease was recorded as best response in 15 patients. The overall response rate (intention-to-treat) was 42% (95% CI 29% to 56%). The median duration of response was 7.7 months (95% CI 7.5–8.9). The median time to progression was 8.3 months (95% CI 5.8–10).

Pharmacokinetics

Pharmacokinetic evaluation was performed in 17 out of 19 patients recruited to the pharmacokinetic protocol in sequence A (capecitabine preceding irinotecan) and in 15 out of 16 patients recruited in sequence B (irinotecan preceding capecitabine). The doses administered in these patients were 250 mg/m^2 irinotecan and 2000 mg/m^2/day capecitabine in both sequences, with the exception of four patients who received 300 mg/m^2 irinotecan and 2000 mg/m^2/day capecitabine (sequence A) (Figure 1).

The mean (± SD) irinotecan and SN-38 pharmacokinetic parameters are summarised in Table 5A and B, and the mean (± SD) capecitabine and its metabolites pharmacokinetic parameters are reported in Table 5C. Irinotecan was rapidly metabolised, with SN-38 peak plasma concentrations achieved within 0.83–1 h (median time after the start of infusion), in both sequences.

Interpatient variability of irinotecan pharmacokinetic parameters was generally higher in the initial sequence (A) than the reverse sequence (B) (e.g. 40% to 54% versus 27% for AUC), with the exception of C_{max}, for which it was in the same range (10% to 22%). For SN-38, the interpatient variability was high and similar in both sequences (48% to 124%).

The CL and V_{ss} were 14 l/h/m^2 and 80 l/m^2 when irinotecan was administered as a 30-min infusion at 250 mg/m^2 prior to capecitabine (sequence B), and 12.6 l/h/m^2 and 83 l/m^2, respectively, when capecitabine was administered 2 h before irinotecan infusion (sequence A). C_{max} and AUC_{0-t} of SN-38 were 62 μg/l and 396 μg·h/l, respectively, when irinotecan was administered prior capecitabine, and 48.2 μg/l and 269 μg·h/l, respectively, when capecitabine was administered 2 h before irinotecan infusion at 250 mg/m^2.

Statistical analysis showed no statistically significant differences in pharmacokinetic parameters for both irinotecan and SN-38 between the two groups, but mean pharmacokinetic parameters of SN-38 were lower in patients taking capecitabine first (sequence A), with large confidence intervals (Table 5D).

Table 4. Phase I grade 3 and 4 non haematological toxicity worst grade by patient and dose level

Adverse event	Total (n =43)	Dose level 1: 200/1500[a] (n =3)	Dose level 2: 250/1500 (n =6)	Dose level 3: 250/2000 (n =19)	Dose level 4: 300/2000 (n =9)	Dose level 5: 300/2500 (n =6)
Alopecia [n (%)]						
Grade 1	10 (23)	0 (0)	1 (17)	7 (37)	2 (22)	0 (0)
Grade 2	20 (47)	2 (67)	4 (67)	5 (26)	6 (67)	3 (15)
Anorexia [n (%)]						
Grade 1	17 (40)	1 (33)	4 (67)	8 (42)	4 (44)	0 (0)
Grade 2	6 (14)	1 (33)	0 (0)	2 (11)	1 (11)	2 (33)
Grade 3	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)
Grade 4	1 (2)	1 (33)	0 (0)	0 (0)	0 (0)	0 (0)
Dehydration [n (%)]						
Grade 3	3 (7)	0 (0)	0 (0)	3 (16)	0 (0)	0 (0)
Diarrhoea [n (%)]						
Grade 3	12 (28)	1 (33)	0 (0)	6 (32)	4 (44)	1 (17)
Grade 4	5 (12)	1 (33)	1 (17)	1 (5)	1 (11)	1 (17)
Dyspepsia [n (%)]						
Grade 3	2 (5)	0 (0)	0 (0)	1 (5)	0 (0)	1 (17)
Fatigue [n (%)]						
Grade 3	4 (9)	0 (0)	0 (0)	0 (0)	1 (11)	3 (50)
Grade 4	1 (2)	1 (33)	0 (0)	0 (0)	0 (0)	0 (0)
Febrile neutropenia [n (%)]						
Grade 3	3 (7)	1 (33)	0 (0)	0 (0)	1 (11)	1 (17)
Grade 4	1 (2)	0 (0)	0 (0)	0 (0)	1 (11)	0 (0)
Hand–foot syndrome [n (%)]						
Grade 3	3 (7)	0 (0)	0 (0)	1 (5)	0 (0)	2 (33)
Nausea [n (%)]						
Grade 3	4 (9)	0 (0)	0 (0)	1 (5)	1 (11)	2 (33)
Stomatitis [n (%)]						
Grade 3	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)
Vomiting [n (%)]						
Grade 3	3 (7)	0 (0)	0 (0)	1 (5)	2 (22)	0 (0)
Weight loss [n (%)]						
Grade 3	2 (5)	2 (67)	0 (0)	0 (0)	0 (0)	0 (0)

[a]Irinotecan (mg/m^2)/capecitabine (mg/m^2/day).

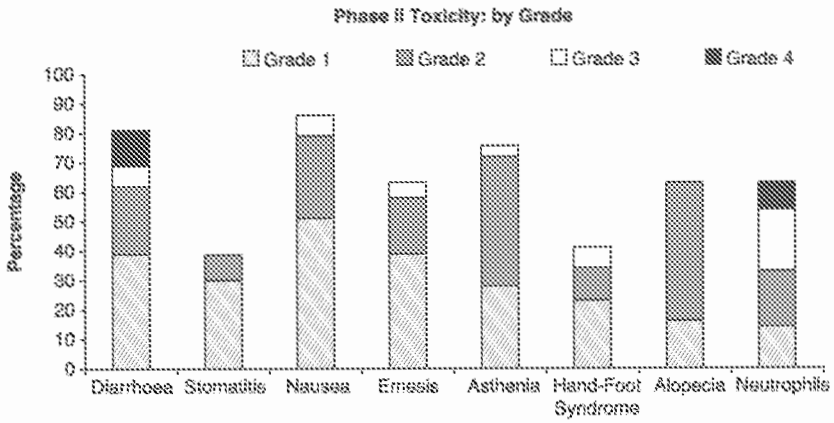

Figure 2. Toxicity in phase II cohort.

Table 5A. Pharmacokinetic results. Pharmacokinetic parameters of irinotecan on day 1 at cycle 1 [expressed as mean (SD)]

Dose, irinotecan (mg/m²)/capecitabine (mg/m²/day)	Sequence A						Sequence B					
	n	C_{max} (mg/l)	AUC (mg·h/l)	t_1λz (h)	CL (l/h/m²)	V_{ss} (l/m²)	*n*	C_{max} (mg/l)	AUC (mg·h/l)	t_2 (h)	CL (l/h/m²)	V_{ss} (l/m²)
250/1000	13	4672 (1023)	19 372 (7777)	6.88 (1.22)	12.6 (4.21)	83.0 (26.3)	15	4449 (951)	16 640 (4564)	6.71 (1.21)	14.0 (4.18)	80.0 (16.4)
300/1000	4	5465 (566)	21 708 (11 720)	6.99 (0.83)	14.0 (6.0)	85.9 (23.6)	–	–	–	–	–	–

AUC, t_2, CL and V_{ss} estimated in three patients (dose 300 mg/m², sequence A) and in 14 patients (sequence B).
C_{max}, peak concentration; AUC, area under the curve from 0 to infinity; t_2, half-life of the terminal phase; CL, plasma clearance; V_{ss}, volume of distribution at steady state.

Table 5B. Pharmacokinetic parameters of SN-38 on day 1 at cycle 1 [expressed as mean (SD)]

Dose (mg/m²) (irinotecan/ capecitabine)	Sequence A					Sequence B				
	n	T_{max}[a] (h)	C_{max} (mg/l)	AUC_{0-t} (mg·h/l)	Metabolic ratio	*n*	T_{max}[a] (h)	C_{max} (mg/l)	AUC_{0-t} (mg·h/l)	Metabolic ratio
250/1000	13	0.83 (0.45–2.00)	48.2 (26.5)	269 (128)	0.0152 (0.0065)	15	1.00 (0.42–4.50)	62.0 (37.4)	396 (247)	0.0234 (0.0132)
300/1000	4	0.85 (0.42–2.25)	77.6 (64.5)	479 (595)	0.0182 (0.0153)	–	–	–	–	–

[a]Median (range) for T_{max}.
AUC_{0-t} and metabolic ratio estimated in three patients (dose 300 mg/m², sequence A) and in 14 patients (sequence B).
T_{max}, time to C_{max}; C_{max}, peak concentration; AUC_{0-t}, area under the curve from 0 to time (t) the last quantifiable concentration.

Capecitabine pharmacokinetics were characterised by a rapid absorption after oral dosing, with maximum plasma concentrations reached at 0.5 h when capecitabine was given 2 h prior to irinotecan (sequence A) or 2 h after the infusion of irinotecan (sequence B).

Capecitabine was thereafter rapidly metabolised into 5′-DFCR, 5′-DFUR, 5-FU and FBAL, with peak levels occurring between 0.5 and 1 h post dosing for 5′-DFUR, 5′-DFCR and 5-FU, and at ~2 h for FBAL.

The main circulating compounds were capecitabine primary metabolite 5′-DFCR and FBAL (5-FU metabolite), in most of the patients in both groups.

A moderate to high interpatient variability was observed in capecitabine and its metabolites for C_{max}, and to a lesser extent for AUC, the coefficient of variation varying from 33% to 63% for C_{max} and from 13% to 45% for AUC of capecitabine, 5′-DFCR, 5′-DFUR and 5-FU. The interpatient variability of the exposures (C_{max} and AUC) of FBAL was lower (18% to 27%).

The statistical analysis showed a statistically significant difference between the two sequences for C_{max} and AUC of capecitabine, with an increased C_{max} of 64% and an increased AUC of 72% in patients receiving irinotecan first (sequence B). The AUC of 5′-DFCR was found to be significantly 17.2% lower in sequence B while C_{max} was also 18.5% lower but in a non-statistically significant manner.

No significant difference in pharmacokinetic parameters of 5′-DFUR, 5-FU and FBAL was detected between the two sequences (Table 5D).

The proportion of patients experiencing any grade 3 or 4 clinical toxicity over the first cycle of treatment in patients treated at the recommended dose was similar, with 21% with sequence A and 20% in sequence B.

Discussion

This study has established the safety profile of combining irinotecan and capecitabine as first-line treatment for advanced colorectal cancer when irinotecan is administered i.v. on day 1 in combination with capecitabine administered as a twice daily dose on days 1–14 every 3 weeks. The phase I component of this study has established the MTD of irinotecan as 300 mg/m² and capecitabine as 2000 mg/m²/day, with diarrhoea as the most common DLT. The recommended dose for the phase II study of irinotecan 250 mg/m² i.v. followed by capecitabine 2000 mg/m²/day was well tolerated, with diarrhoea the most frequently observed toxicity. Grade 3–4 diarrhoea occurred in 19% of patients. A concern with irinotecan used in single-agent studies at higher doses or in combination with bolus doses of 5-FU is the combination of severe diarrhoea and simultaneous myelosuppression. The diarrhoea, indicative of damage to the colonic mucosa, providing an entry portal for gut acquired systemic infection, can result in the potential lethal combination of uncontrolled sepsis and dehydration. In our study we encountered only moderate myelosuppression. Compared with larger studies with infusional 5-FU–leucovorin (LV) regimens in combination with irinotecan, the incidence of grade 3–4 neutropenia of 30% in our

Table 3C. Pharmacokinetic parameters of capecitabine and its metabolites on day 1 at cycle 1 (expressed as mean (SD)) in patients receiving 1000 mg/m^2 twice daily capecitabine with 250 or 300 mg/m^2 irinotecan

Compound	Sequence A (n = 17)					Sequence B (n = 14)				
	T_{max}[a] (h)	C_{max} (mg/l)	AUC_{0-t} (mg·h/l)	AUC (mg·h/l)	$T_{\frac{1}{2}}$ (h)	T_{max}[a] (h)	C_{max} (mg/l)	AUC_{0-t} (mg·h/l)	AUC (mg·h/l)	$T_{\frac{1}{2}}$ (h)
Capecitabine	0.63 (0.47–3.00)	6999 (3416)	7521 (3410)	7880 (3519)	0.660 (0.350)	0.50 (0.33–2.03)	11 330 (4953)	13 131 (4210)	13 138 (4208)	0.590 (0.207)
5′-DFCR	0.98 (0.50–3.00)	8615 (3056)	14 693 (3334)	14 936 (3391)	0.856 (0.143)	0.75 (0.33–3.00)	6959 (226)	12 446 (3236)	12 460 (3242)	0.801 (0.118)
5′-DFUR	0.98 (0.50–3.00)	8171 (4869)	11 902 (4437)	11 894 (4495)	0.832 (0.232)	1.00 (0.50–3.00)	7026 (3197)	11 385 (1469)	11 486 (1489)	0.713 (0.113)
5-FU	0.98 (0.50–3.00)	366 (200)	488 (186)	484 (186)	0.836 (0.230)	0.50 (0.33–2.03)	309 (195)	413 (118)	419 (115)	0.806 (0.137)
FBAL	2.27 (1.00–4.00)	4717 (839)	22 414 (5434)	23 764 (6903)	2.84 (0.23)	2.06 (1.00–4.00)	4339 (839)	19 543 (4779)	22 673 (4378)	2.53 (0.41)

[a]Median (range) for T_{max}.

Sequence A: $t_{\frac{1}{2}}$ and AUC estimated in 15 patients for capecitabine and FBAL and in 16 patients for 5′-DFUR, 5′-DFCR and 5-FU.

Sequence B: $t_{\frac{1}{2}}$, AUC and AUC_{0-t} estimated in 13 patients.

T_{max}, time to C_{max}; C_{max}, peak concentration; AUC, area under the curve from 0 to infinity; AUC_{0-t}, area under the curve from 0 to time (t) the last quantifiable concentration. 5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxyfluorouridine; 5-FU, 5-fluorouracil; FBAL, α-fluoro-β-alanine.

Table 3D. Statistical analysis of pharmacokinetic parameters

Compound	Parameters	P value	Estimate (90% CI)
Sequence A versus sequence B			
Irinotecan	C_{max}	0.5424	104.8 (92.1–119.2)
	AUC[a]	0.4067	111.0 (89.9–137.2)
	CL	0.3809	89.6 (72.6–110.5)
	V_{ss}	0.8487	101.8 (86.8–119.5)
SN-38	C_{max}	0.3206	82.5 (59.8–114.0)
	AUC_{0-t}	0.3524	77.4 (48.9–122.7)
Metabolic ratio	AUC_{0-t} SN-38/ irinitecan	0.1101	71.3 (50.3–101.1)
Sequence B versus sequence A			
Capecitabine	C_{max}	0.0068	164.1 (122.9–219.1)
	AUC[a]	0.0005	172.2 (136.2–217.9)
5′-DFCR	C_{max}	0.1027	81.5 (66.4–100.2)
	AUC[a]	0.0386	82.8 (71.5–96.0)
5′-DFUR	C_{max}	0.6400	91.7 (67.3–125.1)
	AUC[a]	0.8806	101.6 (85.4–120.8)
5-FU	C_{max}	0.3455	81.2 (56.2–117.4)
	AUC[a]	0.3761	89.3 (72.1–110.6)
FBAL	C_{max}	0.2283	91.7 (81.4–103.3)
	AUC[a]	0.2466	89.7 (76.8–104.9)

[a]Similar results were obtained for AUC_{0-t}.

CI, confidence interval; C_{max}, peak concentration; AUC, area under the curve from 0 to infinity; CL, plasma clearance; V_{ss}, volume of distribution at steady state; AUC_{0-t}, area under the curve from 0 to time (t) the last quantifiable concentration; 5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxyfluorouridine; 5-FU, 5-fluorouracil; FBAL, α-fluoro-β-alanine.

study is similar to the study of Tournigand et al. [10] (24%) and to the weekly schedule in the study by Douillard et al. [5] (29%), but somewhat less than the 2-weekly schedule (46%). Compared with the incidence of 19% grade 3–4 diarrhoea in our study these figures are 13% [10], 44% and 13% [5], respectively. In our study, cases of severe diarrhoea were accompanied by neutropenic sepsis in only one patient. This observation, and the absence of any treatment-related mortality in the phase II study, is reassuring, and is parallel to the experience of irinotecan in combination with infusional 5-FU–LV regimens [5, 10]. Patient education is essential in limiting the impact of toxicity, and clear instructions should be provided to patients on the management of diarrhoea and the importance of seeking specialist advice if marked diarrhoea occurs. The incidence of any grade of hand–foot syndrome was 41%, including 7% of grade 3, which is less than experienced with single-agent capecitabine at 2500 mg/m^2/day [7]. The combination of irinotecan and capecitabine is highly active, with a response rate in our phase II series of 42% (95% CI 29% to 56%). The response rate and median time to progression (8.3 months) are comparable to the results for combinations of 5-FU and irinotecan in randomised studies: Saltz et al. [4] (bolus 5-FU) reported 39% and 7 months,

Table 6. Studies with irinotecan plus capecitabine in advanced colorectal cancer

Reference	Type of study	Patients (*n*)	Irinotecan schedule	Capecitabine schedule	Length of cycle
Park et al. [14]	Phase II	39	240 mg/m^2 day 1	2000 mg/m^2/day days 1–14	3 weeks
Bajetta et al. [15]	Phase II	140	240 mg/m^2 day 1 or 120 mg/m^2 days 1+8	2000 mg/m^2/day days 2–15	3 weeks
Goel et al. [16][a]	Phase I	34	275 mg/m^2 day 1	2300 mg/day days 2–15	3 weeks
Tewes et al. [17]	Phase I	37	70 mg/m^2 weekly ×6	2000 mg/m^2/day days 1–14, 22–35	7 weeks
Borner et al. [18]	Phase II	75	70 mg/m^2 days 1, 8, 15, 22, 29 or 300/240 mg/m^2 days 1+22	2000 mg/m^2/day days 1–14, 22–35	6 weeks
This study	Phase I/II	100	250 mg/m^2 day 1	2000 mg/m^2/day days 1–14	3 weeks

[a]Patients with gastrointestinal cancer.
For phase I studies, the recommended dose is presented.

Douillard et al. [5] (infusional 5-FU) 41% and 6.7 months, and Tournigand et al. [10] (infusional 5-FU) 56% and 8.5 months, respectively. Obviously, a true comparison can only be made in a prospective randomised study.

The pharmacokinetics of irinotecan were similar whatever the sequence of administration used, and are consistent with published data obtained from phase I trials of irinotecan administered as a single agent [11, 12]. The administration of capecitabine 2 h before irinotecan did not modify the pharmacokinetics of irinotecan and its metabolite SN-38, suggesting the absence of a pharmacokinetic interaction of capecitabine on irinotecan with this schedule of administration.

However, irinotecan administration 2 h before administration of capecitabine increased the exposure of capecitabine by 72% and decreased the exposure of 5′-DFCR by 17%. The exposure of 5′-DFUR, 5-FU and FBAL appeared to be not affected by the administration of irinotecan.

Of note, no major differences were observed in 5-FU AUC, whatever the administration order used, and the systemic exposures of 5-FU were consistent with published data when capecitabine is given alone [13]. The evaluation of pharmacokinetic interaction was of particular interest because of the complexity of the metabolism of both irinotecan and capecitabine. The principle issue of interest is that the carboxylesterases metabolise irinotecan into its active metabolite SN-38, and also capecitabine into 5′-DFCR. Consequently, a pharmacokinetic drug–drug interaction is theoretically possible.

The pharmacokinetic evaluation performed in this study has not identified a significant effect of capecitabine on irinotecan pharmacokinetics, which contrasts with the observations with infusional 5-FU reported by Falcone et al. [9]. These authors reported a reduced clearance of irinotecan when administered in combination with 5-FU and an associated clinical impact with increased toxicity when 5-FU was administered first. Capecitabine and its immediate metabolites appear not to produce the same interaction, and the systemic levels of 5-FU encountered with clinically effective doses of capecitabine appear to have no significant effect on irinotecan metabolism. Unless very dramatic or long lasting, effects of irinotecan on capecitabine pharmacokinetics would not be expected to give rise to clinically observable effects, since capecitabine administration covers a 14-day period. The effect of irinotecan on capecitabine and capecitabine metabolites is, however, complex; capecitabine levels were higher when preceded by irinotecan but this was not reflected in lower concentrations of downstream metabolites, in particular blood 5′-DFUR and 5-FU and FBAL levels were not affected. The lower conversion of capecitabine into 5′-DFCR could be explained by a competition for irinotecan conversion into SN-38 by carboxylesterases.

An interaction of either drug on intracellular drug or metabolite concentrations of the other cannot be excluded, but any such effect is unlikely to be clinically relevant since we were unable to detect any major differences in toxicity profile in relation to the order of drug administration. We can conclude from this study that the timing and order of drug administration on day 1 of this regimen is not as critical as has been demonstrated for irinotecan in combination with 5-FU.

Several studies on the combination of irinotecan and capecitabine have been reported (Table 6). Differences in schedule concern the administration of irinotecan (weekly, weekly ×2 followed by 1 week rest, or 3-weekly) and the starting day of capecitabine (day 1 or 2). Our results are similar to the study by Park et al. [14], who administered irinotecan at 240 mg/m^2 and capecitabine at 2000 mg/m^2. This study reported a response rate of 44% with a time to progression of 6.7 months. The toxicity profile was similar to that seen in our study. Bajetta et al. [15] initially used in their 3-weekly irinotecan schedule a dose of irinotecan 300 mg/m^2 and capecitabine 2500 mg/m^2/day, but since this was associated with unacceptable toxicity doses were reduced to irinotecan 240 mg/m^2 and capecitabine 2000 mg/m^2/day. This arm of their randomised study reported a response rate of 47% and progression-free survival of 8.3 months. Toxicity was more manageable when irinotecan was administered only at day 1 compared with day 1 and 8 of a 3-weekly schedule. Goel et al. [16] explored day 1 irinotecan dosing in combination with capecitabine days 2–15 on a 21-day cycle in a mixed population of patients with advanced gastrointestinal tumours. In their study using fixed dosing of capecitabine, the recommended dose was irinotecan 275 mg/m^2 with capecitabine 2300 mg/m^2/day. Diarrhoea and fatigue were the predominant DLTs. Tewes et al. [17] administered irinotecan as a weekly administration for 6 weeks. Diarrhoea, neutropenia and asthenia were the DLTs, and the response rate was

38%. In a recent randomised phase II study by Borner et al. [18], a 3-weekly schedule of irinotecan had a comparable response rate compared to a weekly schedule in combination with capecitabine (35% and 34%, respectively), but the 3-weekly schedule seemed advantageous in terms of toxicity (except for alopecia) and survival, although the study was not designed for these parameters.

Taking these results together, no outright preference for any schedule in terms of efficacy or toxicity is obvious. However, our 3-weekly schedule with irinotecan on day 1 and capecitabine on days 1–14 does appear more convenient compared with other irinotecan/capecitabine schedules, and certainly when compared with 5-FU/FA infusional schedules, which involve ambulatory pumps, permanent vascular access devices and more frequent patient visits. Small but potentially clinically significant differences in efficacy may exist, and phase III studies are currently ongoing to more accurately compare the relative efficacy and toxicity of this regimen in comparison with irinotecan in combination with infusional 5-FU–FA. Our recommended dose of capecitabine and irinotecan is currently being tested in the CAIRO study of the Dutch Colorectal Cancer Group (DCCG), which investigates the sequential versus concomitant use of capecitabine, irinotecan and oxaliplatin. Accrual of 820 patients was completed in December 2004. This regimen is also currently under investigation in the adjuvant context in the recently opened UK Quasar II study. These and other studies will provide relevant data for the use of capecitabine–irinotecan as part of the standard treatment of colorectal cancer.

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Safety Science Article

HER2-targeted liposomal doxorubicin displays enhanced anti-tumorigenic effects without associated cardiotoxicity

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ABSTRACT

Anthracycline-based regimens are a mainstay of early breast cancer therapy, however their use is limited by cardiac toxicity. The potential for cardiotoxicity is a major consideration in the design and development of combinatorial therapies incorporating anthracyclines and agents that target the HER2-mediated signaling pathway, such as trastuzumab. In this regard, HER2-targeted liposomal doxorubicin was developed to provide clinical benefit by both reducing the cardiotoxicity observed with anthracyclines and enhancing the therapeutic potential of HER2-based therapies that are currently available for HER2-overexpressing cancers. While documenting the enhanced therapeutic potential of HER2-targeted liposomal doxorubicin can be done with existing models, there has been no validated human cardiac cell-based assay system to rigorously assess the cardiotoxicity of anthracyclines. To understand if HER2-targeting of liposomal doxorubicin is possible with a favorable cardiac safety profile, we applied a human stem cell-derived cardiomyocyte platform to evaluate the doxorubicin exposure of human cardiac cells to HER2-targeted liposomal doxorubicin. To the best of our knowledge, this is the first known application of a stem cell-derived system for evaluating preclinical cardiotoxicity of an investigational agent. We demonstrate that HER2-targeted liposomal doxorubicin has little or no uptake into human cardiomyocytes, does not inhibit HER2-mediated signaling, results in little or no evidence of cardiomyocyte cell death or dysfunction, and retains the low penetration into heart tissue of liposomal doxorubicin. Taken together, this data ultimately led to the clinical decision to advance this drug to Phase I clinical testing, which is now ongoing as a single agent in HER2-expressing cancers.

Introduction

Anthracyclines have served as the backbone of breast cancer therapy for decades. Despite consistent clinical benefit observed with anthracycline-based regimens in breast cancer, significant toxicities that include acute and/or chronic cardiac dysfunction have limited more expansive therapeutic use, specifically with HER2-suppressing agents. Doxorubicin-induced cardiac damage is irreversible, resulting in acute injury and also damage that can manifest itself years after treatment (Lipshultz et al., 2008). Exposure to cumulative concentrations of doxorubicin above 550 mg/m^2 increases the potential for cardiomyopathy and heart failure (Lefrak et al., 1973; Von Hoff et al., 1979). The development of HER2-directed therapy for the treatment of HER2-positive breast cancer has led to the investigation of the clinical benefit of the combination of doxorubicin (D) and trastuzumab (H) (Slamon et al., 2001). The clinical efficacy of D + H was superior to that of paclitaxel plus trastuzumab, however, there was an increased incidence of cardiac toxicity observed on the D + H arm of the study, and the combination was not approved by the FDA. The proposed mechanism of action responsible for the cardiotoxicity associated with this combination is the simultaneous induction of stress by doxorubicin being exacerbated by trastuzumab-mediated inhibition of HER2 signaling pathways necessary to respond to this stress (Chien, 2006; Feldman et al., 2000; Gianni et al., 2007). The clinical benefit of anthracycline-based therapy, specifically in HER2-positive breast cancer, remains controversial.

Liposomal encapsulation of drugs has enabled delivery of potent cytotoxic agents with an improved therapeutic index. Pegylated liposomal doxorubicin (PLD) alters the tissue distribution and pharmacokinetic profile of doxorubicin. PLD has demonstrated a significantly lower rate of left ventricular cardiac dysfunction and symptomatic congestive heart failure as compared to therapy with conventional doxorubicin, alone and in combination with trastuzumab in anthracycline-naive and previously treated patients (Chia et al., 2006; O'Brien et al., 2004). A proposed mechanism for the reduced cardiotoxicity of PLD is that its greater size relative to conventional doxorubicin prevents it from

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crossing the endothelial barrier in the heart, thereby minimizing doxorubicin exposure to heart tissue.

HER2-targeted liposomal doxorubicin is a HER2-targeted, pegylated liposome designed to deliver doxorubicin directly to HER2-overexpressing cancers. HER2-targeted PLD deposits in tumors through the enhanced permeability and retention effect similar to PLD (Drummond et al., 1999; Kirpotin et al., 2006; Peer et al., 2007). HER2-targeted liposomal doxorubicin contains 45 copies of mammalian-derived F5-scFv (anti-HER2) per liposome, whereas previous studies used a formulation containing bacterially-derived F5-scFv with only 30 copies per liposome. The F5-scFv was selected for its ability to internalize while not affecting HER2 signaling (Neve et al., 2001). Once in the tumor microenvironment, we and others have demonstrated that targeting HER2-overexpressing cells with HER2-targeted PLD results in superior efficacy relative to PLD in preclinical models (Nielsen et al., 2002; Park et al., 2002).

While HER2-targeted liposomal doxorubicin can potentially improve upon the clinical efficacy demonstrated by conventional anthracyclines and PLD for HER2-positive cancers, there are two primary safety considerations that need to be addressed. First, human cardiomyocytes have been reported to express low levels of HER2 (Fuchs et al., 2003), which therefore have the potential to mediate uptake of HER2-targeted liposomal doxorubicin. Therefore, it is important to demonstrate that HER2-targeted liposomal doxorubicin is not effectively taken up by human cardiomyocytes via low-level expression of HER2. Second, HER2-directed therapies such as trastuzumab cause cardiotoxicity (Ewer et al., 2005). This toxicity is believed to be mediated through the blockade of heregulin-mediated signaling via the HER2-HER4 dimer (Gianni et al., 2007). Therefore, it will be important to show that HER2-targeted liposomal doxorubicin does not cause any appreciable inhibition in HER2-signaling that could lead to direct cardiotoxicity or exacerbate doxorubicin-mediated cardiotoxicity.

Materials and methods

Materials. Doxorubicin was from SIGMA-ALDRICH, Inc. (St. Louis, MO). FITC-conjugated lectin was purchased from Vector Laboratories, Inc. (Burlingame, CA). Acetic acid, Methanol, and Acetonitrile were from EMD Chemicals Inc. (Gibbstown, NJ). Water and Trifluoroacetic Acid (TFA) were from J. T. Baker (Phillsburg, NJ). Hoechst 33342 trihydrochloride trihydrate, ProLong Gold, and $DiIC_{18}(5)$-DS (DiI5) were from Invitrogen (Carlsbad, CA). Cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (PEG-DSPE) were from Avanti Polar Lipids Inc. Hydrogenated soy phosphatidylcholine (HSPC) was from Lipoid (Newark, NJ). RPMI was from Lonza (Walkersville, MD). Fetal Bovine Serum (FBS) was from Tissue Culture Biologicals and penicillin G/streptomycin sulfate mixture was from GIBCO (Invitrogen).

Preparation of immunoliposomes. Liposomes were prepared and loaded with doxorubicin using an ammonium sulfate gradient as previously described (Kirpotin et al., 2006; Park et al., 2002). The lipid components were HSPC, Cholesterol, and PEG-DSPE (3:2:0.3, mol:mol:mol). The anti-ErbB2 (F5)-PEG-DSPE conjugate was prepared and inserted into the liposome to form immunoliposomes as reported by Nellis et al., (Nellis et al., 2005a; Nellis et al., 2005b). The DiI-5-labeled liposomes, HER2-targeted liposomal doxorubicin-DiI5 and PLD-DiI5, were prepared as above with the difference that the $DiIC_{18}(5)$-DS (DiI5) dye was solubilized with the lipid components at a concentration of 0.3 mol% of total phospholipid. Importantly, in all cases unloaded free doxorubicin was removed using a Sephadex G-75 size exclusion column eluted with Hepes buffered saline (pH 6.5). F5-lipo-DiI5 was prepared in a similar fashion as above but without doxorubicin, and incorporating an aqueous solution of Hepes buffered saline (pH 6.5).

Cell culture. BT474-M3 cells were cultured in RPMI medium containing 10% FBS and 1% penicillin G/streptomycin sulfate in 5.0% CO_2 at 37 °C. Embryonic stem cell-derived (ESCd) cardiomyocytes were obtained from the Zandstra lab at the University of Toronto. These cells have been shown to express appropriate cellular markers of cardiomyocytes such as LIM domain homeobox gene Isl-1, Troponin T, and Myosin Light Chain 2c and were cultured as previously described (Bauwens et al., 2011; Yang et al., 2008). The percentage of Troponin T positive cells was determined following differentiation. Batches containing less than 70% positive for Troponin T were discarded. Induced pluripotent stem cell-derived (iPSd) cells were obtained from Cell Dynamics International and were handled and cultured per the manufacturer's protocol.

Animal studies. 5–7-week-old female nude mice were purchased from Taconic. In accordance with the PHS Policy and the Guide for the Care and Use of Laboratory Animals, all resident colony animals received acceptable standards in their care, use and treatment. The care and treatment of experimental animals were in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Mice were inoculated with BT474-M3 breast cancer cells (15×10^6 in 100 μl RPMI) into the mammary fat pad (m.f.p) of the mice. When the tumors reached an average volume of ~200 mm^3, studies were performed as described below.

Efficacy study. Based on the average tumor volume, mice were randomized into three treatment groups (n = 7/group) that received PBS (control), HER2-targeted liposomal doxorubicin or PLD, dosed at 3 mg/kg (q7d, n = 3 total doses). Tumors were measured twice/week with a caliper. Tumor volumes were calculated using the formula: $width^2\times length\times0.52$. Mice were weighed twice/week to monitor weight loss.

Biodistribution of liposomes. Mice were randomized into 4 groups that received a single i.v. dose of PBS, doxorubicin, HER2-targeted liposomal doxorubicin-DiI5 or PLD-DiI5 (all at 3 mg/kg), respectively. Mice (n = 4/time point/group) were sacrificed at 0.5, 4, 24 and 96 h (doxorubicin) or 168 h (HER2-targeted liposomal doxorubicin-DiI5 and PLD-DiI5) after the single dose. Five minutes before sacrificing, mice were injected i.v. with 100 μl of FITC-lectin, to label the vasculature.

HPLC quantification of doxorubicin. Heart tissues were weighed and disaggregated with 1 mL H_20 using a TissueLyser (Qiagen) for 3 min. Afterwards 100 μl of the homogenate were transferred into a new tube and 900 μl of 1% acetic acid/methanol was added. For the cultured cells, cells were treated with drug, as described, trypsinized and lysed using 1.0% acetic acid in methanol. Lysates were vortexed for 10 s and placed at −80 °C for 1 h. Samples were spun at RT for 10 min at 10,000 RPM. Supernatants and doxorubicin standards were analyzed by HPLC (Dionex) using a C18 reverse phase column (Synergi Polar-RP 80A 250×4.60 mm 4 μm column). Doxorubicin was eluted running a gradient from 30% acetonitrile; 70% 0.1% trifluoroacetic acid (TFA)/H_2O to 55% acetonitrile; 45% 0.1% TFA/H2O during a 7 min span at a flow rate of 1.0 ml/min. The doxorubicin peak was detected at 6.5 min using an in-line fluorescence detector excited at 485 nm, and emitting at 590 nm. The extraction efficiency of doxorubicin from the heart tissue was 83% as determined by a control heart spiked with a known amount of doxorubicin.

Confocal microscopy and image analysis of heart snap-frozen sections. 10 μm-thick heart sections were air-dried for 30 min at RT, counterstained with Hoechst diluted 1:10,000 in mounting media (ProLong Gold) and mounted. Slides were imaged on a LSM 510 Zeiss confocal microscope equipped with Enterprise (351, 364 nm), Argon (458,

477, 488, 514 nm), HeNe1 (543 nm) and HeNe2 (594 nm) lasers with a Plan-Neofluar 40×/1.3 oil DIC objective.

Image analysis and quantification of nuclear doxorubicin was carried out using *Definiens Developer XD* (Definiens, Parsippany, NJ). Nuclei were segmented in the Hoechst channel. Doxorubicin positive nuclei were segmented in the doxorubicin channel. The percentage of doxorubicin positive nuclei was quantified as a ratio of the number of objects in the doxorubicin channel divided by the total nuclei objects in the Hoechst channel. The rule set for the analysis is available upon request.

Receptor quantification. Stem cell-derived cardiomyocytes were trypsinized, washed and stained using a fluorescently-conjugated anti-HER2 antibody from BD Biosciences (Franklin Lakes, NJ). HER2 receptor numbers were determined by assessing the antigen binding capacity (ABC) of the same fluorescently-labeled HER2 antibody from BD Biosciences through quantitative fluorescence activated cell sorting (qFACS). ABC determination was determined using Simply Cellular Quantum Beads from Bangs Labs (Fishers, IN) per the manufacturer's instructions.

Viability and apoptotic response. ESCd cardiomyocytes were treated for 3 h at the indicated concentrations of HER2-targeted liposomal doxorubicin, PLD and doxorubicin. Cells were washed twice with PBS, fresh media was added and the cells were incubated for an additional 24 h. Cell viability was assessed using CellTiter-Glo from Promega (Madison, WI) and the percent of viable cells was determined relative to the untreated population. Effects on the DNA damage marker gamma-H2AX, the cell stress proteins phospho-p53 and phospho-HSP27, and the apoptosis protein cPARP were assessed by High-Content analysis as described below.

Effects on HER2 downstream signaling. iPSd cardiomyocytes were pretreated for 24 h with either 1.0 μg/ml trastuzumab, 1.0 μM lapatinib or a HER2-targeted liposomal doxorubicin molecule not containing doxorubicin (F5-lipo) at an equivalent concentration to 5.0 μg/ml of HER2-targeted liposomal doxorubicin. Effects on phospho AKT and phospho ERK were quantified by High-Content Analysis described in detail below following a 10 min stimulation with 10 and 5 nM of heregulin, respectively,.

High-content analysis. Cardiomyocytes were treated as described. Cells were fixed using 3.7% formaldehyde, washed twice with PBS containing 0.1% Tween-20 (PBS-T), and permeabilized with methanol. Cells were blocked using 1:1 mixture of Licor Odyssey Blocking Buffer (Lincoln, NE) and PBS-T for 1 h at room temperature (RT). Cells were stained with a 1:400 dilution of the indicated primary antibody from Cell Signaling Technology (Beverly, MA) and incubated shaking at 4 °C overnight. Cells were washed and incubated with a 1:2000 dilution of the fluorescently labeled secondary antibody for 1 h at RT. Cells were stained with a 1:10,000 dilution of Hoechst 33342 and 1:1000 dilution of Whole Cell Dye from Pierce (Rockford, IL) for 30 min at RT to allow visualization of DNA and the whole cell, respectively. Plates were scanned using the Applied Precision Instruments ArrayWorx High Content Scanner (Issaquah, WA) with a 10× objective for Hoechst 33342/whole cell stain (460 nm), doxorubicin (595 nm), and APC/DiI5 (657 nm). Images were analyzed using the software *ImageRail* (Millard et al., 2011). An intensity threshold was established for nuclear and whole cell signals. This threshold was then applied to all images and used to segment individual cells. Data is presented as the mean pixel intensity for all cells in a given well for the indicated channel.

Heart exposure kinetic model. The mechanism-based pharmacokinetic model for PLD and conventional doxorubicin is identical in concept and structure to a similar tumor biodistribution model in Hendriks, et al. (Hendriks, submitted for publication). In order to adapt the model to represent biodistribution in the heart, parameters were changed to reflect heart physiology (rather than tumor), while the pharmacokinetic parameters are held fixed at the values determined in Hendriks et al. (cartooned in Fig. 6A) (Hendriks, submitted for publication). Parameters characterizing conventional doxorubicin and PLD transport were estimated using literature data, in a similar manner as described in Hendriks, et al. (Hendriks, submitted for publication) with one alteration: a correction factor had to be incorporated to account for incomplete flushing of drug from the heart capillaries (total heart drug = (drug in interstitial space) + (drug in cells) + (residual blood fraction * drug in capillary space)). All parameters are listed in Supplementary Table 1; all parameters not listed are identical to those in Hendriks, et al. (Hendriks, submitted for publication). Model parameters, values, descriptions, references, reactions and rules are available in *Supplementary Material*. Experimental heart deposition data for conventional doxorubicin or PLD was extracted from literature using a tool from http://code.google.com/p/figdigitizer/.

Results

HER2-targeted liposomal doxorubicin is effectively internalized into HER2 overexpressing tumor cells and significantly inhibits tumor growth in a xenograft model.

To demonstrare effective binding and internalization of HER2-targeted liposomal doxorubicin into HER2 over-expressing tumor cells, BT474-M3 cells (1.2×10^6 HER2/cell) were incubated with HER2-targeted liposomal doxorubicin, PLD or conventional doxorubicin at 15 μg/ml for up to 3 h (Fig. 1A). HER2-targeted liposomal doxorubicin is efficiently taken into tumor cells, as evidenced by total cell binding (Fig. 1A) and nuclear doxorubicin accumulation (Fig. 1B). By contrast, the untargeted analog, PLD, does not show any appreciable total binding (Fig. 1A) or nuclear accumulation (Fig. 1B). Conventional doxorubicin, used as a control, freely enters cells and accumulates in the nucleus.

The anti-tumor activity of HER2-targeted liposomal doxorubicin was evaluated in a breast cancer xenograft model. Mice were inoculated with BT474-M3 cells and when tumor volumes reached an average of 200 mm^3, treatment with PBS, HER2-targeted liposomal doxorubicin or PLD (both at 6 mg/kg) was started (q7d, n = 3 doses). Both HER2-targeted liposomal doxorubicin and PLD significantly inhibited tumor growth relative to control (t-test at day 55; $p<0.0001$). HER2-targeted liposomal doxorubicin resulted in a stronger inhibition of tumor growth relative to PLD (t-test at day 55; $p = 0.0310$) (Fig. 1C). At study termination, 3 complete regressions were observed with HER2-targeted liposomal doxorubicin and only 1 with PLD. To demonstrate that the improved efficacy was a direct result of HER2-targeting and not prolonged exposure, the pharmacokinetic profiles of HER2-targeted liposomal doxorubicin and PLD were measured and found to be similar (data not shown), indicating that the improved efficacy was a result of HER2-targeting, rather than prolonged exposure.

Human cardiomyocytes do not express sufficient HER2 levels to actively take up HER2-targeted liposomal doxorubicin

Characterization of the F5-scFv indicates that it does not cross react with mouse, rat or rabbit HER2 (data not shown). Considering the specificity of F5-scFv for human HER2, ESCd and iPSd human cardiomyocytes were obtained to study the effect of HER2-targeted liposomal doxorubicin on human cardiac cells in vitro. HER2 receptor levels on cardiomyocytes were determined by qFACS to be approximately 70,000 and 200,000 receptors per cell in human ESCd and iPSd cardiomyocytes, respectively (not shown). HER2 expression levels on normal and diseased human heart tissue were measured via quantitative immunohistochemistry and shown to be ≤100,000 receptors/cell, indicating that the ESCd and IPSd cardiomyocytes were representative of real human cardiac tissue (data not shown).

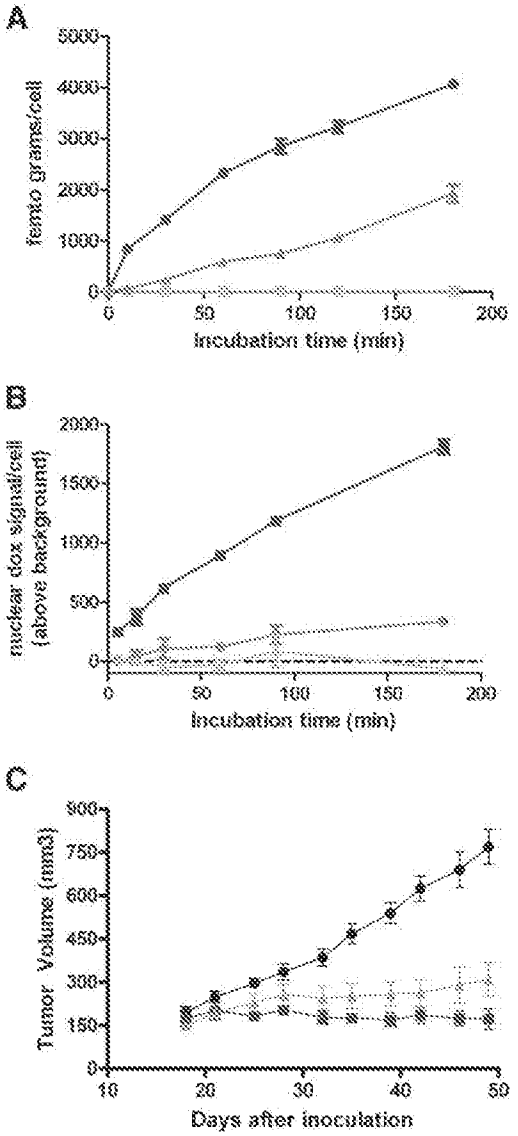

Fig. 1. (A) HER2-overexpressing BT474-M3 cells were treated with 15 μg/ml of HER2-targeted liposomal doxorubicin (blue), PLD (green), and conventional doxorubicin (red) for the indicated times. Total cellular doxorubicin was quantified by HPLC. (B) Nuclear doxorubicin delivery was quantified by high content microscopy 24 h following the indicated incubation times. (C) The anti-tumor activity of HER2-targeted liposomal doxorubicin and PLD was compared in a BT474-M3 orthotopic breast cancer model. Both HER2-targeted liposomal doxorubicin and PLD significantly inhibited tumor growth compared to control (t-test at day 55: $p<0.0001$). HER2-targeted liposomal doxorubicin resulted in a stronger inhibition of tumor growth relative to PLD (t-test at day 55; $p=0.0310$).

To determine if the level of HER2 expression on cardiomyocytes is sufficient to induce uptake of HER2-targeted liposomal doxorubicin, total cellular doxorubicin was quantified by HPLC following treatment of ESCd (Fig. 2A) and iPSd (Fig. 2B) cardiomyocytes with HER2-targeted liposomal doxorubicin, PLD or free doxorubicin. Treatment of both ESCd and iPSd cardiomyocytes with conventional doxorubicin results in doxorubicin cellular accumulation. Treatment with PLD does not result in doxorubicin delivery to either cardiac cell type. In contrast with the observations on HER2-overexpressing cancer cells (see Fig. 1A), the HER2 expression level on cardiomyocytes is not sufficient to promote active uptake of HER2-targeted liposomal doxorubicin. Taken together, these results demonstrate that liposomal encapsulation of doxorubicin in HER2-targeted liposomal doxorubicin minimizes doxorubicin exposure to non-target cells such as cardiomyocytes.

HER2-targeted liposomal doxorubicin does not reduce human cardiomyocyte viability or induce apoptotic responses

Exposure to low levels of doxorubicin can be cytotoxic, and we sought to determine if treatment with HER2-targeted liposomal doxorubicin or PLD affected cardiomyocyte viability. ESCd cardiomyocytes were incubated for 3 h at the indicated concentration followed by washing and incubation in fresh media for 24 h. Treatment with doxorubicin resulted in a loss of viability at concentrations as low as 0.2 μg/ml (Fig. 2C). Conversely, treatment with HER2-targeted liposomal doxorubicin and PLD did not lead to reductions in viability at any concentration tested, including super-physiological concentrations up to 45 μg/ml. These results demonstrate that ESCd cardiomyocytes are sensitive to

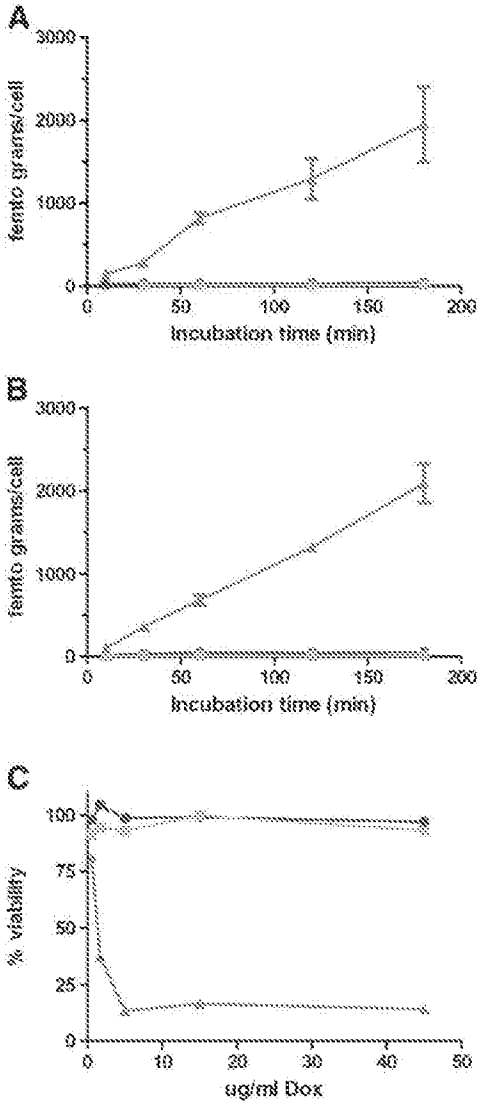

Fig. 2. Uptake of HER2-targeted liposomal doxorubicin (blue), PLD (green) and doxorubicin (red) was measured in human cardiomyocytes. ESCd (A) and iPSd (B) cardiomyocytes were treated with 15 μg/ml of HER2-targeted liposomal doxorubicin, PLD and conventional doxorubicin for the indicated times. Total cellular doxorubicin was quantified by HPLC. (C) ESCd cardiomyocytes were treated for 3 h with drug at the indicated concentrations and incubated for an additional 24 h with fresh media and cell viability was assessed.

doxorubicin, and that treatment with HER2-targeted liposomal doxorubicin and PLD does not provide sufficient doxorubicin exposure to affect cardiomyocyte viability.

Exposure of cells to low levels of doxorubicin may induce subtle cellular changes not revealed by cell viability measurements, including DNA damage, cell stress and apoptosis (Liu et al., 2008). Following treatment with HER2-targeted liposomal doxorubicin, PLD and free doxorubicin, cardiomyocytes were stained for proteins in each of these response pathways and imaged using high-content microscopy. Next, single-cell data was generated and analyzed as described in Materials and methods.

Treatment of cardiomyocytes with conventional doxorubicin resulted in a dose-dependent increase in the DNA damage marker nuclear gamma-H2AX (Fig. 3A). However, treatment with HER2-targeted liposomal doxorubicin and PLD did not increase nuclear gamma-H2AX signal at any concentration tested.

Cardiac cells exposed to conventional doxorubicin demonstrate a dose-dependent increase in phospho-p53 and phospho-HSP27 indicating an induction of cellular stress following treatment (Figs. 3B and C). However, an increase in phospho-HSP27 was not observed in cells treated with either HER2-targeted liposomal doxorubicin or PLD regardless of concentration. In most cases, there did not appear to be an effect on phospho-p53 in cells treated with HER2-targeted liposomal doxorubicin or PLD with the exception of a slight increase in phospho-p53 following treatment with 5.0 μg/ml of HER2-targeted liposomal doxorubicin.

Severe DNA damage and cell stress may promote the initiation of the apoptotic pathway including activation of a caspase cascade, ultimately resulting in the cleavage of the DNA repair protein PARP. Treatment with 5.0 μg/ml of conventional doxorubicin led to an increase in nuclear cPARP (Fig. 3D), correlating with the observed increase in cell death. However, treatment with HER2-targeted liposomal doxorubicin or PLD did not result in increased cPARP demonstrating that treatment under these conditions is not sufficient to induce apoptosis.

HER2-targeted liposomal doxorubicin does not inhibit signaling in human cardiomyocytes

To test the effects of HER2-targeted liposomal doxorubicin on ligand-induced HER2 signaling, iPSd-cardiomyocytes were stimulated with heregulin (HRG) and the effects on the downstream proteins AKT and ERK were quantified. iPSd-cardiomyocytes respond to heregulin stimulation by increasing phosphorylation of AKT (Fig. 4A) and ERK (Fig. 4B). Pretreatment with trastuzumab for 24 h results in a reduction in HRG-mediated phosphorylation of both proteins. Pretreatment with lapatinib (a small molecule HER2 tyrosine kinase inhibitor) led to a reduction in basal phosphorylation of AKT and ERK as well as a complete inhibition of HRG-induced phosphorylation of these proteins. Pretreatment with a HER2-targeted liposomal doxorubicin analog not containing doxorubicin (F5-lipo) does not inhibit HRG-induced phosphorylation of AKT or ERK. These results suggest that despite targeting HER2, HER2-targeted liposomal doxorubicin does not inhibit ligand-induced phospho-AKT and phospho-ERK signaling in cardiomyocytes thus leaving critical signaling pathways functional.

HER2-targeted liposomal doxorubicin has a lower accumulation in mouse heart tissue compared to conventional doxorubicin. Even though the F5-scFv does not recognize mouse HER2 (not shown), one can reasonably investigate whether HER2-targeted liposomal doxorubicin has altered biodistribution from that of PLD. We compared the biodistribution of HER2-targeted liposomal doxorubicin, PLD and doxorubicin in mouse heart tissue (all at 3 mg/kg). Mice (n = 4/time point/group) were injected i.v., hearts were collected at 0.5, 4, 24, and 96 h (for doxorubicin) or 168 h (for HER2-targeted liposomal doxorubicin and PLD) post injection and was doxorubicin quantified by HPLC (Fig. 5A). Injection of conventional doxorubicin resulted in a high peak exposure at 0.5 h (10% of i.d./g tissue) compared to the two liposomal formulations. The clearance of doxorubicin from the heart tissue after conventional doxorubicin injection was faster compared to HER2-targeted liposomal doxorubicin and PLD and at 24 h the amount of detected doxorubicin

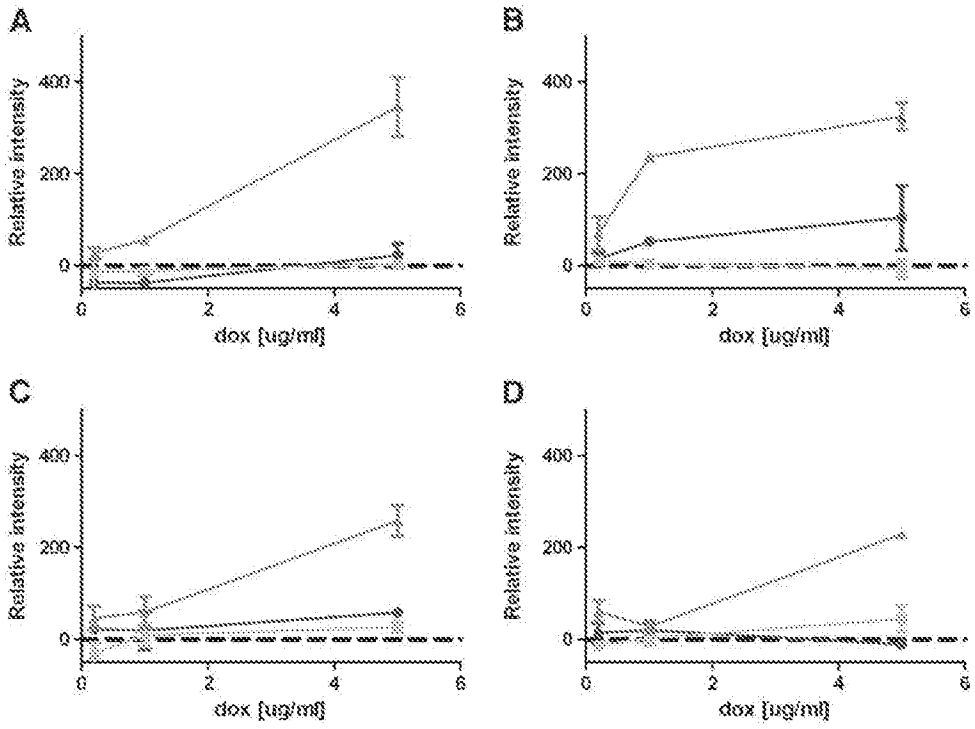

Fig. 3. ESCd cardiomyocytes were treated for 3 h with HER2-targeted liposomal doxorubicin (blue), PLD (green), and conventional doxorubicin (red) at the indicated concentrations and then incubated for an additional 24 h with fresh media. Cells were stained and imaged using high-content microscopy. Single cell intensity for each stain was quantified and represented as the mean relative intensity of individual cells. Cells were stained for the DNA damage marker gamma-H2AX (A). In addition, cells were stained for the cell stress proteins phospho-p53 (B) and phospho-HSP27 (C), and the apoptosis protein cPARP (D).

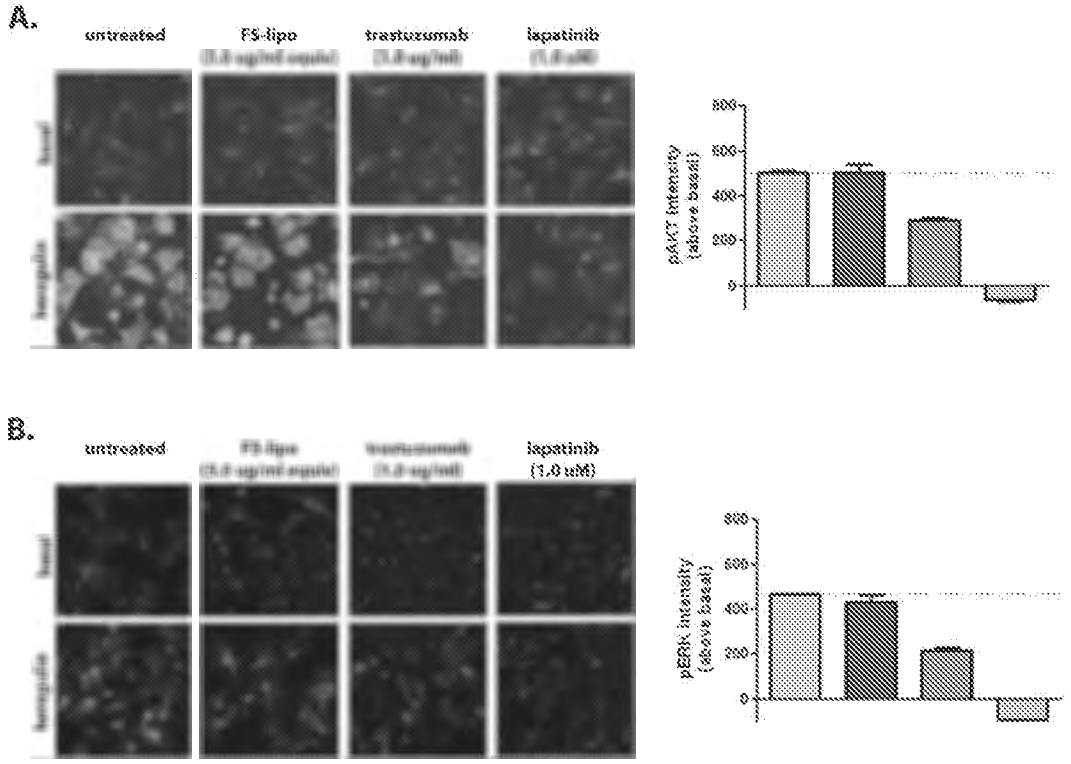

Fig. 4. To address if pretreatment with HER2-targeted liposomal doxorubicin affects HER2-mediated signaling, iPSd cardiomyocytes were exposed to components of HER2-targeted liposomal doxorubicin and the levels of phospho-AKT (pAKT) and phospho-ERK (pERK) were assessed. Cells were pretreated for 24 h with either 1.0 μg/ml trastuzumab, 1.0 μM lapatinib or a HER2-targeted liposomal doxorubicin molecule not containing doxorubicin (FS-lipo) at an equivalent concentration to 5.0 μg/ml of HER2-targeted liposomal doxorubicin. Cells were stained and imaged using high-content microscopy for (A) pAKT (green) and (B) pERK (red) following a 10 min stimulation with 10 and 5 nM of heregulin, respectively. Images were analyzed and individual cells were segmented using Hoechst 33342 (blue). Single cell signal intensity for each stain was quantified following heregulin treatment and represented as the mean relative intensity over basal of individual cells. Heregulin-stimulated only (gray), FS-lipo (blue), trastuzumab (red) and lapatinib (green).

(0.77% of i.d./g tissue) was close to background. Both HER2-targeted liposomal doxorubicin and PLD had a sustained accumulation profile that peaked at 24 h (2.8% and 2.6% for HER2-targeted liposomal doxorubicin and PLD, respectively) while values returned to background at 168 h. These results are in a similar range of previously reported data on the heart biodistribution of other PLD formulations (Kirpotin et al., 2006; Lu et al., 2004) (see also data in Figs. 6B and C). No significant differences were observed between the heart biodistribution of HER2-targeted liposomal doxorubicin and PLD.

HER2-targeted liposomal doxorubicin results in lower nuclear doxorubicin accumulation in mouse tissue compared to conventional doxorubicin. The microdistribution of doxorubicin (naturally fluorescent) and liposomes (DiI5-labeled) was analyzed on cryosections generated from heart tissues of mice injected with either conventional doxorubicin, HER2-targeted liposomal doxorubicin-DiI5 or PLD-DiI5 (all at 3 mg/kg) at 0.5, 4 and 24 h post injection. In order to visualize the heart vasculature, mice were injected i.v. with FITC-lectin 5 min before sacrificing. Heart slices were imaged by fluorescence confocal microscopy. Representative fields for the different treatment groups at the three time points analyzed (0.5, 4 and 24 h) are shown in Fig. 5B. Untreated hearts were also imaged and a representative image is shown in Fig. 5B (left panels). Co-localization of doxorubicin with the nuclear signal is indicated in purple in the bottom panels of Fig. 5B. Higher magnification images of the nuclear doxorubicin signal are shown in Fig. 5C, for both doxorubicin and HER2-targeted liposomal doxorubicin at the 0.5 h time point. It is apparent that, while with HER2-targeted liposomal doxorubicin no doxorubicin signal is visible in the nuclei, with free doxorubicin, the majority of the nuclei appear doxorubicin-positive (indicated in purple). The images were analyzed as described in Materials and methods and the percent of doxorubicin positive nuclei was quantified (Fig. 5D). Injection of conventional doxorubicin resulted in a prominent nuclear accumulation of doxorubicin at 0.5 h, with about 50% of the nuclei positive for doxorubicin. By 4 h, however, only 23% of the nuclei were positive for doxorubicin and the signal returned to basal levels at 24 h.

In the case of the liposomal formulations, little to no signal was detected for the majority of fields of view. Occasional signal in the DiI5 channel (liposome) was detected. In these cases, the liposome signal predominantly co-localized with the FITC-lectin signal, indicating liposomes that had not extravasated into the heart tissue but still remained in the vascular compartment (not shown). Upon HER2-targeted liposomal doxorubicin-DiI5 or PLD-DiI5 treatment, doxorubicin was not detected in the nucleus in the majority of the heart fields analyzed, independent of time point. In one of the four HER2-targeted liposomal doxorubicin-DiI5 hearts collected at 0.5 h and in one of the four HER2-targeted liposomal doxorubicin-DiI5 hearts collected at 4 h, a liposomal signal was detected in the extravascular space and doxorubicin was found in a small percentage of the nuclei. Similarly, in one of four PLD hearts collected at 0.5 h and in two of four PLD heart collected at 24 h, images revealed the extravascular liposomal signal and presence of nuclear doxorubicin. These fields are not presented as representative images, however their values were considered for the quantification shown in Fig. 5B. The area under the curves of both HER2-targeted liposomal doxorubicin and PLD was statistically significantly lower than the doxorubicin AUC ($p<0.001$). No significant differences were observed between the AUCs of HER2-targeted liposomal doxorubicin and PLD.

In order to get a broader visualization of the distribution of doxorubicin and of the liposomes in the heart tissue, full heart section scans were taken. The full section heart scans visually confirmed the results of the

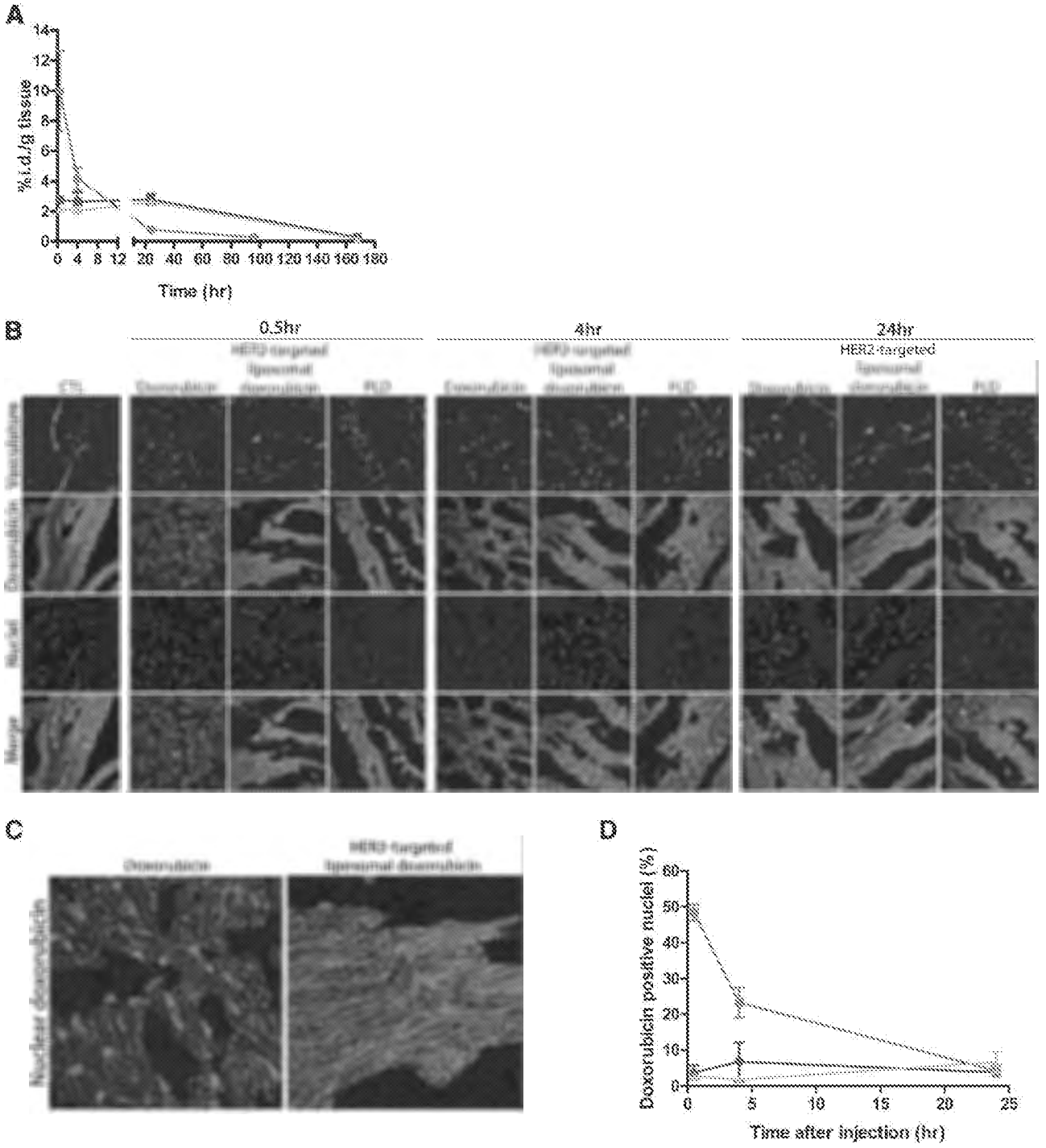

Fig. 5. (A) The biodistribution of HER2-targeted liposomal doxorubicin, PLD and doxorubicin was studied. Mice (n = 4/time point/group) were given a single dose (3 mg/kg) of either doxorubicin, HER2-targeted liposomal doxorubicin or PLD. Mice were sacrificed at 0.5, 4 and 24 h post injection and the doxorubicin accumulation in heart tissue was quantified by HPLC. (B) HER2-targeted liposomal doxorubicin induces lower nuclear doxorubicin accumulation in heart tissue compared to conventional doxorubicin, and comparable to PLD. Nu/nu mice were injected intravenously with HER2-targeted liposomal doxorubicin-Dil5, PLD-Dil5, and conventional doxorubicin at 3 mg/kg (dox equiv.). At the designated time points, hearts were collected for the preparation of cryosections to analyze the microdistribution of liposomes and doxorubicin. FITC-lectin was injected to visualize functional/perfused blood vessels. Heart sections were counterstained with Hoechst and imaged by confocal fluorescence microscopy. Doxorubicin positive nuclei are shown in purple. Doxorubicin positive nuclei are visible in conventional doxorubicin treated samples at 0.5 h and 4 h and not in HER2-targeted liposomal doxorubicin treated samples. (C) A higher magnification (2×) of the overlay of the nuclei and doxorubicin of the above fields of for the 0.5 h time points for doxorubicin and HER2-targeted liposomal doxorubicin is shown. (D) The percent of doxorubicin positive nuclei was quantified using *Definiens Developer XD*.

confocal microscopy, showing a broad doxorubicin distribution with nuclear localization upon conventional doxorubicin injection, and only rare liposome and doxorubicin signals in the hearts of mice injected with either HER2-targeted liposomal doxorubicin or PLD (not shown).

Both treatment with HER2-targeted liposomal doxorubicin and PLD showed significantly lower nuclear doxorubicin accumulation compared to treatment with conventional doxorubicin. In addition, HER2-targeted liposomal doxorubicin did not show any significant

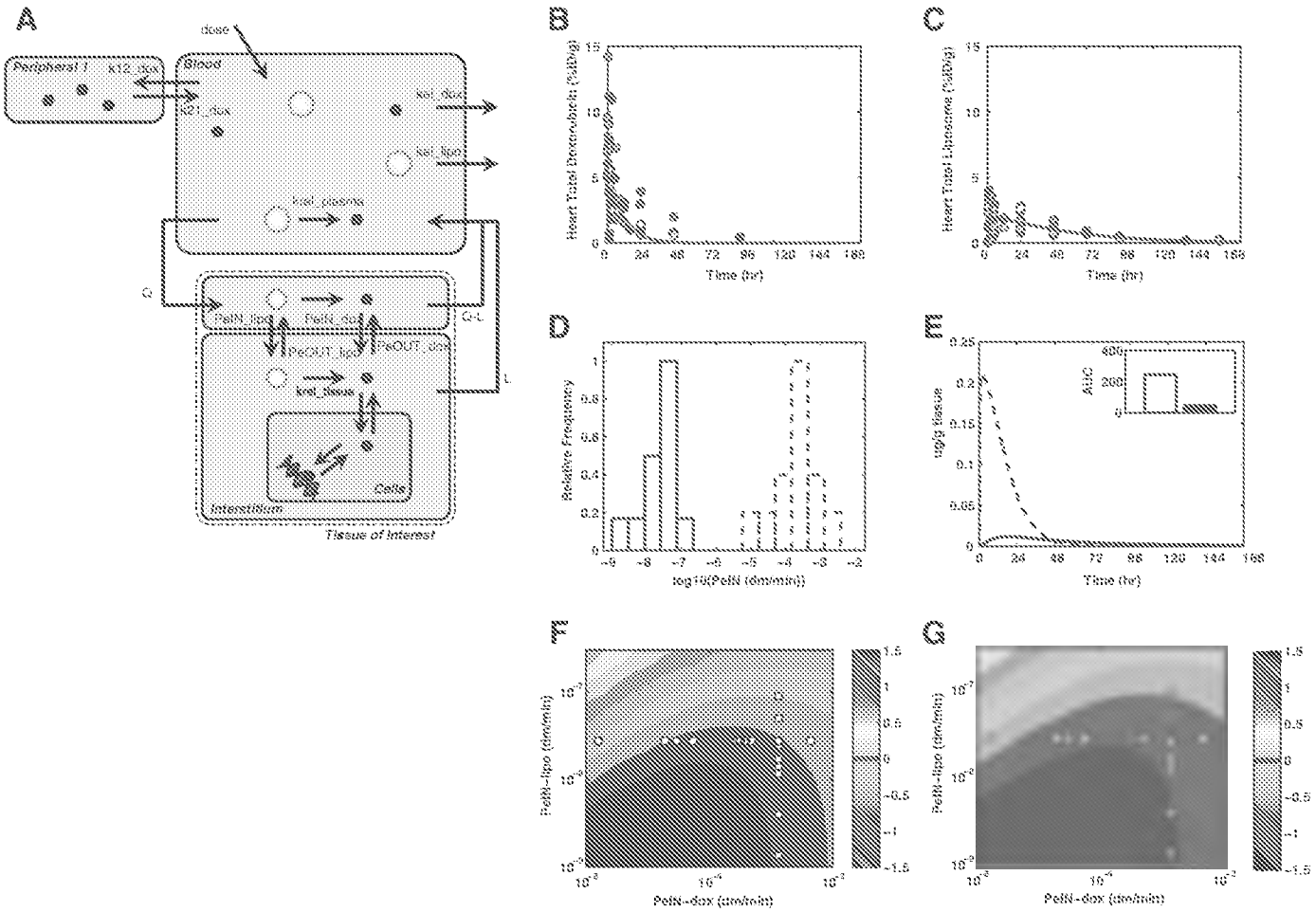

Fig. 6. (A), a schematic diagram of the PBPK model of heart exposure to doxorubicin is shown. The model consists of a PK model for conventional doxorubicin, a PK model for liposomal doxorubicin, and a physiologically-based heart tissue compartment. The PK models are linked via a common central compartment and the rate of release of doxorubicin from the liposome. Transport into the heart, which consists of capillary, interstitial and cellular space, occurs via plasma flow rate into the tissue and then mass transport of conventional doxorubicin and PLD into the interstitial space. Conventional doxorubicin is transported into and out of cells via the mechanism described in Eytan and Kuchel (1999) and binds to nuclear DNA with the heart cells. Kinetic reactions, parameter names, values and references are available in *Supplementary Material* and in Hendriks (submitted for publication). (B), conventional doxorubicin heart deposition data (circles) was compiled from mouse biodistribution studies in the literature. The model was fit to each data set to estimate rates of doxorubicin transport and the mean model fit to data is shown with the solid line. (C), PLD heart deposition data (circles) was compiled from mouse biodistribution studies for liposomes with similar size and surface characteristics to PLD. The model was to each data set to estimate rates of liposome transport into and out of heart tissue. The mean model fit is shown with the solid line. (D), a histogram of estimated values for individual model fits for conventional doxorubicin (dashed line) or PLD (solid line) transport into heart tissue is shown. (E), the model was used to simulate the time course of nuclear bound doxorubicin in the heart cardiomyocyte in response to 3 mg/kg of conventional doxorubicin (dashed line) or PLD (solid line). (F), the effect of variability in conventional doxorubicin and PLD transport into the heart was systematically examined by running mouse simulations across a range of transport values. The relative exposure (AUC) of cardiomyocyte DNA to doxorubicin via either 3 mg/kg PLD vs. conventional doxorubicin was determined over 1 week. Contours represent $\log_{10}$(PLD AUC/conventional doxorubicin AUC). Individual estimated values for conventional doxorubicin and PLD transport are plotted in open circles and squares, respectively, outlining a physiologically-relevant parameter space. (G), the model was scaled to reflect human physiology, as described in Materials and methods, and a similar analysis performed as in (F). The bound doxorubicin AUC over 3 weeks following 40 mg/m^2 of either conventional doxorubicin vs. PLD was calculated and is shown in the contours as $\log_{10}$(PLD AUC/conventional doxorubicin AUC). Since HER2-targeting does not significantly affect tissue deposition and cardiomyocytes do not express sufficient HER2 to effectively take up HER2-targeted liposomal doxorubicin, the systemic behavior of PLD should yield insight into the behavior of HER2-targeted liposomal doxorubicin.

differences compared to PLD in terms of nuclear doxorubicin accumulation in the heart.

Kinetic modeling of heart exposure

There are multiple competing kinetics that influence the extent of heart exposure to doxorubicin when delivered in liposomal form: liposome extravasation and clearance, as well as doxorubicin release and clearance. To better understand these processes, we constructed a mechanism-based pharmacokinetic model of drug exposure to the heart. This model enabled study of how liposome extravasation and drug release in the general circulation affected the exposure of cardiomyocytes to doxorubicin.

Based on our in vivo results (see Fig. 5A), we have demonstrated that HER2-targeted liposomal doxorubicin has a similar extent of total deposition in the heart as untargeted liposomes. Our in vitro results (see Figs. 2A and B) demonstrated that there are insufficient HER2 expressed on cardiomyocytes for efficient cellular uptake of HER2-targeted liposomal doxorubicin. Consequently, one can effectively study HER2-targeted liposomal doxorubicin delivery to the heart by studying untargeted liposomes. This enabled us to build off of related work studying liposomal drug delivery. As described in Materials and methods and cartooned in Fig. 6A, the model developed is a re-application of a tumor delivery model presented in Hendriks, et al. (Hendriks, submitted for publication).

In order to estimate the transport properties for doxorubicin and liposome deposition in the heart, we compiled literature data for heart doxorubicin accumulation, delivered via either conventional or PLD forms (Figs. 6B and C). From the data we were able to quantify the transport of doxorubicin and PLD into the heart (Fig. 6D), indicating that the heart is roughly 1000× less permeable to liposomes than it is to free drug. Further, we are able to simulate the expected time course for nuclear-bound doxorubicin in cardiomyocytes (Fig. 6E),

in qualitative agreement with the nuclear doxorubicin image quantification obtained in Fig. 5B. We infer that liposomal encapsulation significantly protects the heart from peak and total exposure to doxorubicin, but does not completely eliminate it. One can also determine from the model that roughly 30% of nuclear doxorubicin accumulation following liposomal delivery is via doxorubicin releasing into the circulation from PLD and subsequently the free doxorubicin crossing the endothelium into the cellular space (rather than the liposomes depositing into the tissue). This is exclusive of the low frequency 'hot-spots' of liposome deposition that were observed in the histology.

In order to gain insight into differences across animals and potentially patients, the effect of variability in conventional doxorubicin and PLD transport into the heart was systematically examined by running simulations of the mouse model across a range of values for the mass transport coefficients for conventional doxorubicin and liposomal doxorubicin (PetN_dox and PetN_lipo, respectively). For each simulation, the relative exposure (AUC) of cardiomyocyte DNA to doxorubicin via either 3 mg/kg conventional doxorubicin or PLD was determined over one week. Shown in Fig. 6F is the relative delivery of doxorubicin to the heart for conventional doxorubicin and PLD. Liposomal encapsulation significantly and consistently reduces heart exposure to doxorubicin for all expected ranges in liposome and doxorubicin transport rates. Lastly, the model was scaled to reflect human physiology, as described in Materials and methods, and a similar analysis performed. The bound doxorubicin AUC over 3 weeks following 40 mg/m^2 of either conventional doxorubicin or PLD was calculated and is shown in Fig. 6G. As before, liposomal encapsulation is predicted to significantly protect heart exposure in humans, consistent with clinical findings.

Discussion

In this work, we describe in vivo, in vitro, and in silico approaches to demonstrate that HER2-targeted liposomal doxorubicin maintains the favorable cardiac safety profile of untargeted liposomal doxorubicin, supported by its similar pharmacokinetic and biodistribution properties, its lack of uptake into human cardiomyocytes, and its inability to significantly inhibit HER2-mediated signaling in human cardiomyocytes.

One limitation of the mouse model is that the F5-scFv does not recognize murine HER2. However, published data for xenograft tumors show no significant difference in liposome accumulation for the HER2-targeted vs. untargeted PLD (Kirpotin et al., 2006). The tumor cells used in that study express 10–20× greater HER2 than our cardiomyocytes and typical tumor vasculature is at least 10× more permeable to liposomes than cardiac vasculature (Hendriks, submitted for publication), thus it is reasonable to assume that targeting is unlikely to affect the extent of deposition in the mouse (or human) cardiac tissue. In our animal studies there were the occasional 'hot-spots' of liposome deposition in the heart. In some cases, this corresponded to incomplete flushing of the vasculature. In others, it may be a result of micro-damage to the vasculature.

Uptake in our human cardiomyocytes was assessed using two stem cell-derived models. Both models express low levels of HER2 that are not sufficient for HER2-targered liposomal doxorubicin intracellular uptake. Two factors are thought to underlie the dramatic differences in uptake between the cardiomyocytes and the tumor cells (see Figs. 1 and 2). First, is that the F5-scFv is a relatively low affinity scFv (K_D~160 nM) (data not shown). Secondly, the receptor density on cardiomyocytes is too low to achieve an avidity effect via the multiple interactions with the liposome, consistent with previous studies of F5-scFv liposomes on low HER2 expressing cells (Nielsen et al., 2002). Therefore, these studies demonstrate that there is no functional effect of HER2 targeting on cardiomyocytes by HER2-targeted liposomal doxorubicin. Consequently, in human cardiac cells HER2-targeted liposomal doxorubicin behaves like the untargeted PLD in vitro. In addition, the liposomal encapsulation of doxorubicin is sufficient to minimize cardiomyocyte exposure to doxorubicin and minimize the resulting DNA damage, cell stress, apoptosis and loss of viability observed with free doxorubicin.

Since HER2-targeted liposomal doxorubicin is anticipated to behave like PLD with regard to cardiac deposition, heart exposure to doxorubicin is likely driven by release in the general circulation and subsequent competition between deposition in the heart versus clearance. Through the construction of a mechanism-based kinetic model we were able to characterize the competing kinetic steps and gain insight into how variability in rates might affect overall exposure in the heart. Using the model it was further possible to scale to human physiology and gain insight into potential effects in human subjects.

A key difference between HER2-targeted liposomal doxorubicin and other HER2-directed agents currently approved for use is that HER2-targeted liposomal doxorubicin uses HER2 as a means to target delivery of doxorubicin to HER2-expressing cancer cells. Suppressing HER2 signaling is not believed to be part of the mechanism of action of HER2-targeted liposomal doxorubicin, in contrast with agents such as trastuzumab or lapatinib. This is particularly important with regard to the potential cardiac safety profile of HER2-targeted liposomal doxorubicin in the clinic. It is anticipated that HER2-targeted liposomal doxorubicin will have an adverse event profile, including cardiac safety profile, which is consistent with PLD.

HER2-directed therapy is currently approved in both breast and gastric cancer, with clear clinical benefit demonstrated when combined with conventional chemotherapeutics. HER2 overexpression has also been identified in a number of other solid tumors including lung, pancreatic and bladder. Anthracyclines have shown broad-based clinical activity in a number of tumor types and have been incorporated into the standard of care for cancer therapy. HER2-targeted liposomal doxorubicin may provide a safer anthracycline-based therapy over currently-approved agents with an opportunity to improve upon the current clinical benefit of anthracyclines in HER2-overexpressing cancers, as a single agent or in combination with other HER2-directed agents.

Conflict of interest statement

The authors Reynolds, Geretti, Hendriks, Lee, Leonard, Klinz, Noble, Drummond, Olivier Jr., Nielsen, Niyikiza, Agresta and Wickham are all current or former employees of Merrimack Pharmaceuticals.

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Appendix A. Supplementary data

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Anti-tumor activity of liposome encapsulated fluoroorotic acid as a single agent and in combination with liposome irinotecan

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Abstract

To test the hypothesis that co-delivery of synergistic drug combinations in the same liposome provides a better anti-tumor effect than the drugs administered in separate liposomes, fluoroorotic acid (FOA) alone and in combination with irinotecan (IRN) were encapsulated in liposomes and evaluated for their anti-tumor activity in the C26 colon carcinoma mouse model. Fluoroorotic acid was dissolved in 7 M urea to increase its solubility so it could be passively loaded into liposomes at a high concentration. IRN was remote loaded into liposomes that contained the ammonium salt of the multi-valent 1,2,3,4-butanetetratcarboxylic acid with a greater than 90% efficiency and at a drug to lipid ratio of 0.2/1. When the two molecules were loaded into the same liposome, FOA was used to remote load IRN. Modulation of the drug/lipid ratio, temperature, and loading time allowed for consistent co-encapsulation of FOA + IRN at various molar ratios. The anti-tumor activity of L-FOA, L-IRN, L-FOA-IRN (5:1), and the L-FOA + L-IRN mixture (5:1) were examined in the C26 mouse model. The maximum tolerated dose of L-FOA was 10 mg/kg given weekly as compared to 100 mg/kg of the non-encapsulated FOA. Delivering two drugs in the same liposome provided a statistically better antitumor effect than delivering the drugs in separate liposomes at the same drug ratio. However, the synergistic activity of the 5:1 ratio of free drugs measured on C26 cells *in vitro* was not observed in the C26 tumor mouse model. These findings point out the challenges to the design of synergistic treatment protocols based upon results from *in vitro* cytotoxicity studies. L-FOA at 10 mg/kg as a single agent provided the best anti-tumor efficacy which supports previous suggestions that L-FOA has useful properties as a liposome dependent drug.

Keywords

fluoroorotic acid; C26 colon carcinoma; co-encapsulation; remote loading; synergism

1. Introduction

The combination of a fluoropyrimidine, such as 5-fluorouracil (5-FU), and irinotecan (IRN) is widely used for the treatment of colorectal cancer because of the enhanced tumor killing effect exerted by this drug pair. 5-FU is an antimetabolite that inhibits RNA and DNA synthesis via the enzyme thymidylate synthase. IRN is a camptothecin prodrug that acts by

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inhibiting the enzyme topoisomerase I, a process which prevents the re-ligation of DNA after replication and causes single strand breaks. A number of studies report that IRN and fluoropyrimidines can be synergistic [1–4], meaning that the combined effect of these drugs is greater than the additive pharmacological effect of the combination. Although the exact mechanism of synergism of this drug combination is not clearly understood, it is believed that IRN recruits cells in S phase that allows increased fluoropyrimidine incorporation into DNA and induces apoptosis [1, 4, 5].

Certain ratios of drug combinations may be synergistic while other ratios may be additive or antagonistic [6]. Therefore, the therapeutic activity of a drug combination such as IRN and 5-FU depends on maintaining the synergistic ratio at the target site. Because drugs have diverse physico-chemical properties, it can be difficult to control the pharmacokinetics of two drugs in a manner that enables the drugs to reach target cells at the optimal ratio and concentration. Phospholipid bilayer vesicles (liposomes) have been used by many groups to enhance the therapeutic activity of anti-cancer drug combinations [5, 7–14]. Bally, Mayer and coworkers have been at the forefront of this new paradigm to improve combination chemotherapy by controlling drug ratios using liposome drug carriers [15, 16]. They have demonstrated that liposomes are able to maintain the encapsulated drug combination at the synergistic ratio for approximately 24 hr after systemic administration in mice [5, 13, 17]. Liposome drug combinations have significantly more therapeutic activity than free drug combinations [5, 13, 14, 18]. Therefore, liposomes are able to synchronize the pharmacokinetics and biodistribution of drug combinations and to control the ratio and dose of the drugs that reach the target site.

The activity of fluoropyrimidines and IRN can be enhanced via liposome delivery [5, 16, 18, 19]. Liposome irinotecan can efficiently encapsulated by remote loading [20–22] and the resulting liposomal IRN (L-IRN) has greater *in vivo* anti-tumor activity than free IRN. 5-FU, on the other hand, is difficult to retain in liposomes because of its hydrophobicity. FOA is an anionic prodrug of 5-FU that can be encapsulated and retained in liposomes. In *in vitro* cytotoxicity studies, the liposome encapsulated form is more active than the free form[23–25]. FOA also has significant antitumor activity [26]. However, the *in vivo* efficacy of liposomal FOA (L-FOA), has not been tested. Nor have FOA and IRN been combined in a liposome formulation.

In this study, we investigated the use of liposomes to deliver FOA and IRN. We describe our efforts to develop effective liposomal formulations encapsulating FOA alone, IRN alone, and FOA + IRN at synergistic molar ratios in order to test the hypothesis that co-encapsulation of two drugs in the same liposome can enhance the efficacy of synergistic agents compared to two drugs delivered in different liposomes. This study illustrates one example of how to co-encapsulate drugs with disparate physico-chemical properties, demonstrates how modifying the drug loading conditions can affect drug co-encapsulation, and provides insights on designing combination chemotherapy studies for assessing drug synergism.

2. Methods and Method

2.1 Materials

5-Fluoroorotic acid (FOA) was purchased from Research Products International (Mt. Prospect, IL). Irinotecan-HCl Trihydrate (IRN) with 98% purity was purchased from Ivy Fine Chemicals (Cherry Hill, NJ). 1,2,3,4-Butanetetracarboxylic acid (BTCA), cholesterol (Chol) and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO). Distearoylphosphatidylcholine (DSPC) and methoxy-polyethylene glycol (MW2000)-DSPE (mPEG2000-DSPE) were products from Genzyme (Cambridge, MA). The above chemicals

were reagent grade and used as received. C26 and HT29 cells were obtained from the University of California, San Francisco Cell Culture Facility.

2.2 Cell Culture

C26 murine colorectal cancer cells were maintained in RPMI 1640 media supplemented with 10% fetal calf serum. HT29 human colorectal cancer cells were maintained in McCoy's 5A media supplemented with 10% fetal calf serum. The cells were cultured as a monolayer in 5% CO_2 at 37 °C.

2.3 Cytotoxicity Assay

C26 cells were seeded in 96 well plates and incubated for 24 hr at 37 °C to allow for cell attachment. IRN + FOA in a fixed ratio (10:1, 5:1, 1:1, 1:5, and 1:10) were simultaneously added to cells at eight doses that capture the full range of cytotoxicity of the most potent drug. The cells were continuously exposed to the single drugs and pairs of drugs for 72 hr at 37 °C. Each concentration was tested in triplicate per plate. Cytotoxicity was evaluated using the sulforhodamine B assay (SRB) assay [27]. Briefly, the cells were fixed with 50% trichloroacetic acid and stained for 30 min with 0.4% SRB in 1% acetic acid (w/v). The protein bound dye was solubilized with 10 mM unbuffered Tris base, and the absorbance of each well was measured at 564 nm.

2.4 Drug Interaction Analysis

Dose-effect curves consisting of eight data points were generated for each drug alone and in the combinations. The effect for each concentration was normalized to the untreated controls as a percent of cell survival and then converted to fraction of affected cells. CalcuSyn software (Biosoft, Ferguson, MO) was used to analyze the drug interaction between FOA and IRN. This program uses the median effect principle to determine the combination index (CI), a term which quantitatively describes the degree of synergism or antagonism of a drug interaction [28, 29]. Synergism is indicated for $CI < 1$, additivity for $CI = 1$, and antagonism for $CI > 1$.

2.5 Preparing Liposomal FOA

Liposomes were composed of DSPC:Chol:mPEG-DSPE at a 55:40:5 molar ratio. Lipid mixtures were dissolved in chloroform and dried into a thin film under reduced pressure by rotary evaporation then placed under high vacuum overnight. The films were subsequently hydrated with 500 mM FOA in 7 M urea (adjusted to pH 7 with triethylamine or LiOH) at 65 °C and vortexed to obtain a lipid concentration of 50 mM. The multilamellar vesicles were then sonicated at 65 °C. The preparation was added to a dialysis cassette (10,000 MWCO) (Pierce Chemical Co, Rockford, IL) and dialyzed against 500 mL of 5 mM Hepes, 5% Glucose pH 7.4. For comparison, FOA was passively loaded into liposomes following the method of Heath and coworkers [25]. To assay for the encapsulated FOA concentration, an aliquot of liposomes from all preparations were diluted with phosphate buffered saline (PBS; 2.16 g/L Na_2HPO_4 $7H_20$, 0.2 g/L KH_2PO_4, 0.2 g/L KCl, 8.0 g/L NaCl) and mixed with methanol:chloroform (1:1:1 v/v/v), vortexed, and centrifuged at 1,000 rpm for 10 min. Then the upper phase was mixed with 1M HCl. The encapsulated FOA concentration was determined by comparing the absorbance at 284 nm to a standard curve prepared with a solution from a blank lipid extraction. The liposome diameter and particle size distribution were measured by dynamic light scattering (Malvern Instruments, Westborough, MA). The average liposome diameter with encapsulated FOA was ~120 nm.

2.6 Preparing Liposomal IRN

The same lipid mixture was used for IRN encapsulation and was processed as described above. The films were subsequently hydrated with 300 mM BTCA (adjusted to pH 5.0 with NH_4OH) at 65 °C and vortexed to obtain a lipid concentration of 100 mM. The liposomes were then sonicated at 65 °C and extruded through 200 nm and 100 nm polycarbonate membranes (Avestin, Ottawa, CA) at 65 °C. The liposomes were exchanged into 5 mM Hepes, 5% Dextrose pH 6.5 by size exclusion chromatography using a Sephadex G25 column. IRN was loaded by incubating the drug with liposomes (0.2/1 drug to lipid molar ratio) at 65°C for 1 hr. The liposome preparation was exchanged into Hepes Buffer (5 mM Hepes, 140 mM NaCl pH 7.4) by size exclusion chromatography using a Sephadex G25 column. To measure the encapsulated IRN concentration, an aliquot of liposomes was mixed with 1% Triton X-100, heated to 100°C until the cloud point was reached, and cooled down room temperature. The encapsulated IRN concentration was determined by comparing the absorbance at 370 nm to an IRN standard curve in the appropriate buffer. The liposome diameter and particle size distribution were measured by dynamic light scattering (Malvern Instruments, Westborough, MA). The average liposome diameter with encapsulated IRN was ~100 nm.

2.7 Liposome Co-encapsulation of FOA and IRN

The same lipid mixture was processed into thin films as outlined above. The lipid films were subsequently hydrated with 500 mM FOA in 7 M urea (adjusted to pH 7 with triethylamine) at 65 °C and vortexed to obtain a lipid concentration of 25 mM. The resulting multilamellar vesicles were then sonicated at 65 °C. The preparations were added to a dialysis cassette (10,000 MWCO) and dialyzed against 500 mL of 5 mM Hepes, 5% glucose pH 6.5. To load IRN into the FOA containing liposomes and achieve an encapsulated FOA:IRN 5:1 molar ratio, IRN was incubated with the liposomes at drug/lipid molar ratios ranging from 0.025/1 to 0.3/1, at loading temperatures of 40, 45, 50, or 60 °C and for incubation periods of 10, 30 or 60 min. The liposome preparations were exchanged into Hepes buffer (5 mM Hepes, 140 mM NaCl pH 7.4) by size exclusion chromatography using a Sephadex G25 column. To assay the drug content of the liposomes, an aliquot was mixed with 1% Triton X-100, heated to 100°C until the cloud point was reached, and then cooled down room temperature. The encapsulated IRN concentration was determined by comparing the absorbance at 370 nm to a standard curve. A second sample was diluted with PBS and mixed with methanol:chloroform (1:1:1 v/v/v), vortexed, and centrifuged at 1000 rpm for 10 min. Then the upper phase was mixed with 1M HCl. The encapsulated FOA concentration was determined by 1) calculating the absorbance due to FOA in the co-formulation at 284 nm according to the equation $(A284)_{FOA} = (A284)_{FOA+IRN} - R(A284)_{IRN}$ where R = [IRN Dilution Factor/FOA Dilution Factor] and 2) comparing $(A284)_{FOA}$ to a standard curve. The liposome diameter and particle size distribution were measured by dynamic light scattering. The average liposome diameter with co-encapsulated drugs was ~120 nm.

2.8 Animals

Eight to ten week old Balb/c mice (for C26 model and maximum tolerated dose studies) and athymic nu/nu mice (for HT29 model) were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). Animal maintenance and experiments adhered to the NIH principles of laboratory animal care under a protocol approved by the Committee on Animal Research at the University of California, San Francisco.

2.9 FOA and L-FOA MTD Studies in Balb/c Mice

A solution of free FOA was made by dissolving the drug in 50 mM MOPS + 50 mM LiCl (pH adjusted to 7.4 with LiOH). L-FOA was prepared by hydrating liposomes with 500 mM

FOA in 7 M urea (adjusted to pH 7 with triethylamine) as described above. In the first maximum tolerated dose (MTD) study, Balb/c mice (n=2 mice/group) were administered a single intravenous injection of FOA 100 mg/kg or L-FOA 10 mg/kg on Day 0. In another arm of the study, Balb/c mice (n=2 mice/group) were administered FOA 100 mg/kg or L-FOA 10 mg/kg by intravenous injections on a q4d schedule starting on Day 0. In a second MTD study, Balb/c mice (n=2 mice/group) were administered FOA 100 mg/kg by intravenous injections on a q7d schedule starting on Day 0. Mouse weight and overall health were monitored on alternate days. If a mouse's body weight decreased by > 15% of the original weight or if a mouse looked unhealthy, treatments were stopped for the group to which the mouse belonged. Mice were sacrificed due to decrease in body weight > 20% of original weight.

2.10 Liposomal FOA+IRN Combination Therapy in C26 Mouse Model

C26 murine colorectal cells (3×10^5) suspended in 50 μL RPMI 1640 medium were inoculated subcutaneously in the right hind flank of each Balb/c mouse. On Day 8 after tumor implantation, mice were randomly distributed into treatment groups (n = 8). L-FOA-IRN (5:1) was prepared by hydrating liposomes with 500 mM FOA in 7 M urea (adjusted to pH 7 with triethylamine) and incubating IRN with liposomes (0.025/1 drug to lipid molar ratio) at 50 °C for 10 min as described above. Each treatment (~200 μL) was administered by tail vein injection on Day 8 and Day 15. Mouse tumor growth, weight, and overall heath were monitored on alternate days. Tumor volume was determined by measuring the tumor in three dimensions with calipers and calculated using the formula: tumor volume = length × width × height. Mice were sacrificed due to tumor burden (volume ≥ 2000 mm^3) or decrease in body weight (> 20% loss). Mouse survival was analyzed by using MedCalc 8.2.1.0 for Windows (MedCalc Software, Mariakerke, Belgium).

2.11 L-IRN Chemotherapy in HT29 Mouse Model

HT29 human colorectal cells (5×10^6), suspended in 50 μL medium, were inoculated subcutaneously in the right hind flank of each athymic nu/nu mouse. On Day 8 after tumor implantation, mice were randomly distributed into treatment groups (n = 8). Each treatment (~200 μL) was administered by tail vein injection on Days 12, 14, 19, and 21. Mouse tumor growth, weight, and overall health were monitored on alternate days. Tumor volume was determined by measuring the tumor in two dimensions with calipers and calculated using the formula: tumor volume = ½ (length × $width^2$). The percent tumor growth delay (%TGD) was calculated from the equation %TGD = (T-C)/C × 100, where T is the mean time for the tumor volume of a treatment group to reach a designated volume of 300 mm^3 and C is the mean time for the control group to reach the designated volume of 300 mm^3. Mice were sacrificed due to tumor burden (volume ≥ 2000 mm^3) or decrease in body weight (>20% loss). Mouse survival was analyzed by using MedCalc 8.2.1.0 for Windows (MedCalc Software, Mariakerke, Belgium).

3. Results

3.1 Synergism of FOA + IRN

FOA and IRN were screened for synergy in C26 murine colorectal cancer cells at 10:1, 5:1, 1:1, 1:5, and 1:10 molar ratios. To determine whether this combination was synergistic, additive, or antagonistic, we used the median effect method which is the most widely utilized model for analyzing drug interactions[29]. In this method, synergism is indicated for combination index (CI) values < 1, additivity for CI = 1, and antagonism for CI > 1. Table 1 displays the CI values at the EC_{50}, EC_{75}, and EC_{90} for the five molar ratios tested. This drug combination was very synergistic at the 5:1 molar ratio and slightly synergistic at 10:1 molar

ratio over a wide range of concentrations. However, FOA + IRN was mostly antagonistic at the 1:5 and 1:1 molar ratios, and additive at the 1:10 molar ratio.

Since delivery of FOA and IRN in liposomes may enhance the efficacy of the combination by maintaining the drugs at their synergistic ratio to tumor cells, we devised liposome formulations for each individual drug and a liposome formulation of the drugs pairs to examine this hypothesis.

3.2 Formulation Development of L-FOA

FOA is a weak acid and is charged in aqueous solution; thus it is difficult to actively load into pre-formed liposomes. Therefore, passive loading methods for encapsulating FOA within liposomes were investigated. Initially, L-FOA was prepared using an approach developed by Heath and coworkers [25]. In this method, the lipid films were hydrated with 50 mM solution of lithium salt of FOA that resulted in the liposomes encapsulating only 1–3 mM of FOA. This drug concentration is low and would require high injection volumes to achieve a therapeutic drug concentration. Therefore, we focused our efforts on ways to increase FOA concentration in liposomes. One method was to increase the solubility of FOA in order to make a more concentrated drug solution for passive loading. FOA was dissolved by using the chaotropic agent 7M urea and adjusted to pH 7 with either LiOH or TEA. By this tactic, we could increase the solubility by greater than 10 fold, and an FOA concentration as high as 650 mM FOA was obtained. This permitted the preparation of L-FOA formulations encapsulating 4–10 mM FOA by passively loading liposomes with 500 mM of TEA-FO or Li-FOA. Remote loading using zinc acetate or calcium acetate were also investigated [30]; however, we were unable to encapsulate FOA to high internal concentrations using these methods.

3.3 Maximum Tolerated Dose (MTD) Analysis of FOA and L-FOA in Balb/c Mice

The toxicity of free FOA in mice and anti-tumor activity of FOA in murine tumors has been described [26, 31]. However, liposomal FOA has only been evaluated *in vitro* [24, 25, 32]. A MTD study in Balb/c mice is shown in Fig. 1A. The weight of mice did not significantly decrease after one i.v. dose of FOA 100 mg/kg or L-FOA 10 mg/kg during the course of the study. Therefore, one i.v. dose of both formulations was well tolerated. A 2xq4d schedule of FOA 100 mg/kg and L-FOA 10 mg/kg was toxic to the mice.

We then examined the MTD when FOA 100 mg/kg and L-FOA10 mg/kg were administered i.v. on a 3xq7d schedule, starting on Day 0 to Balb/c mice (Fig. 1B). The weight of the mice showed a non-statistically significant downward trend for twenty-one days after initiating dosing. However the weight of the animals then recovered, indicating that this dose and schedule provided an acceptable maximum tolerated dose in non-tumored mice.

3.4 Development of L-IRN Formulation

There are several published methods for formulating liposomal irinotecan by remote loading, a highly efficient technique used to encapsulate drugs into preformed liposomes via a transmembrane ion or pH gradient [20–22, 33]. Encapsulating IRN in liposomes containing 1,2,3,4-butanetetracarboxylic acid (BTCA) resulted in greater than a 90% encapsulation efficiency of IRN. Therefore, L-IRN was prepared with BTCA as the trapping agent for the single agent studies. Fig. S1 (supplementary information) depicts the proposed mechanism of IRN encapsulation with BTCA as the trapping agent.

3.5 Therapeutic Efficacy of L-IRN in HT29 Tumor Model

To verify that the antitumor efficacy of BTCA loaded L-IRN was similar to that observed in previous studies using sucrose octasulfate as the remote loading gradient generating

molecule [20], we determined the anti-tumor activity of L-IRN in HT29 tumor-bearing mice. Free IRN and L-IRN were administered at a dose of 50 mg/kg intravenously to mice twice per week for a total of four doses. This is the MTD of free IRN, and when administered using the dosing regimen is well tolerated [20]. Fig. S2 shows that L-IRN 50 mg/kg had significantly greater tumor growth inhibition than IRN 50 mg/kg ($p < 0.05$). In fact, the %TGD of L-IRN was 114% whereas the %TGD of IRN was 36%. Also, mice treated with L-IRN 50 mg/kg had a slight increase in survival rate when compared to mice treated with free IRN 50 mg/kg (% increase in life span equaling 50% and 29.3% for L-IRN and IRN, respectively). Thus the BTCA loaded L-IRN had a similar efficacy as other liposomal IRN formulations evaluated in a HT29 tumor xenograft model [20]. L-IRN did not adversely affect the weight of the mice, which indicates that there is an acceptable toxicity profile in the mice at this dose (Fig. S3 in the supplementary information).

3.6 Liposome Co-encapsulation of FOA + IRN

To investigate whether liposomes encapsulating a synergistic ratio had better anti-tumor efficacy *in vivo*, liposomes co-encapsulating both drugs were formulated. The approach was to first passively load FOA into the liposomes and then to use the weak acid on FOA to remote load IRN. Cholesterol content, drug/lipid ratio, loading temperature, and incubation time can influence co-encapsulation of drugs into liposomes [22]. Therefore, various IRN drug/lipid ratios, loading temperatures, and incubations times were tested in order to load IRN into the FOA encapsulated liposomes and achieve an FOA/IRN 5:1 molar ratio. The approaches examined are summarized in Table 2. The encapsulated FOA concentration is significantly reduced during IRN remote loading. Decreasing the initial IRN drug/lipid ratio, loading temperature, and incubation time increased the retention of FOA in the liposomes. Whereas, increasing the initial IRN drug/lipid ratio and incubation time generally increased the encapsulated IRN concentration. Loading temperature did not significantly affect IRN encapsulation. Thus, the final loading protocol had to balance the competing tendency of the two drugs to be retained in the liposome.

There is a strong correlation between the initial IRN drug/lipid ratio and: 1) the final encapsulated IRN concentration, 2) the final encapsulated FOA concentration, and 3) the co-encapsulated drug ratio (Fig. 2). A FOA/IRN 5:1 co-encapsulated molar ratio could be consistently achieved when remote loading IRN at a 0.025/1 drug/lipid ratio at 50 °C for 30 min. How the aforementioned parameters as well as cholesterol content would affect co-encapsulation of the drugs at a 1:1 molar ratio were also investigated (Table S2 and Fig. S4 in the supplementary information). Thus, the loading parameters were selected to reproducibly achieve a 5:1 or 1:1 molar ratio in the formulations of FOA/IRN in the liposomes.

The proposed mechanism of co-encapsulation is shown in Fig. 3. We hypothesize that triethylamine present in the liposome internal buffer partitions out of the liposome interior similar to what occurs in other remote loading process [34, 35]. This causes a transmembrane pH gradient across the liposome bilayer (high [H+] in the interior, low [H+] in the exterior). IRN in the external buffer is then able to cross the liposome bilayer and become protonated. IRN within the liposomes that is positively charged (pKa = 8.1) interacts with FOA that is negatively charged (pKa = 2.4) and probably forms a complex [36].

3.7 Anti-tumor Effect in and Survival of C26 tumor-bearing mice treated with Liposomal FOA + IRN

The therapeutic activity of the co-formulation was compared to the single formulations and to mixtures of the single formulations in C26 tumor-bearing mice (Fig. 4). L-FOA 57.4

μmol/kg (10 mg/kg) had a superior tumor growth inhibition and significantly longer survival rate (p=0.0027; log rank test) than L-IRN 73.8 μmol/kg (50 mg/kg). None of the combinations were more effective than L-FOA. Mice treated with drugs in the same liposome, L-FOA-IRN 5:1, had an increased survival compared to mice treated with drugs in separate liposome, L-FOA + L-IRN 5:1 (p=0.0414, log rank test). Furthermore, mice treated with L-FOA + L-IRN 5:1 at twice the dose, which is the same dose given as L-FOA, had a similar survival time as the L-FOA. Mice in both treatment groups lived longer than mice given L-FOA + L-IRN 5:1 at the lower dose (p=0.0015, log rank test). Thus the L-IRN provided no advantage in this combination and perhaps a disadvantage since mice from two groups, L-IRN and L-IRN + L-FOA 5:1, were steadily losing weight after the second i.v. injection (Fig. 4B).

4. Discussion

The objective of this study was to investigate the hypothesis that co-encapsulation of two drugs in the same liposome can enhance the efficacy of synergistic agents compared to two drugs delivered in different liposomes. This required us to devise liposome formulations of FOA, IRN, and FOA + IRN combination.

The combination activity of FOA + IRN was tested in C26 cells. FOA + IRN at a 5:1 molar ratio was synergistic in C26 cells *in vitro* (Table 1). It was surprising that IRN showed good activity since it is a prodrug that has to be activated by a carboxylesterase to the active compound SN38. The results from the screen indicated that the synergism exhibited by FOA and IRN is ratio-dependent. This makes it important to control the ratios *in vivo* in order to achieve maximum therapy.

To prepare L-FOA, a method developed by Heath and coworkers [25] was initially used which allowed encapsulation of 1–3 mM of FOA. Dissolving FOA in 7M urea, which can disrupt hydrogen bonding between molecules, allowed us to make a more concentrated FOA solution. As a result, liposome that encapsulated higher concentrations (~10mM) of FOA could be prepared. With the L-FOA formulation, one could deliver a 10 mg/kg dose to mice in a 200 μL volume.

The MTD of L-FOA was established because: 1) L-FOA has never been tested in mice and 2) we needed to determine whether L-FOA could be administered safely at a schedule similar to an IRN dosing schedule. The maximum tolerated dose of L-FOA is 10 mg/kg, which is a 10 fold less dose than the MTD of free FOA. This enhanced toxicity is probably due to the longer circulation, sustained release, and enhanced accumulation of FOA to sites of toxicity due to the liposome formulation. This increased toxicity for a water soluble anti-metabolite has been observed in liposomal cytosine arabinoside formulations [37]. Balb/c mice were able to tolerate a single i.v. dose of both treatments, but could not endure multiple i.v. doses of FOA 100 mg/kg given four days apart (Fig. 1A). The mice also could not tolerate multiple i.v. doses of 10 mg/kg L-FOA given four days apart. Therefore, L-FOA could not be administered on the schedule followed in the L-IRN therapy study in HT29 mice. The MTD of the two formulations were also evaluated in the Balb/c mice on a 3×q7d dosing schedule (Fig. 1B). This is the schedule that Mayer and coworkers used to evaluate the therapeutic activity of their liposome IRN + floxuridine co-formulations [5]. The formulations were not toxic to the mice at this schedule; therefore, the weekly schedule was selected for use in animal studies with the FOA and IRN liposome formulations.

The therapeutic efficacy of BTCA loaded L-IRN was tested in a HT29 human xenograft mouse model at a similar dose and schedule used by Drummond and coworkers [20]. The

data in Fig. S2 and Fig. S3 (supplementary information) demonstrate that L-IRN was efficacious and safe at the dose and schedule administered.

Lastly, a liposome formulation that encapsulated both FOA and IRN was developed. Co-encapsulating these two drugs in one liposome formulation was challenging because of the disparate physico-chemical properties of these two drugs. Weakly acidic drugs like FOA are traditionally passively loaded into liposomes; while amphipathic drugs like IRN can be actively loaded into preformed liposomes. Passive loading of drugs occurs through hydrating a lipid film with an aqueous solution of drug. This method is inefficient, and the resulting encapsulated drug concentration relies on the maximum solubility of the drug in solution [34]. It is difficult to remote load FOA because it is deprotonated in aqueous solution; therefore, FOA cannot readily cross a lipid bilayer. Whereas, IRN can partition into and diffuse across a bilayer when deprotonated. To co-encapsulate FOA and IRN in liposomes, FOA was passively loaded into vesicles and then was used to remote load IRN (Fig. 3). In this protocol, some of the FOA leaked out during IRN remote loading (Fig. 2, Table 2, as well as Table S1 and Fig. S4 in the supplementary information). Reducing the loading temperature, loading time and IRN drug/lipid ratio enhanced FOA retention but minimized IRN loading. The IRN drug/lipid ratio had the biggest impact on FOA retention and IRN loading, and adjusting the loading conditions allowed us to reproducibly encapsulate FOA + IRN at ratios between 5:1 to 1:1.

We investigated the combination therapy of FOA + IRN when delivered in the same liposome or when delivered together in separate liposomes in C26 tumor-bearing mice. L-FOA (57.4 μmol/kg) was more effective than L-IRN (73.8 μmol/kg). Although IRN + floxuridine, another fluoropyrimidine, co-encapsulated in liposomes had greater anti-tumor activity than the single liposome agents [5], we did not observe the same results. Although delivering the two drugs at the 5:1 ratio in the same liposome was statistically superior than delivering the two drugs at the 5:1 ratio in separate liposome, none of the combinations were more effective than L-FOA alone at 10 mg/kg (Fig. 4). This might have been due to: 1) lower dose of FOA in the co-encapsulated formulations than in the single liposome formulation or 2) co-delivery of the drugs in separate liposomes. The dose of FOA in the co-encapsulated formulation, L-FOA-IRN 5:1, was 28.7 μmol/kg while that in L-FOA was 57.4 μmol/kg. It was challenging to devise a co-encapsulated formulation with this concentration of FOA because of the leakage of FOA due to IRN remote loading in the liposome. To deliver a co-formulation with FOA at the dose of the single agent formulation, we had to deliver the drugs in separate liposomes. The L-FOA + L-IRN 5:1 (double strength) combination was administered at the same dose of FOA as the L-FOA formulation and had slightly but not significantly less, tumor growth inhibition as L-FOA.

It is possible that this co-formulation did not show enhanced efficacy compared to L-FOA because the drugs were in separate liposomes. Delivering drugs in the same liposome formulation may help to coordinate the release of the drugs in the cell such that the encapsulated drugs leak at similar rates [15, 16]. Perhaps the L-FOA + L-IRN 5:1 (double dose) mixture did not deliver the 5:1 synergistic ratio into the cell.

In conclusion, we describe the development and evaluation of liposomal formulations for FOA and IRN alone and in combination. An optimized method for encapsulating FOA into liposomes was developed which allowed us to encapsulate up to 10mM of FOA. This method enabled us to co-encapsulate FOA and IRN, which have disparate physico-chemical properties, at different molar ratios. L-FOA as a single agent has anti-tumor efficacy in the C26 tumor mouse model that was superior in tumor growth inhibition and in the increase in survival time compared to L-IRN 73.8 μmol/kg (50 mg/kg). However, the co-delivery of

IRN with FOA in either the same or different liposomes failed to improve further the anti-tumor activity in the C26 model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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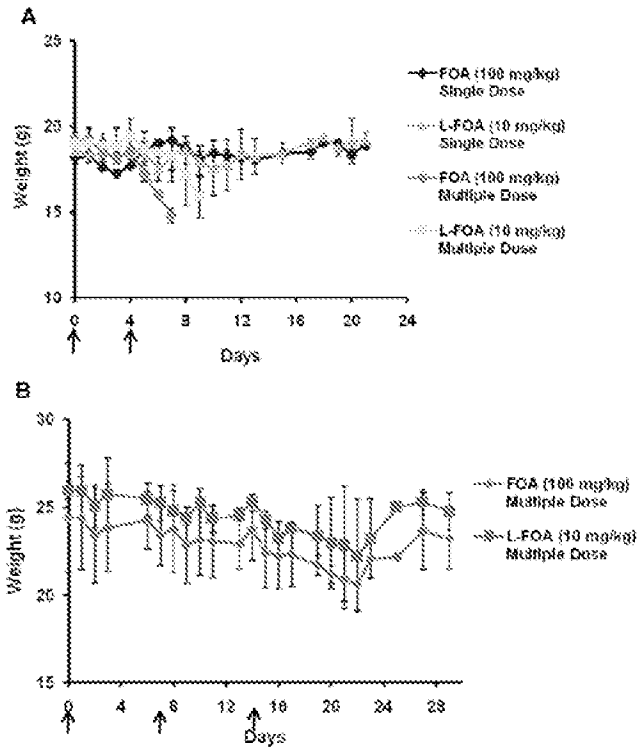

Fig. 1.

Maximum tolerated dose study of FOA 100 mg/kg and L-FOA 10 mg/kg in Balb/c mice. **A**. For single dose, FOA and L-FOA administered i.v. on Day 0. For multiple dose, FOA and L-FOA administered i.v. on Day 0 and four days apart (as indicated by arrows). **B**. FOA 100 mg/kg and L-FOA 10 mg/kg administered i.v. on a 3xq7d schedule starting on Day 0.

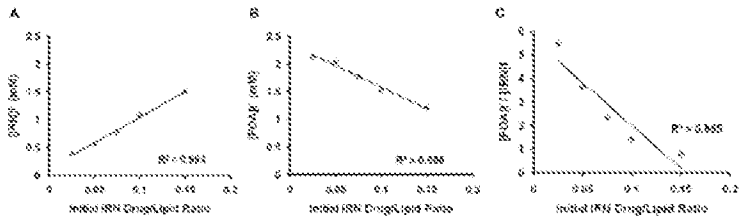

Fig. 2.

Effect of IRN drug to lipid ratio on FOA and IRN co-encapsulation at 5:1 molar ratio. Loading temperature was 50 °C and loading time was 30 min. **A**. Effect of initial IRN drug/lipid ratio on the final encapsulated IRN concentration ($[IRN]_f$). **B**. Effect of initial IRN drug/lipid ratio on the final encapsulated FOA concentration ($[FOA]_f$). **C**. Effect of initial IRN drug/lipid ratio on the final ratio of FOA and IRN in the liposomes ($[FOA]_f/[IRN]_f$).

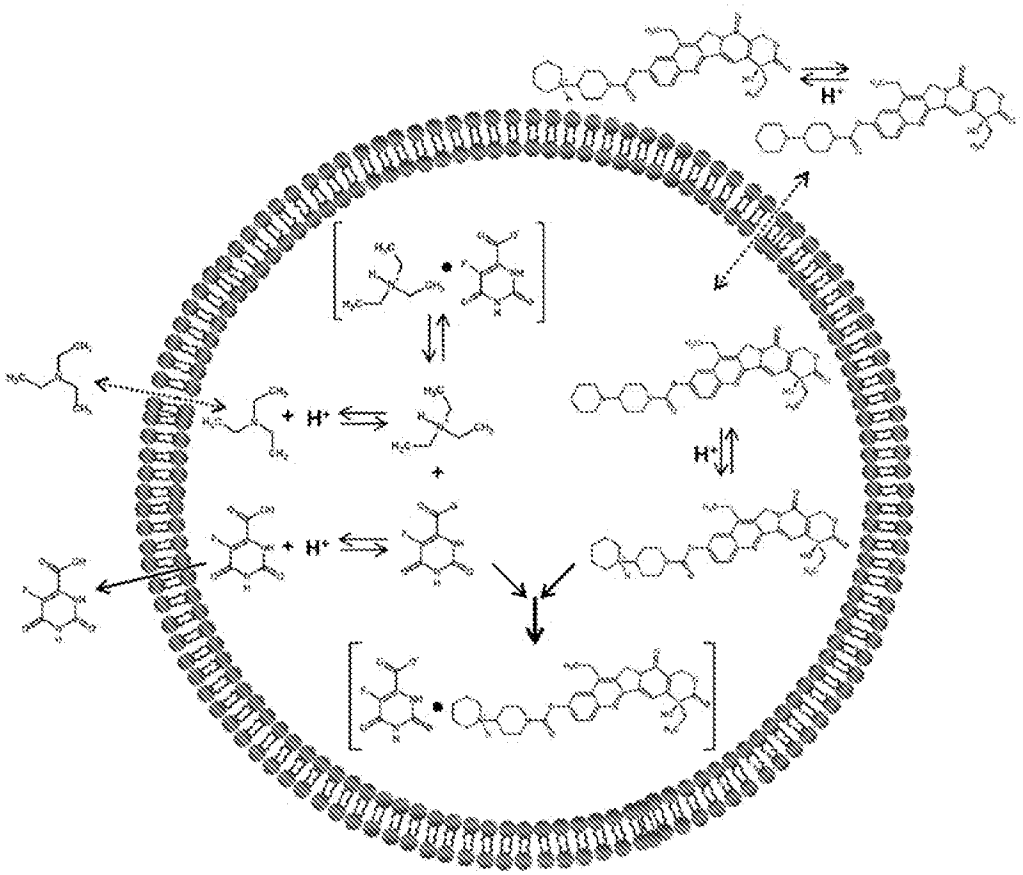

Fig. 3.
Schematic diagram of proposed mechanism of co-encapsulation of FOA + IRN in liposomes.

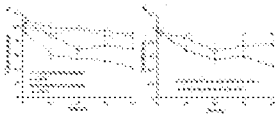

Figure 4.
FOA and IRN release profile from L-FOA-IRN, L-IRN, and L-FOA. **A**. *In vitro* leakage from liposomes during 96 hr incubation in 33% serum at 37°C. **B**. Ratio of IRN and FOA released from the co-encapsulated and the individual liposomes over time. *Statistical significance ($P < 0.05$) between the ratio from co-encapsulated and separate liposomes as measured by Student's t-test.

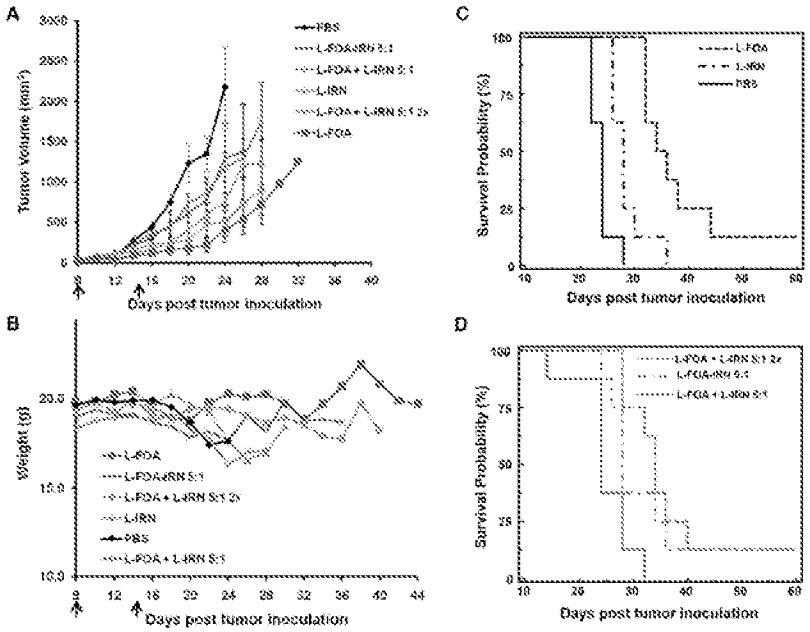

Fig. 5.

L-FOA-IRN combination therapy in C26 tumor-bearing mice. Balb/c mice (n=8) were treated with i.v. injections on Days 8 and 15 (as indicated by arrows). **A**. Anti-tumor activity. Error bars represent SEM. **B**. Effect of combination therapy on weight of C26 tumor-bearing mice. **C** and **D**. Survival curves. The treatment group s are PBS, L-FOA (10 mg/kg; 57.4 μmol/kg), L-IRN (50 mg/kg; 73.8 μmol/kg), L-FOA-IRN 5:1 (5 mg/kg or 28.7 μmol/kg FOA; 3.9 mg/kg or 5.7 μmol/kg IRN), L-FOA + L-IRN 5:1 (5 mg/kg or 28.7 μmol/kg FOA; 3.9 mg/kg or 5.7 μmol/kg IRN), and L-FOA + L-IRN 5:1 double strength (10 mg/kg or 57.4 μmol/kg FOA; 7.8 mg/kg or 11.5 μmol/kg IRN).

Table 1

FOA + IRN Combination Activity in C26 Cells

Drug Combination	Ratios	Combination Index*		
		EC50	*EC75*	*EC90*
FOA + IRN	1:10	1.1	0.99	0.89
	1:5	1.2	1.2	1.2
	1:1	1.3	1.2	1.1
	5:1	0.20	0.16	0.13
	10:1	0.80	0.72	0.66

*Combination Index is a quantity derived from the median effect equation that describes the degree of a drug interaction. Synergism is indicated for CI < 0.9 (Green), additivity for 0.9 < CI < 1.1 (Yellow), and antagonism for CI > 1.1 (Red).

Table 2

Summary of Conditions and Outcomes of FOA + IRN Co-encapsulation into Liposomes at 5:1 Ratio

Trial	$[FOA]_o$ (mM)	IRN D/L Ratio	Load Temp (°C)	Load Temp (min)	$[IRN]_f$ (mM)	$[FOA]_f$ (mM)	FOA/IRN Ratio
i	7.2	0.1/1	50	60	1.21	1.74	1.4/1.0
		0.2/1			2.18	0.94	1.0/2.3
		0.1/1	65		1.05	0.51	1.0/2.1
		0.2/1			1.73	0.21	1.0/8.2
ii	6.13	0.1/1	50	10	1.23	3.7	3.0/1.0
				30	1.14	3.11	2.7/1.0
				60	2.29	2.05	1.1/1.0
iii	8.69	0.1/1	40	30	1.08	2.43	2.2/1.0
		0.2/1			1.93	1.81	1.0/1.1
		0.1/1	45		1.15	3.61	3.1/1.0
		0.2/1			2.1	1.41	1.0/1.5
iv	4.56	0.05/1	50	30	0.56	2.03	3.6/1.0
		0.075/1			0.77	1.78	2.3/1.0
		0.1/1			1.07	1.53	1.5/1.0
		0.15/1			1.51	1.21	1.0/1.2
v	4.77	0.025/1	50	30	0.39	2.13	5.4/1.0

$[FOA]_o$: initial FOA concentration. D/L: drug to lipid ratio. $[IRN]_f$: final IRN concentration. $[FOA]_f$: final FOA concentration.

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Increased liposome extravasation in selected tissues: Effect of substance P

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ABSTRACT **We have used a pharmacologic mediator to open intercellular connections in selected vessels to allow liposomes to escape from the blood stream and to extravasate into tissues that have appropriate receptors. We have examined the effects of substance P (SP), a peptide known to increase vascular permeability in selected tissues, such as trachea, esophagus, and urinary bladder in rats. We used quantitative fluorescence analysis of tissues to measure two fluorescent markers, one attached to the lipid (rhodamine-phosphatidylethanolamine) and another, doxorubicin (an anti-tumor drug), encapsulated within the aqueous interior. We have also examined the deposition of liposomes microscopically by the use of encapsulated colloidal gold and silver enhancement. Analysis of the biochemical and morphological observations indicate the following: (*i*) Injection of SP produces a striking increase in both liposome labels, but only in tissues that possess receptors for SP in postcapillary venules; (*ii*) liposome material in these tissues has extravasated and is found extracellularly near a variety of cells beyond the endothelial layer over the first few hours; (*iii*) 24 h following injection of liposomes and SP, liposome material is found in these tissues, localized intracellularly in both endothelial cells and macrophages. We propose that appropriate application of tissue-specific mediators can result in liposome extravasation deep within tissues that normally do not take up significant amounts of liposomes from the blood. Such liposomes are able to carry a variety of pharmacological agents that can be released locally within selected target tissues for therapeutic purposes.**

Liposomes have been used as carriers for drugs and macromolecules (1, 2), as carriers for DNA to transfect cells *in vitro* (3), and to deliver genes into specific tissues for gene therapy (4, 5). However, the ability of liposomes administered via the bloodstream to reach target cells in various tissues is severely limited by two factors. First, conventional liposomes are cleared rapidly from the circulation by the phagocytic cells of the reticuloendothelial system (6). Thus, following i.v. injection, the majority of conventional liposomes are taken up by liver and spleen within a few minutes to hours, depending on particle size and lipid composition (6, 7). Second, most tissues have an endothelial barrier that does not permit particles of the size of even the smallest liposomes to extravasate (8). The recent development of sterically stabilized liposomes, which have a markedly prolonged residence time in blood (9, 10), has made it possible to deliver liposome-encapsulated drugs to tumors (10–14) and to sites of infection (15, 16) that have increased endothelial permeability to such particles.

An alternative possibility for delivering liposomes to selected tissues is to use a pharmacologic mediator that, by opening intercellular connections in selected vessels, might allow liposomes to escape from the bloodstream and extravasate into the tissues of organs whose postcapillary endothelia contain receptors for the mediator (17, 18). In the present study, we examined the effects of substance P (SP), a peptide that increases vascular permeability only in selected tissues. First, we studied the ability of SP to allow liposomes to escape from the bloodstream and extravasate into the tissues of organs that contain SP receptors (such as trachea, esophagus, and urinary bladder) using rhodamine-phosphatidylethanolamine (PE) as a liposomal marker. Second, we investigated the ability of SP to increase the tissue uptake of a liposome-encapsulated drug ito examine whether the extravasated liposomes can retain their contents for possible pharmacological actions. As a model drug we have chosen doxorubicin, an anti-tumor agent that can be encapsulated with high efficiency within the interior aqueous space of the liposomes. Finally, we studied the anatomic localization of liposomes in tissues using microscopic imaging of encapsulated colloidal gold-containing liposomes. Our findings indicate that the use of specific mediators of inflammation, such as SP, can produce a large increase in the extravasation of liposomes along with their encapsulated contents into specific tissues. These results appear to have general applicability, because liposomes have been used to successfully encapsulate of pharmacological agents including beta-adrenergic antagonists, antibiotics, oligonucleotides, and DNA (1, 2).

MATERIALS AND METHODS

Materials. Hydrogenated soy phosphatidylcholine was purchased from Natterman (Cologne, Germany). Distearoylphosphatidylethanolamine derivatized at the amino position with a segment of polyethylene glycol-PE (molecular weight, 1900) was synthesized as described (9) and was obtained from Sequus Pharmaceuticals (Menlo Park, CA). EggPC, dioleoylphosphatidylethanolamine (DOPE), and *N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine were purchased from Avanti Polar Lipids. Highly purified cholesterol was purchased from Calbiochem. Deferoxamine mesylate (desferal) and dimethyl-dioctadecylammonium bromide (DDAB) were obtained from Sigma. Doxorubicin hydrochloride was obtained from Chiron. SP, purity 98% (HPLC), was purchased from Bachem. Other solvents and chemicals were of analytical grade.

Preparation of Liposomes. Sterically stabilized liposomes composed of hydrogenated soy phosphatidylcholine/cholesterol/polyethylene glycol-PE/*N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine (molar ratio, 30:20:1.8:0.24) were prepared by hydration of thin lipid films with Hepes·NaCl buffer (20 mM Hepes/144 mM NaCl, pH 7.2,

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Abbreviations: SP, substance P; PE, phosphatidylethanolamine.

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300 mosmol) at 57°C. The hydrated lipid suspension was put through freezing (dry ice and ethanol) and thawing (57°C) cycles five times. The multilamellar liposomes thus formed were extruded at 57°C under argon through polycarbonate membranes, through 0.1 μm pore size three times, and then through 0.05 μm pore size five times (19). Then the liposome solution was put through cold water (≈20°C) and hot water (57°C) cycles three times, followed by passage through a Sephadex G-75 column equilibrated with Hepes·NaCl buffer.

Doxorubicin-loaded liposomes contained the same lipid components and molar ratio as the liposomes described above, but no rhodamine-PE. The dry lipid mixture was hydrated with ammonium sulfate solution (250 mM $(NH_4)_2SO_4$/1 mM desferal), followed by freeze-thaw cycles, extrusion, cold-hot water cycles, and a Sephadex G-75 column, as described above. Doxorubicin (1 mg per 10 μmol of phospholipid) was loaded into liposomes by dissolving doxorubicin dry powder in the liposome solution and shaking in a water bath at 57°C for 30 min. (20). Free doxorubicin was then removed on a Sephadex G-75 column. The final preparation contained 0.1 mg of doxorubicin per μmol of phospholipid.

Liposomes containing colloidal gold were prepared according to the published procedure (21). Liposomes composed of EggPC/cholesterol/polyethylene glycol-PE (30:20:1.8) were prepared by reverse-phase evaporation in gold chloride solution (6.36 mM $HAuCl_4$/60 mM citric acid/15 mM K_2CO_3, pH 3.5) at a concentration of phospholipid of 10 μmol/ml. The liposomes obtained from reverse-phase evaporation were extruded through polycarbonate membrane filters (19), 0.1 μm pore size (twice), and 0.05 μm pore size (five times) to obtain liposomes with a narrow size distribution. Immediately after the final extrusion, the colloidal gold formation was promoted by raising the pH of the liposome suspension to 5.5–6.0 by adding NaOH and incubating at 55°C for at least 30 min. The unencapsulated gold particles were separated from gold liposomes by passing the liposome suspension through a Sephacryl S-400 column (Pharmacia). The gold-containing liposomes were characterized by microscopy (21) and were stable at 4°C under argon for at least 1 month.

***In Vivo* Procedures.** Experimental procedures followed in this study were approved by the Committee on Animal Research of the University of California, San Francisco. We used pathogen-free male rats of the F344 strain, which were 10–12 weeks old, 250–300 g, and obtained from Simonsen Laboratories (Gilroy, CA). Three different types of liposomes were used to examine the deposition of liposomes in various tissues.

Experiments with rhodamine-labeled liposomes (three groups of rats). In one group of rats (n = 4), rhodamine-PE labeled liposomes (15 μmol of phospholipid per kg of body weight) were injected into a femoral vein. SP (10 nmol/kg) or 0.9% saline was injected i.v. 5 min later. After another 5 min, rats were perfused with PBS as follows. A cannula was inserted into the ascending aorta through the left ventricle, the left atrium was incised, and perfusion was carried out for 1 min. Then the right atrium was cut and perfusion was continued for another 2 min. In a second group of rats (n = 5), injection of SP was repeated three times at 5, 35, and 65 min after injection of liposomes, to evaluate the effect of multiple injections of SP. In a third group of rats (n = 3), 0.3 ml of blood samples was taken from a femoral artery immediately before and 10 min after the injection of liposomes for measurement of liposome concentration over time.

Experiments with doxorubicin-encapsulated liposomes. In one group of rats (n = 5), doxorubicin-loaded liposomes (15 μmol of phospholipid per kg of body weight) were injected into a femoral vein. SP (10 nmol/kg) or saline was injected i.v. 5 min later. After another 5 min, the rats were perfused as described above. In a second group of rats (n = 3), 0.3 ml of blood samples was taken from a femoral artery at 1, 10, 30, and 60 min after liposome injection. These studies were performed to determine the concentration of doxorubicin-loaded liposomes in the blood over time.

Experiments with liposomes containing colloidal gold. We injected liposomes containing colloidal gold (15 μmol of phospholipid per kg of body weight; n = 4) into a femoral vein, and 5 min later we injected SP (10 nmol/kg) or the vehicle control without SP (0.9% saline). The rats were euthanized and perfused at varying intervals (5 min, 60 min, 6 h, or 24 h) after SP was administered.

Tissue Analysis of Rhodamine-Labeled Lipid and Doxorubicin. The fluorescence intensity of rhodamine was measured after lipids were extracted from tissues. Tissues were first homogenized in 50% methanol, and then the lipids were extracted with chloroform with a volume ratio of 1:1:5.3 (methanol/water/chloroform). Extraction was carried out by shaking for ≈15 h at room temperature. Fluorescence signals were measured using Soex Fluorolog (Edison, NJ) with excitation slits of 1.25 mm and emission slits of 5 mm. Rhodamine in chloroform was excited at 560 nm, and emission was observed at 595 nm.

To measure doxorubicin, tissues were homogenized with 50% ethanol and then mixed with an equal volume of 50% ethanol containing 0.6 M HCl, followed by shaking for ≈15 h at room temperature. In this way, doxorubicin was released from liposomes. Fluorescence measurements were obtained using the same equipment and slits as described above. Doxorubicin was excited at 470 nm, and emission peaks occurred at 555 nm and 590 nm. The fluorescence intensity at 555 nm and 590 nm was converted to the amount of doxorubicin in the tissue by comparing values to a standard curve of doxorubicin. Rats that were only injected with saline provided background fluorescence signals for the measurements of rhodamine and doxorubicin.

Blood samples containing liposomes were treated as follows. We added 0.75 ml of PBS and centrifuged the blood to separate the plasma from red cells. Then we added 0.75 ml of PBS to the cell pellet and centrifuged again. Both supernatants were combined, and rhodamine-PE was extracted with 4 ml of chloroform, and doxorubicin was extracted with ethanol containing 0.6 M HCl.

Light Microscopy Following Silver Enhancement of Colloidal Gold. The tissues were fixed in 3% glutaraldehyde/1% paraformaldehyde overnight at 4°C. The tissues were then rinsed with buffer at 4°C for 30 min and dehydrated with acetone for 15 min each in 70% acetone, 2% dimethyl sulfoxide/95% acetone, 2% dimethyl sulfoxide, and 100% acetone. Following dehydration, the tissues were embedded in JB4 resin obtained from Polysciences, allowed to polymerize, and cut (2.5-μm sections) on a Reichert JB4 microtome. The tissues containing liposome–gold complex were then incubated for 15 min with silver enhancement reagents (Amersham). The sections were counterstained with hematoxylin and examined.

Statistical Analysis. All data are expressed as mean ± SEM. Statistical analysis was performed using a Macintosh computer and the STATVIEW program. Analysis of variance was performed for quantitative data by the Student's t test for unpaired samples. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Localization of Liposomes Labeled with Rhodamine-PE. To study the effect of SP on deposition of liposomes in various tissues, sterically stabilized liposomes were labeled with rhodamine-PE, a lipid-soluble fluorescent probe. SP or saline was introduced into rats 5 min after injection of liposomes. The animals were perfused 5 min later, and rhodamine fluorescence from 10 different tissues was measured.

In the trachea, esophagus, and urinary bladder, the liposome lipid concentration was low in the absence of SP, but the

concentration of liposome marker increased markedly after SP (Fig. 1). Thus, the amount of liposome lipid after SP averaged 24 times greater than control in trachea, 55 times greater in esophagus, and four times greater in urinary bladder. In other tissues, including ileum, skin, kidney, heart, and lung, and in the absence of SP, liposome concentrations were also low, and in these tissues liposome concentrations were also unaffected by SP. In the liver and spleen, liposome concentrations were higher than in other tissues in the control state, but these amounts were also unaffected by SP (Fig. 1).

We also examined the rhodamine fluorescence of blood samples after injection of rhodamine-PE-labeled liposomes to determine whether the concentration of the liposomes in the bloodstream changed over time during the experiments. Ten minutes after injection of liposomes, ≈100% of the original amount of the liposomes remained in the blood (n = 3; data not shown). In another experiment, introduction of SP was repeated three times (5, 35, and 65 min after i.v. injection of liposomes). The uptake of phospholipid was not greater in trachea, esophagus, and bladder than in the animals given only one injection of SP (data not shown).

Localization of Doxorubicin Encapsulated in Liposomes. To determine whether liposome contents are retained within the liposomes during extravasation, we also encapsulated doxorubicin (an anti-tumor drug) into the aqueous interior of liposomes. Doxorubicin-loaded liposomes were injected i.v. into rats, followed by the injection of SP or saline 5 min later. Five minutes after SP or saline, the animals were perfused, and doxorubicin fluorescence was measured in 10 tissues. In blood, the concentration of doxorubicin was unchanged during the experimental procedure (n = 3; data not shown). The findings in trachea, esophagus, and urinary bladder were similar to the results with rhodamine-labeled liposomes. In the control state without SP, doxorubicin concentrations were uniformly low; after SP, doxorubicin increased markedly in all three tissues (Fig. 2), with doxorubicin concentrations increased 52-fold in trachea, 138-fold in esophagus, and 13-fold in urinary bladder. On the other hand, the concentrations of doxorubicin in ileum, skin, kidney, heart, liver, and lung were not affected by SP. In the spleen, the doxorubicin concentration was high in the control state but unaffected by SP (Fig. 2).

Localization of Gold-Labeled Liposomes. We performed morphological studies with gold-labeled, sterically stabilized liposomes. The presence of colloidal gold particles is visualized by silver enhancement and is taken as evidence for the presence of liposomes (11, 13). In the tracheas of rats that received no SP, the colloidal gold/silver particles were visualized only within the lumen of the blood vessels; no liposomes were found in the tracheal tissue (Fig. 3*a*). In rats that received liposomes and then SP 5 min later, the gold label was dispersed diffusely throughout the submucosal tissue between the cartilage and the epithelium, with the highest concentration of gold surrounding the small venules. Except in occasional small foci, the epithelium itself remained free of liposomes (Fig. 3*a*). The pattern of dispersion of gold particles after SP in the esophagus (Fig. 3*b*) and in the urinary bladder (Fig. 3*c*) was similar to the trachea. In all other tissues studied, the liposomes remained only in the vascular lumens, even after SP.

To determine the time sequence for distribution and clearance of gold particles after extravasation, a group of rats were euthanized at varying time intervals after the i.v. injection of the gold-labeled liposomes. Five minutes after administration of SP, the gold label was dispersed diffusely in tracheal tissue (Fig. 4, *Middle*). At 60 min after administration of SP, gold particles were dispersed to approximately the same degree within the trachea as at 5 min (data not shown). However, at

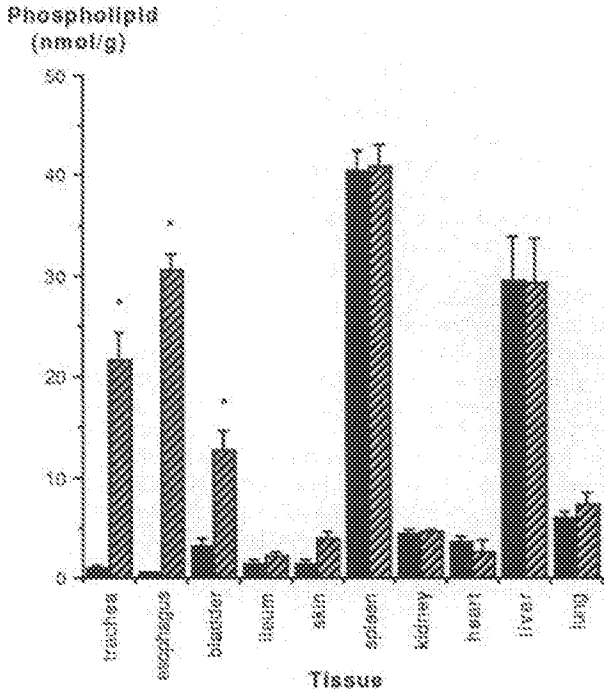

FIG. 1. Effect of SP on the deposition of sterically stabilized liposomes labeled with rhodamine-PE in various tissues of rats. Liposomes (15 μmol of phospholipid per kg of body weight) were injected i.v., and 5 min later either the vehicle control without SP (0.9% saline; solid columns; n = 4) or SP (10 nmol/kg; hatched columns; n = 4) was injected. Animals were euthanized 10 min after liposome injection, and the circulation was perfused with PBS to remove the blood. Liposome concentrations in tissues are expressed as phospholipid in nmol per g of wet tissue. Data reported as mean ± SEM. *, significantly different from controls ($P < 0.05$).

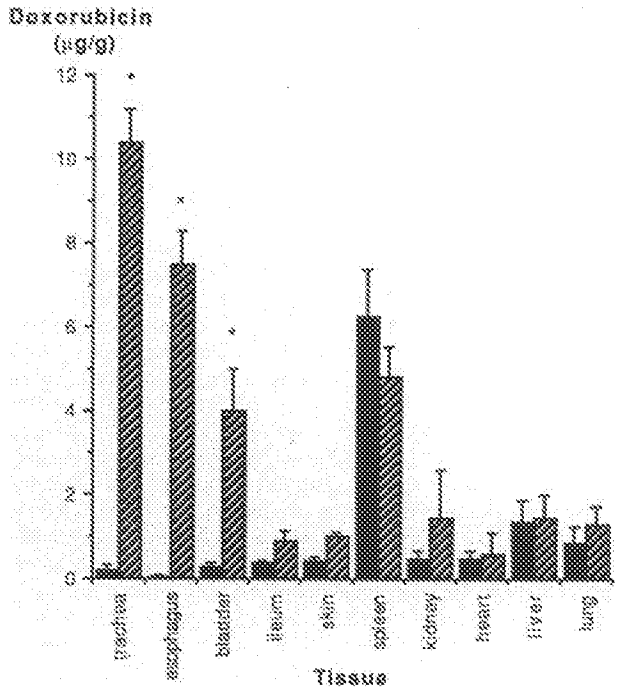

FIG. 2. Effect of SP on the deposition of sterically stabilized liposomes loaded with doxorubicin in various tissues in rats. Liposomes (15 μmol phospholipid per kg of body weight) were injected i.v., and 5 min later either the vehicle control without SP (0.9% saline; solid columns; n = 5) or SP (10 nmol/kg; hatched columns; n = 5) was injected i.v. Animals were euthanized 10 min after liposome injection, and the circulation was perfused with PBS to remove the blood. Doxorubicin concentrations were measured and expressed as μg per g of wet tissue. Data reported as mean ± SEM. *, significantly different from controls ($P < 0.05$).

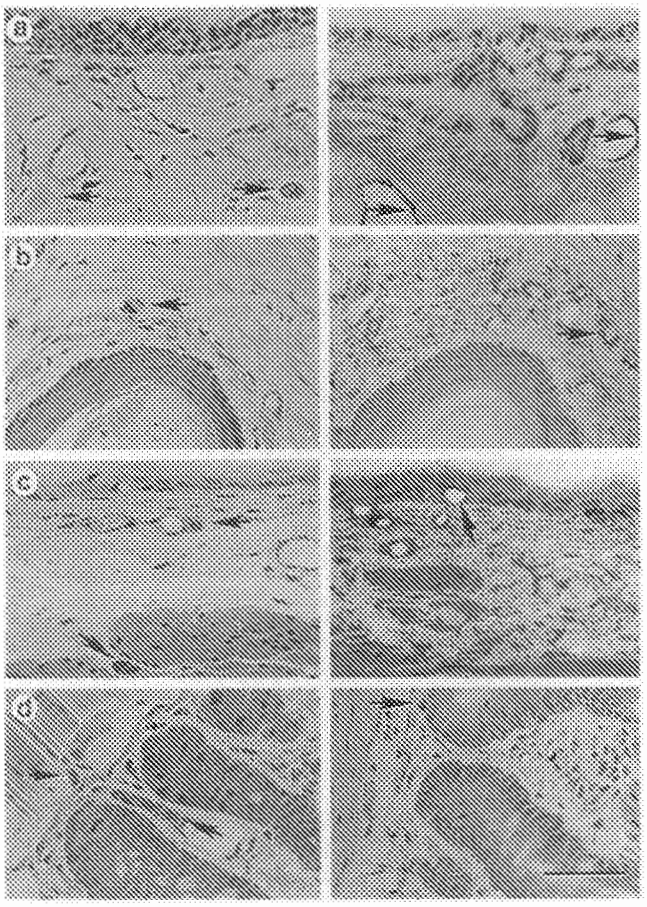

FIG. 3. Effect of SP on the localization of gold-labeled sterically stabilized liposomes in various tissues of rats: *a*, Trachea; *b*, esophagus; *c*, urinary bladder; and *d*, ileum. Animals were euthanized 10 min after liposome injection. When no SP is administered (*Left*), the gold/silver particles representing liposomes are located only in the vascular lumen. In rats given liposomes and then SP 5 min later (*Right*), the particles are dispersed diffusely throughout the submucosal tissue in the trachea, esophagus, and urinary bladder, with the highest concentration of particles surrounding small vessels. Except for occasional small foci, the epithelium remains free of particles. In the ileum, the particles remain in the vascular lumens, even after SP. Staining was performed with hematoxylin. Scale bar = 25 μm. Arrows indicate blood vessels.

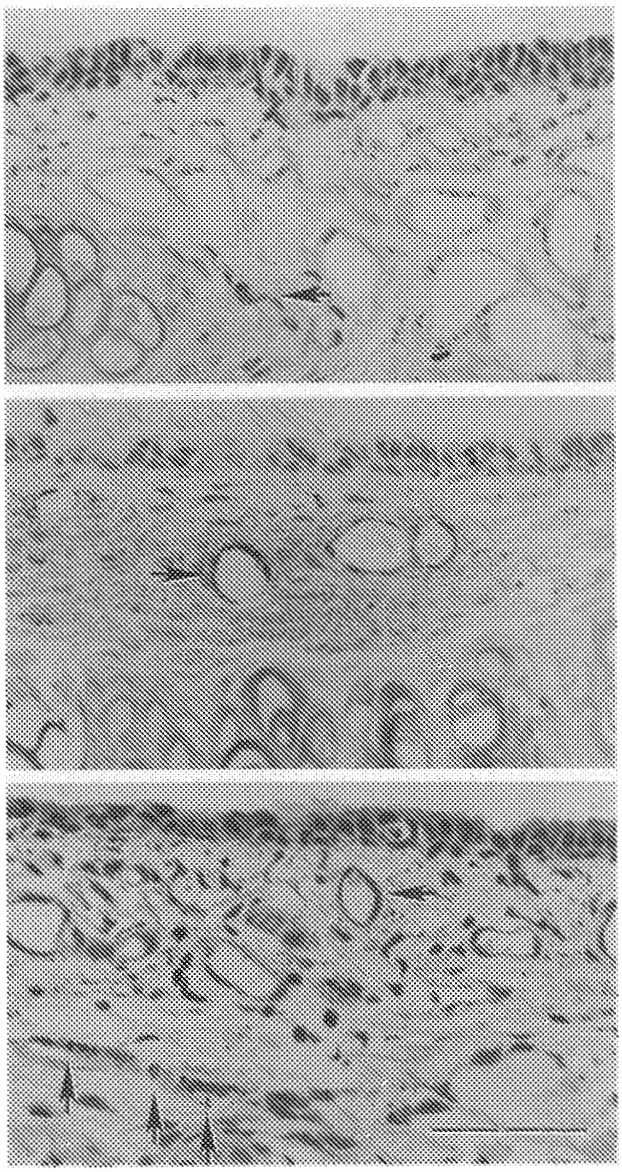

FIG. 4. Time-dependent effect of extravasation and clearance of gold-labeled sterically stabilized liposomes in the trachea of rats. (*Upper*) No SP. The gold/silver particles remain within the vessels. (*Middle*) Five minutes after SP administration, the particles are dispersed diffusely throughout the submucosal tissue. (*Lower*) At 24 h, many of the gold/silver particles are seen in the walls of small blood vessels. Staining was performed with hematoxylin. Bar = 25 μm. Arrows indicate blood vessels.

6 h, there was less widespread dispersion of the gold particles; many particles were localized intracellularly in endothelial cells. This disappearance of widely dispersed particles and their localization in small blood vessels was more evident in the tissue at 24 h (shown at low power in Fig. 4, *Lower*).

In the liver, liposomes were seen intracellularly within the macrophage cells lining the sinusoids, both in control rats and in rats given SP. No difference in the uptake of liposomes was noted between controls and SP-injected rats (data not shown).

DISCUSSION

The purpose of the present study was to examine whether liposomes could be made to "home" into selected tissues by causing an increase in their vascular permeability. We injected SP, a neuropeptide that is known to cause vascular extravasation, in selected tissues whose postcapillary venules possess SP receptors (e.g., trachea, esophagus, and urinary bladder) but not in organs whose postcapillary venules do not possess SP receptors (e.g., ileum, heart, and kidney; refs. 17 and 22). Our results indicate that SP causes striking increases in the tissue concentrations of both rhodamine-PE and doxorubicin in trachea, esophagus, and urinary bladder, but not in other tissues. These results are impressive, especially considering the short period of time that SP is capable of increasing extravasation (22). In fact, we used SP to increase vascular permeability to test whether very short periods of extravasation could allow selective deposition of liposomes in tissues. It is known that when SP is injected i.v., extravasation in the trachea occurs only for a short time (half-time, 2.4 min; ref. 23). Thereafter, there is long-lasting desensitization to SP. Other studies suggest that the rapid desensitization to SP is due, at least in part, to rapid internalization of SP receptors (24). In the airways, internalization of NK1 receptors is believed to play a role in limiting the duration of SP-induced extravasation (25).

It has been recognized for some time that various mediators of inflammation (e.g., histamine and SP) cause increased vascular permeability by opening gaps in the postcapillary venular endothelium (26); this allows plasma proteins and other macromolecules (either resident in the blood or injected

into the circulation as "markers") to escape into the extravascular tissue. One such marker is Evans blue dye, which binds spontaneously to plasma albumin and thus tests the permeability of blood vessels to molecules of ≈60 kDa. When vascular permeability is increased, the dye extravasates into the extravascular tissue and moves rapidly through the connective tissue after extravasation. Particulate tracers of much larger dimensions, such as Monastral blue (greatest dimension, ≈300 nm) have also been injected into the bloodstream (27). Monastral blue normally remains in the circulation and is cleared slowly by the reticuloendothelial system. However, after injection of SP, the particles of dye leak through the endothelium in organs that contain SP receptors. These large particles are reported to remain trapped in the endothelial basal lamina (27).

Sterically stabilized liposomes (diameter, ≈100 nm) remain in the peripheral blood for long periods of time (10) and uptake by normal tissues is limited, except for tissues with resident macrophage cells such as the liver and spleen (10). Other tissues reported to accumulate long-circulating liposomes are tumors (11–14) and *Klebsiella pneumoniae*-infected lung tissue (15, 16). In the present study, we examined the "homing" of liposomes to selected tissues by increasing vascular permeability selectively. For quantitative estimation of liposome deposition in tissues, we used fluorescence spectroscopy, using two different fluorescent labels: rhodamine attached covalently to phosphatidylethanolamine was used as a marker for the uptake of lipid material, and doxorubicin encapsulated into the aqueous interior of liposomes was used as a marker for the presence of liposome contents. Previous studies have shown that doxorubicin can be stably encapsulated in sterically stabilized liposomes, with a half-life in blood of 14–18 h in rodents (10). Its presence in various tissues is an indication for the uptake of liposomes still containing at least some of their encapsulating contents, as has been seen previously in some tumors implanted in mice (11–14). The biochemical markers allowed us to show that SP permits liposomes to leave the bloodstream and to enter selected tissues. We performed morphological studies using liposome-encapsulated colloidal gold to examine the anatomic localization of liposomes in tissues. Previous studies showed that 24 h after i.v. injection of gold-containing liposomes in mice, the ratio of gold-containing to unlabeled liposomes in plasma was unchanged, suggesting that the gold particles in tissue represent intact liposomes (11). Furthermore, electron microscopy has established the presence of intact liposomes, still encapsulating gold, within tissues in mice after 24 h. (13). In the present studies, we used silver enhancement of colloidal gold to localize liposomes in hematoxylin-stained sections of various tissues. Using this technique, silver-coated gold particles could be visualized as black dots by regular light microscopy within the lumens of blood vessels or within tissue, either extracellularly or intracellularly (Figs. 3 and 4).

Analysis of the biochemical and morphological observations described above indicate the following points. (*i*) SP produces a large effect in the tissues whose postcapillary venules possess receptors for SP (trachea, esophagus, and urinary bladder) where the uptake of liposome material per g of tissue weight is strikingly increased after SP, whereas the uptake observed in liver and spleen, (the organs that are normally the main recipients for liposome uptake) is unaffected by SP. (*ii*) In tissues containing SP receptors, extravasation of liposomes is localized in the extracellular spaces of these tissues beyond the postcapillary venular endothelium. In contrast, the uptake in the liver is intracellular, within the macrophage cells lining the sinusoids. (*iii*) Extravasated liposome material in SP-sensitive tissues extends to the vicinity of a variety of cells well beyond the endothelial layer. Thus, in the trachea, the early deposition of liposomes extends from the border of the cartilage to the tissue immediately beneath the epithelium. However, very little uptake of gold particles by epithelial cells was observed. (*iv*) Over a period of many hours after SP, most of the gold particles eventually appear to become concentrated intracellularly, both in tissue macrophages and in endothelial cells. However, it is not certain whether the intracellular gold particles remain encapsulated or released from liposomes at these later time periods. Further studies are required to investigate longterm changes in concentrations of various liposome constituents in tissues where liposomes are deposited.

From the biochemical and morphological findings described above, we conclude that sterically stabilized liposomes, which normally remain in the bloodstream for long periods of time, can be taken up by selected tissues following the injection of substances that modify vascular permeability. In contrast to Monastral blue (23), the liposomes are not confined to the basal lamina but appear to be distributed widely in the affected tissues. The exact mechanisms responsible for the dispersion of particles in the tissues following extravasation are unknown. In addition to physical dimensions, their movement may be related to physicochemical characteristics, such as surface charge and hydrophilicity. After crossing the endothelium, movement beyond the basal lamina cannot be completely limited by physical dimensions, because neutrophils normally migrate across the endothelium and through the tissue without restraint. The dynamics of movement of particulates through tissue is an important, largely unexamined phenomenon.

As shown by the present biochemical and morphological findings, organs such as liver (Kupfer cells), which normally take up liposomes show no change in liposome uptake after SP. Morphologic studies in the liver before and after SP showed that liposomes were only found in the phagocytic cells and within the vascular lumens; none were found adjacent to hepatocytes.

In this study, we have used SP to produce extravasation of liposomes and to increase the deposition in selected tissues. Studies of extravasation using different mediators of inflammation (e.g., histamine, serotonin, and bradykinin) indicate that extravasation sites can be selected by the choice of mediator (22). In the present study, we injected the mediator (SP) i.v., which may have undesirable side effects. We envision the use of the extravasation-enhancing mediator in anatomically restricted areas, such as into the urinary bladder following instillation via a catheter or into airways following inhalation. In such cases, extravasation can be localized to single tissues such as the trachea (28). In this way, the effect of the mediator in enhancing the uptake of circulating liposomes can be expressed in the specific tissues desired without systemic effects.

Our demonstration that the delivery of doxorubicin in the trachea, esophagus, and urinary bladder can be strikingly increased by SP is an example of enhanced local drug delivery, which could be applied to various therapies. In the future, this technique may be useful for such diverse therapies as the delivery of antiinflammatory drugs to selected postcapillary venules or the delivery of genes to selected tissues.

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Anti-CD166 single chain antibody-mediated intracellular delivery of liposomal drugs to prostate cancer cells

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Abstract

Targeted delivery of small-molecule drugs has the potential to enhance selective killing of tumor cells. We have identified previously an internalizing single chain [single chain variable fragment (scFv)] antibody that targets prostate cancer cells and identified the target antigen as CD166. We report here the development of immunoliposomes using this anti-CD166 scFv (H3). We studied the effects of a panel of intracellularly delivered, anti-CD166 immunoliposomal small-molecule drugs on prostate cancer cells. Immunoliposomal formulations of topotecan, vinorelbine, and doxorubicin each showed efficient and targeted uptake by three prostate cancer cell lines (Du-145, PC3, and LNCaP). H3-immunoliposomal topotecan was the most effective in cytotoxicity assays on all three tumor cell lines, showing improved cytotoxic activity compared with nontargeted liposomal topotecan. Other drugs such as liposomal doxorubicin were highly effective against LNCaP but not PC3 or Du-145 cells, despite efficient intracellular delivery. Post-internalization events thus modulate the overall efficacy of intracellularly delivered liposomal drugs, contributing in some cases to the lower than expected activity in a cell line–dependent manner. Further studies on intracellular tracking of endocytosed liposomal drugs will help identify and overcome the barriers limiting the potency of liposomal drugs. [Mol Cancer Ther 2007;6(10):2737–46]

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Introduction

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related death of men in industrialized countries (1, 2). Androgen deprivation and androgen receptor blockade have been the mainstays of treatment for prostate cancer. Although initially effective, hormone therapy fails for the majority of initial responders, as subpopulations of tumor cells undergo mutations and gain capacity to proliferate in an androgen-deprived environment (3, 4). Until recently, the therapeutic alternatives for hormone-refractory prostate cancer were limited (4).

Monoclonal antibodies against tumor markers can be exploited for the development of targeted therapeutics. Phage antibody display is an effective way to generate tumor-specific human antibody fragments (5, 6). For example, internalizing single chain variable fragments (scFv) targeting erbB2 and epidermal growth factor receptor (EGFR) has been identified and used to deliver liposomal drugs to breast cancer cells (7–9). Recently, we have selected a nonimmune phage antibody library containing more than 100 million members on live tumor cells and identified a panel of internalizing scFvs targeting prostate cancer cells (10). We have identified a subset of unique antibodies that (*a*) recognize hormone-refractory cell lines; (*b*) deliver therapeutic payloads efficiently and specifically to tumor cells; and (*c*) stain tumor cells in tissue slides by immunohistochemistry (10, 11). Using immunoprecipitation and mass spectrometry, we have identified the antigen for one of the selected scFv antibodies (H3) as CD166, also known as MEMD or activated leukocyte cell adhesion molecule (11, 12). CD166, a member of the immunoglobulin superfamily, was found to be expressed on cultured prostate cancer cells and specifically in prostate cancer tissues (13–15). Immunohistologic studies detected overexpression of CD166 in 86% of prostate carcinomas (13). In addition, CD166 was shown to undergo ligand-induced endocytosis (14). An internalizing scFv targeting CD166 could thus be used for intracellular delivery of various therapeutic agents to prostate cancer cells.

Liposomal nanocarriers represent one of the most efficient and well-characterized drug delivery strategies for small molecules (16, 17). Liposomal encapsulation of a drug can improve the toxicity profile and therapeutic efficacy of the encapsulated agent by enhancing its localization to solid tumors and optimizing the rate at which it becomes bioavailable due to release from the nanocarrier (16, 18). Therapeutic immunoliposomes contain encapsulated chemotherapeutic drugs and have antibody fragments conjugated to their surface to impart delivery specificity and preferably endocytosis, leading to improved delivery of bioavailable drug and antitumor efficacy.

In this study, we used the internalizing anti-CD166 human scFv to target immunoliposomes to prostate cancer cells. We have constructed H3-targeted immunoliposomes encapsulating small-molecule drugs that disrupt DNA metabolism (topotecan, doxorubicin, and mitoxantrone) and affect microtubule dynamics (vinorelbine) and key post-translational modifications, such as histone deacetylation (LAQ824). The internalization and cytotoxicity of the various scFv-targeted immunoliposomal drug formulations were compared with nontargeted liposomal formulations of the same drugs. We found that H3 scFv-targeted immunoliposome drugs were efficiently delivered intracellularly to prostate cancer cells. However, different immunoliposome drugs exhibited varying degrees of efficacy when compared with the corresponding nontargeted liposomes or free drugs. Anti-CD166 (H3 scFv) immunoliposome topotecan, once delivered intracellularly, was effective in killing prostate cancer cells, whereas nontargeted liposome topotecan or free topotecan were not. Other immunoliposome drugs, such as doxorubicin, however, displayed modest improvements in targeted cytotoxic activity when compared with the nontargeted liposome drugs despite significant intracellular uptake by prostate cancer cells. Thus, events after internalization may significantly modify the efficacies of immunoliposome drugs.

Materials and Methods

Materials

Pepsin A, 2-mercaptoethylamine, 2-mercaptoethanol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich. $DiIC_{18}(3)$-DS was purchased from Molecular Probes/Invitrogen. Distearoylphosphatidylcholine and poly(ethylene)glycol (PEG_{2000})–derivatized distearoyl-phosphatidylethanolamine ($mPEG_{2000}$-DSPE) were purchased from Avanti Polar Lipids. Cholesterol was purchased from Calbiochem and maleimide-derivatized PEG-DSPE (Mal-PEG-DSPE) from Shearwater Polymers. Topotecan was a kind gift of the Taiwan Liposome Co. and LAQ824 of Novartis Oncology. Mitoxantrone dihydrochloride was purchased from LKT Laboratories. Doxorubicin hydrochloride (Bedford Laboratories) and vinorelbine (GlaxoWelcome) were obtained from the pharmacy. Sucrose octasulfate (sodium salt) was purchased from Toronto Research Chemicals, Inc. Sepharose CL-4B and Sephadex G-75 size exclusion resins, Dowex 50W-8X-200 cation exchange resin, and triethylamine were purchased from Sigma-Aldrich.

Cell Lines

All cell lines (PC3, Du-145, LNCaP, and SKBR3) were obtained from American Type Culture Collection. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2.

Production of H3 scFv′

The coding region of the H3 scFv was spliced into an expression vector imparting a cysteine and a hexahistidine tag at the COOH terminus as described (10). Following IPTG induction, antibody fragments were purified from bacterial periplasmic space on Ni^+-NTA beads (Qiagen) as described (10).

Preparation of Fluorescence-Labeled and Drug-Loaded Liposomes

Fluorescently labeled unilamellar liposomes were prepared according to the repeated freeze-thawing method of Szoka and Papahadjopoulos (19). Liposomes were composed of distearoylphosphatidylcholine, cholesterol, mPEG-DSPE, and the lipophilic fluorescent marker, $DiIC_{18}(3)$-DS, combined in a 200:133:1:1 molar ratio. Liposomes were subsequently extruded 10 to 15 times through polycarbonate filters with defined pore sizes of 0.1 μm, yielding liposomes of 100 to 120 nm diameter as determined by dynamic light scattering. Liposomal phospholipid concentrations were determined using a standard phosphate assay (20).

For encapsulation of doxorubicin, a gradient-based drug loading method using diethylammonium sulfate was used (21, 22). First, the dried lipids distearoylphosphatidylcholine/cholesterol/mPEG-DSPE (molar ratio, 3:2:0.015) were dissolved in ethanol and heated to 60°C. The ethanolic lipid solution was subsequently injected into a heated solution (60°C) of 200 mmol/L diethylammonium sulfate (pH 5.5), followed by extrusion of the hydrated lipid suspensions at 60°C through polycarbonate filters with uniform pore sizes ~0.1 μm. Free diethylammonium sulfate was removed by size-exclusion chromatography using a Sephadex G-75 column eluted with HEPES-buffered saline [5 mmol/L HEPES, 145 mmol/L NaCl (pH 6.5)]. Liposomes were then incubated with doxorubicin (150 μg drug/μmol phospholipid) for 30 min at 60°C, and unencapsulated doxorubicin was removed by gel filtration chromatography using a Sephadex G-75 column eluted with HEPES-buffered saline [5 mmol/L HEPES, 145 mmol/L NaCl (pH 7.2)]. The drug mitoxantrone was loaded into liposomes using the same method.

Liposomal topotecan of an identical lipid composition was prepared using a novel intraliposomal drug stabilization strategy as described (23). One modification from the published method (23) was that the drug entrapping solution was diethylammonium sucrose octasulfate [0.65 mol/L DEA (pH 5.5)]. Diethylammonium sucrose octasulfate was prepared from the commercially obtained sodium salt by ion exchange chromatography on the Dowex 50Wx8-200 resin in the H^+ form, immediately followed by titration with neat diethylamine. Following extrusion, unentrapped diethylammonium sucrose octasulfate was removed on a Sepharose CL-4B size exclusion column eluted with HEPES-buffered dextrose [5 mmol/L HEPES, 5% dextrose (pH 7.0)]. Topotecan was then added at 350 g drug/mol phospholipid and the pH was adjusted to 6.0 to 6.5 with 1 N HCl before initiating loading at 60°C for 30 min. The resulting liposomal topotecan was subsequently placed on ice for 15 min and purified on a Sephadex G-75 column to remove unencapsulated drug. The histone deacetylase inhibitor LAQ824 and the *Vinca* alkaloid, vinorelbine, were loaded using methods described previously (24, 25).

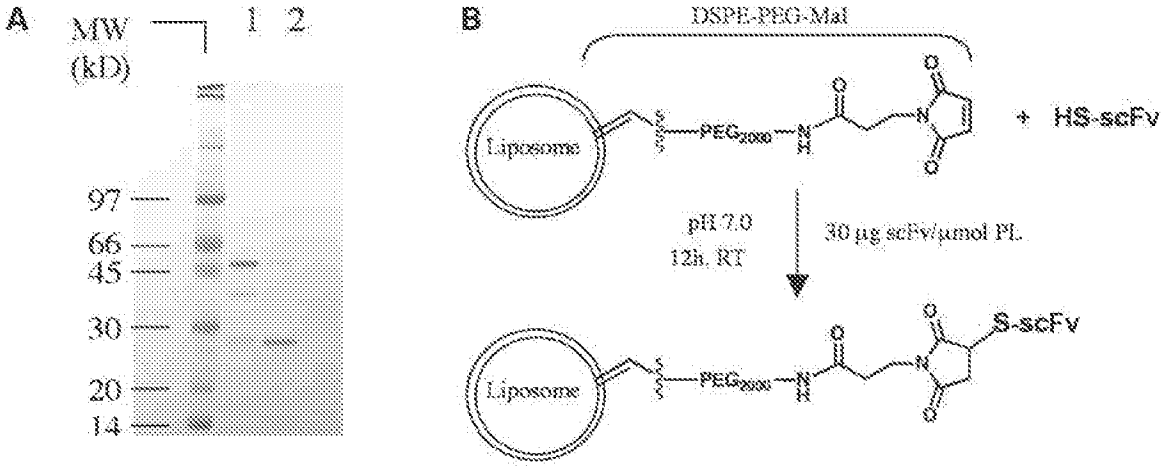

Figure 1. Preparation of H3 scFv-targeted immunoliposomes. **A**, analysis of scFv reduction by nonreducing SDS-PAGE. *Lane 1*, unreduced scFv preparation; *lane 2*, scFv reduced with 2-mercaptoethylamine. *MW*, molecular weight. **B**, outline of conjugation of the reduced scFv containing a reactive sulfhydryl group with DSPE-PEG-Mal–activated liposomes. Thirty micrograms of scFv were conjugated per micromole of phospholipids (*PL*), corresponding to ~60 scFvs per liposomal particle. *RT*, room temperature.

Construction of H3 scFv-Targeted Immunoliposomes

Purified H3 $(scFv')_2$ were reduced with 20 mmol/L mercaptoethylamine by incubation at 37°C for 45 min in PBS containing 2 mmol/L EDTA (pH 6.0) deoxygenated by bubbling argon through it. Reduced scFvs were subsequently recovered by purification on a Sephadex G-25 gel filtration column eluted with HEPES-buffered saline [5 mmol/L HEPES, 145 mmol/L NaCl, 3.4 mmol/L EDTA (pH 7.0)]. Reduction efficiencies were evaluated by SDS-PAGE. Typically >90% of scFv dimers were reduced. The liposomes were activated by incorporation of Mal-PEG-DSPE as follows: Mal-PEG-DSPE was dissolved in water and added to the preformed liposomes at 0.5 mole% of the liposome phospholipid, and the mixture was incubated at 60°C for 30 min (26, 27). The pH was increased to 7.0 by titration with a HEPES buffer [0.5 mol/L (pH 7.0)]. Reduced scFv was incubated with activated liposomes overnight at room temperature at 30 μg/μmol of phospholipids, corresponding to ~60 scFv/liposome. The ligands were thus attached to the outer lipid monolayer of preformed liposomal therapeutic or fluorescent liposomes via hydrophilic polymers linked to hydrophobic DSPE domains. An excess of 2-mercaptoethanol (2 mmol/L final concentration) was added to inactivate all unreacted maleimide groups. Unincorporated conjugates, unconjugated scFv, and any released free small-molecule drugs were separated from the resulting immunoliposomes using a Sepharose CL-4B gel filtration column eluted with HEPES-buffered saline (pH 6.5; 5 mmol/L HEPES, 145 mmol/L NaCl).

Quantification of encapsulated drugs was done using a photometric method: the liposomes sample (5–20 μL) was dissolved in a 1 mL total solution of acid isopropanol [70% 2-propanol (v/v), 30% double-distilled water (v/v), and 20 mmol/L HCl] for doxorubicin and LAQ824, or acidic methanol [90% methanol (v/v) and 10% 0.1 mol/L H_3PO_4 (v/v)] for topotecan, vinorelbine, and mitoxantrone, and the absorbance was read at 375 nm (topotecan), 278 nm (vinorelbine), 498 nm (doxorubicin), 664 nm (mitoxantrone), or 277 nm (LAQ824) and compared with a standard solution of free drug (0–20 μg/mL). Each sample was analyzed in triplicate. Liposome matrix was quantified by phosphate assay (20).

Internalization Studies by Fluorescence Microscopy

Prostate cancer cells (PC3, Du-145, and LNCaP) grown to 80% confluency in 24-well plates were coincubated with H3 scFv-targeted immunoliposomes or nontargeted liposomes labeled with $DiIC_{18}(3)$-DS, at concentrations of 0 to 50 μm phospholipids, for 4 h at 37°C. The cells were washed twice with PBS and examined through a Nikon Eclipse 300 inverted fluorescence microscope (Nikon) with a 540/25 nm bandpass filter for excitation and a long pass filter at 565 nm for emission.

Flow Cytometry Analysis

$DiIC_{18}(3)$-DS–labeled liposomes were used for these studies. Cells grown to 80% confluency in 12-well plates were coincubated with $DiIC_{18}(3)$-DS–labeled immunoliposomes or nontargeted liposomes, at concentrations of 0 to 50 μmol/L phospholipids, for 4 h at 37°C. After washing with either PBS or 100 mmol/L glycine/150 mmol/L NaCl (pH 2.8), cells were analyzed by fluorescence-activated cell sorting (BD LSRII). The ratios of the mean fluorescence intensity between the glycine-washed and PBS-washed cell samples were used to calculate the percentage of internalized liposomes. To reproduce conditions used in the drug studies, cells were further incubated in fresh medium without immunoliposomes for an additional 8 h to determine fraction internalized (typically >85%) by glycine treatment. The fraction released into the medium during the 8-h chase (typically <10%) was determined by comparing total cell-associated mean fluorescence intensity values before and after the 8-h chase.

Cellular Uptake of Immunoliposome Drugs

Prostate cancer cells were seeded in 12-well plate at 350,000 cells per well and incubated in triplicates with liposomal drugs (immunoliposomes and nontargeted liposomes at 10 μg/mL) for 4 h at 37°C. After 4-h incubation, cells were washed thrice with PBS and lysed with 1% Triton X-100 (100 μL/well) for 10 min at 37°C. For the samples containing doxorubicin, 70% acid isopropanol was added, whereas for topotecan and vinorelbine, a mixture of acetonitrile/methanol 1:1 and acidic methanol (500 μL/well) was added. The plates were shaken for 30 min at room temperature. The samples were then centrifuged at 10,000 rpm for 2 min and the supernatants were measured by fluorescence spectroscopy (Fluorolog II, Jobin Yvon Spex) for doxorubicin ($\lambda_{Ex/Em}$ = 470/590 nm) and topotecan ($\lambda_{Ex/Em}$ = 382/530 nm). The concentration of topotecan or doxorubicin was calculated by extrapolation using a standard curve and normalized to the cell number, and cellular uptake was expressed as nanogram drug per million cells. For vinorelbine, the samples were loaded on a C_{18} reverse-phase silica column (Supelco C-18 column, 250 × 4 mm i.d., particle size of 5 μm) preceded by a C-18 guard column. The column was eluted isocratically with aqueous 50 mmol/L triethylammonium acetate (pH 5.5) and acetonitrile (58:42, v:v) at a flow rate of 1.0 mL/min and detected by absorbance at 280 nm. A typical retention times for vinorelbine was 9.1 min.

In vitro Cytotoxicity

Prostate cancer cells were seeded in 96-well plates (6,000 cells per well for PC3 and Du-145 cells; 10,000 cells per well for LNCaP cells) and incubated with liposomes (immunoliposomes and nontargeted liposomes) or free drugs (0–10 μg/mL) for 4 h at 37°C. Cells were washed twice with supplemented RPMI 1640 to remove drugs and incubated with fresh medium for an additional 72 h at 37°C. Cell viabilities of PC3 and Du-145 cells were assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining as described (28), and

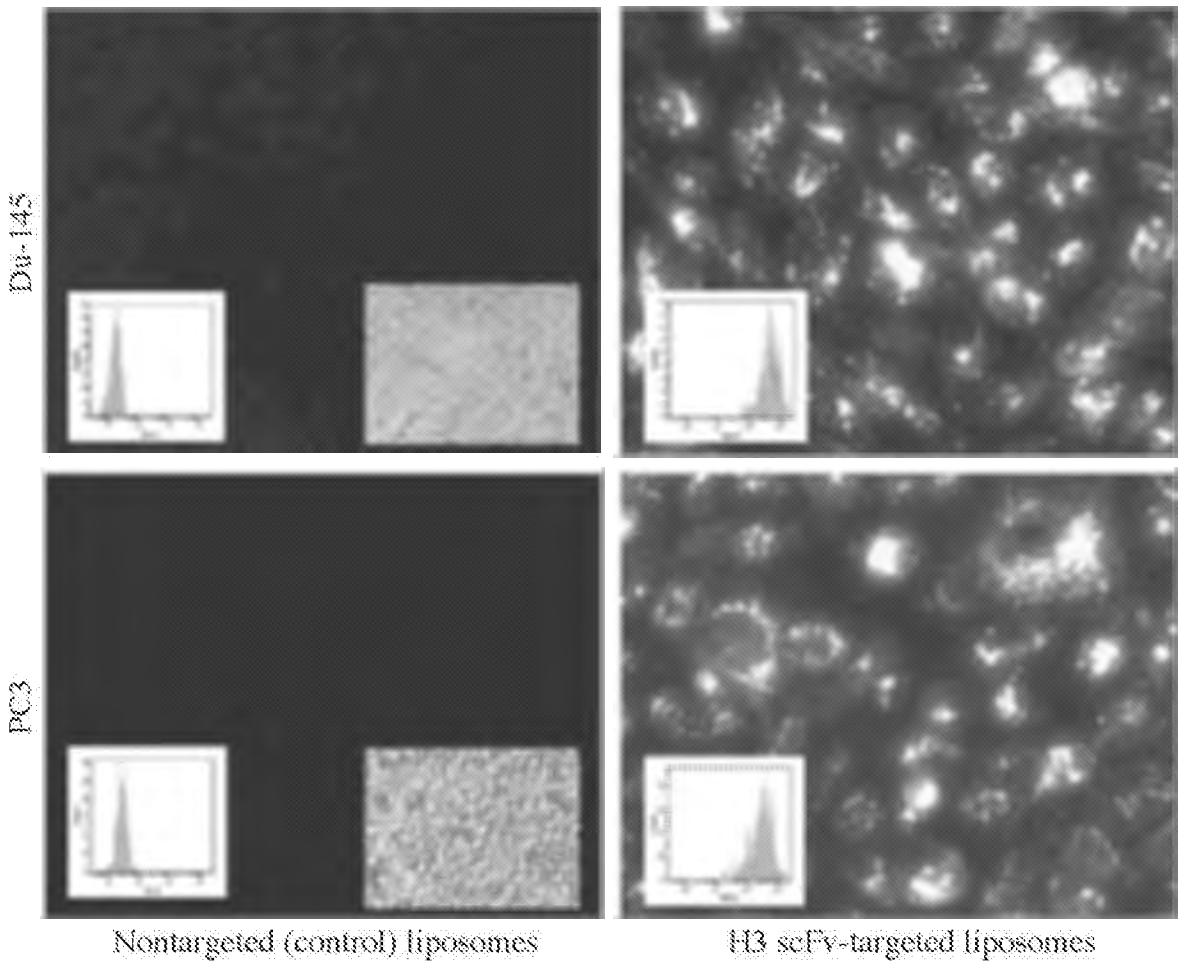

Figure 2. Internalization of H3 scFv-targeted immunoliposomes. Du-145 and PC3 cells were treated with H3 scFv-targeted $DiIC_{18}(3)$-DS–labeled immunoliposomes (25 μmol/L phospholipids) or control nontargeted liposomes at 37°C for 4 h, and uptake was examined by fluorescence microscopy. *Insets*, fluorescence-activated cell sorting analysis of fluorescence signals and images of phase-contrast microscopy to indicate the presence of cells.

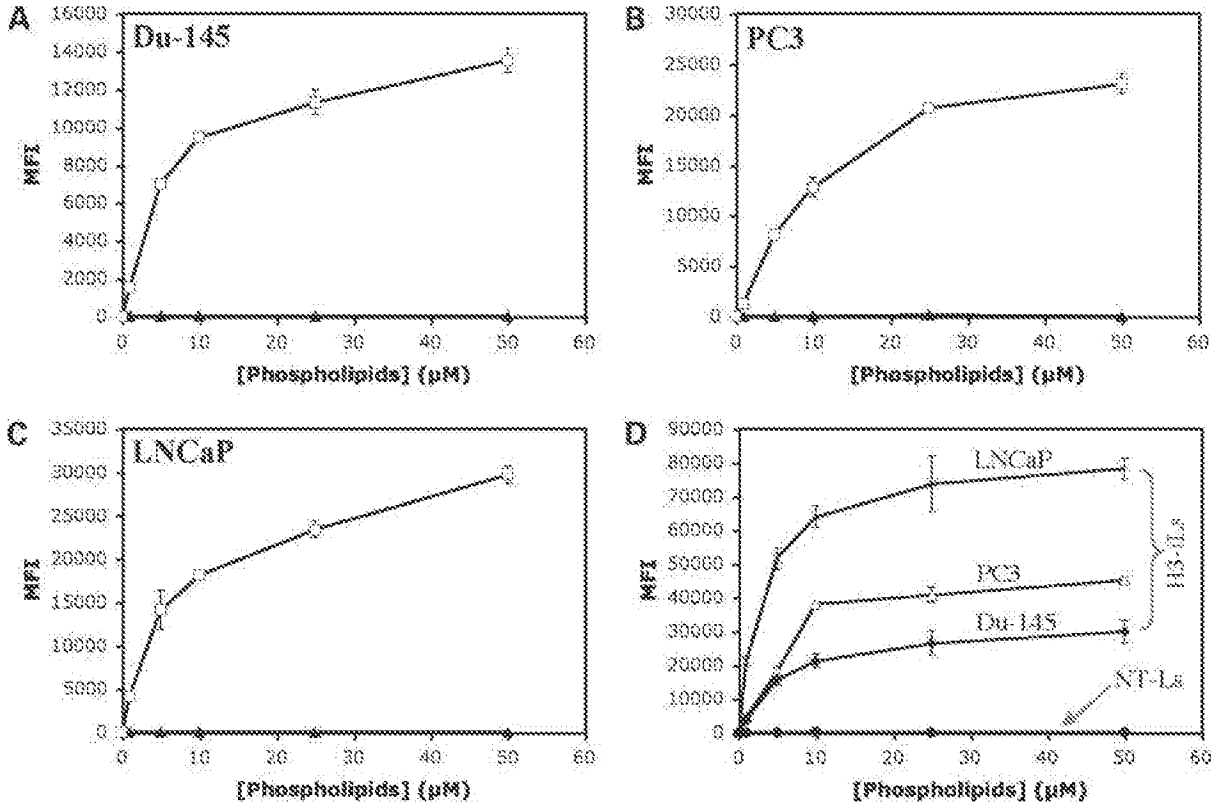

Figure 3. Binding and uptake of H3 scFv-targeted immunoliposomes to prostate cancer cell lines. A to C, binding of immunoliposomes (□) or nontargeted liposomes (▲) to Du-145 (A), PC3 (B), and LNCaP cells (C) at 4°C over a range of phospholipid concentrations was determined by fluorescence-activated cell sorting. The K_d's are calculated to be 4.5, 5.0, and 7.0 μmol/L phospholipids for Du-145, PC3, and LNCaP cells, respectively. Because 10,000 immunoliposomes contain about 1.32 fmol phospholipids (22), the K_d's correspond to 75, 83, and 116 pmol/L immunoliposomes. D, concentration-dependent liposomal uptake after 4-h incubation at 37°C by Du-145 (♦), PC3 (△), and LNCaP (×) cells. There was no significant uptake of nontargeted liposomes (*NT-Ls*, ●) by any of the three prostate cancer cells studied. *Points*, mean; *bars*, SD.

the results were read at 570 nm using a microtiter plate reader (SpectraMax 190, Molecular Devices). The cell viability of LNCaP cells was determined using the Cell counting kit-8 (Dojindo) according to the manufacturer's instructions.

Results

Construction of Immunoliposomes Targeting Prostate Cancer

Liposomes were prepared using a lipid molar ratio distearoylphosphatidylcholine/cholesterol/mPEG-DSPE 3:2:0.015, and 0.5 mole% Mal-PEG-DSPE was further inserted for antibody conjugation. Liposomes were either labeled with the fluorescent lipid $DiIC_{18}(3)$-DS or loaded with a chemotherapeutic drug, such as doxorubicin, topotecan, vinorelbine, mitoxantrone, or LAQ824. The gene encoding the H3 scFv was spliced into a bacteria expression vector to create an in-frame fusion with the amino acid cysteine and the hexahistidine tag (10). For conjugation to liposomes, the $(scFv')_2$ were purified and reduced using 2-mercaptoethylamine. Approximately 90% of H3 $(scFv')_2$ were in a reduced form as estimated from a nonreducing SDS-PAGE gel (Fig. 1A). The reduced scFv was then conjugated via its cysteine to maleimide-modified termini of PEG chains located on the external surface of the liposomes (Fig. 1B), producing 100 to 120 nm diameter liposomes bearing ~60 scFvs on the surface.

Internalization of scFv-Targeted Immunoliposomes in Prostate Cancer Cell Lines

Internalization of H3 scFv-targeted immunoliposomes in prostate cancer cell lines was evaluated by fluorescence microscopy and flow cytometry. For this purpose, liposomes were fluorescently labeled with $DiIC_{18}(3)$-DS and conjugated with the reduced H3 scFv. Fluorescence microscopy showed significant uptake of H3 scFv-targeted immunoliposomes by prostate cancer cell lines (Fig. 2). Without scFv, nontargeted liposomes were not significantly endocytosed (Fig. 2), showing the importance of scFv on the liposome surface for targeted delivery. We further quantified liposome binding and uptake by fluorescence-activated cell sorting. H3 scFv-targeted immunoliposomes bound to prostate cancer cells in a concentration-dependent manner (Fig. 3A–C). H3-targeted immunoliposomes bound to all three prostate cancer cell lines with K_d values in the range of 4.5 to 7 μmol/L phospholipids (corresponding to 75–116 pmol/L immunoliposomes). At saturating

concentrations (50 μmol/L phospholipids), more immunoliposomes were bound by LNCaP than Du-145 and PC3 cells, suggesting a higher level of surface expression of CD166 on LNCaP cells. In all experiments, nontargeted liposomes did not show significant binding to prostate cancer cells, consistent with the results of the fluorescence microscopy studies.

We further did a dose-response experiment at 37°C, which permits receptor endocytosis (Fig. 3D). Below ~10 μmol/L phospholipids, the amount of immunoliposomes associated with prostate cancer cells was linearly related to the amount of liposomes added. Above 15 μmol/L, a plateau was observed for all prostate cancer cells studied (Fig. 3D). There was no association of nontargeted liposomes to any of the prostate cancer cells studied across all the concentrations tested (Fig. 3D).

Uptake of H3 scFv-Targeted Immunoliposome Drugs by Prostate Cancer Cells

Three liposomal drugs were studied for cell uptake: doxorubicin, topotecan, and vinorelbine. In all experiments, nontargeted liposome drugs were poorly taken up by prostate cancer cells (Fig. 4A and B). In contrast, delivery of drugs by H3 scFv-targeted immunoliposomes to prostate cancer cells was efficient, and high uptake ratios of immunoliposome drugs versus nontargeted liposome drugs were obtained. The level of uptake varied depending on the cell lines and specific liposomal drugs. LNCaP cells took up more liposomal doxorubicin than PC3 and Du-145 cells (1,802 ng drug/million LNCaP cells versus 277 ng drug/million cells for Du-145 cells and 177 ng drug/million cells for PC3 cells; Fig. 4A). The uptake of H3 scFv-targeted immunoliposomes encapsulating topotecan was similar in the three cell lines (between 731–1,299 ng drug/million cells; Fig. 4B) and ranged from 20- to 38-fold greater than observed for the corresponding nontargeted liposomes. Similarly, H3 scFv-targeted immunoliposome vinorelbine was efficiently taken up by prostate cancer cells, with uptake reaching 559 ng drug/million Du-145 cells, 1,173 ng drug/million PC3 cells, and 2,670 ng drug/million LNCaP cells (data not shown). The targeting by H3 scFv thus promoted efficient drug delivery to prostate cancer cells. All these data are consistent with the results obtained using DiI-labeled H3 scFv-targeted immunoliposomes (see Fig. 2).

In vitro Cytotoxicity Studies

Five anticancer drugs were studied, targeting various cellular functions. Topotecan, doxorubicin, and mitoxantrone

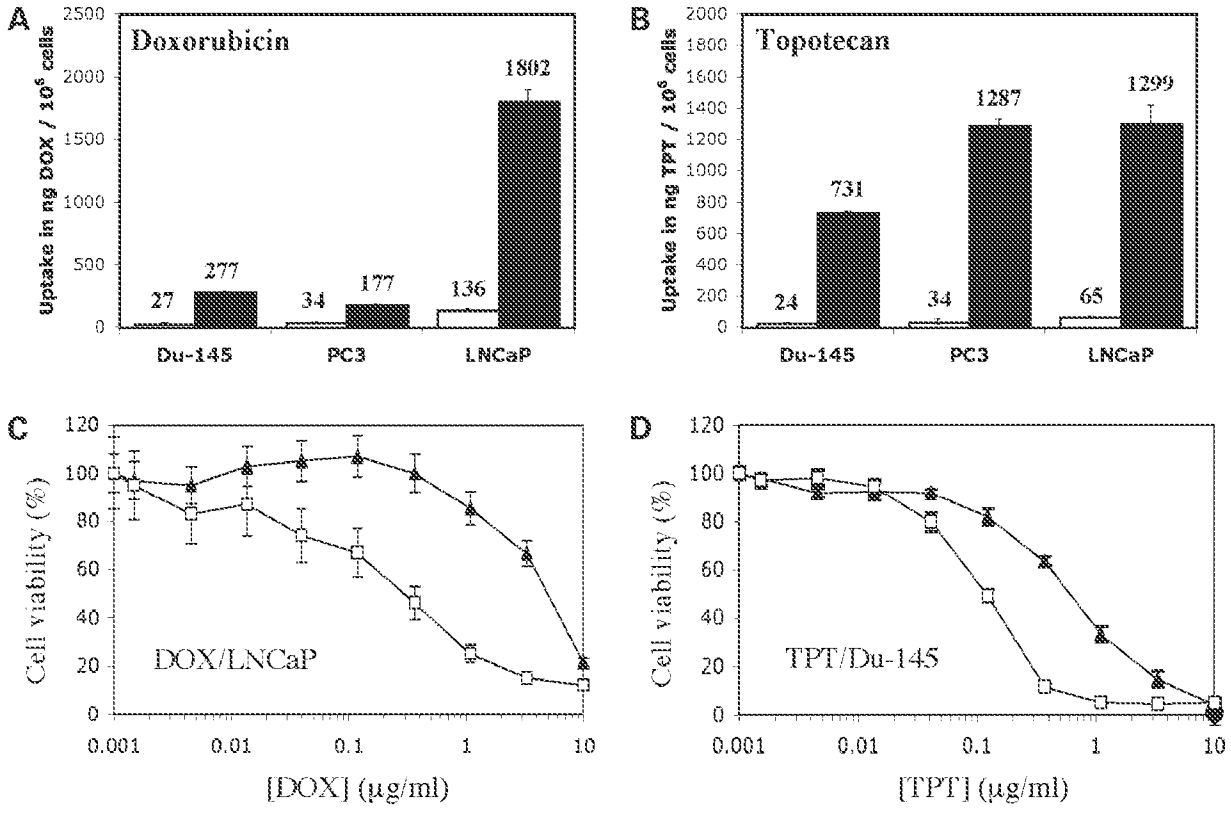

Figure 4. Uptake and *in vitro* cytotoxicity of H3 scFv-targeted immunoliposome drugs in prostate cancer cell lines. For uptake studies, immunoliposome and nontargeted liposome drugs were incubated with prostate cancer cell lines at concentrations of 10 μg drug/mL for 4 h at 37°C. After lysing the cells, doxorubicin (*DOX*; **A**) and topotecan (*TPT*; **B**) were quantified by fluorescence. *White columns*, uptake of nontargeted liposomes; *black columns*, uptake of H3 scFv-targeted immunoliposomes. *Columns*, mean; *bars*, SD. Data are given in nanogram drug per million cells. For *in vitro* cytotoxicity studies, LNCaP (**C**) and Du-145 (**D**) cells were treated with nontargeted liposomes (▲) or H3 scFv-targeted immunoliposome (□) drugs at varying concentrations. Cytotoxicity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability was calculated as a percentage of living cells. *Points*, mean; *bars*, SD.

Table 1. Cytotoxic effects (IC_{50}, μg/mL) of various drugs in free, liposomal, or H3 scFv-targeted immunoliposomal forms, on prostate cancer and control cell lines

Drug		PC3		Du-145		LNCaP		SKBR3	
		IC_{50}	Ratio	IC_{50}	Ratio	IC_{50}	Ratio	IC_{50}	Ratio
TPT	Free	3.4		0.50		3.2		0.85	
	NT-Ls	>10		0.6		0.14		0.75	
	H3-iLs	1.6	>6	0.13	5	0.029	5	0.70	1.07
DOX	Free	0.15		0.086		0.047		0.080	
	NT-Ls	>10		9.0		7.4		8.2	
	H3-iLs	7.3	>1.5	6.4	1.5	0.19	39	7.5	1.09

NOTE: The IC_{50} ratios (nontargeted liposomes/immunoliposomes) are calculated to indicate enhancement in cytotoxicity of H3-targeted immunoliposome over nontargeted liposome drugs.
Abbreviations: TPT, topotecan; Dox, doxorubicin; NT-Ls, nontargeted liposomes; iLs, immunoliposomes.

target DNA metabolism, vinorelbine disrupts microtubule function, and LAQ824 inhibits histone deacetylation. We first studied sensitivity of prostate cancer cells to free drugs. Other than topotecan, which has a poor membrane permeability (29, 30), the other drugs in their free forms showed potent cytotoxicities toward prostate cancer cells (Supplementary Table S1).[6] The cytotoxic effects (IC_{50}) of the same drugs in liposomal forms, either H3 scFv targeted or nontargeted, are shown in Table 1. In most instances, drug-loaded nontargeted liposomes showed a weak cytotoxicity toward the cells studied. Some nontargeted liposomes showed cytotoxicity at the highest concentrations tested, probably due to a small amount of weakly associated drug or leakage of drug from the liposomes during incubation with prostate cancer cells. H3 scFv-targeted immunoliposome drugs showed various levels of *in vitro* cytotoxicity. Treatment with H3 scFv-targeted immunoliposome topotecan was 5-fold more efficient in killing Du-145 cells than nontargeted liposome topotecan (IC_{50}, 0.13 versus 0.6 μg/mL; Fig. 4D; Table 1). Similar improvements in cytotoxicity were obtained for LNCaP cells (0.029 μg/mL immunoliposomes versus 0.14 μg/mL nontargeted liposomes) and PC3 cells (1.6 versus >10 μg/mL; Table 1). There was no significant improvement (immunoliposome topotecan versus nontargeted liposomes) in cytotoxicity on the control cell line SKBR3 (Table 1; the CD166 expression on SKBR3 was shown in Supplementary Fig. S1). The immunoliposome topotecan is thus a promising therapeutic for the treatment of prostate cancer.

Treatment with H3 scFv-targeted immunoliposome doxorubicin was 39-fold more potent in killing LNCaP cells than nontargeted liposome doxorubicin (IC_{50}, 0.19 versus 7.4 μg/mL; Fig. 4C; Table 1). However, H3 scFv-targeted immunoliposome doxorubicin did not show significant improvement in cytotoxicity in PC3 or Du-145 cells, when compared with nontargeted liposome doxorubicin, although both cell lines were sensitive to free doxorubicin (Table 1). The sensitivity of LNCaP cells to free doxorubicin was similar to that of Du-145 cells (Table 1). It is interesting to note that LNCaP cells took up about six times more immunoliposome doxorubicin (see Fig. 4A) than Du-145 cells, possibly due to a higher level of CD166 expression on those cells (see Fig. 3). No improvement in cytotoxicity (immunoliposomes versus nontargeted liposomes) was observed in the control cell line SKBR3 (Table 1).

The improvement of other immunoliposomes over nontargeted liposome drugs is rather moderate. H3 scFv-targeted immunoliposome vinorelbine showed a 4-fold improvement in cytotoxicity compared with nontargeted liposome vinorelbine in Du-145 cells, 2-fold in PC3 cells, and 3.5-fold in LNCaP cells (data not shown). The improvement in cytotoxic activity for immunoliposome mitoxantrone and LAQ824 was about 1.5- to 3-fold over nontargeted liposome drugs (data not shown).

The above studies show that although H3 scFv-targeted immunoliposome drugs were efficiently delivered intracellularly into prostate cancer cells, their potencies vary depending on the drug and the cell line used. The immunoliposome topotecan was the most effective among the various drugs that we have studied for cytotoxic activity in all three prostate cancer cell lines.

Potency of a Liposomal Drug versus a Free Drug

The aforementioned studies revealed discrepancies between cytotoxicity profile and uptake for some of the drugs studied. For example, H3 scFv-targeted immunoliposome doxorubicin was readily taken up by PC3 and Du-145 cells but its cytotoxicity was not significantly improved compared with nontargeted liposomes. To understand the relationship between the amount of liposomal drug accumulated in the cells and its cytotoxic effects, we compared viabilities of cells taking up equal amounts of free versus immunoliposome drug. We first measured concentration-dependent uptake of free doxorubicin in Du-145 cells (Fig. 5A). For example, when incubated with 1.0 μg/mL free doxorubicin, 277 ng doxorubicin was taken up per 10^6 cells. This amount of cellular uptake of free drug was sufficient to kill 91% of Du-145 cells (Fig. 5B and C). Approximately the same amount of liposomal doxorubicin/10^6 cell was delivered to the Du-145 cells after 4-h incubation of H3 scFv-targeted immunoliposome doxorubicin (at 10 μg drug/mL; Fig. 4A). If doxorubicin is efficiently liberated from its liposomal carrier on internalization, then one liposomal doxorubicin molecule should be as potent as one free doxorubicin molecule, and the amount delivered should theoretically be sufficient to cause similar cytotoxic effects in Du-145 cells. However, only 58% killing of Du-145 cells was achieved (Fig. 5B), indicating that at equal intracellular concentration, liposome-encapsulated doxorubicin was less efficient in cell killing compared with free drug. Thus, to achieve similar levels of cell killing, more immunoliposome doxorubicin was needed to be delivered into target cells than free drug.

[6] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

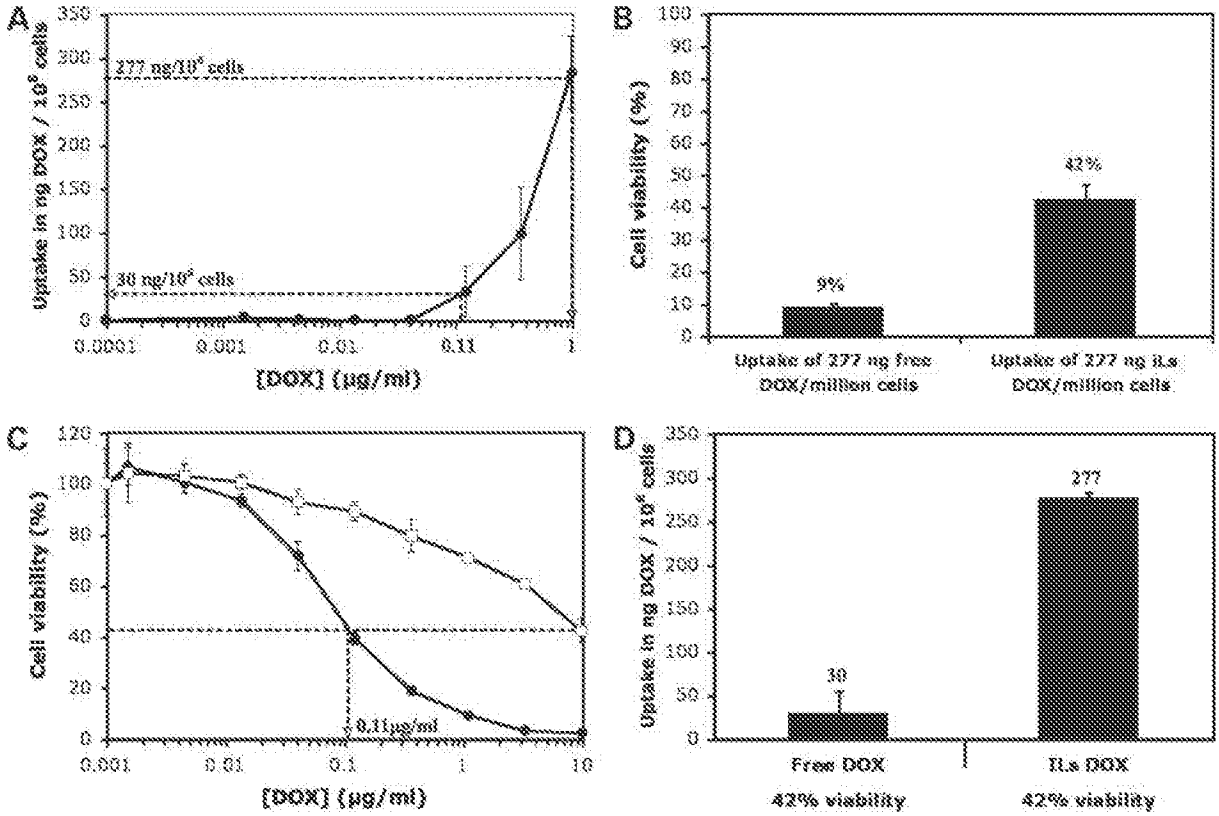

Figure 5. Efficacy of intracellularly delivered immunoliposome doxorubicin versus free drug. **A**, uptake profile of free doxorubicin in Du-145 cells. Free doxorubicin (●) was incubated with Du-145 cells at different drug concentrations, for 4 h at 37°C. After lysing the cells, doxorubicin was quantified by fluorescence (λ_{Ex} = 470 nm; λ_{Em} = 590 nm). Results are expressed as nanogram of doxorubicin per 10^6 cells. **B**, killing efficiencies of identical amounts (277 ng drug/10^6 cells) of immunoliposome (*IL-s*) doxorubicin and free drug delivered intracellularly to Du-145 cells. After incubation of 1.0 μg/mL free doxorubicin or H3 scFv-targeted immunoliposome doxorubicin at 10 μg/mL with cells for 4 h at 37°C, 277 ng drug/10^6 cells were delivered intracellularly. **C**, cytotoxicity profile of free doxorubicin and immunoliposome drug. To achieve 58% killing (42% viability), Du-145 cells needed to be incubated with 10 μg/mL immunoliposome drug or 0.1 μg/mL free drug, corresponding to 277 ng immunoliposome drug/10^6 cells or 30 ng free drug/10^6 cells, were delivered intracellularly. **D**, amounts of intracellularly delivered immunoliposomes (277 ng immunoliposome doxorubicin/10^6 cells) and free doxorubicin (30 ng free drug/10^6 cells) needed to achieve similar levels of cell killing. *Points* and *columns*, mean; *bars*, SD.

For example, to achieve 58% killing (42% cell viability), 277 ng of immunoliposome doxorubicin were needed to be delivered per 10^6 cells, whereas only 30 ng of free doxorubicin per 10^6 cells were needed to achieve the same effect, a difference in efficacy of about 9-fold (Fig. 5D).

Discussion

Targeted cancer therapy requires the combination of a tumor recognition and a tumor killing function in a single therapeutic entity. We have previously identified an internalizing scFv, H3, which targets prostate cancer cells. The H3 scFv binds to an internalizing epitope of CD166 (12), which is overexpressed in both primary and metastatic prostate carcinomas (13, 31, 32). We developed H3 scFv-targeted immunoliposomes and studied cytotoxicity profiles of a panel of liposomal small-molecule drugs following intracellular delivery to prostate cancer cells. The use of scFv instead of whole IgG molecule prevents Fc receptor-mediated clearance *in vivo* (33, 34), protecting liposomes from rapid clearance by macrophages of the reticuloendothelial system (25, 35).

We constructed immunoliposomes encapsulating a panel of small-molecule drugs and studied their cytotoxic activity in prostate cancer cells following H3 scFv-targeted delivery. These drugs target different cellular functions. Topotecan, doxorubicin, and mitoxantrone disrupt DNA metabolism, whereas vinorelbine and LAQ824 disrupt microtubule assembly and inhibit histone deacetylation, respectively. We first studied uptake of fluorescently labeled immunoliposomes and found a significant increase in intracellular delivery compared with fluorescently labeled nontargeted liposomes. We next quantified uptake of liposomal drugs by prostate cancer cells. We found that, like the fluorescently labeled immunoliposomes, H3 scFv-targeted immunoliposome drugs were efficiently internalized by the three prostate cancer cells studied. Nontargeted liposome drugs were not efficiently taken up by prostate cancer cells, showing the importance of a targeting function in mediating efficient payload delivery to tumor cells.

Although scFv targeting the CD166 epitope was effective in mediating intracellular delivery of liposomal drugs, the

cytotoxic effects of the drugs differed considerably between drugs. H3 scFv-targeted delivery of liposomal topotecan achieved significantly greater cell killing than either nontargeted liposome or free topotecan, showing the benefit of a targeting mechanism. Unlike the other drugs studied, free topotecan did not cross cell membranes readily, and there was no measurable uptake in any of the prostate cancer cells studied even at 10 μg/mL (data not shown). This is consistent with the poor membrane permeability of the carboxylate form of the drug at neutral pH and thus poor cellular uptake and cytotoxicity (29, 30). Therefore, the use of a liposome carrier for topotecan is particularly relevant to its therapeutic effects.

For other drugs, however, efficient intracellular delivery did not readily predict their potency in cell killing. For example, immunoliposome doxorubicin was efficiently delivered to all three prostate cancer cells, but it was only in LNCaP cells that we observed a significant increase in potency compared with its nontargeted liposome counterpart. The total uptake of immunoliposome doxorubicin by LNCaP cells was several times higher than that of Du-145 and PC3 cells, possibly due to higher surface expression of CD166 on LNCaP cells as revealed by our fluorescence-activated cell sorting binding studies. Nonetheless, the amount of doxorubicin molecules delivered to Du-145 cells by H3 scFv-targeted immunoliposomes reached the level that should have been sufficient to kill those cells, as predicted from the uptake and cytotoxicity profile of free drug. It is thus evident that intracellularly delivered liposomal doxorubicin, on a mole-per-mole basis, is less potent in cell killing than free doxorubicin. We have shown that for Du-145 cells, 9-fold more immunoliposome doxorubicin than free drug needs to be delivered intracellularly to achieve the same level (58%) of cell killing.

Several factors may account for this observed discrepancy. For example, subcellular localization of endocytosed immunoliposomes and the release rate of encapsulated drugs may all have profound influence on the efficiency of liposomal drugs. Endocytosed immunoliposomes are often routed through the endosome and lysosome pathway (9, 36, 37). For encapsulated doxorubicin, release from liposomes and further escape from endosomes/lysosomes are required for translocation to the nucleus to disrupt DNA metabolism (38). Chloroquine and omeprazole increase endosome pH and have been reported to increase the rate of release of free doxorubicin trapped inside the endosome (39). Treatment of prostate cancer cells with chloroquine or omeprazole, either concurrent to or immediately after liposomal drug delivery, however, did not lead to significant changes in cytotoxicity of immunoliposome doxorubicin (data not shown), indicating that doxorubicin molecules remained encapsulated inside the liposomes. Further development of pH-sensitive liposome formulations may facilitate the collapse of endosome-trapped liposomes, allowing more efficient release of encapsulated drug (38, 39).

In conclusion, conjugating liposomes with a scFv targeting the internalizing epitope of CD166 is an effective strategy to deliver small-molecule drugs to prostate cancer cells. Once delivered inside the cells, liposomal drugs exhibited different cytotoxic effects compared with equal amounts of their corresponding free drugs. It is evident that post-internalization events modify the potency of liposomal drugs and in many cases reduce their efficacies compared with their free drug counterparts. This reduced efficacy, however, helps create a threshold effect that may increase the specificity of targeted killing by liposomal drugs as many tumor cell surface antigens are also expressed on normal cells at lower densities. In practice, the threshold is likely to vary depending on cell types, drugs used, and the formulation of liposomes. From the perspective of tumor cell surface antigens, the threshold hypothesis predicts that simultaneously targeting multiple epitopes will achieve greater potency than targeting a single epitope alone. Constructing such multitargeting immunoliposomes may offer one way to improve efficacy and specificity of tumor cell killing. Nonetheless, the demonstrated levels of prostate cancer uptake and cytotoxicity of the anti-CD166 scFv-targeted immunoliposome drugs warrant further investigation *in vivo* using tumor models, as targeting may further improve efficacy by increasing intratumoral distribution of the immunoliposome drugs (40).

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Molecular Cancer Therapeutics

Anti-CD166 single chain antibody-mediated intracellular delivery of liposomal drugs to prostate cancer cells

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Alternative Dosing Schedules for Irinotecan

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Most of the clinical experience with irinotecan (CPT-11 [Camptosar]) has been with either a weekly or an every-3-week schedule. Recent phase I trials have explored new routes and schedules of administration. One approach

ABSTRACT: Most of the clinical experience with irinotecan (CPT-11 [Camptosar]) has been with either a weekly or an every-3-week schedule. Recent phase I trials have explored new routes and schedules of administration. One approach attempts to maximize dose frequency and intensity by giving irinotecan every 2 weeks. A phase I trial of this approach is now complete and has led to a phase II trial in patients with recurrent colorectal cancer. Data suggest that smaller doses of a topoisomerase I inhibitor administered repeatedly may result in greater antitumor activity than larger doses administered intermittently. A phase I trial has been performed in adults in which irinotecan was administered daily for 5 consecutive days, followed by 2 days off, for 2 weeks out of 3. Similar trials are under way in children. Oral administration, another strategy that has undergone phase I testing, has several theoretical advantages: (1) The acidic pH of the stomach favors maintenance of irinotecan in the active lactone ring form. (2) Irinotecan is more rapidly and extensively converted to SN-38 by tissue carboxylesterases found in high concentrations in the gut and liver. (3) Low doses can be delivered over a protracted period. (4) The oral route enhances patient convenience. These alternative dosing schedules may facilitate integration of irinotecan into combination chemotherapy and combined-modality treatment regimens. [ONCOLOGY 12(Suppl 6):68-71, 1998]

Introduction

Four factors must be taken into account in the development of new methods of administration for a topoisomerase I inhibitor (Table 1), such as irinotecan hydrochloride (CPT-11 [Camptosar]). First, the dosing schedule should optimize *biological* effects resulting in topoisomerase inhibition. These include not only exposure of the

cells to the topoisomerase I inhibitor during the S-phase of the cell cycle and prolonged stabilization of the cleavable complex, but also periodic release of inhibition to avoid the downregulation of cellular topoisomerase I levels as a mechanism for drug resistance.[1]

The second factor that should be considered is camptothecin *pharmacology*. In the case of irinotecan, this would mean selection of a dosing schedule that generates the highest (and potentially most effective) plasma and intracellular levels of SN-38, the active metabolite of irinotecan. The schedule would also take into account ways in which the conversion of irinotecan to SN-38 could be maximized and the balance between SN-38 and its inactive metabolite, SN-38 glucuronide (SN-38G) could be optimized.

The third consideration in selecting a schedule of drug administration is toxicity. Some schedules may be associated with less severe (or different) toxicity profiles without a loss of efficacy.

Lastly, pragmatic factors should be taken into account, including the regimen's cost, convenience, and ease of compliance. An additional pragmatic consideration is how well the schedule accommodates other drugs and/or modalities, such as radiation, that will be given with irinotecan.

Since the introduction of irinotecan into clinical trials more than a decade ago, a number of clinical strategies have been pursued in an attempt to identify the schedule with the optimal balance between clinical activity, safety, and convenience. The two schedules that have been used most often in phase II and III testing are: (1) the weekly schedule (most popular in the United States and Japan), in which the drug is administered once a week for 4 consecutive weeks, followed by 2 weeks of rest; and (2) the once-every-3-week schedule (the most commonly used schedule in Europe) [2]

Irinotecan, like all of the camptothe- cins, is considered a cell-cycle-specific drug. The lethal lesion is created when the DNA replication fork collides with a single-strand DNA break that has been created by topoisomerase I and has been stabilized (ie, not allowed to reseal) by the camptothecin analog. When the replication fork encounters the stabilized cleavable complex, the single-strand break is converted into an irreversible--and lethal--double-strand DNA break. [3] This is referred to as the "fork-collision" model. Given that cells must be in S-phase of the cell cycle for the fork collision to occur and that, at any given time, only a small percentage of cells are in S-phase, one could argue that more frequent (ie, weekly) dosing would be most desirable.

On the other hand, many drugs, including irinotecan, have a clear dose-response relationship in vitro.[4] This suggests that irinotecan should be given at the highest single dose possible in order to achieve maximal

antitumor effect. This approach has been taken in the development of irinotecan in Europe, where the drug is most commonly administered at a dose of 350 mg/m^2 once every 3 weeks. In addition to exploiting the dose-response relationship, this approach has the added advantage of greater patient convenience, as it entails less frequent dosing than is required on a weekly schedule.

Alternative Dosing Strategies

As our knowledge of and experience with topoisomerase I inhibitors have increased, several new routes and schedules of drug administration have been explored in phase I trials.

Protracted Intravenous Dosing Schedule

One strategy that has been tested recently is a protracted intravenous (IV) dosing schedule.[5] Since the DNA-topoisomerase I cleavable complex is readily reversible, and the plasma half-life of SN-38, the active metabolite of irinotecan, is 13 to 15 hours, daily dosing may approximate the level of topoisomerase I inhibition achievable with continuous infusion but without the requirement for a central line or infusion pump. In addition, lower doses of irinotecan, given more frequently, could allow more complete glucuronidation to take place and result in decreased toxicity.[6] Lastly, in vitro and in vivo data suggest that camptothecin analogs may have enhanced activity and reduced toxicity when lower doses are administered more frequently.[7,8]

Saltz and colleagues from Memorial Sloan-Kettering Cancer Center performed a phase I trial in which patients were given intravenous irinotecan on a daily × 5 basis.[5] When the starting dose of 10 mg/m^2/d for 5 days every 3 weeks proved to be tolerable, the duration of treatment was extended to 2 weeks, with treatment given on a Monday-through-Friday schedule (ie, 5 days on, 2 days off) every 3 weeks. A total of 21 patients were treated at dose levels of 10 to 22 mg/m^2/d. Late-onset, grade 3 diarrhea and neutropenic fever were the dose-limiting toxicities (DLTs) at the 22-mg/m^2/d dose level. Two patients with colorectal cancer had objective partial responses, and an additional six patients (five with colorectal cancer and one with soft-tissue sarcoma) achieved stable disease as their best response. The maximum tolerated dose (MTD)/recommended phase II dose was determined to be 17 mg/m^2 in heavily pretreated patients.

A follow-up study combining this daily IV dosing schedule with pelvic radiation in patients with locally advanced rectal cancer has been initiated.

Two ongoing trials are examining the toxicity and tolerability of low-dose, daily IV irinotecan in children. The Pediatric Oncology Group is evaluating irinotecan given daily × 5 every 3 weeks, while researchers at St. Jude Children's Research Hospital are testing a daily × 5 schedule given every week for 4 consecutive weeks,

followed by a 2-week rest. Both studies are performing detailed pharmacokinetic assessment.

An alternative approach with a similar rationale is being explored in an ongoing phase I trial at the National Cancer Institute. In this trial, irinotecan is being given as a 96-hour continuous IV infusion for 2 out of 3 weeks.

Every-Two-Week Dosing Schedule

The finding of similar antitumor activity, but slightly different toxicity profiles, with the weekly and every-3-week schedules piqued interest in administering irinotecan once every 2 weeks to determine whether toxicity could be modified while antitumor activity was retained. A total of 51 patients were treated with irinotecan at dose levels ranging from 125 to 325 mg/m^2 administered every 2 weeks. Of the 51 patients, 41 (80%) had received prior chemotherapy and 20 (40%), prior radiation. The median number of prior chemotherapy regimens was 3 (range, 1 to 10).

Dose-limiting toxicity, in the form of grade 4 neutropenia and fever, occurred in two of two patients treated at the 300-mg/m^2 dose level. The study was amended to include granulocyte colony-stimulating factor (G-CSF, filgrastim [Neupogen]), 5 mg/kg subcutaneously on days 2 through 13. A second DLT of delayed, grade 3 diarrhea (one patient) and grade 3 vomiting despite optimal antiemetics (one patient) was defined for irinotecan at 325 mg/m^2 every 2 weeks (with G-CSF).

Non-dose-limiting grade 3 vomiting (acute and delayed) occurred at the 275-mg/m^2 dose level. A serotonin antagonist plus dexamethasone given before and for 3 days after chemotherapy effectively prevented this toxicity. Grade 3 diarrhea occurred in only 1 of 10 patients treated at the MTD/recommended phase II dose of irinotecan of 250 mg/m^2 (without G-CSF) and in none of 6 patients treated at the MTD/recommended phase II dose of irinotecan of 300 mg/m^2 (with G-CSF).

Pharmacokinetic analysis revealed a linear relationship between irinotecan dose and area under the concentration-time curves (AUCs) for irinotecan and, to a lesser extent, SN-38, suggesting that the peripheral conversion of irinotecan to SN-38 is not saturated at these doses. The AUC for SN-38G appeared to increase with irinotecan dose, suggesting that hepatic glucuronidation is not saturated at doses up to 325 mg/m^2.

Severe diarrhea was not a frequent toxicity with this schedule. No pharmacodynamic relationship between diarrhea and the AUCs for irinotecan, SN-38, or SN-38G was identified, and biliary index did not predict the occurrence of grade 3 or 4 diarrhea. Two patients with recurrent colorectal cancer achieved partial responses, lasting 6.8 and 13.4 months, and 26 patients had stable disease as their best response.

Although the spectrum of toxicity with the every-2-week schedule appeared to be similar to that observed with other irinotecan schedules, neutropenia and vomiting appeared to be more common in this trial, while delayed diarrhea seemed to be less common. The every-2-week schedule has now been tested in a phase II trial in patients with fluorouracil-refractory colorectal cancer and has demonstrated antitumor activity similar to that attained with a weekly or every-3-week schedule.[9]

Oral Dosing Schedule

As noted above, low-dose, daily administration of irinotecan has been shown to be more effective and less toxic than higher-dose, intermittent administration in vitro and in vivo.[7,8] However, the oral route of administration has several additional advantages. First, the oral route takes advantage of low gastric pH, which may favor retention of the drug in the active lactone ring configuration. Second, high concentrations of tissue carboxylesterases in the liver and gut should promote presystemic conversion of irinotecan to SN-38. This, coupled with the first-pass effect, should result in high concentrations of SN-38 in the liver, the most common site of colon cancer metastases. Lastly, the oral route should provide a convenient, cost-effective option for protracted dosing of irinotecan.

A phase I trial of short-course, oral irinotecan has now been completed. [Drengler RL et al, unpublished data, 1998] In this trial, the intravenous preparation of irinotecan was administered orally once daily for 5 consecutive days every 3 weeks. The safety, pharmacokinetics, and antitumor activity of irinotecan were assessed in 28 patients.

Late-onset, grade 4 diarrhea, with or without concomitant grade 4 neutropenia, was the DLT. Different MTDs were defined for patients above and below the age of 65 years; the MTD (and recommended phase II dose) was 66 mg/m^2/d for patients ≤ 65 years old and 50 mg/m^2/d for those > 65 years old.

Several provocative observations were made regarding the pharmacokinetic behavior of irinotecan given by the oral route. There was an association between irinotecan dose and SN-38 pharmacokinetics, implying that there was no saturation of the enzymes responsible for the conversion of irinotecan to SN-38 when irinotecan was administered orally. There was a very high ratio of SN-38 AUC (both the total and glucuronide forms) to irinotecan AUC, suggesting that the oral route resulted in substantial presystemic conversion of irinotecan to SN-38. Lastly, more than two thirds of all the SN-38 present during the first 24 hours following drug administration was detectable in the active lactone ring form.

In addition to these important pharmacokinetic observations, clinical antitumor activity was also observed. One patient with previously treated colorectal cancer and liver metastases achieved a

confirmed partial response, and an additional 17 patients experienced stable disease lasting from 2.5 to 14.7 months.

Several follow-up phase I studies using an encapsulated oral form of irinotecan have now been initiated.

Conclusions

The sound scientific rationale for exploring alternative dosing routes and schedules of irinotecan is being borne out by the data emerging from phase I trials. Although the *spectrum* of toxicity seen with new routes and schedules of administration does not appear to differ from that observed with the more traditional administration schedules, the relative *frequency* and *severity* of irinotecan-induced toxicities seem to vary with different routes and schedules.

Antitumor activity has been observed in phase I trials using these newer approaches. As we gain more experience with the newer routes and schedules of drug administration, we can expect to see the emergence of more convenient, more effective combination chemotherapy and combined-modality treatment regimens using irinotecan.

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Related Content:
News

FDA Approves Margetuximab-cmkb, Chemo Combo for Pretreated Metastatic HER2+ Breast Cancer

December 16, 2020
Hannah Slater

The FDA approved margetuximab-cmkb (Margenza) in combination with chemotherapy for the treatment of adult patients with metastatic HER2-positive breast cancer who have received 2 or more prior anti-HER2 regimens, at least 1 of which was for metastatic disease.

The FDA has approved margetuximab-cmkb (Margenza) in combination with chemotherapy for the treatment of adult patients with metastatic HER2-positive breast cancer who have received 2 or more prior anti-HER2 regimens, at least 1 of which was for metastatic disease, according to MacroGenics, the developer of the agent.

The approval was based on safety and efficacy results observed in the pivotal, randomized, open-label, phase 3 SOPHIA trial (NCT02492711). The trial compared margetuximab-cmkb plus chemotherapy to trastuzumab (Herceptin) plus chemotherapy in patients with HER2-positive metastatic breast cancer who had previously been treated with anti-HER2-targeted therapies.

Overall, the study demonstrated a statistically significant 24% reduction in the risk of disease progression or death with margetuximab-cmkb plus chemotherapy compared with trastuzumab plus chemotherapy (HR, 0.76; 95% CI, 0.59-0.98; *P* = .033; median progression-free survival [PFS], 5.8 vs 4.9 months). The objective response rate (ORR) for margetuximab-cmkb plus chemotherapy was 22% and 15% for trastuzumab plus chemotherapy.

The final overall survival (OS) analysis is expected to be reported in the second half of 2021.

"Early detection and treatment have had a positive impact on the survival of patients with breast cancer, but the prognosis for people diagnosed with metastatic breast cancer remains poor, and additional treatments are needed," Hope S. Rugo, MD, professor of Medicine and director of Breast

Oncology and Clinical Trials Education at the University of California San Francisco Helen Diller Family Comprehensive Cancer Center, said in a press release. "As the only HER2-targeted agent to have shown a PFS improvement versus trastuzumab in a head-to-head Phase 3 clinical trial, MARGENZA with chemotherapy represents the newest treatment option for patients who have progressed on available HER2-directed therapies."

A total of 536 patients were randomized 1:1 to receive either margetuximab-cmkb (n = 266) given intravenously at a dose of 15 mg/kg every 3 weeks or trastuzumab (n = 270) given intravenously at a dose of 6 mg/kg (or 8 mg/kg for loading dose) every 3 weeks in combination with 1 of 4 chemotherapy agents (capecitabine, eribulin, gemcitabine, or vinorelbine) given at the standard doses. All of the study participants had previously received trastuzumab, all but 1 had previously received pertuzumab, and 91% had previously received ado-trastuzumab emtansine (T-DM1).

Of note, an intent-to-treat PFS analysis was planned following 265 PFS events.

The dual primary end points of the study are sequentially assessed PFS, determined by blinded, centrally reviewed radiological review, followed by overall survival (OS). Additional key secondary end points are PFS by investigator assessment, ORR, and duration of response. Moreover, tertiary end points include ORR by investigator assessment and safety. PFS and ORR were evaluated according to Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1).

Regarding safety, adverse events (AEs) occurring in greater than 20% of patients with margetuximab-cmkb in combination with chemotherapy were fatigue/asthenia (57%), nausea (33%), diarrhea (25%), and vomiting (21%).

Importantly, the margetuximab-cmkb US prescribing information includes a boxed warning for left ventricular dysfunction and embryo-fetal toxicity.

Additionally, margetuximab-cmkb may cause infusion related reactions (IRRs); IRRs occurred in 13% of patients treated with margetuximab-cmkb, though most IRRs were reported as grade 2 or less. Grade 3 IRRs occurred in 1.5% of patients.

In addition to the phase 3 SOPHIA trial, margetuximab-cmkb is also being evaluated in combination with checkpoint blockade in the phase 2/3 MAHOGANY trial for the treatment of patients with HER2-positive gastroesophageal cancer (NCT04082364), and in combination with tebotelimab (PD-1 × LAG-3 bispecific DART molecule) in various HER2-positive tumors (NCT03219268).

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Related Content:
News | HER2-Positive Breast Cancer

Laurie H. Sehn, MD, MPH, Discusses the Extension Arm of the Phase 2 GO29365 Trial in DLBCL

December 16, 2020
Laurie H. Sehn, MD, MPH

Conference | ASH
DLBCL

The ongoing GO29365 study of combination regimens containing polatuzumab vedotin (Polivy) for patients with relapsed or refractory diffuse large B-cell lymphoma added an additional 106 patients to confirm preliminary findings of safety and efficacy.

Initial findings from the randomized cohort of GO29365 (NCT02257567), a phase 1b/2 study that examined polatuzumab vedotin (Polivy) plus bendamustine (Bendeka) and rituximab (Rituxan; BR) versus BR alone for patients with relapsed or refractory diffuse large B-cell lymphoma, led to the FDA granting accelerated approval to the antibody-drug conjugate in June 2019. Results from that study showed improved progression-free survival and overall survival for patients in the polatuzumab arm.

In an interview with CancerNetwork®, Laurie H. Sehn, MD, MPH, of the BC Cancer Centre for Lymphoid Cancers, talked about the rationale behind amending the GO29365 study to include an additional extension cohort.

Transcription:

The study that was presented was an update of the [trial comparing] polatuzumab vedotin [Polivy] and bendamustine/rituximab [BR] combination versus BR alone. This was a randomized phase 2 trial that documented the effectiveness of polatuzumab vedotin. But in addition to a follow-up of the original randomized phase 2, we also presented data on an extension cohort of an additional 106 patients who were treated with that combination.

So, the original trial was a randomized phase 2 trial and rolled 40 patients in each arm, so it included 40 patients treated with [polatuzumab vedotin and BR]. The extension arm was really designed to further evaluate the merit of that combination by adding an additional 106 patients.

Related Content:
ASH DLBCL | News | Conference | Diffuse Large B-Cell Lymphoma

Vikram M. Narayan, MD, on Nadofaragene Firadenovec Efficacy Across Different Patient Subgroups for NMIBC

December 16, 2020
Vikram Narayan, MD

Conference | SUO

At the 2020 SUO Meeting, Vikram M. Narayan, MD, spoke about the preliminary finds of his work with nadofaragene firadenovec.

Vikram M. Narayan, MD, spoke with *CancerNetwork®* regarding the findings from a handful of posters presented at the 21st Annual Meeting of the Society of Urologic Oncology researching investigating nadofaragene firadenovec as a treatment of patients with non–muscle invasive bladder cancer (NMIBC).

Transcription:

With respect to the projects that we did, specifically in our first project, our goal was to analyze efficacy in various subgroups. That is to say, we wanted to look at factors like age, gender, and prior treatments that patients received, including the number of courses of bacillus Calmette-Guérin [(BCG)] or non-BCG therapies that they may have had. We wanted to understand whether these factors effected how patients responded to nadofaragene. So, the study that was published met its primary end point in that 53% of patients who had CIS [carcinoma in situ] achieved a complete response by 3 months. But our first poster looked at, – if you were to look at subgroups, do [whether] certain patients do better or worse and can we use that information to guide decision making?

The first take home message was that we found that in our analysis was that there were no significant differences in the response rates to nadofaragene, both at 3 months and at 15 months, when comparing between males and females, different age groups, [or] whether or not they had gotten different courses of BCG or non-BCG treatments. And what this tells us is that regardless of the patient population, nadofaragene appears to be similarly efficacious which allows it to be potentially applicable to a broader range of patients.

Related Content:
SUO | News | Conference | Bladder Cancer

MammoScreen AI Tool Improves Diagnostic Performance of Radiologists in Detecting Breast Cancer

December 16, 2020
Hannah Slater

This study demonstrated that the concurrent use of this new artificial intelligence tool alongside mammography improved the diagnostic performance of radiologists in the detection of breast cancer without prolonging their workflow.

A clinical investigation published in *Radiology: Artificial Intelligence* demonstrated that the concurrent use of a new artificial intelligence (AI) tool improved the diagnostic performance of radiologists in the detection of breast cancer by mammography without prolonging their workflow.[1]

Researchers used MammoScreen, an AI tool designed to identify regions suspicious for breast cancer on 2D digital mammograms and determine their likelihood of malignancy. The system produces a set of image positions with scores for suspicion of malignancy that are extracted from the 4 views of a standard mammogram.

"The results show that MammoScreen may help to improve radiologists' performance in breast cancer detection," Serena Pacilè, PhD, clinical research manager at Therapixel, where the software was developed, said in a press release.[2]

In this multireader, multicase retrospective study, a dataset including 240 digital mammography images were analyzed by 14 radiologists by a counterbalance design, where each half of the dataset was read either with or without AI in the first session and vice versa for a second session, with the 2 sessions separated by a washout period. End points assessed by the investigators included area under the receiver operating characteristic curve (area under the curve [AUC]), sensitivity, specificity, and reading time.

Overall, the average AUC across readers was 0.769 (95% CI, 0.724-0.814) without the use of AI and 0.797 (95% CI, 0.754-0.840) with AI. The average difference in AUC was 0.028 (95% CI, 0.002-0.055; $P = .035$). The investigators said these data indicate greater interreader reliability with the aid of AI, resulting in more standardized results.

Further, average sensitivity was increased by 0.033 when AI support was utilized ($P = .021$). Reading time changed dependently with the AI-tool score.

For those with a low likelihood of malignancy (< 2.5%), the time was about the same in the first reading session and slightly decreased in the second reading session. For those with a higher likelihood of malignancy, the reading time was generally increased with the use of AI.

"It should be noted that in real conditions, additional factors may have an impact on reading time (ie, stress, tiredness, etc), and that those factors were obviously not considered in the present analysis," explained the authors.

Importantly, the main limitation of this study was that the used dataset was not representative of normal screening practices. Specifically, a high rate of false-positive readings may have resulted due to readers awareness of the dataset being enriched with cancer cases, causing a laboratory effect. Moreover, because readers had no access to prior mammograms of the examined patients, other images, or additional patient information,

the assessment was more challenging than a typical screening mammography reading workflow.

"...the overall conclusion of this clinical investigation was that the concurrent use of this AI tool improved the diagnostic performance of radiologists in the mammographic detection of breast cancer," wrote the authors. "In addition, the use of AI was shown to reduce false negatives without affecting the specificity."

In March, the FDA cleared MammoScreen for use in the clinic, where it could aid in reducing the workload of radiologists. Moving forward, the investigators plan to continue to explore the behavior of the AI tool on a large screening-based population and its ability to detect breast cancer earlier.

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News | Screening | Technology

Lisa La on Areas of Interest Within Multiple Myeloma

December 16, 2020
Lisa La

The clinical researcher discussed areas of multiple myeloma which she believes deserve further assessment.

In an interview with *CancerNetwork®*, Lisa La, director of clinical research in the Center for Cancer Care at White Plains Hospital, spoke about what areas of multiple myeloma she believes deserve further evaluation.

Transcription:

The last 5 or 6 years have really focused on heavily treated relapsed/refractory myeloma patients. And I think it's because a lot of the times these patients don't really have any options left, right? You've done all of the concoctions of different combinations of chemotherapy and you've given them multiple transplants in hopes to prolong their quality of life and also their life in general.

So, I think that it would be interesting to see if we can look into the earlier stages of myeloma, right, perhaps looking more at smoldering myeloma patients or newly diagnosed patients, maybe we can find some sort of treatment that could really nip it in the bud there instead of waiting until it becomes heavily pretreated relapsed/refractory myeloma. I know there's a lot of complications with smoldering and newly diagnosed myeloma, you run into a lot of insurance issues and the budgets for those studies seem to be much more expensive, and so on, so forth. But I think if we can catch it earlier on, I think that would be an interesting research area to look into. And also, there's a lot of excitement about translational research in myeloma, looking at different targets, looking at possibly personalized medicine. I know that's been in the talks for some time, but I think that that'd be interesting as well.

Related Content:
News | Multiple Myeloma Year in Review

Phase 3 KEYNOTE-775/Study 309 Trial Meets Dual Primary End Points in Advanced Endometrial Cancer

December 16, 2020
Hannah Slater

The pivotal phase 3 KEYNOTE-775/Study 309 trial evaluating the investigational use of pembrolizumab (Keytruda) plus lenvatinib (Lenvima) met its dual primary end points of overall and progression-free survival in patients with advanced endometrial cancer.

The pivotal phase 3 KEYNOTE-775/Study 309 trial (NCT03517449), designed to evaluate the investigational use of pembrolizumab (Keytruda) plus lenvatinib (Lenvima), met its dual primary end points of overall survival (OS) and progression-free survival (PFS), as well as its key secondary end point of objective response rate (ORR) in patients with advanced endometrial cancer following at least 1 prior platinum-based regimen, according to Merck and Eisai, the developers of the agents.

Importantly, these positive results were reported in the mismatch repair proficient (pMMR) subgroup and the intent-to-treat (ITT) study population, which includes patients with endometrial carcinoma that is pMMR and patients whose disease is microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR).

"We are encouraged by the data observed in KEYNOTE-775/Study 309, which represent a possible step forward for patients impacted by advanced endometrial carcinoma and support the results seen in the advanced endometrial cancer cohort of KEYNOTE-146/Study 111," Takashi Owa, vice president, chief medicine creation officer, and chief discovery officer of the Oncology Business Group at Eisai, said in a press release. "As more clinical data from the LEAP (Lenvatinib And Pembrolizumab) program are revealed, we cannot help but be energized by the trajectory of our collaboration with Merck and the benefits we hope to provide to patients together. Most importantly, we are grateful for the trust that the patients and healthcare professionals who participated in this trial have shown us."

The multicenter, randomized, open-label, phase 3 trial enrolled a total of 827 patients. The total study cohort consisted of 697 patients with tumors that were non-MSI-H or pMMR, and 130 patients with tumors that were MSI-H or dMMR.

The study's dual primary end points are OS and PFS, as assessed by Blinded Independent Central Review (BICR) per Response Evaluation Criteria in Solid Tumors Version (RECIST) v1.1. Key secondary end points include ORR by BICR per RECIST v1.1 and safety/tolerability.

Patients were randomized 1:1 to receive either pembrolizumab at a dose of 200 mg intravenously (IV) every 3 weeks for up to 35 cycles (approximately 2 years) in combination with lenvatinib at a dose of 20 mg orally once daily, or chemotherapy with physician's choice of either doxorubicin at a 60 mg/m2 dose by IV every 3 weeks for up to a maximum cumulative dose of 500 mg/m2 or paclitaxel at a 80 mg/m2 dose by IV on a 28-day cycle (3 weeks of receiving weekly paclitaxel and 1 week of not receiving paclitaxel).

According to an analysis conducted by an independent data monitoring committee, pembrolizumab plus lenvatinib demonstrated a statistically significant and clinically meaningful improvement in OS, PFS, and ORR versus chemotherapy (treatment of physician's choice of doxorubicin or paclitaxel).

Regarding safety, the safety profile of combination treatment with pembrolizumab plus lenvatinib was found to be consistent with previously reported studies.

Moving forward, Merck and Eisai intend to discuss these data with regulatory authorities worldwide. The companies also plan to submit marketing authorization applications based on these results and present these results at an upcoming medical meeting.

Of note, KEYNOTE-775/Study 309 is the confirmatory trial for KEYNOTE-146/Study 111, which supported the FDA accelerated approval of the pembrolizumab plus lenvatinib combination in 2019 for the treatment of patients with advanced endometrial carcinoma that is not MSI-H or dMMR, who have disease progression following prior systemic therapy, and are not candidates for curative surgery or radiation. More specifically, this accelerated approval was based on tumor response rate and durability of response and was the first approval granted under Project Orbis, an initiative of the FDA Oncology Center of Excellence that provides a framework for concurrent submission and review of oncology drugs among its international partners. Health Canada and Australia's Therapeutic Goods Administration (TGA) also granted conditional and provisional approvals, respectively, under Project Orbis for this indication.

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A randomized phase II study of PEP02 (MM-398), irinotecan or docetaxel as a second-line therapy in patients with locally advanced or metastatic gastric or gastro-oesophageal junction adenocarcinoma†

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Background: PEP02 is a novel highly stable liposomal nanocarrier formulation of irinotecan. This randomized phase II study evaluated the efficacy and safety of single agent PEP02 compared with irinotecan or docetaxel in the second-line treatment of advanced oesophago-gastric (OG) cancer.

Patients and methods: Patients with locally advanced/metastatic disease who had failed one prior chemotherapy regimen were randomly assigned to PEP02 120 mg/m^2, irinotecan 300 mg/m^2 or docetaxel (Taxotere) 75 mg/m^2 every 3 weeks. The primary end point was objective response rate (ORR). Simon's two-stage design was used and the ORR of interest was 20% ($\alpha = 0.05$, type II error $\beta = 0.10$, null hypothesis of ORR was 5%).

Results: Forty-four patients per arm received treatment, and 124 were assessable for response. The ORR statistical threshold for the first stage was reached in all arms. In the intent-to-treat (ITT) population, ORRs were 13.6% (6/44), 6.8% (3/44) and 15.9% (7/44) in the PEP02, irinotecan and docetaxel arms, respectively. The median progression-free survival (PFS) and overall survival were similar between the trial arms. Commonest grade 3–4 adverse event reported was diarrhoea in the PEP02 and irinotecan groups (27.3% versus 18.2%).

Conclusion: The ORR associated with PEP02 was comparable with docetaxel and numerically greater than that of irinotecan. PEP02 warrants further evaluation in the advanced gastric cancer setting.

Key words: docetaxel, irinotecan, liposomal irinotecan, oesophago-gastric cancer, phase II, second line

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introduction

Oesophago-gastric (OG) cancer represents a significant global health problem with an estimated one million cases diagnosed every year worldwide [1]. Several randomized trials and meta-analyses have established the role of combination chemotherapy in the first-line treatment of advanced OG cancer with prolongation of OS and improvement in the quality of life [2].

Currently there are no standard second-line treatments in this setting [3, 4], although a trend exists towards increased use of second- and third-line treatments, with a significant geographical variation seen in both the therapeutic approach and the uptake of second-line treatment. In large first-line clinical studies, the rates of uptake of subsequent chemotherapy were 14% in the UK REAL 2 study, 42% in the international ToGA trial and 75% in the Japanese SPIRITS trial [5–7]. Recent phase III trials have demonstrated a survival benefit associated with the use of irinotecan or docetaxel (Taxotere) compared with best supportive care (BSC) alone in patients who have failed one or two prior lines of treatment [8, 9]. More recently, a randomized study from Japan demonstrated comparable results with either weekly paclitaxel (Taxol) or irinotecan in second-line therapy [10].

PEP02

PEP02, also known as MM-398 (Merrimack Pharmaceuticals, Inc.), is a highly stable liposomal nanocarrier formulation of irinotecan hydrochloride (CPT-11) [11]. This liposomal formulation is associated with preferentially increased tumour exposure to irinotecan and therefore, local release and conversion to SN-38 as a result of prolonged circulation in the bloodstream, longer half-life, increased area under the curve (AUC), slower clearance and reduced volume of distribution compared with the free drug [11]. In a phase 1 study of a variety of solid tumours, the maximum tolerated dose (MTD) of PEP02 as a single agent was found to be 120 mg/m^2 once every 3 weeks [12].

This randomized three-arm phase II study was designed to assess objective response rate (ORR) with single agent PEP02, or irinotecan or docetaxel in patients with locally advanced or metastatic gastric and gastro-oesophageal (GEJ) adenocarcinomas in the second-line setting.

methods

patients

Eligible patients were aged ≥18 years of age with histologically or cytologically confirmed locally advanced or metastatic gastric or GEJ junction adenocarcinoma. Patients had to have at least one measurable lesion and have failed one prior systemic chemotherapy (including patients with disease recurrence within 6 months of (neo)adjuvant chemotherapy).

Additional eligibility criteria included Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0–2, adequate organ function, life expectancy >3 months, no concurrent uncontrolled medical condition, no other active malignancy, no known brain metastasis, no prior irinotecan/taxane treatment and no history of allergic reactions to liposomal products. The trial was conducted in accordance with the Declaration of Helsinki and had ethical approval. A written informed consent was obtained from each patient before study entry. The institutional review boards of all participating centres reviewed and approved the protocol (ClinicalTrials.gov identifier NCT00813072).

treatment

Eligible patients were randomly assigned 1:1:1 to receive PEP02: 120 mg/m^2 (90-min infusion on day 1 of each cycle), irinotecan: 300 mg/m^2 (90-min infusion on day 1 of each cycle) or docetaxel (Taxotere): 75 mg/m^2 (60-min infusion on day 1 of each cycle) intravenously as monotherapy administered every 3 weeks. In the PEP02 arm, a protocol-specified dose level increase to 150 mg/m^2 was allowed for patients who did not have a ≥grade 1 adverse event. Treatment was continued until disease progression, unacceptable toxicity or withdrawal of consent. Treatment was delayed by 1 week (maximum of 2 weeks) if the neutrophil count was $<1.5 \times 10^9$/l or the platelet count was $<100 \times 10^9$/l. The severity of adverse events was graded according to NCI-CTCAE v 3.0.

assessments

Medical history, vital signs and PS were documented within seven days before randomization, and the patients underwent ECG, urinalysis and routine blood tests (including creatinine clearance) during this timeframe. Physical examination, haematology, biochemistry and urinalysis were repeated at the beginning of each cycle.

Baseline tumour assessment [computed tomography (CT) scan of chest, abdomen, and pelvis] was carried out within 28 days before randomization and CT scans were repeated after every two treatment cycles until disease progression. Response and progression were evaluated using the RECIST version 1.0 [13] criteria and all responses were confirmed with a second CT scan carried out 1 month later. The survival status was assessed every 2 months following the completion of trial treatment. Safety assessments were carried out on the day of treatment administration and at 30 days following the last exposure to trial treatment. The severity of adverse events was graded according to NCI-CTCAE v 3.0. An independent data monitoring committee regularly reviewed study safety and efficacy data.

pharmacokinetic and pharmacogenetic analysis (non-UK sites)

Pharmacokinetic (pK) studies were carried out in the PEP02 and irinotecan arms (supplementary Appendix SA. I. 1, available at *Annals of Oncology* online). An optional pharmacogenetic (pGx) study was also conducted, with analysis being carried out on samples from consenting patients in the PEP02 or irinotecan arms (see supplementary Appendix SA. I. 2, available at *Annals of Oncology* online).

statistical considerations

The primary end point was ORR and was analysed in both the intent-to-treat (ITT) and assessable populations (AP). The ITT population was defined as all recruited subjects who received any study medication. The AP, a subset of ITT, was defined as patients who had received at least two cycles of treatment and were assessable for response.

The study was not powered to allow statistical comparison of efficacy and toxicity between the three treatment arms. For the primary end point, a Simon's two-stage design was used and the response rate of interest was set at 20% ($\alpha = 0.05$, type II error $\beta = 0.10$) with a null hypothesis rate of 5%. For each arm, two responses within the first 21 assessable patients were required to proceed to the second stage, and five responses among 41 assessable patients in both the stages were required to reject the null hypothesis. Based on these calculations, 41 assessable patients were planned to be enrolled in each arm of the study.

The secondary end points included progression-free survival (PFS; time from the date of first study treatment to the date of disease progression or death, overall survival (OS; time from the date of first study treatment to the date of death), and 1-year survival rate.

results

Between January 2008 and June 2010, 135 patients were randomly assigned from 19 sites in the UK, Spain, Taiwan, Croatia, Korea and Bosnia. Overall, 54% (73/135) of the patients were recruited from Europe and 46% (62/135) were recruited from Asia. Three patients (one per arm) were ineligible and were withdrawn before receipt of any study medication, leaving 132 patients (44 in each arm) in the ITT population (Figure 1). Eight patients did not receive at least one post-treatment tumour assessment, leaving 124 patients in the AP (PEP02 $n = 41$, irinotecan $n = 43$, docetaxel $n = 40$). The baseline characteristics were well balanced between the treatment arms, the majority of patients were male (78%), had metastatic disease (94%) and PS 0–1 (92%). (Table 1)

The mean number of treatment cycles was 4.4 in the PEP02 arm (range 1–18), 4.6 in the irinotecan arm (range 1–12) and 4.7 in the docetaxel arm (range 1–12). In the PEP02 arm, five patients without ≥grade 1 toxicity received a dose of 150 mg/m^2. The median relative dose intensity by cycle was high in all the three treatment arms (>0.90) and the proportion of patients requiring dose reduction was also similar between the treatment arms [20.5% (9 of 44) with PEP02, 25% (11 of 44) with irinotecan and 22.7% (10 of 44) with docetaxel]. The primary reason for treatment discontinuation was disease progression (68.9%) followed by adverse events (13.6%) and investigators' decision (9.8%).

efficacy

Within the first assessable 21 patients recruited to each arm, responses were noted in 4, 2 and 5 patients treated with PEP02, irinotecan and docetaxel, respectively. The ORR threshold for the first stage of Simon's two-stage was, therefore, reached in all the three arms and the trial continued to full accrual. In the ITT population, the ORR was 13.6% (6/44; 95% CI 5.2–27.4) in the PEP02 arm, 6.8% (3/44; 95% CI 1.4–18.7) in the irinotecan arm and 15.9% (7/44; 95% CI 6.6–30.1) in the docetaxel arm. (Table 2) Additionally, the response rate of PEP02 at 150 mg/m^2 ($n = 5$) was 60% (3 PR). The DCRs for the three arms were PEP02 59.1% (26/44), irinotecan 61.4% (27/44), and docetaxel 52.3% (23/44), respectively. A pre-specified subgroup analysis demonstrated a numerically better ORR in Asian versus European patients in the PEP02 and docetaxel arms [20% versus 8.3% for PEP02 and 26.3% versus 8.0% for docetaxel (supplementary Table SA.1, available at *Annals of Oncology* online)].

survival

In the ITT population, the median OS was 7.3 months (95% CI; 3.84–9.17) in the PEP02 arm, 7.8 months (95% CI; 4.90–9.20) in the irinotecan arm and 7.7 months (95% CI; 5.32, 12.32) in the docetaxel arm. Kaplan–Meier estimates of 1-year survival rates were 21.3%, 30.8% and 40.4% in those three treatment arms, respectively (Table 2, Figure 2A). Median PFS was similar in all the three arms [2.7 months (95% CI; 1.54–3.65) with PEP02, 2.6 months (95% CI; 1.48–4.34) with irinotecan and 2.7 months (95% CI; 1.41–5.45) with docetaxel] (Table 2, Figure 2B). A trend towards better overall survival was observed in Asian patients (median OS 8.9 m versus 6.0 m, HR 1.40, 95% CI 0.97–2.16, $P = 0.065$) (supplementary Figure S1, available at *Annals of Oncology* online). Median PFS and OS of patients who received PEP02 at 150 mg/m^2 were numerically higher than patients who received that at 120 mg/m^2 group (PFS: 6.0 m versus 2.5 m; OS: 7.8 m versus 6.0 m, respectively).

toxicity

Table 3 demonstrates treatment-related grade 3–4 toxic effects. Treatment was well tolerated; the overall incidence of grade

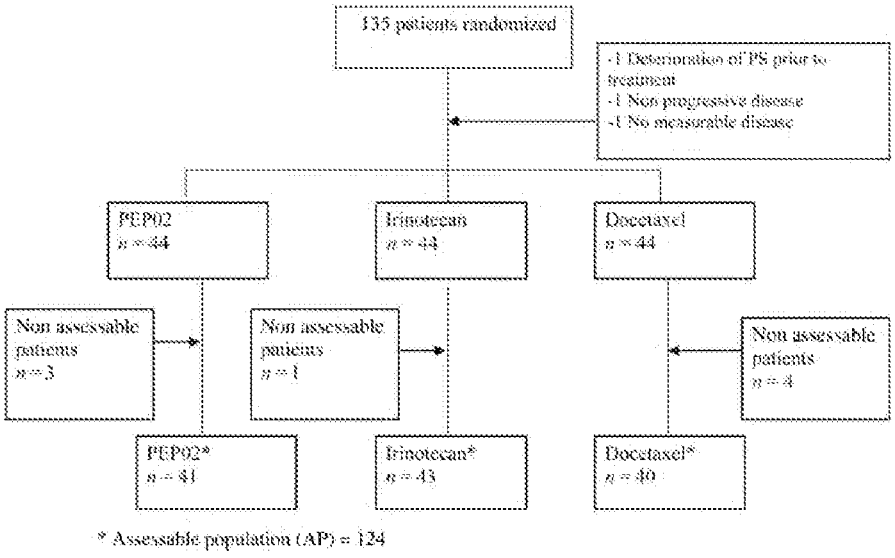

Figure 1. Consort diagram.

Table 1. Baseline characteristics

Baseline characteristics	PEP02 N = 44		Irinotecan N = 44		Docetaxel N = 44	
	n	%	n	%	n	%
Sex						
% of males	35	79.5%	34	77.3%	34	77.3%
Age						
Median	56		62		58	
Range	38–81		33–79		33–81	
Eastern Cooperative Oncology Group performance status (ECOG PS)						
0–1	41	93%	41	93%	40	91%
2	3	7%	3	7%	4	9%
Geographical region ($n = 45$ each group)						
Asia	21	47%	21	47%	20	44%
Europe	24	53%	24	53%	25	56%
Previous treatment						
Prior Radiotherapy	9	20.5%	6	13.6%	7	15.9%
Prior Surgery	31	70.5%	31	70.5%	37	84.1%
Prior Chemotherapy	44	100%	44	100%	44	100%
Primary tumour site						
Gastric	37	84%	35	80%	30	68%
GO Junction	7	16%	9	20%	14	32%
Extent of disease						
Metastatic	43	97.7%	40	91%	43	97.7%

3–4 adverse events was 38.6% in the PEP02 arm, 34.1% in the irinotecan arm and 15.9% in the docetaxel arm. No treatment-related deaths were observed. Diarrhoea was the most common toxicity noted in the PEP02 and irinotecan arms (all grade toxicity 72.7% versus 68.2%, respectively). The most frequent toxicity in the docetaxel group was alopecia (52.3% all grade toxicity). Overall, PEP02 was associated with an increased frequency of grade 3–4 diarrhoea and nausea, with similar rates of vomiting, neutropaenia and febrile neutropaenia compared with irinotecan and docetaxel. In the five patients treated at the dose of 150 mg/m^2, no clinically relevant toxicity difference was noted. Treatment-related toxic effects led to discontinuation of the study drug in six patients in each arm.

pharmacokinetic/pharmacogenetic evaluation

Sixty-four patients were included in the pK analysis. The effect of treatment on pK parameters is summarized in supplementary Table SA.1, available at *Annals of Oncology* online (supplementary Appendix, available at *Annals of Oncology* online). The pGx sub-study was undertaken in 71 patients of which 37 were treated with PEP02 and 34 with irinotecan.

pharmacokinetics of the active metabolite, SN-38

The mean T_{max} values of SN-38 were 10.2 and 2.1 h after infusion of 120 mg/m^2 PEP02 and 300 mg/m^2 irinotecan, respectively. The dose-normalized C_{max} value following PEP02 treatment was lower than that of irinotecan, and correspondingly the dose-normalized C_{max} value for the formation of SN-38 from CPT-11 following infusion of PEP02 was ~50% less than after infusion of irinotecan. However, the dose-normalized AUC_{0-t} and $AUC_{0-\infty}$ values of SN-38 in the PEP02 treatment group were 3.30 and five times higher, respectively, than those seen with irinotecan. The mean $T_{1/2}$ and $MRT_{0-\infty}$ values of PEP02 treatment were four and five times higher, respectively, than those associated with irinotecan. The pK parameters of CPT 11 and SN-38G are detailed in the supplementary Appendix, available at *Annals of Oncology* online (see supplementary Appendix SA.II, available at *Annals of Oncology* online).

pharmacogenetic analysis

The genotype frequencies of the genetic polymorphisms of the UGT1A family were analysed. Forty-three (61.4%) patients were found to be wild type for *UGT1A1*28* (TA_6TA_6), 26 (37.1%) patients had a heterozygous polymorphism (TA_7TA_6) and only one (1.4%) patient was found to have homozygous mutation (TA_7TA_7). Genotype frequencies for the other UGT1A polymorphisms are summarized in supplementary Table SA, available at *Annals of Oncology* online. 2 (supplementary Appendix, available at *Annals of Oncology* online).

UGT1A1 variants were correlated with toxicity. Thirty-six patients from the PEP02 group and 34 patients from the irinotecan group were included in this analysis. In the PEP02 arm, the frequency of grade 3–4 neutropaenia was higher for *UGT1A1*6* heterozygotes compared with the wild-type genotype [3% (1 of 30) for wild type versus 40% (2 of 5) for heterozygotes, $P = 0.0220$]. Higher rates of grade 3–4 neutropaenia was also observed in heterozygotes for the genotype *UGT1A1*27* in the irinotecan arm, when compared with wild type [13% (4 of 31) for wild type versus 66% (2 of 3) for heterozygotes, $P = 0.0197$]. No other association between gene polymorphisms and toxic effects was significant. No correlation between UGT1A gene polymorphism and PEP02/irinotecan pK was demonstrated. (supplementary Appendix SA II. 4, available at *Annals of Oncology* online)

Table 2. Summary table of main efficacy results (ITT, $n = 132$)

	ITT population	Disease response CR + PR n (%)	DCR n (%)	1 Year survival rate % (95% CI)	PFS Median 95% CI	OS Median 95% CI
PEP02	44	6 (13.6)	26 (59.1)	21.3% (6.6, 36.0)	2.7 (1.54, 3.65)	7.3 (3.84, 9.17)
Irinotecan	44	3 (6.8)	27 (61.3)	30.8% (16.6, 45.1)	2.6 (1.48, 4.34)	7.8 (4.90, 9.20)
Docetaxel	44	7 (15.9)	23 (52.3)	40.4% (25.8, 55.1)	2.7 (1.41, 5.45)	7.7 (5.32, 12.32)

ITT, intent to treat; CR, complete response; PR, partial response; DCR, disease control rate (CR + PR + SD).

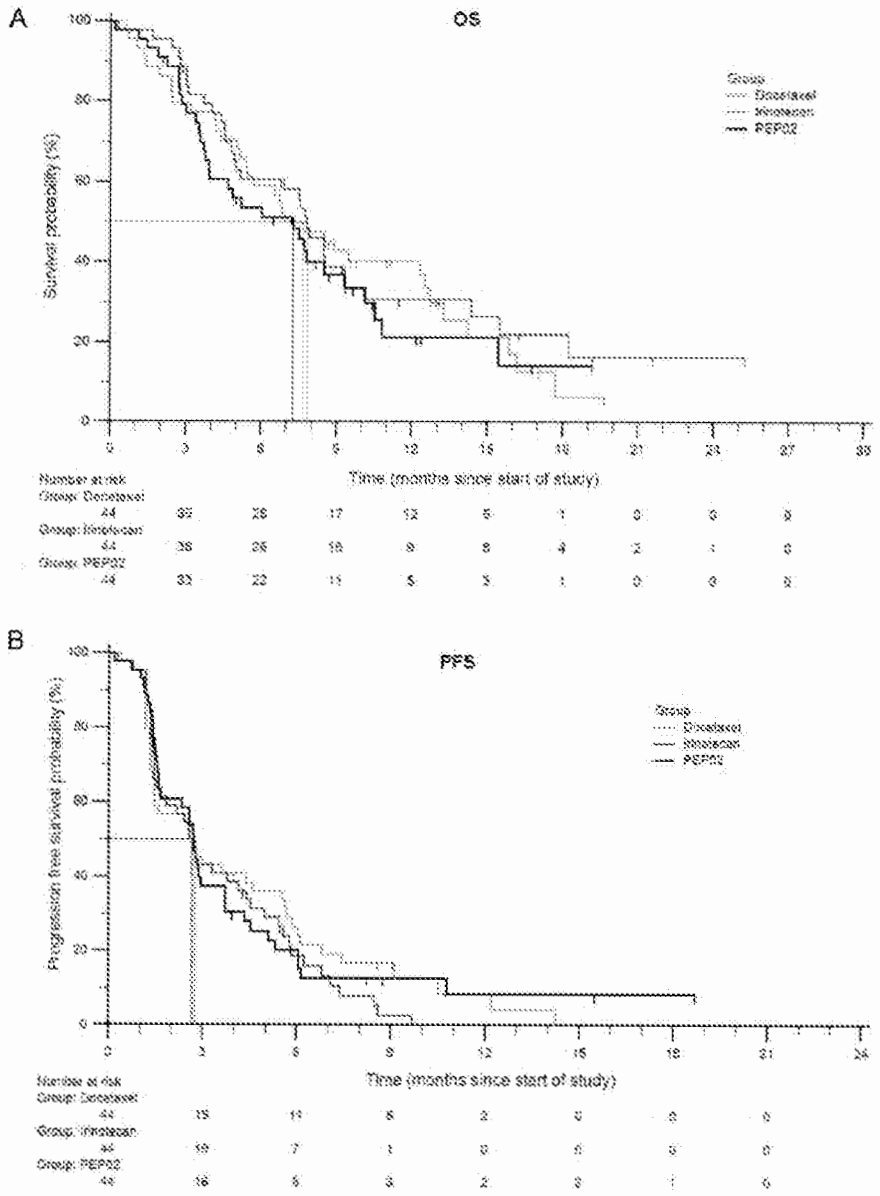

Figure 2. (A) Kaplan–Meier estimates of OS in the intent-to-treat population. (B) Kaplan–Meier estimates of PFS in the intent-to-treat population.

Table 3. Most common grade 3–5 adverse events

	Most common grade 3–5 adverse events					
	PEP02		Irinotecan		Docetaxel	
	N	%	n	%	n	%
Anaemia	2	4.5	2	4.5	3	6.8
Neutropaenia	5	11.4	7	15.9	2	2.6
Thrombocytopaenia	1	2.3	1	2.3	0	0
Febrile neutropaenia	3	6.8	5	11.3	2	2.6
Diarrhoea	12	27.3	8	18.2	1	2.3
Nausea	5	11.4	2	4.6	0	0
Vomiting	2	4.6	6	13.6	3	6.8
Anorexia	3	6.8	3	6.8	0	0
Fatigue	2	4.6	1	2.3	1	2.3

discussion

This randomized phase II trial represents the first study comparing a novel highly stable liposomal nanocarrier formulation of irinotecan (PEP02, MM-398) with docetaxel and irinotecan in the treatment of locally advanced or metastatic OG cancer after failure of first-line treatment.

The study's primary end point was ORR and in Simon's two-stage design only the PEP02 and docetaxel arms met the protocol-specified primary end point of five or more patients with confirmed tumour response in a total of 41 assessable patients. PFS, 1-year survival rate and OS were similar in the three arms. Other stratification factors such as geographical region, gender, ECOG and disease status (locally advanced versus metastatic) did not affect ORR or survival outcomes.

CPT-11 is mainly present in encapsulated form in the plasma after administration of PEP02 [12, 14]. In our study, pK results were consistent with previously reported profiles of PEP02 and free irinotecan [14, 15]. This study also confirms that following infusion of PEP02, there is a higher AUC, lower clearance and smaller volume of distribution for total and encapsulated irinotecan compared with the published pharmacokinetic data for free irinotecan [15, 16]. Multiple pre-clinical models have demonstrated that extended circulation of PEP02 leads to increased tumoural drug retention which permits local release and enzymatic conversion of irinotecan into SN-38. This sustained-release effect of the drug provides longer effective concentrations and AUC of the active metabolite (SN-38) in plasma and consequently a potentially beneficial longer duration of anti-tumour activity. Although the mechanism of release is not fully understood, it is assumed that once irinotecan is released from the liposomes either passively or from active breakdown potentially by Kupffer cells in the liver, it is metabolized in a similar fashion to the conventionally administered irinotecan. Therefore, genetic polymorphisms affecting toxicity and efficacy of irinotecan should be relevant to the study drug PEP02.

In this study, the percentages of observed toxic effects were consistent with the previously reported toxicity profiles of irinotecan and PEP02 while lower rates of PEP02 related diarrhoea were observed in other studies [8, 9, 12, 14, 17]. Of note, irinotecan and docetaxel doses used in our study were higher than those in the Korean and the German Arbeitsgemeinschaft Internistische Onkologie (AIO) studies. However these doses were based on the available evidence and expert clinical recommendation at the time of trial design [18, 19]. Liposomal irinotecan is not known to accumulate in many of the target organs and therefore, theoretically results in lower tissue exposure to the free drug and reduced toxicity while maintaining a greater anti-tumour potency [11]. However, overall toxicity and rates of grade 3–4 diarrhoea in our study were numerically higher than expected. We speculate that the lower clearance and higher AUC of PEP02 and SN-38 could explain this unexpected toxicity.

The frequency of homozygosity for *UGT1A1*28* allele is higher in Caucasians (5.8%–9.0%) and is associated with decreased UGT1A1 expression and activity [20–22]. The presence of homozygous mutation is known to critically impact on the glucuronidation of SN-38 resulting in severe neutropenia and diarrhoea in patients who receive irinotecan [21]. The majority of patients in this study were wild type (TA_6TA_6) for this mutation and only 1 (1.4%) Caucasian patient was found to harbor the homozygous mutation (TA_7TA_7). In Asian patients, the *UGT1A1*28* is a rare allele [23, 24] and genetic polymorphisms of *UGT1A1*6* are more frequent that may have an association with irinotecan-related grade 3–4 neutropenia and other toxic effects [25]. In this study, we found no significant associations between gene polymorphisms and pK parameters; however as previously described [26], there did seem to be an association between the heterozygote alleles of the prominent genetic polymorphisms and treatment-related grade 3–4 toxicity.

The recent phase III trial results reported by the AIO and the South Korean groups confirm the benefits associated with second-line chemotherapy in an advanced OG cancer population [8, 9]. The AIO study randomized metastatic OG cancer patients who had failed one prior line of treatment to irinotecan or BSC. The trial was terminated prematurely due to poor accrual. However, irinotecan was associated with a statistically significant OS benefit of 1.6 months (hazard ratio, HR 0.48, 95% CI 0.25–0.92, $P = 0.012$). Similarly, Kang et al. randomized 202 previously treated advanced OG cancer patients with a good PS in a 2:1 fashion to salvage chemotherapy (docetaxel or irinotecan as per investigators' choice) or BSC. In the ITT population, an OS benefit was noted in favour of chemotherapy (5.1 months versus 3.8 months, HR 0.63; $P = 0.004$) and more patients in the chemotherapy arm received further salvage chemotherapy compared with the BSC arm (40% versus 22%, respectively; $P = 0.011$). Median OS with PEP02 in this study is comparable and therefore encouraging. However, clearly with trial results demonstrating median OS of consistently <10 months, there are still significant improvements required to improve the outcomes for this patient group.

The potential advantages of nanoparticle liposomal delivery of irinotecan are several, and include bypassing solubility limitations of irinotecan, extending the circulation time, increasing tumour accumulation via the enhanced permeability and retention effect, and decreased organ toxicity. The results from recent phase I studies and this phase II study demonstrate that PEP02 is well tolerated and also has a comparable efficacy to docetaxel and irinotecan in patients with prior treatment of advanced gastric and GEJ cancer. Interestingly, patients who received PEP02 at 150 mg/m^2 had a numerically better response rate and PFS/OS compared with the patients who received 120 mg/m^2, suggesting a higher antitumour activity and this dose is worthy of further evaluation in future studies of PEP02. However, due to small numbers in this cohort and a potential selection bias for good PS patients, a significant conclusion cannot be made from these data at this time. Although toxicity especially diarrhoea associated with PEP02 appears to be high in this study, the results from ongoing studies of PEP02 as monotherapy and in combination with other cytotoxic [27] or targeted agents in other tumour types will be crucial to establish this novel agent's utility in the cancer therapeutics armamentarium.

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funding

PharmaEngine (Sponsor) provided a research grant to conduct this study. PharmaEngine and Fisher Clinical Services UK Ltd supplied PEP02 to the Asian sites and to the European sites, respectively. Docetaxel and irinotecan were reimbursed by the sponsors. PharmaEngine was involved in study design and data analysis. They were not involved in the manuscript preparation.

Scientific and Ethical committee as well as regulatory approvals were obtained at each institute and country.

PEP02 is designated as MM-398 by Merrimack Pharmaceuticals, Inc. (Cambridge, MA, USA).

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disclosure

ADG and LTC are consultants or have received honorarium from PharmaEngine. CGY holds stock of PharmaEngine, the makers of PEP02. ACR, SRP, DC, YKK, YC, CR, HYL, JT, FJR, MK and MBC have no relevant competing interest to declare.

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Gd-Labeled Liposomes for Monitoring Liposome-encapsulated Chemotherapy:

Quantification of Regional Uptake in Tumor and Effect on Drug Delivery[1]

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Kathryn J. Stevens, MD, Charles A. Gooding, MD, Philipp Lang, MD

RATIONALE AND OBJECTIVES

Recently developed sterically stabilized liposomes are used clinically for delivery of anticancer chemotherapy (currently the drug doxorubicin, Doxil®). In investigational drug development they are studied as targeted (by surface attached antibody or receptor ligands) and nonspecific drug delivery strategies for a variety of pathologies (cancer, arthritis, AIDS). Liposomes have also been considered as potential MRI contrast media vehicles, primarily for blood pool enhancement (1) and for contrast enhancement in the diagnosis and characterization of tumors (2–7). However, little work has been aimed at using liposomal MRI contrast material for purposes of image-based tracking of drug delivery of liposome-drug formulations.

Intravenously administered liposomes are distributed passively by flowing blood and extravasate, usually slowly, in territories where vascular endothelium is either (a) very leaky due to large intracellular gaps such as in inflammatory zones, tumors and healing wounds; or (b) fenestrated such as liver or spleen. Selective concentration of liposomes in tumors is slow-typically requiring day(s) for maximal accumulation—which imposes certain limitations on strategies for external image-based detection, as well as upon the liposome itself. Non-sterically stabilized liposomes are cleared from the blood within a few hours via the reticuloendothelial system and accumulation in tumor is obviated kinetically. Sterically stabilized liposomes are successful for antitumor application because they remain in the circulation much longer, with plasma half-lives on the order of days, and substantial accumulation in tumor is possible. Similarly for image-based external detection strategies, the use of PET or SPECT tracers bound to liposomes would fail to the short radioactive half-lives of acceptable radionuclides. Consequently, only liposomal-bound CT or MRI contrast media would be viable candidates for this purpose.

In the current study, sterically stabilized liposomes labeled with encapsulated GdDTPA-BMA were examined as probes for quantitative tracking of liposomal drug delivery in implanted osteogenic sarcoma model. The purpose of the study was to evaluate dose-response in the context of intravenously administered dose versus the response of $\Delta R1$ accumulation in the tumor.

MATERIALS AND METHODS

GdDTPA-BMA-liposomes (80 nm dia) were prepared from egg phosphatidyl choline (EPC), cholesterol, and polyethylene glycol-phosphatidyl ethanolamine (PEG-PE) (ratio 3:2:0.3) and 0.25 M GdDTPA-BMA by the reverse phase evaporation method followed by extrusion (8). Inclusion of PEG-PE provided steric stabilization. Each batch was characterized by assay of phosphate content and MR determination of Gd content (spectroscopic inversion recovery measurement of $\Delta R1$ of intact and detergent solubilized liposomes, with and without GdDTPA-BMA). Encapsulated volume was calculated using assumption that all Gd was encapsulated at 0.25 M concentration.

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Thirty nude athymic rats (NCI, 80–110 gm, 5-weeks old) were implanted with osteogenic sarcoma (cell line UMR 106 E IV) by subcutaneously injecting 3–5 million cells while under ketamine-xylazine anesthesia. When palpable tumors were approximately 0.6–0.8 cm diameter (6 to 10 days later) animals were imaged.

MRI was conducted at 2 Tesla using a Bruker Omega system (Bruker Instruments, Inc., Fremont, CA). Animals were anesthetized by exposing to isoflurane (5% to induce and 1.7–2% to maintain) mixed into 100% O_2. Animals were placed supine on a holder covered by a heated water pad (38 ± 1°C), inserted into a 5 cm birdcage rf coil and placed in the magnet. A tail vein was catheterized for administration of liposomes. GdDTPA-BMA liposome was infused (5–10 min) at Gd doses of 0.025, 0.05, 0.1 and 0.2 mmol/kg body weight (n = 6). MRI was conducted before and 24 and 48 hr after liposome administration. Contrast enhancement was measured from standard multislice T1 weighted spin echo (TR/TE = 500/13 ms) images. Tumor size and morphology were determined from magnetization transfer (3D spoiled GRE, TR/TE = 100/3 ms, flip = 30 deg) and/or diffusion weighted images (TR/TE = 2500/44 ms with diffusion gradients set to 1, 5, 8 and 10 G/cm, b = 14, 366, 938, and 1465 s/mm^2). Regional T1 values were measured before and after contrast administration by either IR EPI (TR/TE = 10 s/10 ms, single slice) or IR snapshot FLASH (TR/TE = 3/1.5 ms, flip = 10 deg, centric phase ordering, 6–10 slices) with TI's ranging from 50–3000 ms.

The remaining six rats were administered GdDTPA-BMA (0.1mmol/kg bolus, 0.004 mmol/kg/hr infusion) and imaged as described above. Regional T1 values were measured using IR snapshot flash MRI after 30 min of infusion. Blood samples were taken before and during infusion and plasma R1 was measured by spectroscopy. Distribution volume of GdDTPA-BMA was calculated from the ratio of ΔR1tumor/ΔR1plasma.

RESULTS AND CONCLUSIONS

Maximum contrast enhancement was observed at 24 hours after injection of Gd-liposomes. The ΔR1 values of central tumor regions measured at 24 hours increased with dose until 0.1 mmol/kg. Higher dose of Gd-liposomes produced no further increase in ΔR1. Significantly greater ΔR1 was measured in the tumor periphery for all doses of Gd-liposomes, and the peripheral region showed no plateau at the higher doses (Table).

ΔR1 value (±SEM) at 24 Hours, in the Tumor, after Injection of Different Doses of Gd-Liposomes. Fractional Volume is 100 × Liposome-encapsulated Volume Divided by the Distribution Volume of GdDTPA-BMA Measured in Similar Regions of Different Animals

	Central Region of the Tumor		Peripheral Region of the Tumor	
Dose (mmol/kg)	ΔR1 ± SEM (sec^{-1})	Fractional Volume	ΔR1 ± SEM (sec^{-1})	Fractional Volume
0.025	0.08 ± 0.02	0.11%	0.22 ± 0.04	0.11%
0.05	0.16 ± 0.03	0.24%	0.28 ± 0.03	0.15%
0.1	0.36 ± 0.04	0.53%	0.61 ± 0.06	0.33%
0.2	0.36 ± 0.02	0.53%	0.78 ± 0.06	0.42%

The liposomal Gd relaxivity among batches was between 3.4–3.7 s^{-1} mM^{-1}. These values led to calculated values of liposome-encapsulated volume within tumor pixels of 0.09 to 0.43 microL/mL in the central region and 0.25 to 0.88 microL/mL in the periphery.

The distribution volume of GdDTPA-BMA was 79 ± 1 microL/mL in the central region and 213 ± 2 microL/mL in the peripheral region of the tumor. Thus, although the high liposome dose produced a saturation of the ΔR1 response in the central region, the volume occupied by the liposome (estimated from the encapsulated volume) was very small in comparison to available extracellular space, as estimated from the distribution volume of GdDTPA-BMA (Table 1). This suggests that the liposome poorly penetrates the tumor interstitium.

In comparison to liposome doses used in clinics, the saturation dose of 0.1mmol/kg found in this study is approximately 100 times a single dose of Doxil® (liposome-encapsulated doxorubicin). From this it can be concluded that typical clinical doses can be increased greatly with commensurate increased accumulation in the tumor.

Moreover, this study supports the proposition that Gd-liposomes are a powerful tool for probing the distributional behavior of liposome-based therapies, and may be very useful in clinical applications.

ACKNOWLEDGMENTS

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Phase 1 Expansion Study of Irinotecan Liposome Injection (nal-IRI) in Patients With Metastatic Breast Cancer (mBC)

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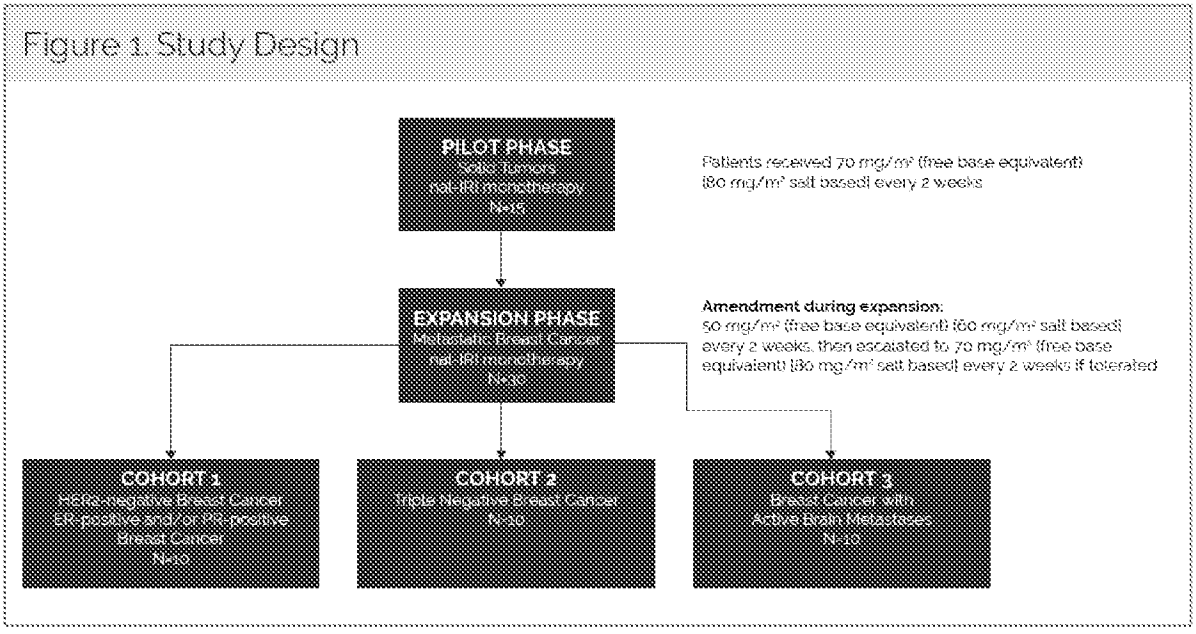
INTRODUCTION

- In 2018, it was estimated that 268,670 people aged 18 and older (266,120 women and 2,550 men) in the United States were diagnosed with invasive breast cancer. Despite greatly improved outcomes in recent years, the 5-year survival rate for women with metastatic breast cancer (mBC) is 27%.[1]
- Irinotecan (a topoisomerase-1 inhibitor) has a distinct mechanism of action from other drugs commonly used to treat patients with mBC and has limited cross-resistance.
  - Irinotecan is commonly used to treat patients with advanced breast cancer, with a single-agent response rate of 5–23%.[2]
- A liposomal formulation of irinotecan (nal-IRI), encapsulated in a nanoliposomal delivery system, was developed to maximize efficacy and reduce toxicities commonly associated with non-liposomal irinotecan.
  - This nanoliposomal delivery system reduces systemic exposure and increases drug accumulation within the tumor.[3-5]
  - Tumor-associated macrophages (TAMs) appear to play a key role in the deposition, retention, and activation of nal-IRI within the tumor microenvironment.[6]
- In clinical studies conducted with nal-IRI, 6 patients had a confirmed diagnosis of mBC.[7,8] In those studies, 2 patients achieved stable disease and 1 patient achieved a partial response as best overall response.
- To further investigate the potential efficacy signal, this open-label, nonrandomized expansion of the phase 1 study NCT01770353 was conducted to evaluate nal-IRI efficacy and safety in patients with mBC.

METHODS

- This was an open-label, nonrandomized expansion phase of a phase 1 trial (Figure 1).

Figure 1. Study Design



Expansion Phase Key Inclusion Criteria

Cohort 1:

- HER2-negative breast cancer
- Hormone receptor–positive breast cancer patients with estrogen receptor (ER)–positive and/or progesterone receptor (PR)–positive tumors

Cohort 2:

- Triple-negative breast cancer (TNBC) with HER2-negative, ER-negative, PR-negative tumors

Cohort 3:

- Any sub-type of mBC with active brain metastases
- Radiographic evidence of new or progressive central nervous system (CNS) metastases after radiation therapy with ≥1 lesion ≥1 cm in longest dimension on gadolinium-enhanced magnetic resonance imaging (MRI)

Cohorts 1–3

- ECOG performance status ≤1
- Adequate organ function
- ≥1 to ≤5 prior lines of cytotoxic therapy in metastatic setting
  - TNBC patients could have progressed within 12 months of adjuvant therapy
  - No limit to prior lines of hormonal therapies in ER+/PR+ tumors
- Topoisomerase-1 inhibitor (irinotecan-derived and topotecan) naïve
- ≥1 measurable lesion

Treatment

- Cohorts 1–3: Treatment continued until Response Evaluation Criteria in Solid Tumors (RECIST)[9] v1.1 progression or unacceptable tolerability.
- Cohort 3: Treatment could be discontinued for radiographic evidence of CNS-progressive disease (PD). Treatment continuation with non-CNS progression or symptomatic CNS progression without radiographic confirmation was allowed.

Dosing

- At the start of the Expansion Phase, the protocol-defined dosing regimen was 70 mg/m^2 free-base equivalent (FBE) every 2 weeks.
- Protocol Amendment 5 (November 3, 2016) reduced the starting dose of irinotecan liposome injection in the Expansion Phase to 50 mg/m^2 FBE from 70 mg/m^2 FBE, with allowance for dose escalation of irinotecan liposome injection from 50 mg/m^2 FBE to 70 mg/m^2 FBE as per patient tolerance.

Efficacy Assessments

- Tumor assessments by computed tomography (CT) or MRI were performed every 8 weeks.
- Cohorts 1–3: RECIST v1.1 was utilized for all patients with non-CNS disease.
- Cohort 3: Modified RECIST[10] criteria was utilized for patients with CNS disease.

Safety Assessments

- All adverse events were tabulated using NCI CTCAE.

Statistical Analyses

- Descriptive statistics are reported.

RESULTS

Patients

- A total of 30 patients (10 per cohort) were enrolled. (Table 1)
  - 29 patients received at least 1 dose of nal-IRI 50 mg/m^2 FBE.
- In each cohort, most patients had ≥3 prior cytotoxic anti-cancer regimens.
- A total of 13 patients started at a dose of 70 mg/m^2 FBE, 15 patients started on 50 mg/m^2 FBE, and 1 patient was started at a dose of 30 mg/m^2 FBE.
- In all patients, irinotecan liposome injection monotherapy was administered by IV infusion over 90 minutes.

Table 1. Demographics and Baseline Characteristics

Demographics	Cohort 1 n=10	Cohort 2 n=10	Cohort 3 n=10	Total Population N=30
Gender, Female, n (%)	10 (100%)	10 (100%)	10 (100%)	30 (100%)
Age, Years				
Median (range)	56 (49–68)	52.5 (37–70)	46.5 (29–63)	53 (29–70)
Race, n (%)				
White	8 (80%)	8 (80%)	7 (70%)	23 (77%)
Black/African	0	1 (10%)	1 (10%)	2 (7%)
Other	2 (20%)	1 (10%)	2 (20%)	5 (16%)
Months Since Metastatic Diagnosis				
Median (range)	63.7 (16–87)	20.7 (0–34)	32.4 (6–56)	24.0 (0–87)
Number of Prior Cytotoxic Anti-cancer Regimens				
Median (range)	3.0 (1–6)	3.0 (0–5)	3.0 (1–6)	3.0 (0–6)

*Four triple-negative, 3 ER-positive or PR-positive and HER2-positive, 2 ER-positive or PR-positive and HER2-negative, 1 ER-negative or PR-negative and HER2-positive.

EFFICACY

Response to Treatment:

- At least 30% of patients in each of the 3 cohorts achieved partial response (PR).
- In Cohort 3, 30% of patients had CNS PR.
- No patient in any cohort experienced complete response (CR).

Table 2. Response to Treatment

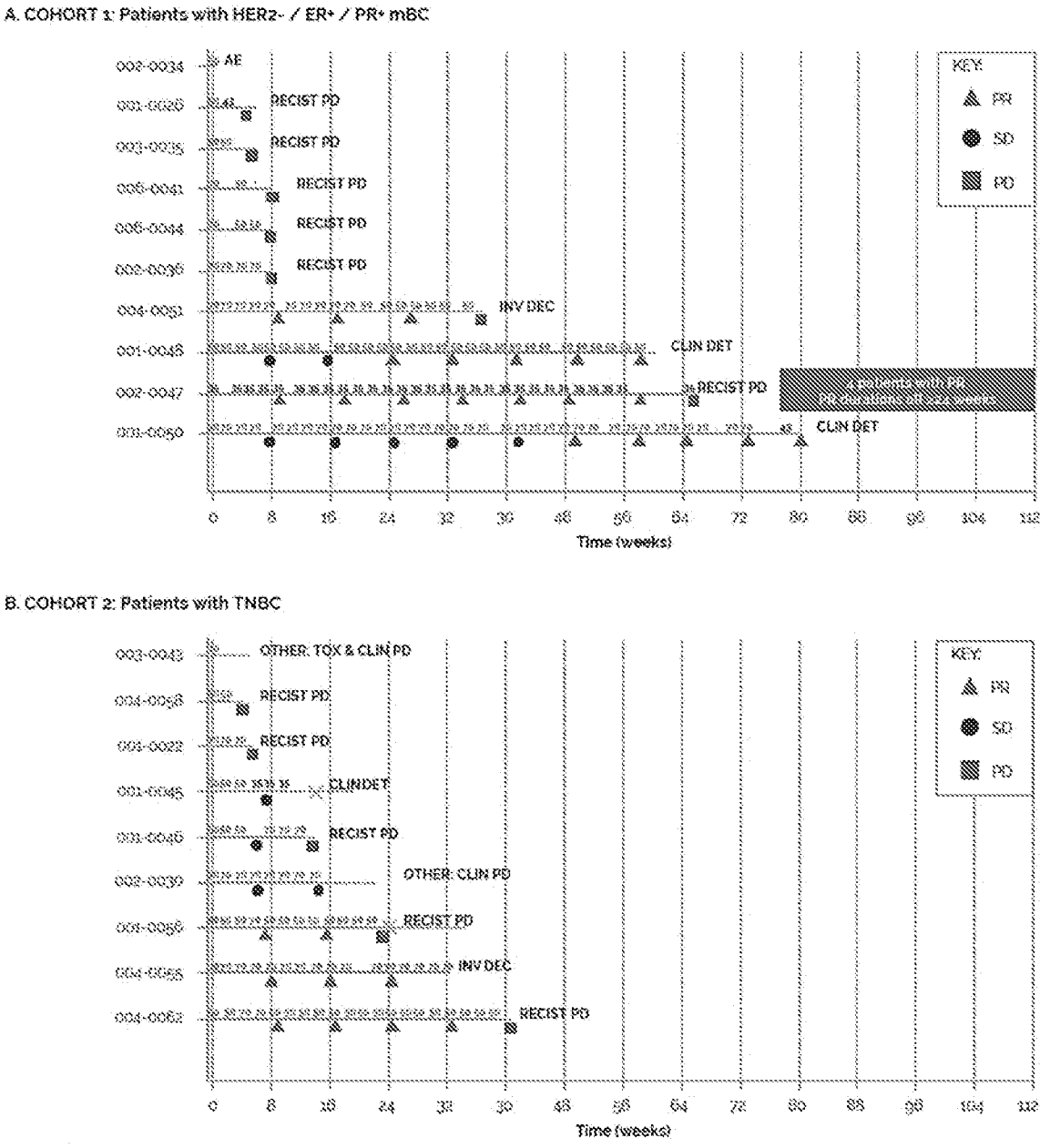
	Cohort 1 n=10	Cohort 2* n=9	Cohort 3 n=10	
Best Overall Response				
	RECIST 1.1	RECIST 1.1	RECIST 1.1	mRECIST
Complete Response	0	0	0	0
Partial Response	4 (40%)	3 (33%)	3 (30%)	3 (30%)
Stable Disease	0	3 (33%)	2 (20%)	3 (30%)
Progressive Disease	5 (50%)	2 (22%)	3 (30%)	2 (20%)
Not Evaluable	1 (10%)	1 (11%)	2 (20%)	2 (20%)
Objective Response Rate				
Complete response + partial response (95% CI)	4 (40%) 12.2–73.8	3 (33%) 7.5–70.7	3 (30%) 6.7–65.3	3 (30%) (6.7–65.3)
Duration of Objective Response				
	RECIST 1.1	RECIST 1.1	RECIST 1.1	mRECIST
Months Median (range)	7.5 (6.4–13.0)	5.6 (3.7–7.4)	4.1 (0.0–22.2)	1.8 (0.0–1.9)

*1 patient was enrolled but not treated and is not included in any safety or efficacy assessment.
Non-CNS tumor response was evaluated according to RECIST v1.1.
CNS tumor response was evaluated according to modified RECIST (mRECIST).
CI, confidence interval; CNS, central nervous system; RECIST, Response Evaluation Criteria in Solid Tumors.

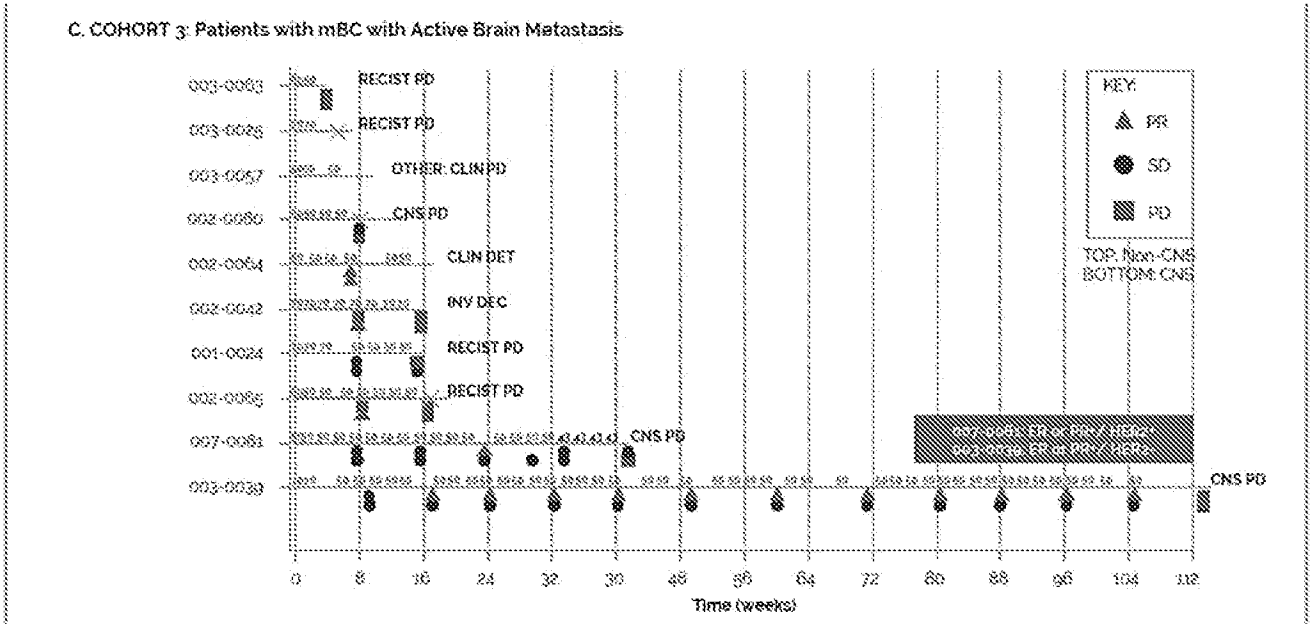
Durability of Response (Figure 2):

- Durability of response for all three cohorts is identified in Figures 2A, B, & C.
- Cohort 1: In all 4 patients with PR, response lasted ≥24 weeks, and 3 patients lasting more than 50 weeks.
- Cohort 2: In 3 patients with PR, 2 patients had a response duration ~24 weeks and 1 had a response duration of 40 weeks.
- Cohort 3: Three patients achieved CNS PR at the Week 8 assessment; 1 patient achieved non-CNS PR starting at Week 16 and continuing through Week 114 with a best CNS response of stable disease (SD), which was maintained over that period. The 2 patients treated for ≥40 weeks were estrogen receptor-positive/ progesterone receptor-positive.

Figure 2. Response to nal-IRI Over Time to Treatment Discontinuation



One patient in Cohort 2 was enrolled but discontinued before receiving treatment.

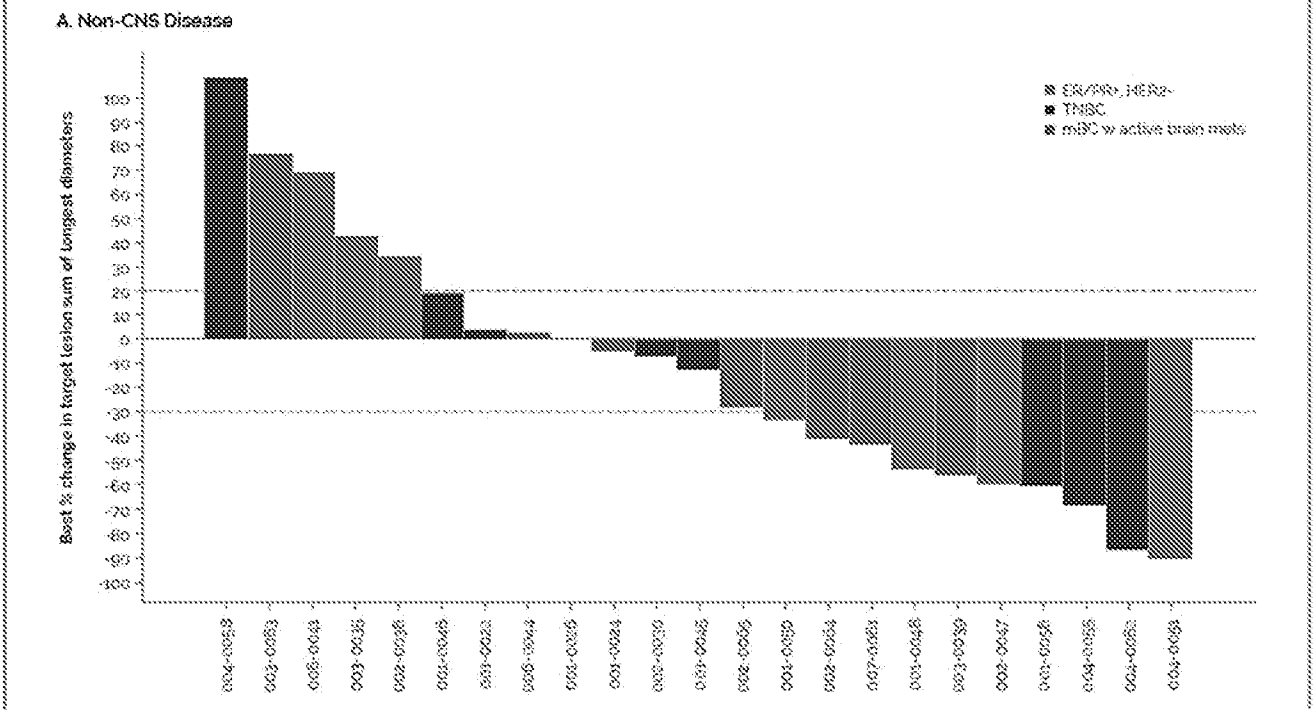


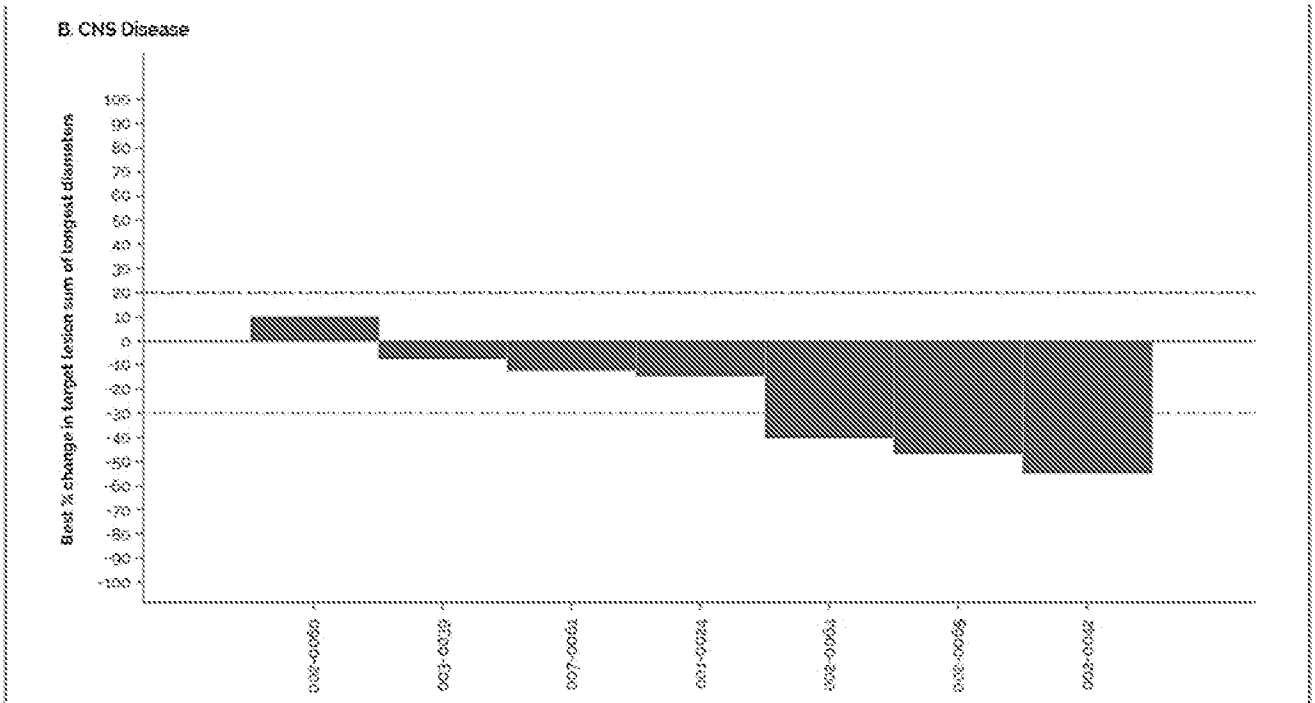
AE, adverse event; CLIN, clinical; CNS, central nervous system; INV DEC, investigator decision; PD, progressive disease; PR, partial response; SD, stable disease; TOX, toxicity.
35-nal-IRI 35 mg/m² infusion FBE; **43**-nal-IRI 43 mg/m² infusion FBE; **50**-nal-IRI 50 mg/m² infusion FBE; **70**-nal-IRI 70 mg/m² infusion FBE.

Tumor Lesion Size

- A reduction in sum of target lesions, in both non-CNS and CNS (Cohort 3 only) disease, was observed for a majority of patients with evaluable follow-up.
- Compared to the baseline, tumor shrinkage varied from 5% to 90% from non-CNS lesions, and from 7% to 55% for CNS lesions.

Figure 3. Percent Reduction in Tumor Lesion Size in Patients With Non-CNS and CNS Disease*





*Only patients who had an evaluable post-baseline tumor assessment for designated target lesions are included in the waterfall plots.

SAFETY

- Most frequent treatment-emergent adverse events (TEAEs, all grades, ≥25%) were gastrointestinal disorders, including diarrhea (90%), fatigue (62%), nausea (55%), vomiting (41%), hypokalemia (38%), and decreased appetite (31%).
  - Diarrhea (Cohort 1, 100%; Cohort 2, 100%; Cohort 3, 70%)
  - Nausea (Cohort 1, 80%; Cohort 2, 56%; Cohort 3, 30%)
  - Vomiting (Cohort 1, 60%; Cohort 2, 33%; Cohort 3, 30%)
- Most common grade 3 TEAEs (≥10%) were diarrhea (28%), nausea (17%), fatigue (14%), hypokalemia (10%), and asthenia (10%), with none reported as grade 4 (Table 3).
- The main related serious adverse events (SAEs, ≥10%) were diarrhea (14%), nausea (10%), and fatigue (10%).
- Grade 3/4 neutropenic events were observed in <5% of treated patients.
- No grade 5 TEAEs were reported.

Table 3. Patient Safety Analysis

Safety Population	Cohort 1 n=10	Cohort 2 n=9	Cohort 3 n=10	Total Population N=29
Exposure				
Treatment Duration, Weeks				
Median (range)	6.1 (0.1–79)	12.3 (0.1–49)	13.9 (1.0–105)	12.3 (0.1–105)
Adverse Events				
Any TEAE	10 (100%)	9 (100%)	10 (100%)	29 (100%)
Grade 3	8 (80%)	6 (67%)	6 (60%)	20 (69%)
Grade 4	1 (10%)	0	2 (20%)	3 (10%)
TEAE Related to nal-IRI	10 (100%)	8 (89%)	10 (100%)	28 (97%)
Grade ≥3	6 (60%)	3 (33%)	3 (30%)	12 (41%)
TEAE Related to nal-IRI Leading to Dose Adjustment*	7 (70%)	3 (33%)	4 (40%)	14 (48%)
SAEs	6 (60%)	4 (44%)	7 (70%)	17 (59%)
SAE Related to nal-IRI	4 (40%)	1 (11%)	1 (10%)	6 (21%)

*n=1 patient in Cohort 1 discontinued treatment due to TEAE related to treatment with nal-IRI.
nal-IRI, irinotecan liposome injection; SAE, serious adverse event; TEAE, treatment-emergent adverse event.

CONCLUSIONS

- Among heavily pretreated patients with mBC, nal-IRI monotherapy every 2 weeks was generally well tolerated (and in line with the known safety profile of nal-IRI).
- Treatment with nal-IRI monotherapy was associated with an improvement of at least 30% ORR for patients with non-CNS as well as CNS disease.
- These results suggest that further clinical assessment of nal-IRI in patients with mBC is warranted.
- GI toxicities, particularly diarrhea, were the most common TEAEs. Proactive management of this toxicity in future trials may improve the risk benefit profile of nal-IRI.

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CANCER RESEARCH

Clinical Trials

Abstract CT048: Phase I expansion study of irinotecan liposome injection (nal-IRI) in patients with metastatic breast cancer (mBC)

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Abstract

Background

nal-IRI is a liposomal formulation of irinotecan (topoisomerase-1 inhibitor), using intraliposomal stabilisation technology to enable high drug load and in-vivo stability. The expansion of Phase I NCT01770353 evaluated nal-IRI in patients (pts) with mBC.

Methods

This Phase I expansion study enrolled mBC pts (Cohort 1: ER+ and/or PR+ HER2 BC (C-1); Cohort 2: Triple Negative BC (TNBC, C-2); Cohort 3: BC with active Brain Metastasis (C-3)). Key inclusion criteria: ECOG ≤1; adequate organ function; received ≥1 to ≤5 prior lines of cytotoxic therapy in metastatic setting, except TNBC pts who could have progressed within 12 months of adjuvant therapy. Pts received nal-IRI 50 mg/m^2 (Free-base equivalent, FBE) q2w iv infusion, escalating to 70 mg/m^2 FBE q2w, if tolerated.

Results

30 pts (10 per cohort) were enrolled: 29 received ≥1 dose nal-IRI 50 mg/m^2 FBE (median age 53 yrs, range 29-70 yrs). Median number of cytotoxic therapies prior to study entry was 3. The most frequent treatment emergent adverse events (TEAEs) were gastrointestinal disorders (all cohorts, 100%), primarily diarrhea (C-1, 100% / C-2, 100% / C-3, 70%), nausea (C-1, 80% / C-2, 56% / C-3, 30%), and vomiting (C-1, 60% / C-2, 33% / C-3, 30%). The incidence of neutropenia was low (C-1, 10% / C-2, 0% / C-3, 10%), all grade 1 / 2. Most common grade 3 TEAEs were diarrhea (28%), nausea (17%), fatigue (14%), hypokalemia (10%) and asthenia (10%), with none reported as grade 4. No grade 5 TEAEs were reported. Partial response (per RECIST) was observed in 10 pts for an objective response rate (ORR) of 35% (C-1, 40% / C-2, 33%) / C-3, 30%). Stable disease was observed in 5 pts (17%). Duration of Response (median, months) was C-1, 7.5 (6.4-13.0) / C-2, 5.6 (3.7-7.4) / C-3, 4.1 (0.0-22.2, extracranial: RECIST) / C-3, 1.8 (0.0-1.9, intracranial: mRECIST).

Demographics	Cohort 1 ER+ and/or PR+ Breast Cancer n=10	Cohort 2 Triple Negative Breast Cancer n=10	Cohort 3 Breast Cancer with Active Brain Metastasis n=10	Total Population N=30
Gender, Female, n (%)	10 (100%)	10 (100%)	10 (100%)	30 (100%)
Age, Years Median (range)	56 (49-66)	52.5 (37-70)	46.5 (29-63)	53 (29-70)

Race, n (%)	8 (80%)	8 (89%)	7 (70%)		23(77%)
White	0	1 (10%)	1 (10%)		2 (7%)
Black/African	2 (20%)	1 (10%)	2 (20%)		5(16%)
Other					
Months Since Metastatic diagnosis, Months Median (Range)	63.7 (16-67)	20.7 (0-34)	32.4 (0-68)		24.0 (0-67)
Number of Prior Cytotoxic Anti-cancer Regimens Median (Range)	3.0 (1-6)	3.0 (0-6)	32.4 (1-6)		3.0 (0-6)
Safety Population	Cohort 1	Cohort 2	Cohort 3		Total Population
	ER+ and/or PR+ Breast Cancer	Triple Negative Breast Cancer	Breast Cancer with Active Brain Metastasis		N=29
	n=10	n=9	n=10		
Exposure					
Treatment Duration, Weeks Median (range)	8.1 (0-78)	12.3 (0-49)	18.9 (1-105)		12.3 (0-105)
Adverse Events					
Any TEAE	10 (100%)	9 (100%)	10 (100%)		29 (100%)
Grade 3	8 (80%)	6 (67%)	10 (100%)		20 (69%)
Grade 4	1 (10%)	0	0 (80%)		3 (10%)
TEAE Related to nal-IRI	10 (100%)	8 (89%)	10 (100%)		28 (97%)
Grade ≥ 3	6 (60%)	3 (33%)	3 (30%)		12 (41%)
TEAE Related to nal-IRI	7 (70%)	3 (33%)	4 (40%)		14 (48%)
Leading to Dose Adjustment*					
SAEs	6 (60%)	4 (44%)	7 (70%)		17 (59%)
SAE Related to nal-IRI	4 (40%)	1 (11%)	1 (10%)		6 (21%)
Duration of Objective Response					
	RECIST 1.1	RECIST 1.1	RECIST 1.1	mRECIST	
Months Median (Range)	7.5 (6.4-13.0)	5.8 (3.7-7.4)	4.1 (3.0-22.2)	1.84 (0.0-1.9)	
Best Overall Response					
	RECIST	RECIST	RECIST	mRECIST	
Complete Response (CR)	0	0	0	0	
Partial Response (PR)	4 (40%)	3 (33%)	3 (30%)	3 (30%)	
Stable Disease (SD)	0	3 (33%)	2 (20%)	3 (30%)	
Progressive Disease (PD)	5 (50%)	2 (22%)	3 (30%)	2 (20%)	
Not Evaluable (NE)	1 (10%)	1 (11%)	2 (20%)	2 (20%)	
Objective Response Rate					
CR+PR	4 (40%)	3 (33%)	3 (30%)	3 (30%)	
(95% CI)	12.2 - 73.8	7.5-70.7	6.7-65.3	(6.7-65.3)	

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*n=1 patient in Cohort 1 discontinued treatment due to TEAE related to treatment with nal-IRI CI, Confidence Interval; nal-IRI, Irinotecan Liposome Injection; SAE, Serious Adverse Event; TEAE, Treatment Emergent Adverse Event

Systemic tumor response was evaluated according to RECIST version 1.1

CNS tumor response was evaluated according to mRECIST (Ardem C. et al., Breast Cancer Res Treat. 2014; 146(3):587-95)

Table 1.

Study Demographics, Adverse Events, and Best Overall Response

Conclusion

nal-IRI monotherapy 50g/m^2 FBE Q2W appears well tolerated, and achieved ≥30% ORR for systemic and CNS disease among heavily pre-treated mBC pts regardless of receptor status.

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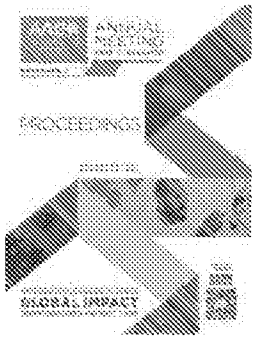

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Effective Irinotecan (CPT-11)-containing Liposomes: Intraliposomal Conversion to the Active Metabolite SN-38

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Irinotecan hydrochloride (CPT-11) is a prodrug of SN-38, which is an active metabolite with antitumor activity and side toxicity. The activities of CPT-11 and SN-38 depend on the closed lactone ring form of SN-38. We have examined the tissue distributions of the closed and open forms of CPT-11 and SN-38 in Lewis lung carcinoma-bearing mice after the administration of liposomal CPT-11 (S-Lip) and polyethyleneglycol (PEG)-modified S-Lip (S-PEG). The plasma concentrations of closed CPT-11 and SN-38 were increased by liposomalization, and their blood circulation was prolonged by the PEG modification. The concentrations of closed CPT-11 and SN-38 in tumors were elevated by both the liposomalization and PEG modification. The closed/total ratio of SN-38 in the tumors of the S-PEG group was greater than that of the CPT-11 solution (Sol) group. Thus, SN-38 was thought to be generated in intact liposomes containing CPT-11. The bile concentration of closed SN-38, which is responsible for CPT-11-induced intestinal disorder, was decreased by liposomalization. In an *in vitro* experiment, the SN-38/CPT-11 ratio in the tumor cells of the S-Lip group was found to be higher than that of the Sol group, and the ratio of the closed form of SN-38 was increased by the liposomalization. Laser scanning confocal microscopy showed the generation of SN-38 in the liposomal membrane after the incubation of S-Lip with carboxylesterase. It is therefore considered that a part of CPT-11 is converted to SN-38 in the intact liposomes.

Key words: Antitumor activity — Irinotecan — SN-38 — Targeting — Conversion

Liposomes are used as models of cell membranes to examine drug permeability and as drug carriers for antitumor agents. Liposomes have also recently been found to be safe, non-viral vectors in gene targeting. Some antitumor agents encapsulated in liposomes have been used clinically and have been shown to be effective against Kaposi's sarcomas in patients with AIDS in the USA and Europe.[1)] However, there is little information available on the efficacy of liposomal prodrugs.

Irinotecan hydrochloride (CPT-11) is a prodrug that can be converted to SN-38, an active metabolite with antitumor activity and side toxicity.[2)] CPT-11 has strong activity against lung carcinomas clinically.[3)] The activities of CPT-11 and SN-38 are known to depend on the closed lactone ring form of SN-38.[4)] We previously reported that the use of liposomal CPT-11 improved the tissue distribution of CPT-11 and increased the antitumor activity of CPT-11 in Ehrlich ascites carcinoma-bearing mice.[5, 6)] However, the effect of the liposomalization on the closed-open reaction of the lactone ring of CPT-11 or SN-38 is not clear. In this study, we examined the tissue distributions of the closed and open forms of CPT-11 and SN-38 in Lewis lung carcinoma-bearing mice after the administration of liposomal CPT-11. The conversion of CPT-11 to SN-38 in the tumor is thought to result in an increase in antitumor activity and a decrease in the side toxicity of CPT-11; it is thus expected that SN-38 generation in tumors is necessary for effective antitumor activity of CPT-11. We therefore investigated SN-38 generation from CPT-11 encapsulated in liposomes *in vitro*.

MATERIALS AND METHODS

Drugs Irinotecan hydrochloride (100 mg/5 ml vial), used to prepare CPT-11 solution (Sol), was purchased from Daiichi Pharmaceutical Co., Tokyo. Distearoylphosphatidylcholine (DSPC) and dimyristoylphosphatidylglycerol (DMPG), used to prepare liposomes, were purchased from Nichiyu Liposome Co., Tokyo. CPT-11, used to prepare liposomes, was kindly provided by Yakult Honsha Co., Tokyo. 1-Monomethoxypolyethyleneglycol-2,3-dimyristoylglycerol (PEG-DMG) containing PEG with an average molecular weight of 2,000 was a gift from Nippon Oil & Fats Co., Tokyo. Carboxylesterase (EC 3.1.1.1) was obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of liposomes Liposome preparation was performed according to the method of Bangham *et al.*[7)] Namely, DSPC/cholesterol/DMPG (100:100:60 μmol) and 10 mg of CPT-11 (15 μmol) were dissolved in a chloroform/methanol mixture (2:1, v/v). The chloroform and methanol were completely evaporated off under a stream

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of nitrogen gas. The resultant thin lipid film was evacuated in a desiccator, then hydrated with 8.0 ml of 9.0% sucrose in 10 m*M* lactate buffer (pH 4.0) in a water bath at 50–60°C for 10 min. The suspension was sonicated for 20 min above the phase transition temperature (*Tc*) after nitrogen gas bubbling. The liposome suspension was extruded through two stacked polycarbonate membrane filters with 0.2 μm pores, followed by extrusion five times through 0.1 μm pore filters above the *Tc*, to afford a homogeneously sized liposome suspension. PEG-modified S-Lip (S-PEG) and liposomal CPT-11 (S-Lip) were prepared by adding 2.0 ml of 9.0% sucrose in 10 m*M* lactate buffer (pH 4.0) with or without 5.0 mol% PEG-DMG, respectively, to 8.0 ml of the liposome suspension, followed by sonication of the mixture. The liposome suspension was dialyzed against 9.0% sucrose in 10 m*M* lactate buffer (pH 4.0) for 16 h. The trapping ratio of CPT-11 in all liposomes was above 90%.

Animal experiments Male CDF_1 mice (body weight, 20–25 g; 5 weeks old) were obtained from Japan SLC (Hamamatsu). Lewis lung carcinoma cells (5×10^5 cells/animal) were subcutaneously transplanted onto the backs of the mice. In the distribution study, on the 21st day after transplantation, tumor-bearing mice were injected via a tail vein with Sol, S-Lip or S-PEG at a dose of 10 mg/kg as CPT-11. At 1, 2 and 8 h after injection, mice were killed by cervical dislocation, and blood was collected from the heart. The liver, gall bladder and tumor were immediately removed and washed. The CPT-11 and SN-38 concentrations in the plasma and each tissue were determined as follows. The tissues were homogenized in saline to obtain a 5.0% homogenate. One milliliter of tissue homogenate or 10% plasma was added to 0.5 ml of 0.1 *M* lactate buffer (pH 4.0) followed by mixing. This mixture was added to 3.0 ml of 1-butanol as an extracting solvent, and the whole was shaken in a vortex mixer then centrifuged at 1,200*g* for 15 min. After centrifugation, total CPT-11 and total SN-38 (i.e., open plus closed forms) in the upper layer were quantitated by fluorescence spectrophotometry (CPT-11, *Ex*: 374 nm, *Em*: 435 nm and SN-38, *Ex*: 380 nm, *Em*: 556 nm). The levels of closed CPT-11 and SN-38 were determined by the same procedure except for the addition of 0.1 *M* Hepes buffer (pH 7.5) in place of lactate buffer.[8] The level of each open form was obtained as total form minus closed form.

To study the effect of liposomalization on the efficacy of CPT-11 in Lewis lung carcinoma-bearing mice, male CDF_1 mice were divided into 4 groups, each consisting of 10 mice. Lewis lung carcinoma cells (5×10^5 cells/animal) were subcutaneously transplanted onto the backs of the mice. On the 15th, 18th and 21st days after inoculation, tumor-bearing mice were injected via a tail vein with Sol, S-Lip or S-PEG at a dose of 10 mg/kg/day×3 days. The numbers of live and dead animals were recorded daily.

Uptake of CPT-11 by tumor cells Ehrlich ascites carcinoma cells (1.0×10^6 cells/animal) were intraperitoneally transplanted into CDF_1 mice. The ascites fluid was collected on the 7th day after transplantation. The ascites carcinoma cells were washed twice and then resuspended (5.0×10^6 cells/ml) in Eagle's MEM medium containing 10% fetal bovine serum. Cell suspensions containing Sol, S-Lip or S-PEG (CPT-11 concentration : 30 nmol/ml (20 μg/ml)) were incubated at 37°C. After incubation, each cell suspension was cooled on ice and then centrifuged at 150*g* for 3 min. The cells were washed and then resuspended in ice-cold phosphate buffer (pH 7.8). The determination of CPT-11 was performed as described above.

Conversion of liposomal CPT-11 to SN-38 *in vitro* The reaction mixture contained Sol or S-Lip (CPT-11: 10 μg/ml) and carboxylesterase (32.5 U/ml) in 0.1 *M* Tris-HCl buffer (pH 7.5). The generation of SN-38 was investigated in this reaction mixture at 37°C after 3 h incubation. In a preliminary experiment, we confirmed that the liposomes were not disrupted under these conditions. The CPT-11 and SN-38 closed-form and open-form concentrations were determined as described above. The level of SN-38 generation was calculated using a reaction mixture without the enzyme as a control. The location of SN-38 in the liposomes was examined by observation of these liposomes by laser scanning confocal microscopy (ACAS Ultima 575 UVC; Meridian Instruments Far East, Tokyo). For this microscopic observation, liposomes were prepared according to the above method without extrusion, so that the size of the liposomes was about 1–5 μm, which could be easily observed microscopically.

Statistical analysis Statistical analysis was carried out by using analysis of variance (ANOVA), Student's *t* test or Wilcoxon's test.

RESULTS

Tissue distribution of liposomal CPT-11

Plasma (Fig. 1): The concentrations of closed CPT-11 and closed SN-38 in plasma were increased by liposomalization, and PEG modification prolonged the circulation time in the blood. In particular, at 8 h after administration, SN-38 was not detected in the plasma of the Sol group mice, whereas in the S-PEG group, SN-38 was still detectable in the blood (closed SN-38 concentration; 0.34 μg/ml plasma).

Liver and bile: At 2 h after injection, the concentration of closed CPT-11 in the liver of the S-Lip group was 3.5-fold ($P<0.001$) higher than that of the Sol group. The concentration in the S-PEG group was similar to that of the Sol group. The changes in the concentration of closed SN-38 in the liver showed the same tendency as that of CPT-11. At 2 h after administration, the closed/total ratios of the SN-38 concentration in the liver in the Sol, S-Lip

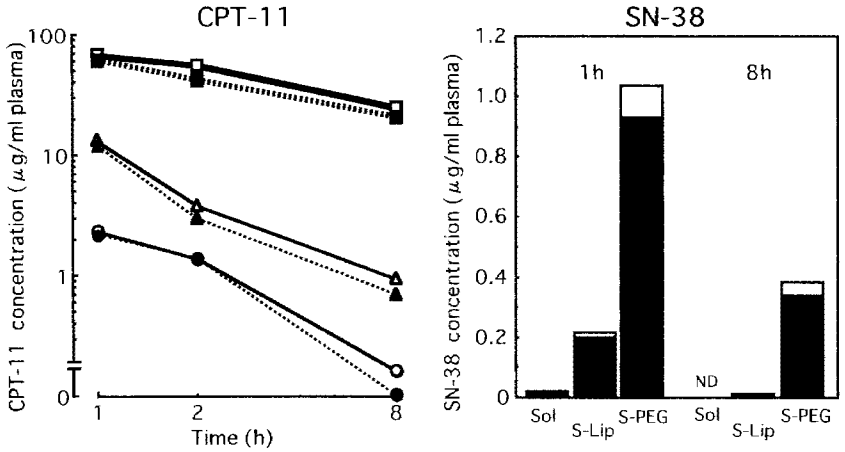

Fig. 1. CPT-11 and SN-38 concentrations in the plasma. Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, S-Lip or S-PEG. The data represent the means for three mice, each with duplicate data with no more than 10% variation between them. Open and closed columns indicate open and closed SN-38, respectively. Total level is open level plus closed level. Each point and each column for the S-Lip and S-PEG groups showed significant differences ($P<0.001$) compared to the corresponding data for the Sol group. Total: ○ Sol, △ S-Lip, □ S-PEG; closed: ● Sol, ▲ S-Lip, ■ S-PEG.

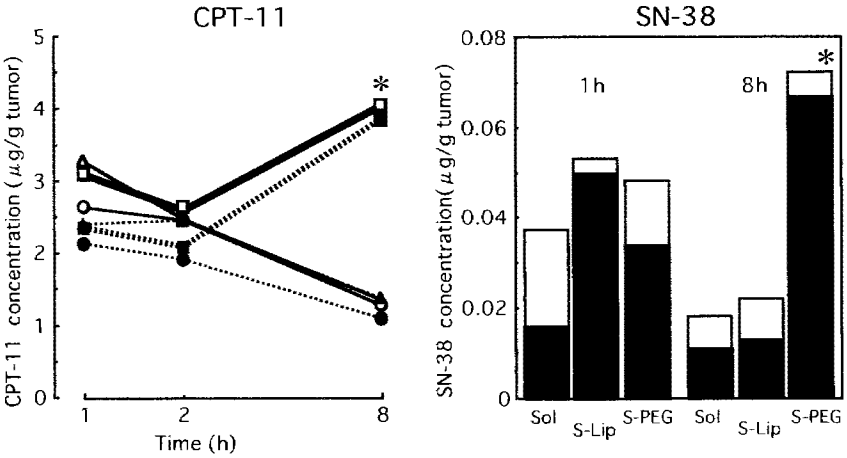

Fig. 2. CPT-11 and SN-38 concentrations in the tumor. Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, S-Lip or S-PEG. The data represent the means for three mice, each with duplicate data with no more than 10% variation between them. Open and closed columns indicate open and closed SN-38, respectively. Total level is open level plus closed level. A significant difference from the Sol level is indicated by * $P<0.001$. Total: ○ Sol, △ S-Lip, □ S-PEG; closed: ● Sol, ▲ S-Lip, ■ S-PEG.

and S-PEG groups were 0.201, 0.674 and 0.451, respectively. Therefore, this ratio was elevated by the liposomalization (figure not shown).

In the bile, the CPT-11 and SN-38 concentrations were decreased by the liposomalization. The concentrations of closed SN-38 in the bile of the S-Lip and S-PEG groups were 60% and 52% ($P<0.01$) of that of the Sol group, respectively (figure not shown).

Tumor (Fig. 2): At 8 h after administration, the concentration of closed CPT-11 in the tumors of the S-PEG group was increased 3.5-fold ($P<0.001$) compared to that of the Sol group. At the same time, the SN-38 concentration in the tumor was increased by the liposomalization and further increased by the PEG modification. In particular, the closed SN-38 concentration in the tumor at 8 h after S-PEG administration was 6.1-fold ($P<0.001$) higher than that in the Sol group. The closed/total ratio in the Sol group was 0.611, whereas that in the S-PEG group was 0.931.

Effect of liposomalization on the CPT-11-induced prolongation of survival in mice with Lewis lung carcinoma (Fig. 3) In mice inoculated with Lewis lung

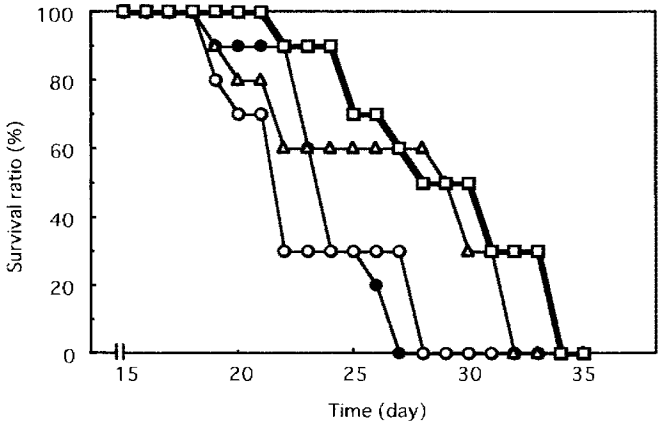

Fig. 3. Survival of tumor-bearing mice after administration of Sol, S-Lip or S-PEG. Lewis lung carcinoma cells (5×10^5 cells/animal) were subcutaneously transplanted onto the backs of the mice. On the 15th, 18th and 21st days after inoculation, tumor-bearing mice were injected via a tail vein with Sol, S-Lip or S-PEG at a dose of 10 mg/kg/day×3 days. Each group consisted of 10 mice. ● control, ○ Sol, △ S-Lip, □ S-PEG.

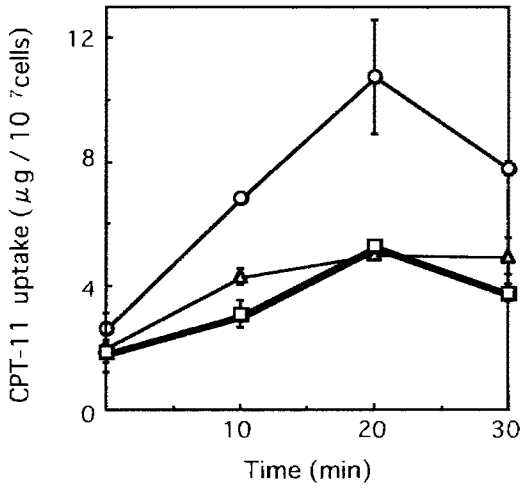

Fig. 4. Time course of uptake of CPT-11 by Ehrlich ascites carcinoma cells. The cell suspensions (5×10^6 cells/ml) in Eagle's MEM medium containing Sol, S-Lip or S-PEG (CPT-11 concentration: 20 μg/ml) were incubated at 37°C. After incubation, cells were collected and their CPT-11 level was determined. The data represent the means±SD for three experiments. ○ Sol, △ S-Lip, □ S-PEG.

carcinoma, the median number of survival days in the control group was 23.0 days. Sol administration had no effect on the survival period. In contrast, survival in the S-Lip and S-PEG groups was increased to 28.5 and 30.0 days ($P<0.01$ compared to the levels in the control and Sol groups), respectively. No significant difference between the S-Lip and S-PEG groups was observed.

Effect of liposomalization on the uptake of CPT-11 by tumor cells Regarding the uptake of Sol, S-Lip and S-PEG by Ehrlich ascites carcinoma cells, the CPT-11 concentrations in the tumor cells at 20 min after the addition of S-Lip and S-PEG were about half that in the Sol group (Fig. 4). As shown in Fig. 5A, with CPT-11 at 40 μg/ml, the CPT-11 concentration in the cells of the S-Lip group was half that of the Sol group, whereas in the case of CPT-11 at 5 μg/ml, the concentration in the S-Lip group was not significantly different from that in the Sol group. Thus, the cell uptake of CPT-11 in the Sol group increased with increase of CPT-11 concentration, whereas the elevation of the cell uptake in the S-Lip group showed a smaller concentraion dependence. However, the ratio of CPT-11 converted to SN-38 in the tumor cells was higher in the S-Lip than in the Sol group (Fig. 5B).

Conversion of liposomal CPT-11 to SN-38 *in vitro* Incubation of S-Lip with carboxylesterase resulted in the generation of SN-38 without disruption of the liposomes (Fig. 6A). The closed/total ratio of CPT-11 or SN-38 was higher in the S-Lip than in the Sol group. In the S-Lip group, these ratios of CPT-11 and SN-38 were 100% and 89%, respectively (Fig. 6B). The results of observation of

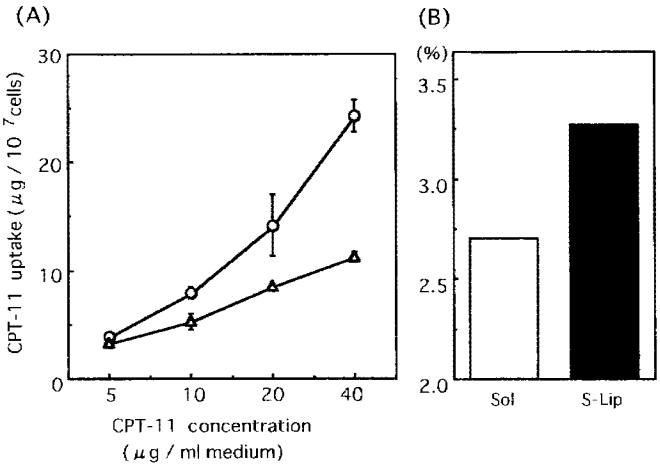

Fig. 5. Uptake of CPT-11 by Ehrlich ascites carcinoma cells. (A) Cell suspensions (5×10^6 cells/ml) in Eagle's MEM medium were exposed to Sol or S-Lip containing various CPT-11 concentrations for 20 min at 37°C. After the incubation, cells were collected and the CPT-11 level was determined. The data represent the means±SD for three experiments. ○ Sol, △ S-Lip. (B) SN-38/CPT-11 ratio with 40 μg/ml of CPT-11.

S-Lip by laser scanning confocal microscopy after incubation of S-Lip with carboxylesterase are shown in Fig. 7. In the photographs, the red coloration in the liposome indicates CPT-11, and the green indicates SN-38. Only the red color was observed in the liposomes incubated in

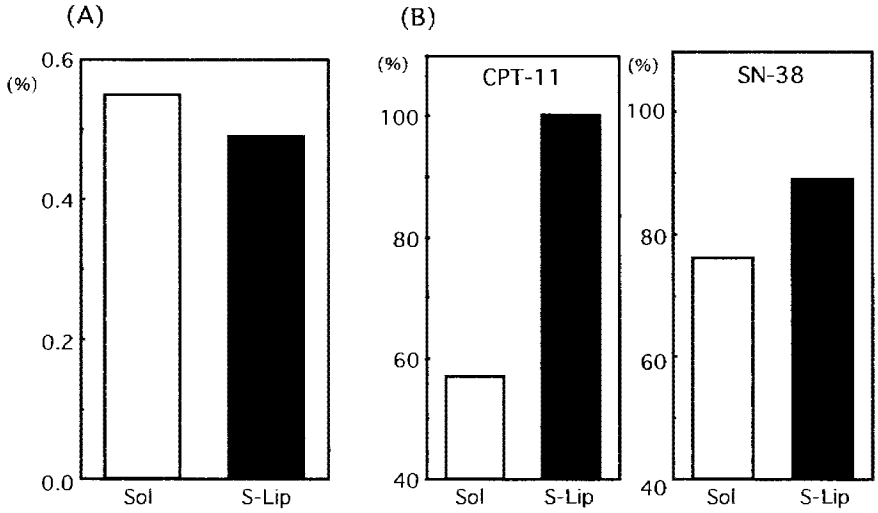

Fig. 6. The conversion of CPT-11 to SN-38 in liposomes. The reaction mixture contained Sol or S-Lip (CPT-11: 10 μg/ml) and carboxylesterase (32.5 U/ml) in 0.1 *M* Tris-HCl buffer (pH 7.5). The generation of SN-38 in this reaction mixture at 37°C was investigated after 3 h incubation. The CPT-11 and SN-38 closed-form and open-form concentrations were determined. (A) SN-38/CPT-11, (B) closed/total.

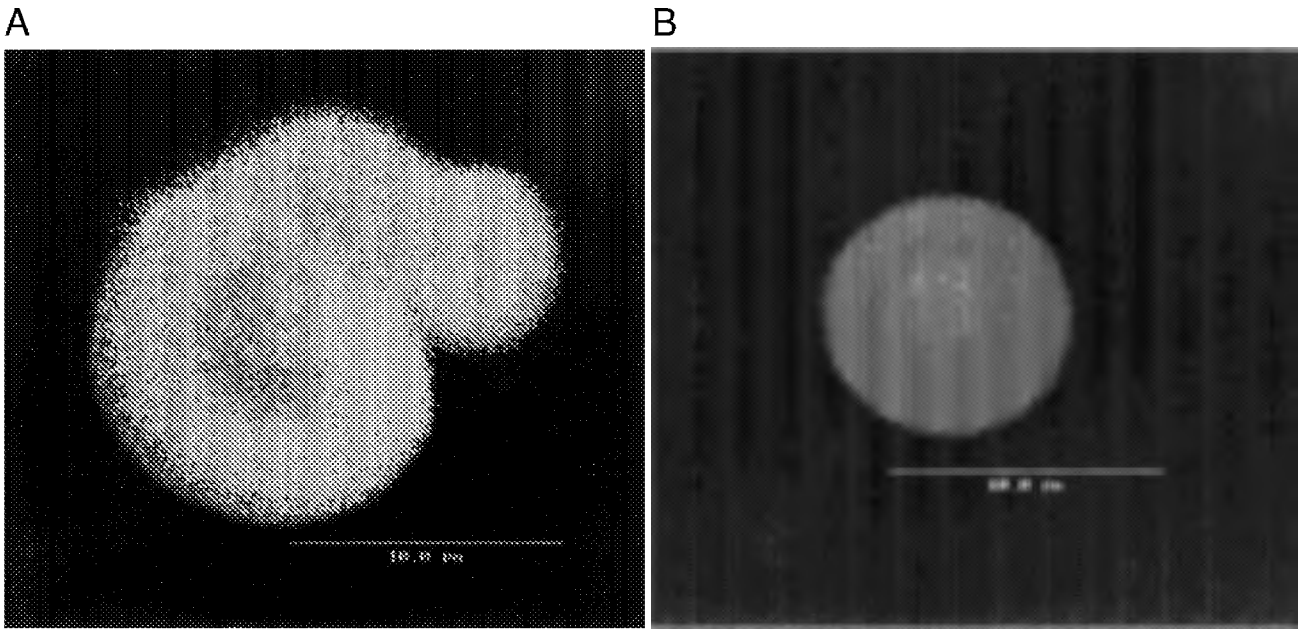

Fig. 7. Laser scanning confocal microscopic images of liposomal CPT-11 incubated with (A) or without (B) carboxylesterase. For this microscopic observation, liposomes were prepared as described in the text but without extrusion, so that their size was about 1–5 μm, which could be easily observed on microscopy.

buffer without carboxylesterase. Therefore, SN-38 did not exist in them. However, the liposomes incubated with carboxylesterase had green coloration on their surface. SN-38 is thus thought to have been generated from CPT-11 in the liposomal membrane in the latter case.

DISCUSSION

The lactone ring of CPT-11 is nonenzymatically hydrolyzed under neutral and basic conditions, and an equilibrium is reached between the open ring (carboxyl acid

form) and closed ring (lactone form) forms. The closed form of the lactone ring is essential for antitumor activity. In addition, the rate and equilibrium conditions of the open-closed reaction are changed on the binding of CPT-11 and SN-38 with serum albumin.[9–12] The closed lactone ring form of administered CPT-11 is cleaved gradually in the body. However, through encapsulation of CPT-11 in liposomes containing acidic water (pH 4.0), it was expected that the more stable lactone form of CPT-11 could be targeted to tumors. The concentrations of closed CPT-11 and SN-38 in the plasma of the Lewis lung carcinoma-bearing mice in the present study were increased by the liposomalization, and prolongation of circulation in the blood was produced by the PEG modification. In addition, the concentrations of closed CPT-11 and SN-38 in the tumors were elevated by both types of liposomes. Burke *et al.* reported that the lactone ring of camptothecin exists stably in the liposomal membrane.[13] Similarly, if the lactone ring of CPT-11 enters the liposomal membrane, the closed lactone ring could be stabilized. Since the closed/total ratio of SN-38 in the tumors of the present S-PEG group was larger than that of the Sol group, it is suspected that SN-38 was generated in the intact liposomes containing CPT-11. This effect, in conjunction with the targeting of loposomes to tumors, may enhance the antitumor activity while decreasing the intestinal side effect due to the closed form of SN-38. In fact, the closed SN-38 concentration in bile was decreased by the liposomalization of CPT-11 in the present study.

In the experiment involving Lewis lung carcinoma-bearing mice, the survival period of the Sol group was not prolonged, whereas those of the S-Lip and S-PEG groups were prolonged at the same dose. However, there was no significant difference in survival between the S-Lip and S-PEG groups. This experimental dose is chemotherapeutic and not toxic; namely, normal mice did not die or show decreased body weight, irrespective of the formulation. Therefore, the deaths of the mice were induced by the Lewis lung carcinomas, not by CPT-11-induced toxicity.

If CPT-11 is released from disrupted liposomes and then converted to SN-38, the closed/total ratio of SN-38 in the S-PEG group should be the same as that in the Sol group. However, increases in the closed SN-38 concentration and closed/total ratio of SN-38 in the tumors occurred upon liposomalization of CPT-11. This supports the idea that SN-38 is generated in intact liposomes.

The cellular uptake of CPT-11 in the Sol group increased depending on the CPT-11 concentration, whereas the concentration dependence was less in the S-Lip group. It is known that the amount of administered lipid affects the tissue distribution of liposomes[14, 15] and that liposome uptake by the liver is saturable.[16–18] With the use of 30–40 μg/ml of CPT-11 (this level is expected to be a lipid uptake saturation level), the lipid concentration was 78–104 m*M*, whereas with 5 μg/ml of CPT-11 (this level is below the uptake saturation level, and there was no difference in cellular uptake between the Sol and S-Lip groups), the lipid concentration was 13 m*M*. The CPT-11 concentration in the tumors *in vivo* was below 5 μg/g tumor, and the lipid concentration was below 13 m*M*. Namely, the lipid level did not reach the saturation level for cellular uptake *in vitro*, and there was no difference in the cellular uptake between the Sol and S-Lip groups. The CPT-11 concentration in the tumors of the S-Lip group was higher than that of the Sol group *in vivo*. Therefore, liposomal CPT-11 is more effective than a CPT-11 solution. In contrast, the CPT-11 concentration in the liver *in vivo* was about 30 μg/g liver and the lipid concentration was 78 m*M*, i.e., the uptake saturation level. Under these conditions, the liposomal uptake by the liver may be suppressed. In addition, the generation ratio of SN-38/CPT-11 in the tumor cells of the S-Lip group was higher than that of the Sol group. Thus, liposomalization resulted in significantly enhanced accumulation of SN-38 in the tumor relative to the liver.

CPT-11 is constituted from a hydrophilic piperidino-piperidinocarboxyl moiety and lipophilic SN-38, and is converted to SN-38 by carboxylesterase in the body, particularly in the liver.[19] We have confirmed that the concentration of closed SN-38 increases with the liposomalization of CPT-11. *In vitro*, under the absence of cells and without disruption of the liposomes, SN-38 was generated after the incubation of S-Lip with carboxylesterase despite the absence of CPT-11 in the medium (extra-liposomal fraction). As CPT-11 adsorbed on the surface of the liposomes was removed by NaCl, we suspect that SN-38

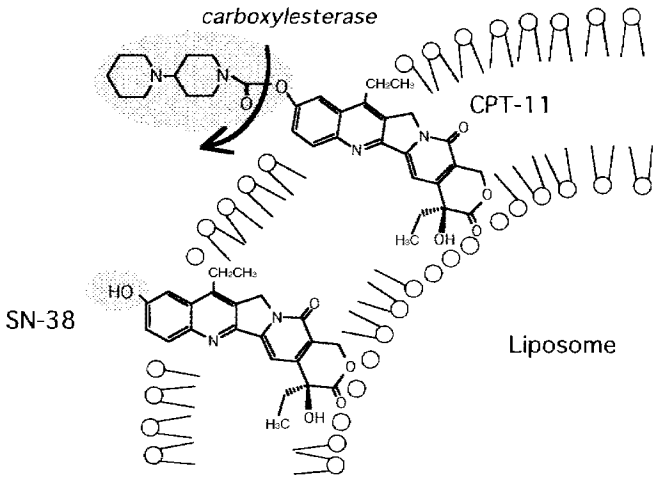

Fig. 8. A model of the conversion of CPT-11 to SN-38 in liposomes.

was generated from CPT-11 present in the liposomal membranes which were in contact with carboxylesterase in the medium. Furthermore, in the laser scanning confocal microscopy study, generation of SN-38 in the liposomal membranes was observed after the incubation of S-Lip with carboxylesterase. The structural change of the surface of liposomes following elimination of the piperidinopiperidinocarboxyl moiety is thought to result in a change in the ζ potentials of S-Lip during the incubation with carboxylesterase (data not shown). Based on these results, as shown in Fig. 8, the closed liposomal CPT-11 is speculated to enter, at least partly, the double lipid membranes of the liposomes in such a manner that the piperidinopiperidinocarboxyl moiety projects into the extra-liposomal fraction (medium). When these liposomes are distributed in some tissues, we suspect that CPT-11 in the membranes of the liposomes is exposed to carboxylesterase activity and generates closed SN-38 in the intact liposomes. Since the SN-38 thus generated exists in the membranes of liposomes, it is not affected by the physiological pH in the body. This closed SN-38 behaves like the liposomes, i.e., is preferentially distributed in the tumor. In mice and rats, carboxylesterase activity in the serum is high and the SN-38/CPT-11 ratio is also high compared with that in humans.[19] However, SN-38 generation by the carboxylesterase in the tumor of mice is similar to that of humans.[19] Therefore, the results in this paper have clinical relevance.

In conclusion, the liposomalization of CPT-11 increased the closed/total ratios of CPT-11 and SN-38 (i.e., favored the generation of closed SN-38) and elevated the antitumor activity of CPT-11 against Lewis lung carcinomas. In liposomal CPT-11, it is considered that a part of the prodrug CPT-11 is converted to SN-38 within the intact liposomes.

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Pharmacokinetically Guided Dose Adjustment of 5-Fluorouracil: A Rational Approach to Improving Therapeutic Outcomes

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Chemotherapy dosing of the fluoropyrimidine 5-fluorouracil (5-FU) is currently based on body surface area. However, body surface area–based dosing has been associated with clinically significant pharmacokinetic variability, and as such, dosing based on body surface area may be of limited use. The clinical activity of 5-FU is modest at standard doses, and in general, dosing is limited by the safety profile, with myelosuppression and gastrointestinal toxicity being the most commonly observed side effects. Various strategies have been developed to enhance the clinical activity of 5-FU, such as biochemical modulation, alterations in scheduling of administration, and the use of oral chemotherapy. Studies that have shown an association between plasma concentration with toxicity and clinical efficacy have shown that pharmacokinetically guided dose adjustments can substantially improve the therapeutic index of 5-FU treatment. These studies have shown that only 20%–30% of patients treated with a 5-FU–based regimen have 5-FU levels that are in the appropriate therapeutic range—approximately 40%–60% of patients are underdosed and 10%–20% of patients are overdosed. To date, 5-FU drug testing has not been widely used because of the lack of a simple, fast, and inexpensive method. Recent advances in testing based on liquid chromatography–mass spectroscopy and a nanoparticle antibody–based immunoassay for 5-FU may now allow for routine monitoring of 5-FU in clinical practice. We review the data on pharmacokinetically guided dose adjustment of 5-FU and discuss the potential of this approach to advance therapeutic outcomes.

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The fluoropyrimidine 5-fluorouracil (5-FU) has been used in daily clinical oncology practice for nearly 50 years, and it has been well established that a good correlation exists between 5-FU plasma levels and the biological effects of 5-FU treatment, both in terms of clinical efficacy and toxicity (1–6). Although 5-FU pharmacokinetic studies using cell-based and physical detection methods have been conducted since the mid-1960s (7–14), the application of 5-FU pharmacokinetic monitoring to clinical practice has become more realistic and practical since 5-FU administration via infusion schedules evolved to become the standard of care over the past 5–8 years (15,16).

Clinical studies that were conducted during the past 20 years have demonstrated reduced toxicity and improved clinical outcomes with pharmacokinetic dose management. These pharmacokinetically guided studies have identified an optimal target therapeutic range for 5-FU and have recommended dose-adjustment algorithms to bring plasma concentrations into the optimal range (17–29). Recent work has shown that many patients who are currently being treated with 5-FU are not being given the appropriate doses to achieve optimal plasma concentration. Of note, only 20%–30% of patients are treated in the appropriate dose range, approximately 40%–60% of patients are being underdosed, and 10%–20% of patients are overdosed. Studies that have shown associations between 5-FU plasma concentration with toxicity and clinical efficacy have demonstrated that pharmacokinetically guided dose adjustments substantially improve these biological effects, which are associated with 5-FU therapy. However, 5-FU monitoring has not been widely used, at least not in the United States, and certainly not outside the clinical research setting, given the absence of simple, fast, and inexpensive testing methods for 5-FU monitoring. Recent developments with testing based on liquid chromatography–mass spectrometry (LC-MS/MS) and a nanoparticle antibody–based immunoassay, as discussed later in this review, may facilitate routine monitoring of 5-FU in daily clinical practice (12–14).

The Rationale for Pharmacokinetically Guided 5-FU Monitoring

Current Dosing of 5-FU

The standard approach for calculating 5-FU drug dosage, as with many anticancer agents, has been to use body surface area (mg/m^2). Unfortunately, there is no rigorous scientific basis for this strategy, and the body surface area–based dose for 5-FU and the majority of other anticancer agents is generally recommended according to the maximum dose tolerated that had been established in early-phase clinical trials. Dosing based on body surface area is associated

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with considerable variability in plasma 5-FU levels by as much as 100-fold (29–31), and such interpatient and intrapatient pharmacokinetic variability is a major contributor to toxicity and treatment failure. Several potential sources of interindividual pharmacokinetic variation exist, including pharmacogenetic differences in absorption, distribution, metabolism, and excretion of anticancer drugs (31–35). Other factors that must also be considered include performance status, age, sex, weight, and circadian diurnal variation. Certain drug-specific factors can lead to pharmacokinetic variability. These factors include mode and schedule of drug administration; dietary status and interactions with food; interactions with prescription and nonprescription drugs, nutritional supplements, and herbal medicines; and compliance with oral agents (36).

The potential relationship between body surface area and 5-FU pharmacokinetics has been investigated by two main groups in Europe. In a series of 81 patients with metastatic colorectal cancer (mCRC), Gamelin et al. (37) documented a complete lack of association between body surface area and 5-FU clearance (Figure 1). Milano et al. (38) observed a similar lack of association between body surface area and 5-FU clearance in a study of 380 patients with head and neck cancer who were administered 5-FU plus cisplatin chemotherapy. They also showed that 5-FU clearance followed a Gaussian distribution and that the interindividual variability for clearance could vary as much as 100-fold. In addition, they found that 5-FU clearance was lower in women than in men but did not vary with age.

Taken together, these data support the view that dosing of 5-FU according to body surface area is of only limited use. Direct monitoring of 5-FU blood levels with appropriate dose adjustments may represent a more rational approach for 5-FU dosing. Using a pharmacokinetically based dosing approach would aid the oncologist in "personalizing" the 5-FU dose so as to obtain optimal systemic drug exposure that, in the end, would be more effective and less toxic for the individual patient. In a review of the role of clinical pharmacokinetics and drug monitoring in cancer therapy, Chabner (39) provided further support for this approach when he suggested that cancer patients would benefit from pharmacokinetic dose management.

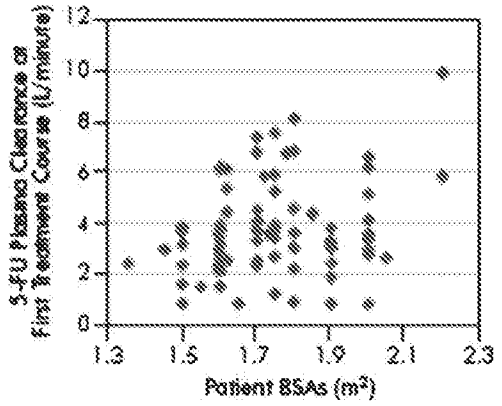

Figure 1. Distribution of 5-fluorouracil (5-FU) plasma clearance vs body surface area (BSA) during the first treatment course of 81 colorectal cancer patients (37). Reprinted with permission. Copyright 2008, American Society of Clinical Oncology. All rights reserved.

Pharmacokinetic Variability of 5-FU and Association With Biological Effect

5-FU was initially synthesized in the 1950s, and it continues to be the cornerstone of all major CRC treatment regimens for adjuvant therapy and for advanced metastatic disease. When it was first developed in the United States, 5-FU monotherapy was usually administered via a bolus schedule. However, during the past 5 years, many bolus schedules have been replaced by infusional regimens based on the work of de Gramont et al. (15,16) in France and Europe. The main treatment regimens used in the United States in the first-, second-, and third-line treatment settings are presented in Table 1 (40,41). The many treatment options now available to patients have transformed mCRC from an acute disease resulting in near-certain short-term mortality into a chronic illness, with median overall survival in the range of 24–28 months. As a result, individual patient characteristics are increasingly being considered in developing treatment strategies to ensure patient safety, allow longer-term systemic therapy, and improve patient quality of life while maintaining treatment intensity to maximize clinical efficacy.

One of the potential limitations of 5-FU therapy is the considerable pharmacokinetic variability that has been documented for both bolus and infusional schedules of administration. Despite this variability, a strong association has been identified between 5-FU plasma levels and biological effect, as it relates to toxicity and efficacy (Table 2). The pharmacokinetic parameter that has been most closely associated with biological effect is total drug exposure or area under the curve (AUC) drug concentration (1–6,17–28). Determination of AUC for bolus 5-FU schedules is somewhat difficult because of the number of samples that must be collected in a short period. In contrast, determination of AUC levels with infusional schedules of 5-FU is considerably simpler because only one sample, which is usually collected at steady state (2 hours into the infusion and throughout the infusion until the end), is required. With infusional regimens, the AUC can be simply calculated from the steady-state concentration (Css) by the relationship with time of continuous infusion in hours (T_{CI}):

$$AUC/T_{CI} = Css$$

or

$$Css \times T_{CI} = AUC.$$

The studies presented in Table 2 highlight the wide variability of 5-FU drug levels with infusional-based 5-FU regimens, whether it be 2-, 3-, or 5-day continuous infusion schedules, and they document the association between AUC levels and toxicity and response. The testing method most often used to measure 5-FU plasma levels has been high-performance liquid chromatography (HPLC) (11–13). In general, AUC levels greater than 25 mg·h/L were associated with an increased risk for developing toxicity in patients with CRC (1–6,23,24). Of note, other studies focusing on patients with head and neck cancer (42–44) found that AUC levels greater than 30 mg·h/L were associated with the development of toxicity, suggesting that target AUCs for 5-FU may differ based on the particular solid tumor type. With respect to CRC, the hematologic toxic effect most often associated with AUC was neutropenia, and the nonhematologic toxic effects associated with AUC included diarrhea, stomatitis, and hand–foot syndrome. In terms

Table 1. Use of chemotherapy regimens for metastatic colorectal cancer in the United States*

Regimen	Use as first-line therapy, %	Use as second-line therapy, %	Use as third-line therapy, %
FOLFOX† or FOLFIRI‡ plus bevacizumab§	44.3	23.8	3.3
FOLFOX†	24.0	5.9	0.6
Capecitabine based‖	13.8	11.3	13.2
Infusional 5-FU/LV¶	4.7	2.2	0.0
FOLFIRI	3.5	9.5	0.2

* Source: *Data Monitor*, 2006

† FOLFOX4 = 5-FU 400 mg/m² followed by 600 mg/m² infusion for 22 hours on days 1 and 2; LV 200 mg/m² IV on days 1 and 2; oxaliplatin 85 mg/m² IV on day 1. Each cycle is repeated every 2 weeks. FOLFOX6 = 5-FU 400 mg/m² on day 1, followed by 2400 mg/m² infusion for 46 hours; LV 400 mg/m² IV on day 1; oxaliplatin 100 mg/m² IV on day 1. Each cycle is repeated every 2 weeks. FOLFOX7 = 5-FU 2400 mg/m² infusion for 46 hours; LV 200 mg/m² IV on day 1; oxaliplatin 130 mg/m² IV on day 1. Each cycle is repeated every 2 weeks. FOLFOX7 = 5-FU 3000 mg/m² infusion for 46 hours; LV 200 mg/m² IV on day 1; oxaliplatin 100 mg/m² IV on day 1. Each cycle is repeated every 2 weeks. LV = leucovorin, IV = intravenous.

‡ FOLFIRI = 5-FU 400 mg/m² on day 1, followed by 2400 mg/m² infusion for 46 hours; LV 200 mg/m² IV on day 1; irinotecan 180 mg/m² IV on day 1. Each cycle is repeated every 2 weeks.

§ Bevacizumab = 5 mg/kg every 2 weeks or 7.5 mg/kg every 3 weeks. Each cycle is repeated every 2 weeks.

‖ Capecitabine monotherapy = 1000–1250 mg/m² orally twice a day on days 1–14. Each cycle is repeated every 3 weeks. Capecitabine combination therapy = 850–1000 mg/m² orally twice a day on days 1–14. Each cycle is repeated every 3 weeks.

of clinical activity, an early study by Hillcoat et al. (1) showed a close association between AUC and response rate and stable disease, whereas a more recent study by DiPaolo et al. (28) investigating a bolus schedule of 5-FU/leucovorin (LV) as adjuvant therapy of early-stage colon cancer identified a strong association between AUC and disease-free interval.

Practical Considerations for Pharmacokinetically Guided 5-FU Dose Management

Testing Methods

In the mid-1960s, cell-based assays were used for conducting pharmacokinetic studies of 5-FU (7,8). By the mid-1970s, this semiquantitative method was replaced with gas–liquid chromatography and gas chromatography–mass spectrometry (10). HPLC subsequently replaced gas–liquid chromatography, and it is currently the most commonly used method to measure 5-FU drug levels in the clinical setting (9–12). Liquid chromatography–mass spectrometry (LC-MSMS) has become increasingly popular, given its higher sensitivity (13). Although these methods are highly sensitive, they require sophisticated instrumentation and as such are expensive, labor intensive, and not readily amenable for widespread clinical use.

To further address the issue of 5-FU testing, an immunoassay for 5-FU has been recently developed (14). Novel, highly selective monoclonal antibodies for 5-FU were developed and then covalently attached to 200-nm nanoparticles, which serve as a label in a homogeneous competitive immunoassay format. The specificity of these antibodies to 5-FU was confirmed when it was shown that cross-reactivity was less than 1% for dihydro-5-FU, one of the main catabolites for 5-FU, 0.05% for capecitabine, and 0.23% for tegafur. This immunoassay requires only a small amount of plasma (<10 μL); takes only about 11 minutes to perform; and when using an Olympus AU400 ImmunoAnalyzer, up to 400 patient samples can be analyzed per hour. Compared with chromatography, the instrumentation required for this test is inexpensive and requires minimal technical training. Moreover, Beumer et al. (45) measured 5-FU drug levels in patients with colorectal cancer and head and neck cancer who were being treated with 5-FU–based regimens and found that the immunoassay results were consistent with those generated by other validated methods, including HPLC and LC-MS/MS. Thus, this novel technology has important advantages in

Table 2. Clinical studies highlighting 5-FU variability and relationship of AUC to biological effect in colorectal carcinoma*

Author	Country	Year	Biological effect
Hillcoat et al. (1)	Canada	1978	Association between AUC and clinical efficacy (RR and SD)
Au et al. (3)	United States	1982	Association between AUC and toxicity (myelosuppression)
van Groeningen et al. (4)	Netherlands	1988	Association between AUC and toxicity
Yoshida et al. (5)	Japan	1990	Association between AUC and toxicity
Trump et al. (6)	United States	1991	Association between AUC and toxicity (stomatitis and myelosuppression)
Gamelin et al. (19)	France	1998	Association between AUC and toxicity (diarrhea, hand–foot syndrome) and clinical efficacy (RR and OS)
Ychou et al. (23,24)	France	1999, 2002	Association between AUC and toxicity (myelosuppression, diarrhea, hand–foot syndrome)
Gamelin et al. (22)	France	2008	Association between AUC and clinical efficacy (response rate, PFS, and OS)
DiPaolo et al. (28)	Italy	2008	Association between AUC and clinical efficacy (DFS)

* 5-FU = 5-fluorouracil; AUC = area under the drug concentration curve; DFS = disease-free survival; OS = overall survival; PFS = progression-free survival; RR = response rate; SD = stable disease.

terms of speed, small sample size, minimal sample handling, and ready application on automated instrumentation. Taken together, the immunoassay test has the potential for making 5-FU testing practical, cost-effective, and widely available for routine clinical practice.

Establishing Target AUCs With the Newer Infusion Regimens

The development of an optimal target therapeutic range for cytotoxic agents, and for 5-FU specifically, is based on a balancing of toxic effects that result from higher levels of drug concentrations and compromised clinical efficacy resulting from suboptimal dosing. The main goal was to establish a target drug concentration that will be just below that which will induce grade 3–4 toxicity in the majority of treated patients, thereby ensuring a balance between the positive and the negative biological effects of 5-FU therapy.

Much of the work to establish the appropriate target 5-FU range was based on identifying an association between AUC levels and toxicity. Gamelin et al. (17–22,25–27) in France have been the leaders in conducting pharmacokinetically guided clinical studies with 5-FU infusion-based regimens for mCRC because the de Gramont leucovorin LV5FU2 regimen was first adopted as the standard of care in Europe and the United States. Recently, Di Paolo et al. (28) reported on the results of a phase II pharmacokinetic study of patients who were treated with a bolus 5-day 5-FU/LV regimen in the adjuvant therapy of patients with stage II/III colon cancer. They observed a strong association between 5-FU AUC levels and disease-free survival, with a relative improvement in 10-year disease-free survival of 50% in the group with an AUC value greater than 8.4 mg·h/L. This AUC value of 8.4 mg·h/L, when multiplied for the number of treatment days per month, corresponds to the same monthly target exposure value that Gamelin et al. have identified in studies using infusion-based regimens. In the United States, where infusion-based regimens have become the standard of care in the past 5–8 years, monitoring studies have been less common, mainly because of the absence of a simple, fast, and reliable testing method.

Evidence exists to suggest that the therapeutic AUC range of 5-FU may differ depending on the specific regimen that is being used and the particular cytotoxic agent that is being administered with 5-FU (46). For example, when the reduced folate LV is combined with 5-FU, as in the case of infusional LV5FU2, the optimal range for 5-FU AUC is 20–25 mg·h/L. In contrast, when 5-FU is combined with cisplatin, a regimen that is widely used for head and neck cancer, the target range for 5-FU AUC is higher, on the order of 25–30 mg·h/L. However, given the unavailability of suitable drug assay methods, therapeutic drug monitoring with dose modification is used only rarely in cancer treatment and usually only in the context of a clinical trial setting. Currently, the only example where drug monitoring is being used in clinical practice is with high-dose methotrexate therapy, in which methotrexate drug levels are routinely monitored to determine the subsequent dose and scheduling of LV rescue.

A consistent target range of AUC for all of the newer 5-FU/LV infusion–based regimens has been established as 20–25 mg·h/L, despite different administration modes (bolus vs infusion) and schedules (several hours to several days) (21–24,27,28). The LV5FU2 regimen on which these studies were based is the foundation for the FOLFOX and FOLFIRI combination regimens. Established target steady-state levels for LV5FU2 have provided guidance for studies evaluating the target range of 5-FU in the FOLFOX and FOLFIRI regimens. To date, it appears that the target AUC is consistent across all treatment regimens as long as 5-FU is administered via an infusion schedule.

Clinical Value of Pharmacokinetically Guided 5-FU Dose Management

Dose Monitoring in Clinical Practice

Gamelin et al. (21) at the Paul Papin Cancer Center in Angers, France, routinely monitor patients who are treated with 5-FU–based chemotherapy for CRC. This group treats approximately 5000 CRC patients each year with the various 5-FU–based regimens (LV5FU2, FOLFOX, FOLFIRI), and 5-FU drug levels are measured in all patients using standard HPLC methods. The dose of 5-FU administered with the first cycle is based on body surface area, with all subsequent doses based on pharmacokinetic-guided dose adjustment. Their testing protocol is simple in that only one patient sample is taken, usually toward the end of the 5-FU infusion. The 5-FU plasma levels are then used to recommend a 5-FU for the next dose of 5-FU, and this treatment algorithm is presented in Table 3. In a subset of 802 patients with CRC treated with this approach, they found that 345 (42.7%) patients had 5-FU levels that were at the target level and did not require further dose adjustment, 373 (46.3%) patients had 5-FU levels that were below the target level and needed dose adjustment upward, and 88 (11%) patients had 5-FU levels that were above the target level and required dose reduction (21). Using this pharmacokinetically guided approach, they were able to substantially reduce the incidence of grade 3 or 4 toxic effects resulting from 5-FU therapy, the need for hospitalization and supportive care, and the need for treatment interruption and dose delays. One of the potential limitations, however, is that the HPLC method used for 5-FU testing is labor intensive, which limits turnaround time and the number of patients that can be monitored.

Dose Management to Improve Clinical Efficacy and Safety Profile

A randomized, phase III multicenter clinical trial was conducted using an infusion regimen of 5-FU/LV and compared standard dosing with therapeutic dose monitoring. Each arm consisted of 104 patients with mCRC, and patients were treated with 5-FU at a somewhat unconventional dosing schedule of 1500 mg/m^2 given as an 8-hour infusion on a weekly basis with 400 mg/m^2 LV (22). In group A, patients were dosed according to body surface area, and in group B, patients were dosed based on body surface area in the first cycle with subsequent doses adjusted to a target level of 20–24 mg·h/L based on drug levels, as determined by HPLC-based monitoring in the previous cycle. To achieve the prescribed target concentration levels, 18 (17.3%) patients in group B had their dose adjusted downward, 15 (14.4%) patients were at target level, and 71 (68%) patients in group B required an increase in dose to achieve the target levels. Thus, 85% of the patients who were randomly assigned to the dose-adjusted group did not receive an optimal dose of 5-FU. On average, four cycles of therapy were

Table 3. Dose-adjustment algorithm for 5-fluorouracil (5-FU) with FOLFOX6*

	Toxicity grade = 0/1		
5-FU plasma concentration, μg/L	AUC, mg h/L	Dose adjustment (± percent of previous dose)	Toxicity grade ≥2
<110	AUC<5	+150	Grade 2: dose reduction of 200 mg
110–220	5<AUC<10	+100	
220–330	10<AUC<15	+25	
330–450	15<AUC<20	+15	
450–550	20<AUC<25	Unchanged	Grade 3: stop for 1 week; subsequent dose reduction of 300 mg
550–650	25<AUC<30	−10	
650–750	30<AUC<35	−15	
750–850	35<AUC<40	−20	

* AUC = area under the drug concentration curve. Reprinted with permission. Copyright 2008, American Society of Clinical Oncology. All rights reserved (22).

required to achieve the target concentration, and impressively, the therapeutic range for 5-FU was eventually achieved in 94% of patients.

With respect to safety profile, the overall incidence of grade 3–4 toxicity was substantially reduced (Table 4). In particular, grade 3–4 diarrhea was markedly reduced in patients receiving pharmacokinetically guided 5-FU when compared with body surface area–dosed 5-FU (4% vs 18%). Of note, the 58% of patients who were below target concentration and needed dose adjustment upwards did not experience a clinically significant increase in grade 3–4 toxicity over the entire treatment period, except for hand–foot syndrome, which was higher in group B. Given the reduced toxicity in group B, patients were treated for longer periods (7.9 months) when compared with patients who were treated in group A with standard dosing (6.8 months). In terms of clinical efficacy, patients who received 5-FU with pharmacokinetically guided dosing had an overall response rate of 34%, which was twofold higher than the 17% response rate seen in patients dosed by body surface area (Table 4). This difference was highly statistically significant (P = .004). An improvement in median overall survival was observed from 16 months for the body surface area–dosed patients to 22 months for the pharmacokinetically guided group. Although this difference did not reach statistical significance (P = .08), it should be noted that overall survival was not an endpoint of the study nor was the trial sufficiently powered for overall survival. Moreover, one would predict that the subsequent salvage therapies would have been well balanced in both arms, thereby diluting out any potential effects of pharmacokinetic dose adjustment on survival.

Gamelin et al. (26) recently presented data of two patient cohorts treated in parallel for mCRC in the front-line setting. In this study, patients were treated with a modified FOLFOX 4 (mFOLFOX4) regimen (oxaliplatin dose: 85 mg/m^2 every 2 weeks, 5-FU: 400 mg/m^2 bolus + 2500 mg/m^2 over 44 hours every 2 weeks and folinic acid 200 mg/m^2 every 2 weeks). Group A consisted of 39 patients whose 5-FU dose was calculated based on body surface area. Group B consisted of 118 patients, and although their initial 5-FU dose was determined by body surface area, all subsequent 5-FU doses were adjusted based on 5-FU drug levels, which were determined by HPLC. In group A, the dose of 5-FU remained unchanged, except in patients who experienced grade 3–4 toxicity. In contrast, for patients treated in group B, the dose was adjusted individually to achieve a target concentration of 600 μg/L based on a dose-adjustment algorithm using the 5-FU plasma concentration measured during 5-FU administration. In group B, dose adjustment was required in greater than 80% of the patients treated to achieve the 5-FU target concentration, and of this group, nearly 50% required dose adjustments of greater than 20%.

Patients treated with 5-FU in the dose-monitoring group (group B) experienced much less overall grade 3–4 toxicity than those in group A who were treated with standard dosing of mFOLFOX4. Specifically, the incidence of grade 3–4 diarrhea, mucositis, and neutropenia were 1.7%, 0.8%, and 18%, respectively, which is much lower than what was observed in patients in group A, whose incidence was 12%, 15%, and 25%, respectively. In terms of clinical efficacy, overall response rate was increased in the group B patient cohort (69.5% vs 46%), and median progression-free survival (16 vs 10 months) and overall survival (28 vs 22 months) were also higher for patients who were treated with 5-FU dose adjustment. Although this study was not randomized and the patient numbers are not well balanced, the findings from this study provide further evidence that therapeutic drug monitoring of 5-FU represents a controlled and safe approach to dose adjustment with potential benefits both in terms of efficacy and toxicity.

Table 4. Gamelin phase III study: summary of toxicity and efficacy*

	Outcome				
	Toxicity, %				
Dosing	Diarrhea	Mucositis	Hematologic	n	P
BSA	18	2	2	104	.003
Pharmacokinetically guided	4	2	0	104	
	Response rate, %				
BSA	18.3				.004
Pharmacokinetically guided	33.6				
	Median OS, mo				
BSA	16				.08
Pharmacokinetically guided	22				

* BSA = body surface area; OS = overall survival. P values (two-sided) were calculated using Fisher exact test.

Dihydropyrimidine Dehydrogenase (DPD) Deficiency

DPD is the rate-limiting enzyme involved in the catabolism of 5-FU. Up to 80%–85% of an administered dose of 5-FU is broken down by this enzyme to inactive metabolites. The presence of DPD deficiency results in a reduced ability to metabolize and clear 5-FU, and the half-life of the drug, which is normally approximately 10–15 minutes, can be markedly prolonged to up to 159 minutes (46–54). A pharmacogenetic syndrome has been identified in which partial and compete deficiency in the DPD enzyme has been observed in 3%–5% and 0.1% of the general population, respectively (50,51,55,56). In this setting, patients experience excessive severe toxicity in the form of myelosuppression, diarrhea and mucositis, and neurotoxicity. To date, more than 30 sequence variations in the *DPD* gene have been identified, and these complex genotypes are inherited in an autosomal codominant fashion.

Of note, even in patients who express normal DPD activity, there is wide interindividual variability in activity of up to 20-fold (52). The association between low DPD activity and high 5-FU plasma levels is strong in individuals who exhibit profound deficiency. However, it is now increasingly appreciated that *DPD* mutations are unable to account for all of the observed cases of DPD deficiency because as many as 50% of patients who experience increased 5-FU toxicity have no documented alterations in the *DPD* gene. Moreover, individuals who have normal enzyme activity may also be diagnosed with high plasma levels of 5-FU, resulting in increased toxicity. It is clear, therefore, that factors other than DPD status may contribute to 5-FU metabolism and eventual 5-FU toxicity.

Several cancer centers and reference laboratories in the United States provide testing services to detect the major *DPD* genotype associated with the deficiency. Unfortunately, there is no approved test for measuring DPD enzyme activity and/or protein expression, although such testing is being performed at certain select centers in the United States. Although identification of a deficient genotype identifies a person at potential risk for toxic effects, it is clearly not the only risk factor. It has been suggested that fewer than 50% of individuals who experience grade 3–4 toxicity have mutations in the *DPD* gene and/or have diminished DPD activity that would identify individuals with low 5-FU clearance (51,55,57). It is now known that 5-FU clearance is a function of several factors in addition to DPD. A recent report by Bocci et al. (58) described using a test dose of 5-FU to identify individuals at risk for developing toxic effects. This study identified three of 188 patients tested who displayed low drug clearance. Interestingly, all three individuals exhibited normal DPD activity, and as such, they would have been missed with a genotype and/or enzyme activity assay for DPD, highlighting the importance of monitoring the actual phenotype, which in this case would be the 5-FU drug level.

Role of Thymidylate Synthase (TS)

TS catalyzes the enzymatic reaction that provides the sole de novo intracellular source of thymidylate, an essential precursor for DNA synthesis. As such, this enzyme has been an important target for cancer chemotherapy for more than 40 years, and specifically for 5-FU, capecitabine, and other antifolate-based TS inhibitor compounds, such as pemetrexed, raltitrexed, and pralatrexate. Several studies (59–61) have shown that TS levels in tumor tissue may be associated with response to 5-FU–based chemotherapy, in that low levels of TS expression in the tumors of mCRC patients were associated with clinical response and improved survival. It has also been well documented that the promoter enhancer region of the TS gene, *TYMS*, is polymorphic (62,63). The promoter region usually contains a double-tandem repeat (2R) or a triple-tandem repeat (3R), and the importance of these polymorphisms is that homozygous 3R/3R tumors express higher levels of TS mRNA and TS protein compared with homozygous 2R/2R tumors. A study conducted by Lecomte et al. (64) showed that patients with the 2R/2R genotype experienced statistically significantly higher grade 3–4 toxicity than patients with the 3R/3R genotype (43% vs 3%). This study suggested that TS genotyping may help identify patients at increased risk for developing toxicity to 5-FU chemotherapy. Schwab et al. (65) recently provided further evidence supporting the association between the 2R/2R genotype and the 5-FU toxicity. However, the increased risk for toxicity was 1.6-fold, which was much less pronounced than that reported in the study by Lecomte et al. In fact, this study concluded that TS genotyping had only a limited role in 5-FU–related toxicity and that other nongenetic factors, such as sex, mode of administration, and the use of LV might be more biologically relevant.

Role of Circadian Variation

Circadian rhythm has been shown to be a potentially important factor that determines 5-FU pharmacokinetic variability (66). Several circadian mechanisms have been identified that include alterations in the enzyme activities of several key enzymes involved in 5-FU metabolism and in the proliferative activity of bone marrow and intestinal mucosa. An inverse relationship between DPD enzyme activity in peripheral blood mononuclear cells and 5-FU plasma concentrations, and a circadian rhythm in 5-FU drug levels and DPD activity was identified (48–54). Based on this and other clinical data, it was postulated that circadian timing or chronomodulation of drug delivery might allow for an increase in 5-FU dosage.

Levi et al. (67) conducted a randomized phase II study comparing fixed-infusion-rate delivery of oxaliplatin, 5-FU, and LV chemotherapy with chronomodulated delivery of the same chemotherapy regimen in patients with mCRC. In patients who were randomly assigned to the fixed-delivery arm, drug delivery was kept constant during a 5-day period, and in the chronomodulated group, patients received a maximum delivery of oxaliplatin at 4:00 PM and a maximum delivery of 5-FU and LV at 4:00 AM. Chronomodulated delivery of chemotherapy resulted in a marked reduction in toxicity, especially in the form of grade 3–4 mucositis, and a statistically significant improvement in clinical efficacy in terms of overall response rate, progression-free survival, and median overall survival. The results of this study highlight the potential importance of chronomodulation. However, at the same time, this study underscores the complexities involved with using such a dosing strategy. In particular, one issue to consider is how best to identify the peak time for optimal drug delivery. In contrast to the study by Levi et al., other investigators (68,69) have failed to identify consistent circadian patterns in 5-FU pharmacokinetics in cancer patients receiving prolonged drug infusion. As a result, chronomodulated administration of 5-FU, although interesting, would not appear to be a practical approach that can be used for daily clinical oncology practice.

Economic Importance of Pharmacokinetically Based 5-FU Dosing

Pharmacokinetically guided dose adjustment of 5-FU may result in substantial savings, given the reduced incidence of toxic effects and improved clinical outcomes. In a multicenter randomized study of head and neck patients who were treated with 5-FU and cisplatin, Fety et al. (29) demonstrated that the costs associated with toxicity were considerably reduced for patients receiving 5-FU by a dose-managed approach when compared with those treated with standard 5-FU dosing. For the 5-FU dose-adjusted group, the total cost for treatment of toxicity was $6803, whereas the costs of toxicity for the standard treatment group were $21 758, which represents a substantial reduction in medical costs of nearly 70%.

It has now been shown that the use of pharmacokinetically guided dose adjustment with infusional LV5FU2 regimen may achieve the same level of clinical efficacy, in terms of response rate and overall survival, when compared with what has been reported for the more complex FOLFOX and FOLFIRI combination regimens using standard dosing of 5-FU (17,18,22,23). It should be noted that these comparisons involve phase II studies with the pharmacokinetically guided approach vs larger phase III studies with the standard dosing approach. However, these findings may have important pharmacoeconomic implications. If only chemotherapy drug costs are considered, the cost of the infusional LV5FU2 regimen is relatively inexpensive, on the order of less than $500 for a 6-month treatment course. In contrast, the drug costs for either the FOLFOX or the FOLFIRI combination regimens would be substantially more expensive, in the range of $22 000–$30 000 during this same 6-month period, when 5-FU is dosed using the conventional body surface area–based approach (70). The cost that would be required for 5-FU testing with the new 5-FU immunoassay is projected at $3000 during this 6-month period. When this cost is combined with the cost of LV5FU2 chemotherapy, the projected overall cost would still be substantially lower than what is projected for FOLFOX/FOLFIRI chemotherapy. As a result, dose monitoring has the potential to yield substantial savings in overall health-care costs by reducing the costs associated with toxicity and complications and by making less expensive regimens more effective.

In a recent study of dose adjustment with FOLFOX4, Gamelin et al. (26) observed that 5-FU dose adjustment resulted in a significant reduction in overall incidence of grade 3 or 4 toxicity when compared with standard dosing of 5-FU. Although an economic analysis was not conducted as part of this study, the savings from the reduction of treatment of complications would, in theory, have been considerable. In terms of clinical efficacy, dose-adjusted patients achieved a response rate of 70% and median progression-free survival of 16 months, which are impressively high for a cytotoxic chemotherapy regimen alone. As a comparison, in the randomized phase III NO16966 study (71), the combination of FOLFOX plus bevacizumab yielded a median progression-free survival of 9.4 months compared with 8.0 months in patients receiving FOLFOX chemotherapy alone. Meropol and Schulman (70) have estimated that, on average, the cost of 12 months of FOLFOX4 chemotherapy without dose adjustment would be $59 978, whereas the cost of FOLFOX4 without dose adjustment plus bevacizumab would be $107 175. In contrast, the cost of FOLFOX4 without bevacizumab but with 5-FU dose adjustment is projected to be $65 278, which includes the $6000 laboratory cost for 5-FU monitoring. Such a cost analysis suggests that optimizing drug concentrations of conventional cytotoxic chemotherapy regimens may lead to improvements in clinical efficacy such that the newer and more expensive biological agents may not be required, at least upfront. Clearly, rigorous pharmacoeconomic studies are required to more fully elucidate the economic value of 5-FU pharmacokinetic dose management.

Future Developments

A simple, cost-effective, and rapid testing method for 5-FU levels would further help replace body surface area dosing with pharmacokinetically guided 5-FU dose management. Moreover, this approach would add an important and complementary tool for physicians to identify and treat patients who experience 5-FU toxicity and who might have DPD deficiency and/or any other genetic defect that results in alterations in 5-FU metabolism. The advantages for such a test include the following:

1. The 5-FU monitoring test will determine the phenotype, which is the sum of all the factors that contribute to 5-FU drug levels, and it can therefore identify those individuals who may have normal DPD activity but are still unable to metabolize and excrete the drug rapidly.
2. The genotype test for DPD can identify individuals who are DPD deficient, but it does not provide guidance on dose adjustment. When used in conjunction with a genotype test, the 5-FU monitoring assay would further aid therapy by fine-tuning the dosing to achieve optimal drug levels.
3. The 5-FU monitoring test can be used in conjunction with a reduced test dose to identify patients at risk before treatment.
4. 5-FU monitoring can be used within a few hours of the start of infusional 5-FU therapy to quickly identify individuals at increased risk for developing toxicity, such as those with poor drug clearance, so that early adjustments could be made.

To further address the role of therapeutic drug monitoring as it relates to 5-FU chemotherapy, a prospective randomized clinical study is being planned in the United States using the mFOLFOX7 regimen plus the anti-vascular endothelial growth factor antibody bevacizumab in the frontline treatment of mCRC. Patients will be randomly assigned to receive 5-FU using standard body surface area–based dosing or to receive 5-FU using pharmacokinetically guided dose adjustment. The primary endpoints of this study will be time to tumor progression and safety profile. In this study, 5-FU drug levels will be measured using the newly developed nanoparticle antibody–based immunoassay. The goal of this study will be to provide further support for the use of pharmacokinetically guided dose adjustment of 5-FU in everyday clinical practice for patients with mCRC.

Conclusions

There is now a large body of clinical data showing that body surface area–based 5-FU dosing is unable to achieve optimal drug concentrations in a high percentage of patients. In fact, studies

that have been done over the past 30 years support the concept of pharmacokinetically guided 5-FU dose management. There is an unmet clinical need for a simple, fast, and reliable test that can be used on a routine basis to manage 5-FU dosing. The association between toxicity and high 5-FU plasma levels has been reported since the late 1970s, and plasma drug levels that result in grade 3–4 toxicity have been identified. Published studies document the ability to reduce toxicity associated with newer infusion 5-FU treatment regimens through pharmacokinetically guided dose adjustment. Of note, approximately 40%–50% of patients today are underdosed, and based on the recent clinical data, it is becoming increasingly clear that dose escalation can improve response rates and overall survival without increasing toxic side effects. Despite different administration modes (bolus vs infusion), administration schedules (several hours to several days), and various 5-FU–based combination regimens, a therapeutic dose range of 20–25 mg-hr/L has been consistently shown to yield optimal results in terms of clinical efficacy and safety. A dose-adjustment algorithm has been developed to guide practical implementation of 5-FU dose management.

The current standard of practice is that drugs with common patterns of toxicity used in combination therapy regimens are all adjusted downward when toxicity is observed because there is no reliable means of identifying which component is the primary contributor to the toxicity. Monitoring 5-FU plasma levels in 5-FU–based chemotherapy regimens, such as FOLFOX and FOLFIRI, would provide a rational approach for identifying the toxicity-inducing component of the combination regimen and would allow specific dose adjustment of 5-FU without having to modify the doses of other cytotoxic partners. DPD genotype detection testing is currently being offered by a number of reference laboratories in the United States. However, it is now well established that this test is unable to fully address the medical need and identifies only at most 40%–50% of patients at risk for increased 5-FU toxicity. A 5-FU monitoring test that directly measures drug levels would provide a more accurate assessment of 5-FU clearance. Finally, although nearly all of the studies focusing on pharmacokinetic dose adjustment have been done in the context of CRC, the findings would appear to have an even broader application for therapeutic drug monitoring of any treatment regimen that incorporates a fluoropyrimidine.

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Clinical Use of Irinotecan: Current Status and Future Considerations

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ABSTRACT

Irinotecan (CPT-11, Camptosar), a semisynthetic, water-soluble derivative of the plant alkaloid camptothecin, is a drug which has undergone extensive clinical investigation worldwide. It is, at this time, commercially available in the United States for the treatment of fluorouracil-refractory colorectal cancer. In this review, I will discuss the current approved clinical use, discuss the issues of toxicity and its management, and consider some of the ongoing clinical investigations which are exploring possible future uses for this agent. ***The Oncologist 1997;2:402-409***

HISTORICAL BACKGROUND

Camptothecin, the material from which irinotecan is derived, is the active agent isolated from the sap of the stem wood of the Asian tree *Camptotheca acuminata*. Camptothecin was identified in the mid 1960s as having antitumor activity in vitro [1]. Initial clinical trials reported in the early 1970s confirmed this activity, but the toxicities encountered were formidable [2-4]. Myelosuppression and hemorrhagic cystitis were both severe and unpredictable. In retrospect, this unpredictability was probably due to the insolubility of camptothecin. In order to conduct clinical trials, camptothecin was administered as the sodium salt. This disrupted the E-ring lactone portion of the camptothecin molecule, now known to be central to clinical activity, thereby resulting in the need for a tenfold increase in the amount of drug given. The variable dissociation of the sodium salt back into the more active parent compound, especially in the acidic environment of the bladder, most likely accounted for the unpredictable toxicity.

In the mid 1980s, investigators identified the mechanism of action of camptothecin as the inhibition of the nuclear enzyme topoisomerase I [5-9]. The identification of this novel target for therapeutics renewed interest in the camptothecins worldwide, and multiple investigators began working on soluble analogs of camptothecin which could be developed for clinical application [10]. As part of their investigations with soluble camptothecins, scientists at the Yakult Honsha Company in Tokyo, Japan, developed irinotecan, then called CPT-11 (CPT is an abbreviation for camptothecin), and demonstrated that this compound had favorable solubility characteristics and excellent preclinical activity [11-13]. These observations led to extensive phase I and II testing of irinotecan in Japan [14, 15] as well as in the United States and France [16-18]. Activity seen on these phase I trials led to phase II investigations in a number of chemotherapy-refractory solid tumors. Colorectal cancer was among the first tumors demonstrated to be potentially responsive to irinotecan, so development of this drug thus far, at least in North America, has focused on the treatment of colorectal cancer.

IRINOTECAN IN THE TREATMENT OF COLORECTAL CANCER

Antitumor responses in multiple patients with chemotherapy-refractory colorectal cancer were noted in the early phase I trials of irinotecan [14-18]. This finding led Japanes investigators to perform a phase II study of irinotecan in patients with metastatic colorectal cancer, 81% of whom had previously been treated with a fluorouracil-based chemotherapy regimen. Patients were treated with irinotecan at a dose of either 150 mg/m^2 given every 14 days, or 100 mg/m^2 given weekly. A major objective response rate of 27% was reported for the 63 evaluable

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patients treated, with a 22% response rate in those patients who had previously been treated with fluorouracil [19]. A subsequent trial conducted at the University of Texas, San Antonio, confirmed this finding, with a major objective response rate of 23% in 43 patients with fluorouracil-refractory colorectal cancer treated with irinotecan by 90-min infusion weekly for four weeks followed by a two-week rest [20]. A small number of patients on this trial received 150 mg/m^2 as their starting dose, but toxicities were felt to be unacceptable at this level and the majority of patients were treated at a starting dose, of 125 mg/m^2. Twenty-three percent of the patients treated on this trial achieved a major objective response, and an additional 31% of patients demonstrated either stable disease, or a minor clinical regression. Thus, 54% of patients experienced some tangible antitumor activity from receiving irinotecan on this trial.

In order to more fully evaluate the usefulness of irinotecan in fluorouracil-refractory colorectal cancer patients, a large confirmatory effort in the United States was undertaken involving the pooled data from 304 such patients in three essentially identical trials [21]. The initial starting dose of irinotecan in these trials was 125 mg/m^2 weekly for four consecutive weeks, followed by a two-week break. Based on concerns of some early toxicity, this starting dose was later reduced to 100 mg/m^2. The major objective response rate was 15% for the 125 mg/m^2 dose and 8% for the 100 mg/m^2 starting dose, with an overall major objective response rate of 13%. An additional 49% of patients experienced either a minor response or a stabilization of disease. It should be noted that this trial represents a sequential, rather than a randomized comparison of the 125mg/m^2 and 100 mg/m^2 starting dose. As such, one cannot make meaningful comparisons between these doses on the basis of this trial. Overall, the authors concluded that the toxicity at the 125 mg/m^2 dose level was acceptable, and this is the recommended starting dose for routine use on this schedule.

Investigators at Memorial Sloan-Kettering Cancer Center evaluated the activity and tolerability of irinotecan in patients with chemotherapy-naive metastatic colorectal cancer. In this trial, forty-one patients received a starting dose of 125 mg/m^2 weekly for four weeks, followed by a two-week break. The major objective response rate reported was 32%, with an additional 44% of patients demonstrating some more modest evidence of antitumor activity in the form of either minor response or stable disease [22]. At the same time, a cohort of patients with no prior chemotherapy treated on the same schedule at the Mayo Clinic demonstrated a 26% major objective response [23], confirming the substantial single agent activity of irinotecan in chemotherapy-naive colorectal cancer patients.

Development of irinotecan in Europe has largely utilized a single brief infusion given once every three weeks. A trial reported in final form from Institute Gustave Roussey in France utilizing a 350 mg/m^2 starting dose on this every-three-week schedule demonstrated an 18% major objective response rate in 213 enrolled patients [24]. Forty-eight of the patients had had no prior cytotoxic chemotherapy and 165 patients had progressed through one fluorouracil-based chemotherapy regimen. On this trial, the response rate was essentially the same for the chemotherapy-naive and fluorouracil-refractory patients.

A pooling of European trials utilizing a starting dose of 350 mg/m^2 given once every three weeks identified a similar activity rate, with 13% of 455 patients with fluorouracil-refractory colorectal cancer experiencing a major objective response (95% C.I. 9.7%-16.8%), with an additional 42% experiencing tumor stabilization, with a median duration of 7.5 months for response and 5 months for stabilization [25].

Pharmacokinetics

Irinotecan is metabolized by carboxylesterases in vivo to its active metabolite known as SN-38 [26-28]. SN-38 is several hundred-fold more active than irinotecan, and as such irinotecan serves to some degree as a prodrug. The relationship between irinotecan and SN-38, however, is quite complex. While the activity of SN-38 is several orders of magnitude greater than that of irinotecan, the concentration of irinotecan may be several orders of magnitude higher in the plasma during the time after drug administration. Thus, the view of irinotecan merely as a prodrug for SN-38 may be overly simplistic. As with the parent compound, camptothecin, irinotecan exists in plasma in an equilibrium between the closed-ring lactone form and the open-ring carboxy acid form, as does SN-38. The closed-ring form is substantially more active; however, the dynamic equilibrium, which is pH-dependent, makes accurate measurement of the ring-open form difficult and probably of little clinical importance, since the ratio is likely to be shifted to virtually all ring-closed at intracellular pH.

Correlations of pharmacokinetic parameters of irinotecan and SN-38 with either activity or toxicities have been inconsistent. In a phase I trial, *Rothenberg et al.* reported a mean terminal half-life for total irinotecan of 7.9 hours (± 2.8) and a mean half-life of 13.0 hours (± 5.8) for SN-38 [16]. The ratio between the area under the concentration versus time curve (AUC) for the lactone and carboxy acid forms of irinotecan and SN-38 remained constant over all dose levels studied. Linear relationships were shown between dose and peak concentration (C_{max}), and between dose and AUC of irinotecan, but no such linearity was demonstrable between irinotecan dose and C_{max} or AUC of SN-38. Fourteen percent of irinotecan and less than 1% of SN-38 was recovered in the urine, suggesting that renal excretion is not an important mechanism of elimination of this drug. In a phase II trial

reported by the same institution, pharmacodynamic relationships between pharmacokinetic parameters and nausea and vomiting, neutropenia, and response were not seen, although some correlation was suggested between SN-38 AUC on week 3 of treatment and diarrhea [20]. Other investigators, however, looking at pharmacokinetics in chemotherapy-naive colorectal cancer patients on a weekly schedule found no correlation between pharmacokinetic values and either toxicity or response [22].

Yet another group explored the pharmacokinetics of irinotecan on a schedule of 30-min infusions daily for three consecutive days every three weeks. They found irinotecan to have a mean terminal half-life of 8.3 hours (half life of SN-38 was not reported), with both irinotecan and SN-38 pharmacokinetics showing high interpatient variability [26]. Irinotecan and SN-38 rebound concentrations were observed in some patients within an hour after infusions, suggesting an enterohepatic recirculation. Total body clearance did not vary with increased dosage on this phase I study, indicating linear pharmacokinetics within the dosage range studied. On this trial, a modest, but statistically significant correlation between irinotecan AUC and SN-38 AUC was observed ($r = 0.52$, $p < 0.05$). Hematologic toxicity did not correlate with either irinotecan or SN-38 AUC; however, a correlation between irinotecan (but not SN-38) AUC and grade of diarrhea was reported.

In a pooled analysis of pharmacokinetics from 107 patients in three different phase I trials [27], mean terminal half-lives for irinotecan and SN-38 were 10.7 hours and 10.6 hours, respectively. This larger analysis also demonstrated a positive correlation between irinotecan dose and irinotecan C_{max} and AUC ($r = 0.78$ $p < 0.001$), and between irinotecan AUC and SN-38 AUC ($r = 0.75$, $p < 0.001$). Increasing dose within the dose levels studied did not lead to an increase in the fraction of drug metabolized to SN-38 (metabolic ratio), with a mean value of SN-38 AUC/irinotecan AUC = 3%. There were no significant correlations in this study population between drug clearance or metabolic ratio with age, sex, tumor type, body height or weight, or renal function; however, bilirubin elevations had a negative correlation with drug clearance. However, in this report, AUC of both irinotecan and SN-38 correlated with neutropenia, degree of diarrhea, and intensity of nausea and vomiting. The metabolic ratio did not correlate with any pharmacodynamic parameters.

Some investigators have demonstrated a correlation between glucuronidation of SN-38 and gastrointestinal toxicity [28], and others have suggested that an early rise in the bilirubin after treatment predicts for increased toxicity, presumably because the bilirubin is competing with SN-38 for glucuronidation, and those patients with more limited ability to glucuronidate will show a resultant rise in unconjugated bilirubin in the presence of SN-38. A specific enzyme isoform of uridine diphosphate glucuronosyltransferase, UGT 1.1, has been identified as being responsible for SN-38 glucuronidation [29]. Since this isoform is lacking in patients with Gilbert's syndrome, it would be anticipated that patients with Gilbert's syndrome would be highly intolerant of irinotecan. In a sample of 36 evaluable patients that explored the influence of race or gender on the pharmacokinetics and toxicity of irinotecan, no correlatons with these factors were seen [28].

Complicating matters further, other metabolites of irinotecan have been identified. One in particular, known as APC, [30] is a metabolite of irinotecan created by the cytochrome P450-dependent oxidation of irinotecan. While APC cannot be converted into SN-38, it can compete with SN-38 for glucuronidation. As such, the degree to which APC is present may influence the glucuronidation of SN-38, and so potentially influence gastrointestinal toxicity of irinotecan.

Toxicity

The two major dose-limiting toxicities of irinotecan are neutropenia and diarrhea. In the earlier stages of this drug's development, diarrhea appeared to be the major dose-limiting toxicity, but greater familiarity with irinotecan and its side-effect profile, and an understanding of procedures for dealing with this toxicity, have lessened the magnitude of this toxicity as an impediment to clinical use (Table 1).

Table 1. Factors favorably influencing irinotecan toxicity

- Physician experience with irinotecan
- Patient and family education
- Appreciation of need for EARLY intervention
- Adequate loperamide duration and intensity
- Aggressive and proactive follow-up
- Appropriate patient selection

Diarrhea

Diarrhea secondary to irinotecan can be divided into two distinct syndromes: early-onset diarrhea, which occurs during or shortly after irinotecan administration, and late-onset diarrhea, which is of greater concern, and which develops more than 24 h after drug administration. The early-onset diarrhea is part of a cholinergic syndrome which can be seen with irinotecan administration and which is characterized by rapid-onset diarrhea, and may also include abdominal cramping and diaphoresis [31]. This syndrome is relatively unusual and is easily managed. In the 304 patients with fluorouracil-refractory colorectal cancer included in the three phase II pivotal trials, 24 patients (8%) were reported as having experienced

grade III-IV diarrhea within 24 h of irinotecan administration (and were thus adjudicated as having experience early-onset diarrhea) [21]. Twenty-seven (9%) of the 304 patients received atropine 0.25 mg-1.0 mg intravenously as treatment of early-onset diarrhea. While formal data collection on the management of early-onset diarrhea was not done on this study, investigators reported that symptoms rapidly responded to atropine therapy, and only one of the 304 patients discontinued treatment due to early-onset diarrhea. No complications secondary to atropine administration were reported in this population.

Late-onset diarrhea has been a more serious and difficult problem in the management of patients receiving irinotecan, although this toxicity appears to be far more manageable now than it was in the earlier days of irinotecan's development. Late-onset diarrhea was the major problem encountered in initial phase I trials and early into phase II development, with diarrhea beginning any time after the first 24 h following treatment, but most commonly occurring approximately 10 days after the initiation of irinotecan therapy.

In 1993, *Abigerges et al.* reported that intensive administration of loperamide (Table 1) was effective in ameliorating much of the late-onset diarrhea caused by irinotecan [32]. For maximum effectiveness, the loperamide must be started at the first signs of diarrhea and taken at a dose of 2 mg every 2 h (or 4 mg every 4 h during the night) until the patient has gone 12 h without diarrhea. Anecdotally, failure to adhere to this rigorous schedule has been associated with less successful treatment of the diarrhea, and the importance of this antidiarrheal regimen must be stressed to each patient prior to the initiation of therapy.

While randomized evaluations of loperamide in the management of late-onset diarrhea have not been done, there are sequential data which give a strong indication of loperamide's effectiveness. In the trial reported from Memorial Sloan-Kettering cancer center [21] 10 of the first 18 patients treated (56%) required dose attenuations due to the development of grade III-IV diarrhea. The investigators then became aware of the report of *Abigerges et al.* [32], indicating the usefulness of loperamide, and began strictly adhering to the prescribed loperamide schedule. Of the next 23 patients treated, only two (9%) experienced grade III-IV diarrhea ($p = 0.001$).

In the three U.S. pivotal phase II studies of fluorouracil-refractory colorectal cancer, 93 of the 304 patients treated (31%) experienced grade III-IV late-onset diarrhea [21]. Of those 193 patients who received the 125 mg/m^2 starting dose of irinotecan, 65 (34%) developed grade III-IV diarrhea. It is noteworthy that bowel function is often a problem in patients with advanced colorectal cancer, possibly secondary to such factors as a history of bowel surgery, abdominal carcinomatosis, and/or narcotic analgesic use. These factors make accurate quantitation of gastrointestinal toxicity difficult in this patient population. Of the 304 patients evaluated 96 (32%) were taking laxatives during their irinotecan treatments. To what degree this influenced the incidence of late-onset diarrhea is not known. Overall, 83% of the 304 patients treated on the three phase II pivotal studies took at least one dose of loperamide; however, only four patients (1%) discontinued treatment secondary to late-onset diarrhea [21, 33].

To put the late-onset diarrhea into proper perspective, it is appropriate to compare its incidence with the incidence of severe diarrhea in fluorouracil-based treatments. A phase III trial of the two most widely used schedules of fluorouracil and leucovorin in chemotherapy-naive colorectal cancer patients reported dose-limiting diarrhea in 32% and 18% of patients receiving weekly high-dose leucovorin and daily × 5 low-dose leucovorin, respectively [34]. Especially when considering that this phase III population of patients was receiving initial chemotherapy and was thus earlier into their overall, antineoplastic treatment, the incidence of irinotecan-induced severe diarrhea does not appear to be outside of toxicity parameters seen with more traditional chemotherapy regimens.

Myelosuppression

Myelosuppression is the other major dose-limiting toxicity of irinotecan. Granulocytopenia is the major manifestation, with irinotecan being essentially platelet-sparing. In a trial of 41 chemotherapy-naive colorectal patients, six (15%) experienced grade III granulocytopenia, and three (7%) experienced grade IV [22]. In the pooled analysis of 304 phase II fluorouracil-refractory colorectal cancer patients, grade IV neutropenia was seen in 12% of patients, and 3% developed neutropenic fever [21]. No clinically significant thrombocytopenia was reported. One treatment-related death (0.3%), which was secondary to neutropenic sepsis, was reported in these 304 patients. This death rate compares favorably with the treatment-related mortality seen with other chemotherapy regimens. For example, seven deaths out of 372 patients (1.9%) [34] and eight deaths out of 620 patients (1.3%) [35] were reported in two large multicenter trials of fluorouracil-based chemotherapy in first-line colorectal cancer patients.

Other toxicities include nausea and vomiting, alopecia, and asthenia. Grade III/IV nausea and vomiting were reported in 13% of the 304 patients on the three U.S. pivotal phase II trials [21]. A variety of antinausea regimens have been used. Dexamethasone alone, or dexamethasone plus ondansetron or granisetron, is effective antiemesis for most irinotecan patients. Early reports had suggested that irinotecan might increase the likelihood of akathisias resulting from use of prochlorperazine, but the 8.5% incidence of akathisias reported is comparable to that seen with prochlorperazine alone. Some degree of alopecia was reported by 61% of the

304 patients, with complete hair loss being a relatively uncommon phenomenon. Twelve percent of the 304 patients reported ≥ grade III asthenia [21, 32].

Recommendations for Current Practice

Patient Selection

Irinotecan is a good choice for treatment of some patients with fluorouracil-refractory colorectal cancer and a poor choice for others. A key factor in the safe and effective use of irinotecan is proper patient selection, and the importance of this issue cannot be overemphasized. Patients with a favorable performance status (ECOG 0-1, or at least 2), adequate bowel function and oral intake, and the ability and motivation to follow complex directions are those who are more likely to tolerate irinotecan well (Table 2). As is true with virtually all chemotherapies for solid tumors, those patients with severe debilitation and/or inadequate motivation or capacity to follow directions are far more likely to experience severe or even life-threatening toxicity and are far less likely to obtain tangible clinical benefit. A patient who is looking and feeling well after progression through first-line fluorouracil-based therapy may benefit from having irinotecan as a therapeutic option. A patient who is moribund, living a bed-to-chair existence, not maintaining nutritional or fluid intake, or essentially unable to ambulate due to weakness and fatigue is a poor candidate for any cytotoxic chemotherapy, and use of irinotecan in such a patient is likely to have an undesirable result (Table 3). Unfortunately, many patients with fluorouracil-refractory colorectal cancer are not in reasonable shape to receive chemotherapy. One must exercise restraint in the management of such patients. The relevant question is not "Is there a drug that can treat this tumor?" but rather, "Is there a drug that can treat this patient?" One must recall that the response and toxicity data for irinotecan are based on the results of treating patients with the performance status, organ function, and motivation to meet the entry criteria of a clinical trial. Extrapolation of those data to patients who do not meet these criteria is neither reliable nor appropriate. Use of irinotecan as a salvage therapy for a severely debilitated patient is likely to result in serious detriment to that patient's quality of life, and as such, would be inappropriate palliative care.

Table 2. Good candidates for irinotecan treatment

- Good performance status (ECOG 0-2)
- Normal bilirubin
- Adequate nutrition (maintaining weight)
- Gut not obstructed (taking fluids well)
- Motivated patient
- Patient willing and able to follow directions

Table 3. Characteristics of poor candidates for irinotecan

- Poor performance status (ECOG 3-4)
- Poor p.o. intake
- Poor p.o. hydration
- Bowel obstruction
- Elevated bilirubin
- Unable to follow directions
- Apathetic or pushed by family

Dose Modifications

On the weekly ×4 schedule, the recommended starting dose is 125 mg/m². As discussed in the **Pharmacokinetics** section above, the metabolism of irinotecan to SN-38 and other metabolites varies widely from patient to patient. It is therefore reasonable to expect that a relatively wide range of doses will be required, and thus, that a significant number of dose modifications will be required. At present, there is no direct evidence of a steep dose-response curve with this drug, so that it is not at all clear that a higher dose is going to be more efficacious. For these reasons, it is often necessary and appropriate to modify doses as therapy progresses. For patients experiencing more than mild toxicity (grade 2 or greater) in the first week, a dose reduction of 20% or more may be needed in order to improve tolerability and increase the chance of delivering all planned doses. Patients experiencing a life-threatening (grade 4) toxicity often require larger dose reductions, in the 40% range.

Such dose adjustments are, of course, consistent with standard chemotherapy practices with other chemotherapy treatment regimens. For example, in a large randomized study of different fluorouracil-based regimens for first-line treatment of metastatic colorectal cancer, dose reductions for toxicity were required in 37% of patients receiving daily × 5 fluorouracil with low-dose leucovorin and were required in 41% of patients receiving weekly fluorouracil with high-dose leucovorin [35].

While it is important to adjust doses to individual patient tolerance, it is equally important not to dose-reduce in the absence of toxicity. For this reason, it is extremely important to take a detailed toxicity history, especially with regard to bowel movements. Patients with colorectal cancer rarely have normal bowel function. Some patients may have a large number of bowel movements per day as their normal post-surgical baseline. It is therefore important to inquire about changes in bowel habits rather than simply asking about the number or consistency of bowel movements. For example, a patient with a baseline of four bowel movements per day who reports having five to six bowel movements per day on irinotecan is not necessarily experiencing significant diarrheal toxicity and would not necessarily require dose adjustments on the basis of diarrhea.

A number of patients appear to tolerate their first three weeks of irinotecan therapy reasonably well but then exhibit evidence of toxicity (low blood counts, diarrhea, or fatigue) by the day of the planned fourth treatment. For such patients, it is appropriate to omit the fourth week of therapy and count that week as the first of two rest weeks, thereby reducing the treatment cycle to five weeks (three weeks "on," two weeks "off"). Some patients appear to tolerate this five-week schedule better than the six-week schedule, and the difference in dose intensity is quite minimal (patients treated three out of five weeks receive treatment 60% of the time; patients treated four out of six weeks receive treatment 66% of the time).

Future Development

With established antitumor activity against fluorouracil-refractory colorectal cancer, irinotecan is currently undergoing further development efforts to determine its usefulness in other aspects of colorectal cancer treatment, in combination regimens, and in earlier stages of the disease. Studies are also under way investigating the use of irinotecan in the management of other malignancies.

Given the activity of irinotecan in fluorouracil-refractory colorectal cancer, a combination regimen of fluorouracil and irinotecan in front-line management of colorectal cancer would appear to be potentially advantageous. Initial reports from investigators in Tokyo, however, indicated that fluorouracil might interefere with the carboxylesterase that converts irinotecan to SN-38, and so might inhibit the activity of irinotecan [36]. This report was based, however, on historical comparisons of the pharmacokinetics from different sets of patients. Investigators at Memorial Hospital in New York designed a phase I trial to explore the clinical possibility of combining irinotecan, leucovorin, and fluorouracil, and to investigate the pharmacokinetic interactions of these agents [37]. A weekly ×4 bolus schedule was chosen for this trial. On day 1, patients received irinotecan only, and a 24-h pharmacokinetic sampling for irinotecan and SN-38 was performed. On day 2, leucovorin and fluorouracil were given. On the following week (day 8), irinotecan was given, with leucovorin and fluorouracil given immediately thereafter, and a second full pharmacokinetic sampling was performed. At the beginning of the second treatment cycle, the leucovorin and fluorouracil were given before the irinotecan, and a third pharmacokinetic sampling was done. Thus, each patient served as his or her own control for irinotecan and SN-38 levels when irinotecan was given alone, given before fluorouracil and leucovorin, or given after fluorouracil and leucovorin. This trial demonstrated no substantial effect of fluorouracil on the conversion of irinotecan to SN-38. The combination regimen was found to be tolerable with full-dose irinotecan given with near full doses of leucovorin and fluorouracil. A phase III trial evaluating the safety and activity of this regimen against irinotecan alone, and against fluorouracil plus leucovorin alone, is currently ongoing. Recently, other treatment schedules of fluorouracil plus irinotecan have also been reported [38-40], and several of these are scheduled to enter phase III trials. A randomized phase II study to compare different irinotecan-plus-fluorouracil schedules with each other is currently planned as well. It should be emphasized that, at the time of this writing, the relative merits of concurrent administration of fluorouracil and irinotecan are as yet unknown, and such combinations are not, at present, recommended for use outside a clinical trial.

Combination trials with a number of schedules of cisplatin plus irinotecan have also been reported [41, 42]. A trial reported from Memorial Sloan-Kettering Cancer Center [41] uses a novel cisplatin schedule, with both cisplatin and irinotecan given weekly in order to maximize the potential for synergistic interaction between these two drugs. Activity on this phase I study was very encouraging. Based on this phase I experience, and on the reported single-agent activity of irinotecan in other malignancies such as non-small cell and small cell lung cancer [43, 44] and cervical cancer [45], investigations of this combination irinotecan/cisplatin regimen in a number of solid tumors, including lung cancer, esophageal cancer, gastric cancer, and ovarian cancer are currently under way. Trials of irinotecan in combination with other agents such as taxotere [46] and oxaliplatin [47] have also been reported, and investigations in combination with gemcitabine and other antitumor agents are currently under way. Phase I investigations of an oral formulation of irinotecan are planned for initiation in early 1998.

Adjuvant Therapy of Colorectal Cancer

Despite the antitumor activity which has been demonstrated to date, no patient is known to be alive and free of disease because of irinotecan. All of the above-mentioned uses of irinotecan, either alone or in combinations, have been targeted at patients with incurable metastatic disease. Irinotecan has therefore contributed thus far to the palliative care of many patients, and in doing so has provided substantial clinical benefit in many cases, but it has yet to save a life.

The potential for cure with currently available chemotherapy for advanced solid tumors lies in the appropriate use of active agents and regimens in the adjuvant setting, in order to eradicate microscopic metastatic disease and thus cure patients who would be otherwise destined to relapse and die after surgery. Based on the demonstrated activity of irinotecan in the metastatic setting, we at Memorial Sloan-Kettering have begun studying the use of irinotecan in the adjuvant setting of colorectal cancer. We are exploring both concurrent and sequential administration schedules of irinotecan plus fluorouracil and leucovorin for resected stage III and high-risk

stage II colon cancer patients. Based on our prior phase I study of daily low-dose irinotecan [48], we are also exploring the use of concurrent daily irinotecan plus pelvic radiation in the preoperative treatment of locally advanced rectal cancer. It is hoped that incorporation of irinotecan into adjuvant regimens for colorectal cancer will lead to increased cure rates in these earlier-stage patients. These clinical trials are now under way and are actively accruing patients.

Conclusion

Irinotecan is an important new tool in the treatment of cancer. Its current indication is in the second-line therapy of metastatic colorectal cancer, and in this setting it provides the potential for palliation in a patient population with an extremely limited number of viable treatment options. Diarrhea and neutropenia are the major dose-limiting toxicities; however, in appropriately selected patients, these toxicities are usually manageable and are comparable in frequency and severity to other routinely used chemotherapeutic agents. Ongoing studies are exploring the role of this agent in the adjuvant treatment of earlier-stage colorectal cancer, where, it is hoped, the demonstrated antitumor activity of irinotecan can be utilized to improve the cure rate and thus save lives. Other ongoing studies will further clarify the role of this new agent in combination chemotherapy regimens and in the treatment of other malignant tumors. On the basis of preliminary data, there is reason for optimism that irinotecan may become an important weapon in the armamentarium used to combat a number of different human malignancies. Clinical trials investigating and defining the therapeutic potential of irinotecan are currently ongoing on a large scale internationally, and patient participation in these trials is to be strongly encouraged so that we may more accurately define the proper uses for this promising new anticancer agent.

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Phase I Clinical and Pharmacokinetic Study of Irinotecan, Fluorouracil, and Leucovorin in Patients With Advanced Solid Tumors

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Purpose: **To determine the maximum-tolerable dose (MTD) of fluorouracil (5FU) when given with fixed doses of leucovorin and irinotecan (CPT-11), to define the dose-limiting toxicities of this combination, and to evaluate the effect of 5FU on the pharmacokinetics of CPT-11.**

Patients and Methods: **CPT-11, leucovorin, and 5FU were administered in repeated 6-week cycles that consisted of weekly treatment with all three drugs for 4 consecutive weeks followed by a 2-week break. On day 1 of treatment, CPT-11 alone was given by 90-minute infusion, and pharmacokinetic sampling was performed over 24 hours. Leucovorin and 5FU were administered by brief intravenous injection on day 2. On days 8, 15, and 22, CPT-11 infusion was immediately followed by leucovorin and then 5FU. A second 24-hour pharmacokinetic sampling was performed on day 8, which permitted comparison of the pharmacokinetics of CPT-11 with and without 5FU. For the second 6-week cycle, leucovorin was administered first, followed by 5FU and then CPT-11, and a third pharmacokinetic sampling was performed.**

Results: **Forty-two patients were entered onto this trial. The CPT-11 dose was initially fixed at 100 mg/m^2. Leucovorin was fixed at 20 mg/m^2. 5FU doses of 210, 265, 340, 425, and 500 mg/m^2 were studied. When the 500-mg/m^2 dose of 5FU was found to be tolerable, this was then maintained and CPT-11 was escalated to 125 and then 150 mg/m^2. This final CPT-11 dose exceeded the MTD. Neutropenia was the major dose-limiting toxicity. Diarrhea was common, but was rarely dose-limiting. Coadministration of 5FU had no substantial effect on the pharmacokinetics of CPT-11 or SN-38. Among the 38 patients with colorectal cancer, six partial responses (PRs) were seen in this predominantly 5FU-refractory patient population.**

Conclusion: **5FU does not substantially affect the metabolism of CPT-11 to its active metabolite, SN-38. The combination of CPT-11 125 mg/m^2, 5FU 500 mg/m^2, and leucovorin 20 mg/m^2 is feasible and tolerable on this schedule.**

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IRINOTECAN (7-ethyl-10-[4(-1-piperidino)-1-piperidino] carbonyloxy camptothecin), also known as CPT-11, is a semisynthetic derivative of the plant alkaloid, camptothecin.[1] Like camptothecin, CPT-11 appears to exert its cytotoxic action via inhibition of the nuclear enzyme topoisomerase I.[2-6] Topoisomerase I facilitates DNA replication and transcription by causing single-strand protein-bridged breaks in DNA, which thereby permits relief of torsional strain in the double helix ahead of the replication fork.[7,8] CPT-11 has shown encouraging antitumor activity in a number of solid tumor malignancies, including colorectal cancers,[9-11] non–small-cell and small-cell lung cancers,[12,13] and cervical cancer.[14] Unlike camptothecin, CPT-11 is water-soluble, which greatly facilitates its ease of administration, and leads to more predictable and manageable toxicity than was encountered with the parent compound.[15-17]

Fluorouracil (5FU) is an antimetabolite that has been the mainstay of gastrointestinal oncology for more than 30 years.[18,19] 5FU-based chemotherapy, usually in the form of 5FU plus leucovorin, is currently the standard treatment for metastatic colorectal cancer. Based on the observation of antitumor activity of CPT-11 in both chemotherapy-naive and 5FU-refractory patients, we set out to develop a regimen that would combine CPT-11 with 5FU plus leucovorin on a schedule which would be well tolerated and which would permit delivery of substantial doses of both cytotoxic agents. We chose to develop our regimen based on the schedule of CPT-11 that has been most extensively investigated in North America, ie, a 90-minute intravenous infusion given weekly for 4 consecutive weeks followed by a 2-week rest. We used a brief intravenous injection of leucovorin followed by a brief intravenous injection of 5FU, with all drugs given on the same schedule (4 weeks on, 2 weeks off). We chose to fix the leucovorin dose at 20 mg/m^2. Since diarrhea was a major dose-limiting toxicity in initial CPT-11 trials, we

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were concerned that use of high-dose leucovorin might lead to early emergence of dose-limiting diarrhea, and thereby limit the ability to dose-escalate 5FU.

Due to concerns raised by a prior report[20] that suggested 5FU might inhibit conversion of CPT-11 to its active metabolite, SN-38, we designed our pharmacokinetic investigations to evaluate the effect of 5FU and leucovorin on the metabolism of CPT-11.

PATIENTS AND METHODS

Eligibility criteria included documentation of incurable solid tumor malignancy with histologic confirmation of diagnosis at Memorial Sloan-Kettering Cancer Center. All patients were required to have essentially normal hepatic, renal, and bone marrow function, with a WBC count $\geq 4 \times 10^9$/L, platelet count $\geq 150 \times 10^9$/L, serum creatinine concentration $\leq$ 1.5 mg/dL, total bilirubin level $\leq$ 1.5 mg/dL, and AST level $\leq$ three times the upper limit of normal (or five times the upper limit of normal if hepatic metastases were present). A Karnofsky performance status of 70% or better was required. Prior 5FU-based therapy was permitted; however, because we anticipated giving substantial doses of 5FU, patients were excluded if prior 5FU had been discontinued solely due to intolerable toxicity. Patients were permitted to have received up to two prior chemotherapy regimens (with 5FU given on two different schedules counting as two different regimens); however, patients who had received prior mitomycin, carboplatin, or nitrosoureas were excluded. Based on concerns that patients who had undergone pelvic radiotherapy might be at greater risk for CPT-11–induced neutropenia, prior pelvic irradiation was adopted as an exclusion criterion for this study.

Pretreatment evaluation included a complete medical history and physical examination, chest x-ray, ECG, and imaging of measurable or assessable disease. A complete blood cell (CBC) count, biochemical screening profile, and, in colorectal cancer patients, carcinoembryonic antigen (CEA) level were obtained at baseline and at the start of each treatment cycle (each treatment cycle was planned with 4 weeks of treatment and a 2-week rest). CBC counts were also repeated weekly. Tumor imaging was repeated after every other treatment cycle. Response was recorded according to standard response criteria.[21]

Treatment Plan

CPT-11 was supplied by Pharmacia & Upjohn, Inc, Kalamazoo, MI, in 5-mL vials that contained 100 mg of drug and 2-mL vials that contained 40 mg of drug, and was administered in 500 mL of 5% dextrose solution by intravenous infusion pump over 90 minutes. Leucovorin (leucovorin calcium, citrovorum factor, folinic acid) was obtained commercially and administered as a 15-minute intravenous infusion immediately following completion of the CPT-11 infusion (except for cycle 2, in which the order of drug administration was reversed and leucovorin/5FU were given immediately before CPT-11). 5FU was obtained commercially and was administered by rapid intravenous injection immediately following leucovorin administration. All treatments were administered in the outpatient chemotherapy unit of Memorial Sloan-Kettering Cancer Center.

Dose-Escalation Schedule

The dose of CPT-11 was initially fixed at 100 mg/m^2. Leucovorin was fixed at 20 mg/m^2. 5FU doses of 210, 265, 340, 425, and 500 mg/m^2, which represents an approximately 30% dose increase per level, were studied. When the 500-mg/m^2 dose of 5FU was found to be tolerable, this dose was then maintained and CPT-11 was escalated to 125 and then 150 mg/m^2.

Three patients were initially enrolled at each dose level. If none of these three experienced dose-limiting toxicity (defined later), then the next three patients were enrolled at the next highest level. If one patient experienced dose-limiting toxicity, then the treatment level was expanded to six patients. If no more than one of six patients experienced dose-limiting toxicity, then the next cohort of patients was treated at the next highest dose level. If two or more patients at any dose level experienced dose-limiting toxicity, then that level was considered to have exceeded the maximum-tolerable dose (MTD), and the level immediately preceding that level was designated as the MTD. Further patients were then enrolled at the MTD to investigate more fully the tolerability and toxicities of that level.

Evaluation During Treatment

Patients were evaluated for toxicity weekly while on study, and all toxicities were graded using the National Cancer Institute common toxicity criteria. Dose-limiting toxicity was defined as any grade 3 or greater nonhematologic toxicity (except alopecia, nausea, or vomiting), grade 4 hematologic toxicity, neutropenic fever, or inability to receive all planned chemotherapy during the first 6 weeks of study. Diarrhea was graded as maximum toxicity experienced despite the intensive loperamide therapy used in this study.

After initiation of protocol treatment, patients were permitted to proceed with therapy if clinically indicated if, on the scheduled day of treatment, the WBC count was $\geq 3 \times 10^9$, the absolute granulocyte count was $\geq 1 \times 10^9$, the platelet count was $\geq 100 \times 10^9$, and all diarrhea and/or stomatitis had resolved. Patients who did not meet these criteria had therapy delayed for 1 week. If therapy could not be administered, as per these parameters, by the following week, then the patient was adjudicated as having experienced dose-limiting toxicity. If therapy resumed following only a 1-week delay for non–dose-limiting toxicity, and the full planned dose of therapy was administered within 5 weeks, then 1 rest week was given before the second cycle of therapy was started. Since the full planned doses were administered within the prescribed 6-week cycle, and the second cycle of therapy was started on schedule, these patients were adjudicated as having tolerated the dose. Further therapy was continued without dose reductions.

If a patient did experience dose-limiting toxicity, as defined earlier, that patient was permitted, if clinically indicated, to continue to receive treatment at the next lowest dose level, once toxicity had completely resolved. Patients who had non–dose-limiting toxicity that necessitated holding of the week 4 treatment dose were permitted, if clinically indicated, to begin the 2-week rest at that time, thereby shortening that cycle to 5 weeks.

Patients were instructed, verbally and in writing, at the time of protocol entry, to pay particular attention to any changes in their bowel habits while taking CPT-11. At the first sign of increased stool frequency, loose stools, or liquid stools, patients were instructed to take loperamide 2 mg orally and to continue this dose every 2 hours (or 4 mg every 4 hours at night) until 12 hours had elapsed without a bowel movement.

Decadron 10 to 20 mg intravenously was routinely given as a prophylactic antiemetic with each CPT-11 dose. Patients in whom this did not provide adequate control of nausea were given either

ondansetron or granisetron on subsequent cycles as needed. Twenty-three of 42 patients on study received ondansetron or granisetron.

This study was reviewed and approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center. All patients were fully informed of the investigational nature of this protocol, and all gave written informed consent before initiation of treatment.

Pharmacokinetic Methods

To obtain baseline pharmacokinetic data, CPT-11 alone was given on day 1 of the first treatment week; pharmacokinetic samples for CPT-11 and SN-38 were then obtained over 24 hours. Leucovorin and 5FU were given on day 2. On all subsequent weeks in the first cycle, leucovorin and 5FU were given immediately after CPT-11. The pharmacokinetic assessment was repeated on week 2; thus each patient had CPT-11 pharmacokinetics performed with and without 5FU/leucovorin, which allowed each patient to serve as his/her own control. During the dose-escalation phase of the study, on the second 6-week treatment cycle, leucovorin and 5FU were given immediately before CPT-11 and a third pharmacokinetic assessment was performed to evaluate the effects, if any, of the order of drug administration on the toxicity and pharmacokinetics of this regimen. On the third and all subsequent cycles, CPT-11 was followed immediately by leucovorin and then 5FU on each treatment day.

Pharmacokinetic specimens were drawn into iced heparinized assay tubes and centrifuged at 3,000 rpm for 20 minutes. A baseline sample was drawn immediately before the initiation of the CPT-11 infusion. Samples were then drawn at 45 minutes into, and at the end of the CPT-11 infusion, as well as at 5, 10, 15, and 30 minutes, and 1, 2, 4, 6, and 24 hours after the completion of the CPT-11 infusion. Plasma was separated and stored at −20°C. Plasma concentrations of CPT-11 and SN-38 were determined using a high-performance liquid chromatographic (HPLC) technique. Briefly, the method consisted of precipitating proteins from the plasma and simultaneously converting all of the ring-open form of CPT-11 and SN-38 to lactones by adding acidified internal standard in acetonitrile and heating at 40°C for 15 minutes. The sample was then buffered with 0.025 mol/L triethylamine buffer (pH 4.2), mixed, centrifuged, and an aliquot of the supernatant injected into the HPLC system. The HPLC consisted of a Zorbax SB C_8 (250 mm × 4.6 mm; internal diameter, 5 μm) analytical column (MAC-MOD Analytical, Chadds Ford, PA) with a mobile phase of acetonitrile 0.025 mol/L triethylamine buffer (3:7 vol:vol) at 1 mL/min. The column eluant was monitored with a fluorescence detector that operated at an excitation wavelength of 372 nm, with emission wavelengths of 425, 535, and 425 nm for the CPT-11, SN-38, and internal standard (camptothecin) peaks, respectively. Inverse prediction of concentration from peak height ratios using a through-the-origin linear model weighted by 1/concentration^2 of the calibration standards allowed quantitation of both analytes. The lower limit of quantitation of CPT-11 was set to 1.4 ng/mL (expressed as the free base) and to 0.40 ng/mL (expressed as the monohydrate) for SN-38. The upper limit of quantitation for CPT-11 was set to 3,461 ng/mL and to 1,280 ng/mL for SN-38. The mean assay precision expressed as the coefficient of variation was 6% for CPT-11 and 5% for SN-38. Interassay mean recoveries of quality control samples ranged from 92% ± 4% to 102% ± 5% for CPT-11 and from 98% ± 4% to 100% ± 6% for SN-38.

For pharmacokinetic analyses, CPT-11 concentrations were expressed as hydrochloride trihydrate equivalents. The actual time of blood sampling was recorded and this time, relative to the start of drug infusion, was used in calculating areas under the concentration-time curve. The CPT-11 and SN-38 plasma concentration data for the 27 patients in the dose-escalation phase of this study were analyzed by noncompartmental methods.[22] The apparent terminal elimination rate constants (λ_z) were determined by linear least-squares regression of plasma-concentration time points that appeared by visual inspection to lie in the terminal log-linear region of the plasma concentration-time profiles. The apparent elimination half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$.

Peak plasma concentrations (C_{max}) and the time at which they occurred (T_{max}) were determined by inspection of individual patient CPT-11 and SN-38 concentration-time curves. Area under the plasma concentration-time curves ($AUC_{0\text{-}24}$) were determined using the linear trapezoidal rule from time zero to the last sampling time (approximately 24 hours after the end of the 90-minute infusion) at which quantifiable drug concentrations were detected (C_T). Area under the CPT-11 plasma concentration-time curves through infinite time ($AUC_{0\text{-}\infty}$) was calculated by adding C_T/λ_z to $AUC_{0\text{-}24}$. The systemic clearance (CL) and apparent volume of distribution (V_z) of CPT-11 were calculated as dose/$AUC_{0\text{-}\infty}$ and CL/λ_z, where dose is the administered dose of CPT-11. Since CPT-11 was administered at three different dose levels (100, 125, and 150 mg/m^2) on this trial, C_{max} and $AUC_{0\text{-}24}$ were also normalized to a dose of 100 mg/m^2 by dividing these parameters by the dose administered and multiplying by 100.

Pharmacokinetic parameters obtained on each of the 3 treatment days were compared using a univariate repeated measures analysis of variance in which the effects of interest were dose (between-subject effect), period (within-subject effect), and the dose-by-period interaction term. Statistical analyses was performed using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS Institute, Inc, Cary, NC) Least-squares means analysis was used for pairwise comparisons if a significant effect was detected. For all evaluations, statistical significance was defined by a P value less than.05.

RESULTS

Forty-two patients were entered onto this trial. Forty-one are included in this report. One patient developed a bowel obstruction secondary to carcinomatosis and was removed from study after only 2 weeks of chemotherapy. He had no toxicity referable to treatment and therefore toxicity and response data from this patient are not included in this report.

Twenty-seven patients were treated on the dose-escalation portion of this trial. Following identification of the MTD, 15 additional patients (14 assessable) were entered at the MTD to evaluate more fully the tolerability and toxicities of this regimen at this dose. Patient characteristics are listed in Table 1. The median age of patients on this study was 52 years (range, 34 to 73). The median Karnofsky performance status was 80% (range, 70% to 90%). Twenty-five men and 17 women were entered. Due to our interest in this regimen for the treatment of colorectal cancer, the majority of our patients (n = 38) had metastatic colorectal carcinoma. Of these 38 patients,

Table 1. Patient Characteristics

Characteristic	No. of Patients
Median age, years	52
Range	34-73
KPS (%)	
Median	80
Range	70-90
Male:female	25:17
Primary tumor site	
Colorectal	38
5FU-refractory	25
Chemotherapy-naive	14
Unknown primary	2
Chemotherapy-naive	2
Esophageal	1
Chemotherapy-naive	1
Carcinoid nonfunctional	1
Chemotherapy-naive	1

Abbreviation: KPS, Karnofsy performance status.

25 (66%) had received one or more prior chemotherapy regimens.

Toxicity

Neutropenia was the major toxicity encountered on this trial. The 150-mg/m^2 dose level of CPT-11 (leucovorin 20 mg/m^2 and 5FU 500 mg/m^2) proved to be intolerable, with one patient developing grade 4 neutropenia with fever, and one patient developing prolonged neutropenia that precluded continuation of treatment for more than 2 weeks. A third patient tolerated this dose level. Hematologic toxicity data are listed in Tables 2 and 3. Thrombocytopenia was not, for all practical purposes, encountered.

No patient developed grade 3 or 4 diarrhea during the dose-escalation phase of this study.

Following identification of the MTD as 125 mg/m^2 of CPT-11 given in conjunction with 20 mg/m^2 of leucovorin and 500 mg/m^2 of 5FU, 14 assessable patients were subsequently treated at this level. Combined with the initial three patients treated at this level, a total of 17 assessable patients have been treated (nine chemotherapy-naive, eight with prior chemotherapy) at the MTD. Of these 17 patients, five (29%) experienced dose-limiting neutropenia and three (18%) experienced dose-limiting diarrhea. Of the patients who experienced dose-limiting neutropenia, one tolerated treatment well for over 4 months and then developed neutropenic fever on week 5 of her third 6-week cycle. Another developed grade 3 neutropenia and fever at the end of his second 6-week treatment cycle in the setting of clinical disease progression and overall clinical deterioration.

This trial was designed without prophylactic cytokine usage, and no patient received prophylactic cytokine support either during the initial treatment cycle, or on subsequent cycles. Two of the three patients with dose-limiting diarrhea experienced this toxicity in the setting of already declining performance status, and both manifested clinical and radiographic signs of intestinal obstruction during or shortly after their diarrhea, which suggests that mechanical factors may have contributed to their symptoms. No clinical or radiographic evidence of toxic megacolon was seen in patients on this trial. The incidence of diarrhea at each dose level is listed in Tables 4 and 5. Other nonhematologic toxicity was easily manageable and is listed in Tables 6 and 7.

Advanced age did not appear to be a factor in the development of toxicity, as three of the five patients with dose-limiting hematologic toxicity and one of the three with dose-limiting diarrhea were below the age of 45 years. No deaths attributed to treatment-related toxicity occurred on this trial.

PHARMACOKINETICS AND PHARMACODYNAMICS

Mean and median pharmacokinetic parameters for CPT-11 and its active metabolite, SN-38, are listed in

Table 2. Hematologic Toxicity* in 27 Patients Treated on Dose-Escalation Phase of Study

5FU Dose (mg/m^2)	CPT-11 Dose (mg/m^2)	Leucovorin Dose (mg/m^2)	No. of Patients	Total No. of Cycles	WBC Nadir		ANC Nodir		Platelet Nadir	
					Medion	Range	Median	Range	Median	Range
210	100	20	3	5	3.6	3.4-3.9	2.0	2.0-2.7	260	258-286
265	100	20	3	10	3.6	3.1-5.1	2.5	1.5-2.7	194	170-319
340	100	20	3	9	3.5	2.3-8.4	1.9	1.0-6.1	264	225-484
425	100	20	6	36	3.8	1.1-5.5	1.8	0.3-2.4	137	119-259
500	100	20	6	29	2.5	1.4-3.1	1.0	0.6-1.8	169	142-244
500	125	20	3	12	3.5	2.4-4.6	1.9	1.1-2.5	257	202-277
500	150	20	3	9	1.7	0.9-3.1	0.8	0.3-1.1	233	159-335

Abbreviation: ANC, absolute neutrophil count.

*Most severe encountered, any course.

Table 3. Hematologic Toxicity* in 17 Patients Treated at the MTD

5FU Dose (mg/m²)	CPT-11 Dose (mg/m²)	Leucovorin Dose (mg/m²)	No. of Patients†	Total No. of Cycles	WBC Nadir		ANC Nadir		Platelet Nadir	
					Median	Range	Medion	Range	Medion	Range
500	125	20	17	51	2.4	0.2-4.6	0.9	0.1-2.5	212	54-251

*Most severe encountered, any course.
†Includes 3 patients from dose-escalation phase of study.

Table 8. Substantial differences in mean CPT-11 pharmacokinetic parameters when CPT-11 was administered with and without leucovorin/5FU were not observed. A small, but statistically significant reduction in SN-38 C_{max} ($P < .001$) and AUC_{0-24} ($P < .002$) values was observed on week 2 (leucovorin/5FU immediately following CPT-11 administration). The mean percent decrease in SN-38 C_{max} and AUC_{0-24} values among patients was 13.7% and 8.2%, respectively, when compared with the corresponding values determined when CPT-11 was given alone. A comparison of SN-38 AUC_{0-24} when CPT-11 was given alone (week 1) versus when CPT-11 was given immediately following 5FU (week 7) showed no statistically significant difference between these values ($P > .9$).

There was no statistical correlation between SN-38 AUC_{0-24} and absolute granulocyte nadir ($r = .149$) when all cycles were examined. The mean nadir granulocyte counts for cycle 1 (CPT-11 followed by leucovorin and 5FU) and cycle 2 (leucovorin and 5FU followed by CPT-11) for all patients who received cycles 1 and 2 in this manner were 2.12 ± 1.12 and 2.68 ± 2.00 cells/μL, respectively. Although this difference appears small, it does achieve statistical significance ($P < .04$) by two-tailed t test. When we examine the granulocyte nadirs of those patients who received cycles 1, 2, and 3, and in whom cycle 3 was given with the same order of drug administration as cycle 1, with the order reversed in cycle 2 (13 patients), there is no statistically significant difference between the mean granulocyte nadirs of cycles 1 and 2 (1.77 ± 0.60 *v* 2.00 ± 0.79, $P > .1$), cycles 2 and 3 (2.00 ± 0.79 *v* 1.94 ± 0.60, $P > .7$) or cycles 1 and 3 (1.77 ± 0.60 *v* 1.94 ± 0.60, $P > .2$).

Table 4. Diarrhea* in 27 Patients Treated on Dose-Escalation Phase of Study

5FU Dose (mg/m²)	CPT-11 Dose (mg/m²)	Leucovorin Dose (mg/m²)	No. of Patients	Total No. of Cycles	Grade			
					1	2	3	4
210	100	20	3	5	1	1	0	0
265	100	20	3	10	2	0	0	0
340	100	20	3	9	2	1	0	0
425	100	20	6	36	4	1	0	0
500	100	20	6	29	4	1	0	0
500	125	20	3	12	2	0	0	0
500	150	20	3	9	2	0	0	0

*Most severe grade.

RESPONSES

Response was not the primary end point of this phase I study; however, evidence of antitumor activity was observed. Of 38 patients with metastatic colorectal cancer, 35 had measurable disease. Four of 23 patients with measurable disease who had received prior chemotherapy and were assessable for response achieved a partial response (PR) (17%; 95% confidence interval. 2% to 32%). Two of 12 patients (17%; 95% confidence interval, 0% to 38%) with measurable disease and no prior treatment achieved a PR.

Additional evidence of antitumor activity was seen. Of 34 colorectal patients in whom the baseline carcinoembryonic antigen (CEA) level was elevated, 12 (35%) were noted to have substantial (> 50%) decreases in their serum CEA levels. While five of these 12 patients also demonstrated major objective responses by computed tomographic (CT) scan, seven patients had stable disease or minor tumor regression by CT scan. These CEA responses were usually accompanied by clinical improvement and decrease in pain medication requirements.

DISCUSSION

5FU-based chemotherapy has been the standard of care for metastatic colorectal and other gastrointestinal tract cancers for more than 30 years.[18,19] Based on encouraging initial data from phase I and II studies, CPT-11 appears to be among the most active anticancer agents to enter clinical trials in the last several decades. Its novel mechanism of action, coupled with its exciting early activity

Table 5. Diarrhea* in 17 Patients Treated at the MTD

5FU Dose (mg/m²)	CPT-11 Dose (mg/m²)	Leucovorin Dose (mg/m²)	No. of Patients†	Total No. of Cycles	Grade			
					1	2	3	4
500	125	20	17	51	6	4	1	2

*Most severe grade.
†Includes 3 patients from dose-escalation phase of study.

Table 6. Nonhematologic Toxicities Other Thon Diarrhea

Toxicity	No. of Patients	Total No. of Cycles	Grade 1	2	3	4
Nausea/vomiting	27	110	9/6	3/3	1/0	0
Fatigue	27	110	14	4	0	0
Alopecia	27	110	6	1	1	0

NOTE. Highest grade experienced by number of patients. Dose-escalation phase of study.

profile, make CPT-11 an excellent candidate for clinical development. It is one of the few new agents with confirmed activity in advanced colorectal cancer. Since CPT-11 has demonstrated activity in both previously untreated[11] and in 5FU-refractory[9,10] colorectal cancer patients, a degree of non–cross-resistance between CPT-11 and 5FU can be postulated. Preclinical studies have demonstrated an additive cytotoxic effect for both CPT-11 and SN-38 when combined with 5FU.[23] A combination regimen might therefore be expected to provide antitumor activity superior to the individual agents alone, if such a regimen could deliver substantial doses of both cytotoxic agents together, and if pharmacologic antagonism between the agents did not occur.

A previously reported pharmacokinetic study had suggested that 5FU reduced the degree of catabolism of CPT-11 to SN-38,[20] presumably by interference with the function of in vivo carboxylesterases that catalyze this catabolism. However, these data were derived from a relatively small number of patients and were based on comparison of the pharmacokinetic data from patients who received CPT-11 and 5FU versus historical data from other patients who had received CPT-11 alone. We designed our trial to test more rigorously the hypothesis that 5FU influenced the conversion of CPT-11 to SN-38. To that effect, we obtained baseline pharmacokinetics of CPT-11 alone on week 1, and then repeat pharmacokinetics of both CPT-11 followed by 5FU/leucovorin, and then preceded by 5FU/leucovorin. Thus, each patient served as his or her own internal control for CPT-11 and SN-38 levels. There was a small, albeit statistically significant reduction in SN-38 C_{max} (13.7%) and $AUC_{0\text{-}24}$ (8.2%) during the week when CPT-ll was followed by 5FU and leucovorin; however, this reduction was not evident 5 weeks later when pharmacokinetic sampling was performed with CPT-11 administered immediately following 5FU.

Table 7. Nonhematologic Toxicities Other Than Diarrhea

Toxicity	No. of Potients	Total No. of Cycles	Grade 1	2	3	4
Nausea/vomiting	14	39	6/2	1/0	0	1/1
Fatigue	14	39	7	2	0	0
Alopecia	14	39	1	1	0	0

NOTE. Highest grade experienced by number of patients. Patients treated at the MTD after dose-escalation phase of study.

Rothenberg et al[10] have reported that the C_{max} and $AUC_{0\text{-}24}$ achieved with a fixed weekly dose of CPT-11 alone were decreased on week 3 as compared with week 1 in patients with colorectal cancer on a phase II study. In their patients receiving a weekly-times-four dose of 125 mg/m^2, these investigators found the mean SN-38 C_{max} for weeks 1 and 3 to be, respectively, 34.4 ± 15.0 versus 24.7 ± 9.9, and the mean $AUC_{0\text{-}24}$ for weeks 1 and 3 to be 459 ± 218 versus 333 ± 151, respectively. The decreases in both C_{max} and $AUC_{0\text{-}24}$ were reported to be statistically significant ($P = .02$ and $P = .03$ for C_{max} and $AUC_{0\text{-}24}$, respectively). The investigators speculated that exposure to CPT-11 might induce certain metabolic processes that could effect the circulating levels of SN-38. Thus, the small differences seen in C_{max} and $AUC_{0\text{-}24}$ in our study could be attributable, at least in part, to inducible differences in metabolism of CPT-11 and SN-38 on weeks 1 versus 2. Since SN-38 pharmacokinetic parameters determined when CPT-11 was administered immediately following leucovorin/5FU (on week 7, 21 days after previous CPT-11 dose) were similar ($P > .1$) to those when CPT-11 was given alone, we believe it is unlikely that leucovorin/5FU substantially altered the conversion of CPT-11 to SN-38.

We believe our relatively large number of patients studied permits us to achieve statistical significance with rather small differences, and we do not believe that such modest differences as 13.7% in C_{max} or 8.2% in AUC are of clinical importance. Supportive of this opinion is the lack of correlation between granulocyte nadir and SN-38 $AUC_{0\text{-}24}$. The unknown degree to which prior exposure to CPT-11 might induce metabolism of the drug, as well as possible differences in disease status, performance status, nutritional status, etc, limits the interpretability of subtle pharmacodynamic differences between treatment cycles. The small differences in mean granulocyte nadir counts do reach statistical significance between cycles 1 and 2, but are not statistically significantly different between cycles 2 and 3 (CPT-11 given first in cycles 1 and 3; leucovorin and 5FU given first in cycle 2).

Thus, in contrast to previously published clinical reports, our data do not suggest that 5FU exerts a substantial effect on the metabolism of CPT-11 to its active metabolite. This is consistent with recent studies using human

Table 8. Summary of CPT-11 and SN-38 Pharmacokinetic Parameters

Parameter	CPT-11 Alone (n = 26)		CPT-11 Followed by 5-FU/LV (n = 26)		5-FU/LV Followed by CPT-11 (n = 22)	
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
CPT						
C_{max} (μg/mL)	1.33 ± 0.340	1.33	1.30 ± 0.305	1.17	1.25 ± 0.267	1.23
N C_{max} (μg/mL)	1.25 ± 0.338	1.25	1.22 ± 0.262	1.15	1.19 ± 0.223	1.20
AUC_{0-24} (μg·h/mL)	7.38 ± 2.58	6.82	7.15 ± 2.32	6.83	6.94 ± 1.49	6.72
N AUC_{0-24} (μg·h/mL)	6.92 ± 2.29	6.77	6.73 ± 2.15	6.39	6.57 ± 1.17	6.58
CL (L/h/m²)	16.6 ± 12.3		15.2 ± 3.72		14.9 ± 2.79	
V_z (L/m²)	153 ± 150		137 ± 37.1		138 ± 32.8	
$t_{1/2}$ (hours)†	6.0		6.2		6.3	
SN-38						
C_{max} (ng/mL)	20.6 ± 7.61	20.9	17.0 ± 8.41	15.1	20.5 ± 7.59	19.6
N C_{max} (ng/mL)	19.4 ± 7.05	18.6	16.3 ± 8.64	13.1	19.7 ± 7.78	17.7
AUC_{0-24} (ng·h/mL)	166 ± 67.1	149	146 ± 58.2*	130	167 ± 47.4	159
N AUC_{0-24} (ng·h/mL)	156 ± 56.1	146	140 ± 59.4*	130	160 ± 50.1	150
$t_{1/2}$ (hours)†	12.7		12.9*		13.7	

Abbreviations: C_{max}, peak plasma concentration; N C_{max}, peak plasma concentration normalized to CPT-11 dose of 100 mg/m²; AUC_{0-24}, orea under the plasma concentration-time curve from start of infusion to last collection time (ie, ~ 24 hours after the end of the infusion); N AUC_{0-24}, orea under the plasma concentration-time curve from start of infusion to last collection time (ie, ~ 24 hours after the end of the infusion)—normalized to a CPT-11 dose of 100 mg/m²; CL, systemic clearance; Vz, apparent volume of distribution; $t_{1/2}$, apparent elimination half-life.

*N = 25.

†Harmonic mean.

hepatic microsomes, which demonstrated that 5FU did not markedly alter the conversion of CPT-11 to SN-38 in vitro.[24]

Consistent with most published pharmacokinetic data on CPT-11, our pharmacokinetic measurements were performed on total drug (lactone plus carboxylate). We cannot exclude the possibility that 5FU plus leucovorin may have shifted the equilibrium between lactone and carboxylate, and thereby increased or decreased the circulating lactone concentrations of CPT-11 and/or SN-38. However, we consider this possibility to be unlikely given that the equilibrium is pH-dependent and there are no data to suggest that administration of 5FU plus leucovorin has an effect on circulating plasma pH. We also did not investigate the effect of CPT-11 on the pharmacokinetics of 5FU. Although we cannot exclude the possibility of an effect on the pharmacokinetics of 5FU by CPT-11, we have seen no data or evidence to suggest such an interaction, and consider the possibility to be unlikely.

The selection of doses and schedules for the combining of CPT-11 with 5FU and leucovorin required many decisions. To minimize the chance for unexpected toxicities from CPT-11, we chose to design our regimen based on the schedule of CPT-11 that has thus far been evaluated most extensively in North America, ie, weekly-times-four administration every 6 weeks, and to administer all three drugs (CPT-11, leucovorin, and 5FU) on that schedule. We chose initially to fix the doses of CPT-11 and leucovorin, with escalations of 5FU.

The selection of the leucovorin dose and schedule was based on our analysis of multiple factors. The optimal dose and schedule of leucovorin in 5FU-based regimens remains controversial. Several randomized clinical trials have failed to show a superiority of high-dose leucovorin regimens over a low-dose daily-times-five leucovorin schedule,[25-27] although one three-arm study did report a superior response rate for a weekly high-dose leucovorin versus a weekly low-dose leucovorin schedule ($P = .046$, two-tailed pairwise comparison unadjusted for multiple comparisons).[28] No direct comparison of weekly low-dose versus daily-times-five low-dose leucovorin has been reported. We chose to use a low dose of leucovorin to optimize the chance of being able to deliver a more substantial dose of 5FU with our selected fixed dose of CPT-11. The dose-limiting toxicity of CPT-11 was severe diarrhea in early clinical trials, and the higher-dose leucovorin regimens had been shown in randomized studies to cause more diarrhea than lower-dose regimens. We therefore felt that substantial concurrent doses of CPT-11 and 5FU would not be tolerable when given with high-dose leucovorin. The subsequent experience of other investigators has shown this concern to be correct.[29]

We considered a combination of weekly CPT-11 with daily-times-five 5FU plus low-dose leucovorin to be prob-

lematic for several reasons. We were concerned that the logistics of such a regimen would be cumbersome to take forward to broader clinical development. Also, the 6-week CPT-11 cycle (4 weeks on, 2 weeks off) would require that the 5 consecutive days of 5FU and leucovorin be given at 6-week intervals, instead of the usual 4 weeks. This would result in a substantial decrease in 5FU dose-intensity. Finally, we felt that the myelosuppression that would be expected from daily-times-five 5FU plus leucovorin administration was likely to result in substantial limitations on the dose of 5FU that could be given if subsequent weekly doses of CPT-11 were to be maintained.

Other investigators have attempted to combine CPT-11 with 5FU, with less favorable results. One group combined weekly high-dose leucovorin and 5FU.[29] Toxicity was formidable, with dose-limiting diarrhea developing early in the course of this clinical trial. The maximum dose of CPT-11 that could be administered with tolerable toxicity was 25 mg/m^2, which represents only 20% of the usual phase II dose of CPT-11 alone. Another group of investigators has reported in abstract form a combination of CPT-11 with 5FU given by 7-day continuous infusion.[30] The dose of 5FU used was a modest 400 mg/m^2/d. It should be noted that substantially higher doses of 5FU are routinely given when continuous infusion is used instead of bolus administration.

Contrary to our initial expectations, neutropenia was the major dose-limiting toxicity on this clinical trial. Diarrhea, while a frequent event, was clinically manageable and did not interfere with the ability of most patients to receive drug. The manageability of diarrhea was most likely attributable to our rigorous use of aggressive loperamide therapy at the earliest onset of diarrhea. Our finding is consistent with a report,[31] confirmed previously by our group[11] and others, that aggressive loperamide usage greatly reduces the incidence of clinically severe diarrhea. Other nonhematologic toxicities were uncommon and mild. Dexamethasone was administered as a routine premedication, with ondansetron or granisetron being added if dexamethasone alone proved to be inadequate. Nineteen of 42 patients (45%) did not require serotonin antagonist antiemetics.

Although clinical response was not the primary end point of this phase I trial, clinical activity was noted in this phase I trial of largely previously treated patients. It is noteworthy that more than half of the patients on this trial were treated below the MTD. The degree of activity in previously treated colorectal cancer patients was comparable to that reported for CPT-11 alone in this population, which further suggests that 5FU/leucovorin does not unfavorably impact the pharmacokinetics and clinical cytotoxicity of CPT-11.

In summary, the regimen we report herein permits the concurrent administration of substantial doses of both CPT-11 and 5FU with low-dose leucovorin on a schedule that is tolerable and easy to administer. The recommended doses for phase II/III investigation are CPT-11 125 mg/m^2, leucovorin 20 mg/m^2, and 5FU 500 mg/m^2, each given weekly for 4 consecutive weeks followed by a 2-week rest. Pharmacokinetic data from this trial indicate that 5FU does not exert a substantial effect on the conversion of CPT-11 to its active metabolite. These findings, coupled with the antitumor activity demonstrated in this trial, have encouraged us to pursue further clinical development of this regimen in patients with metastatic colorectal cancer. A phase II trial of this regimen in chemotherapy-naive colorectal cancer patients is in progress.

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Pharmacokinetic Assessment of Irinotecan, SN-38, and SN-38-Glucuronide: A Substudy of the FIRIS Study

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Abstract. *Background: We evaluated the pharmacokinetics of irinotecan (CPT-11) and its metabolites in patients with metastatic colorectal cancer receiving the combination of CPT-11/S-1 (IRIS) or 5-fluorouracil (5-FU)/l-leucovorin (LV)/CPT-11 (FOLFIRI) regimens in the FIRIS trial. Patients and Methods: Serum CPT-11, SN-38 (an active metabolite of CPT-11), and SN-38–glucuronide concentrations were compared between the IRIS and FOLFIRI regimens, and between days 1 and 15 of administration. Correlations between pharmacokinetic data and incidence of neutropenia and diarrhea were also assessed. Results: There were no significant differences in the pharmacokinetics of CPT-11 or its metabolites between days 1 and 15. SN-38 concentrations were correlated with the occurrence of neutropenia, which was significantly more frequent in the FOLFIRI group than in the IRIS group. Conclusion: No alterations in CPT-11 pharmacokinetics after repeated IRIS or FOLFIRI administration were observed. Neutropenia was more frequent in the FOLFIRI group than in the IRIS group because exposure to SN-38 was greater in the former group.*

The combination of 5-fluorouracil (5-FU)/l-leucovorin (LV) with either irinotecan (CPT-11) (FOLFIRI) or oxaliplatin (FOLFOX) is established as first-line chemotherapy for metastatic colorectal cancer (mCRC). Initial treatment with FOLFOX followed by secondary FOLFIRI treatment, or *vice versa*, is currently recommended as the standard therapy (1, 2). However, neither long-term continuous intravenous infusion of 5-FU nor implantation of an intravenous port system for 5-FU infusion with FOLFOX or FOLFIRI is convenient. Therefore, some clinical trials have tested the viability of replacing 5-FU infusion with oral 5-FU derivatives.

S-1 is an oral 5-FU derivative anticancer agent, consisting of tegafur, 5-chloro-2,4-dihydropyrimidine (CDHP), and potassium oxonate. In this formulation, tegafur is a pro-drug of 5-FU; CDHP inhibits the 5-FU-degrading enzyme, dihydropyrimidine dehydrogenase, and maintains the blood concentration of 5-FU; and potassium oxonate is an orotate phosphoribosyltransferase inhibitor that reduces gastrointestinal toxicity. In Japan, phase II studies, consisting of CPT-11 plus S-1, combination therapy as first-line treatment for mCRC, achieved a response rate of 52.5-60% and median progression-free survival of 7.8-8.6 months (3-5). Based on these results, the FIRIS study was conducted to verify the non-inferiority of CPT-11/S-1 (IRIS) to FOLFIRI in patients with mCRC who failed first-line chemotherapy (6).

CPT-11 is an extremely important anticancer agent in the treatment of gastrointestinal cancer (7). Because CPT-11 and 5-FU have different mechanisms of action, several clinical trials have assessed the feasibility and safety profile of different combination therapies of CPT-11 and 5-FU (8, 9). However, the pharmacokinetics of CPT-11 show a high degree of interpatient variability, and some reports suggest that this variability may be due to genetic background or drug interactions (10, 11). In one study of weekly administration of CPT-11 in patients with mCRC, decreased

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Key Words: Irinotecan, colorectal cancer, IRIS, S-1, FIRIS.

mean maximum concentrations (C_{max}) and areas under the curve (AUC) for SN-38 were observed in the plasma (12). In a case report, daily oral administration of S-1 markedly reduced the AUC of SN-38 (13). Finally, there is some indication that exposure to CPT-11 or SN-38 is related to the occurrence of neutropenia and diarrhea. Indeed, although the FIRIS trial verified that IRIS is not inferior to FOLFIRI as second-line chemotherapy for mCRC, the trial revealed differences in the adverse event profiles of the two regimens (6). Therefore, better understanding of the pharmacokinetics of these treatments is critical for optimal CPT-11-based chemotherapy.

The current study was undertaken as an additional component of the FIRIS study (6) to evaluate the pharmacokinetics of CPT-11, SN-38, and SN-38 glucuronide (SN-38G) on days 1 and 15. We evaluated the changes in the pharmacokinetics of CPT-11 and its metabolites during the IRIS and FOLFIRI treatment cycles, and we compared the relative exposure of patients in each group to these agents. We also attempted to re-confirm any correlation between drug exposure and the occurrence of neutropenia and diarrhea. This study is registered with ClinicalTrials.gov, number NCT00284258.

Patients and Methods

Patients. The FIRIS study was an open-label, multicenter, randomized, phase II/III study of patients with second-line mCRC receiving either the IRIS or FOLFIRI treatment regimen (6). Of the 40 institutions participating in the FIRIS study, six participated in the present additional study. The inclusion and exclusion criteria were identical to those in the full FIRIS study (6).

The patients provided informed consent to participate in the main FIRIS study and to participate in the pharmacokinetic study before randomization. The protocol was approved by the Institutional Review Board and/or Ethics Committee of each institution.

Treatments. In accordance with the FIRIS study protocol, patients were centrally randomized to receive either the FOLFIRI or IRIS regimens using a minimization method, with stratification by institution, prior therapy (with or without oxaliplatin), and PS (0 or 1). The IRIS group received CPT-11 (125 mg/m^2) intravenously on days 1 and 15, and S-1 (40-60 mg, based on body surface area) twice daily for two weeks from days 1 to 14, followed by two weeks of rest. This dosing regimen was selected on the basis of results from previous phase II studies (14, 15).

In the FOLFIRI group, patients received concurrent administration of LV (200 mg/m^2 over 120 min) and CPT-11 (150 mg/m^2) followed by a bolus injection of 5-FU (400 mg/m^2) on day 1 and subsequent continuous infusion of 5-FU (2,400 mg/m^2) over 46 h, every two weeks (each 4-week cycle was considered a single course). The dose of CPT-11 used in the FOLFIRI group (150 mg/m^2) is an approved clinical dose in Japan (16).

Sample collection. For the pharmacokinetic studies, blood samples (3 ml) were collected on days 1 and 15 in tubes containing sodium heparin anticoagulant. Samples were obtained before CPT-11 administration and at specific intervals (1, 1.5, 2, 2.5, 3.5, 5.5, 8.5, and 24 h) after starting CPT-11 infusion. All blood samples were stored in an ice bath until the plasma was prepared, and they were centrifuged within 30 min of sample collection. The supernatant was collected as the plasma sample and stored at –20°C until analysis.

Analytical methods. Plasma samples for CPT-11 and its metabolites were analyzed at Sekisui Medical Co., Ltd. (Naka-gun, Ibaraki, Japan), using liquid chromatography with fluorescence detection. Plasma concentrations of CPT-11, SN-38, and SN-38G were determined as previously described, with some minor modifications (17). This method was fully validated by Sekisui Medical Co., with quality-control procedures and acceptance criteria based on requirements described by Shah *et al.* (18) and the FDA Guidance for Industry (19). Camptothecin was used as the internal standard.

Pharmacokinetic analyses. Profiles of plasma concentration *versus* time for CPT-11, SN-38, and SN-38G were obtained for each patient, and standard pharmacokinetic parameters were calculated using non-compartmental methods with WinNonlin v5.2 software (Pharsight Corporation, Mountain View, CA, USA). Total AUC was calculated by adding the AUC_{0-24} values on days 1 and 15 for each compound to evaluate the correlation between total AUC values of CPT-11, SN-38, or SN-38G and the occurrence of neutropenia or diarrhoea.

Efficacy and safety assessment. In accordance with the FIRIS study protocol, physical examinations, electrocardiography, performance status (PS), and laboratory tests were performed at baseline and repeated at least every two weeks during treatment. Tumours were assessed at baseline (within one month before enrolment); at two, three, and four months after enrolment; and every two months thereafter until progression. Progression was defined as the occurrence of any of the following three events: (i) progressive disease based on the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 (20); (ii) clinical progression as judged by the investigator; or (iii) death from any cause without progression. Toxicity was evaluated based on the Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v3.0) (21).

Statistical analysis. Summary statistics, such as mean and standard deviation calculations, were performed for all pharmacokinetic parameters for both treatment groups on days 1 and 15. The time-dependent alterations in the pharmacokinetics of CPT-11 and its metabolites after repeated administration were evaluated by applying paired *t*-tests to log-transformed means for each pharmacokinetic parameter (excluding t_{max}) in each treatment group (CPT-11, SN-38, and SN-38G) to compare data between days 1 and 15. Differences in t_{max} between days 1 and 15 were evaluated using the Wilcoxon rank test for paired data. Exposure to CPT-11, SN-38, and SN-38G were compared between the two treatments using Student's *t*-test with log-transformed total AUC values for each compound. To evaluate the correlation between total AUC of CPT-11, SN-38, or SN-38G and the occurrence of neutropenia or diarrhea, log-transformed total AUCs for each compound for patients with and without adverse events were compared using Student's *t*-test. A *p*-value of ≤ 0.05 was considered statistically significant. Data storage and statistical analyses were carried out using SAS v8.02 software (Cary, NC, USA).

Table I. *Pharmacokinetic profiles of irinotecan (CPT-11), SN-38, and SN-38 glucuronide (SN-38G) on days 1 and 15 in the IRIS and FOLFIRI treatment groups.*

	CPT-11		SN-38		SN-38G	
	Day 1	Day 15	Day 1	Day 15	Day 1	Day 15
IRIS						
C_{max} (ng/ml)	1844±584	1631±246	17.96±4.78	19.09±9.36	87.25±46.12	103.39±71
AUC_{0-24} (ng h/ml)	9813±2528	9940±1440	145.7±33.4	159±53.8	1031.1±646.3	1273.1±884.2
$t_{1/2}$ (h)	6.63±0.83	6.74±1.03	13.7±1.95	13.69±3.42	13.18±2.72	13.1±3.55
t_{max} (h)	1.4±0.2	1.5±0	1.7±0.5	1.8±0.4	2.5±0.5	3.2±0.5
MRT (h)	6.98±1.1	7.37±1.22	16.56±3.07	17.3±4.6	18.02±3.94	18.41±5.09
CL (l/h/m^2)	12.5±2.9	11.9±1.8	–	–	–	–
VD_{ss} (l/m^2)	86.2±20.4	86.8±16.4	–	–	–	–
FOLFIRI						
C_{max} (ng/ml)	2079±461	2175±488	36.03±11.59	33.61±13.79	99.14±30.74	94.44±29.67
AUC_{0-24} (ng h/ml)	11037±1775	11239±1912	269.4±102.5	249±86.8	982.9±304.6	974.2±264.2
$t_{1/2}$ (h)	6.2±0.72	5.96±0.47	11.02±1.74	12.24±2.2	10.93±2.13	11.79±3.19
t_{max} (h)	1.6±0.3	1.5±0	2.3±0.5	2.1±0.7	2.5±0.5	2.5±0.5
MRT (h)	6.44±0.89	6.31±0.53	13.05±1.68	14.98±2.78	14.58±3.07	16.07±4.48
CL (l/h/m^2)	13.3±3	13.1±2.6	–	–	–	–
VD_{ss} (l/m^2)	84±14.3	81.8±14	–	–	–	–

IRIS, Irinotecan/S-1; FOLFIR, 5-fluorouracil/l-leucovorin/irinotecan; C_{max}, maximum concentration; AUC, area under the curve; $t_{1/2}$, half-time; t_{max}, time taken to reach maximum concentration; MRT, mean resistance time from 0 to infinity; CL, plasma clearance; VD_{ss}, apparent volume of distribution at steady state.

Results

Patients' characteristics. Among the 426 patients enrolled in the FIRIS study, 18 patients were enrolled in this pharmacokinetic study from May 2007 to January 2008. Six patients (five males and one female) were enrolled into the IRIS group and 12 patients (five males and seven females) to the FOLFIRI group. The median age was 59 years (range=46-72 years) and 57 years (range=41-73 years), respectively. Four and 2 patients in the IRIS group and 10 and two patients in the FOLFIRI group had an Eastern Cooperative Oncology Group (ECOG) PS of 0 and 1, respectively. Four patients in the IRIS group and 6 in the FOLFIRI group had received previous chemotherapy with oxaliplatin. There were no obvious differences in patient characteristics between the patients enrolled in the FIRIS study (6) and those enrolled in this pharmacokinetic study. All of the patients enrolled in the present study were treated in accordance with the FIRIS study protocol (6).

Pharmacokinetic analysis of CPT-11, SN-38, and SN-38G in the IRIS regimen. Following treatment with the IRIS regimen, we recorded the mean plasma concentration–time profiles of CPT-11, SN-38, and SN-38G on days 1 and 15 (Figure 1) and the pharmacokinetic parameters of CPT-11, SN-38, and SN-38G every day (Table I). No statistically significant differences were detected in any of the pharmacokinetic parameters for CPT-11, SN-38 and SN-38G between days 1 and 15. These results suggest that the extent of plasma exposure of CPT-11, SN-38, and SN-38G was not affected by repeated administration of S-1 and CPT-11 in patients with mCRC.

Pharmacokinetic analysis of CPT-11, SN-38, and SN-38G in the FOLFIRI regimen. Following treatment with the FOLFIRI regimen, we recorded the mean plasma concentration–time profiles of CPT-11, SN-38, and SN-38G on days 1 and 15 (Figure 2) and the pharmacokinetic parameters of CPT-11, SN-38, and SN-38G every day (Table I). No statistically significant differences were detected in any of the pharmacokinetic parameters for CPT-11, SN-38, and SN-38G between days 1 and 15. These results suggest that the plasma exposure of CPT-11, SN-38, and SN-38G was unchanged during the course of the study.

Comparison of exposure to CPT-11, SN-38, and SN-38G between the IRIS and FOLFIRI regimens. Figure 3 shows the total AUCs (*i.e.* the total exposure to each compound during one treatment cycle) for CPT-11, SN-38, and SN-38G following the IRIS and FOLFIRI regimens. Although there were no significant differences in the total AUC values for CPT-11 and SN-38G between the two treatment regimens, the total AUC value for SN-38 (an active metabolite of CPT-11) was significantly greater in the FOLFIRI regimen than in the IRIS regimen.

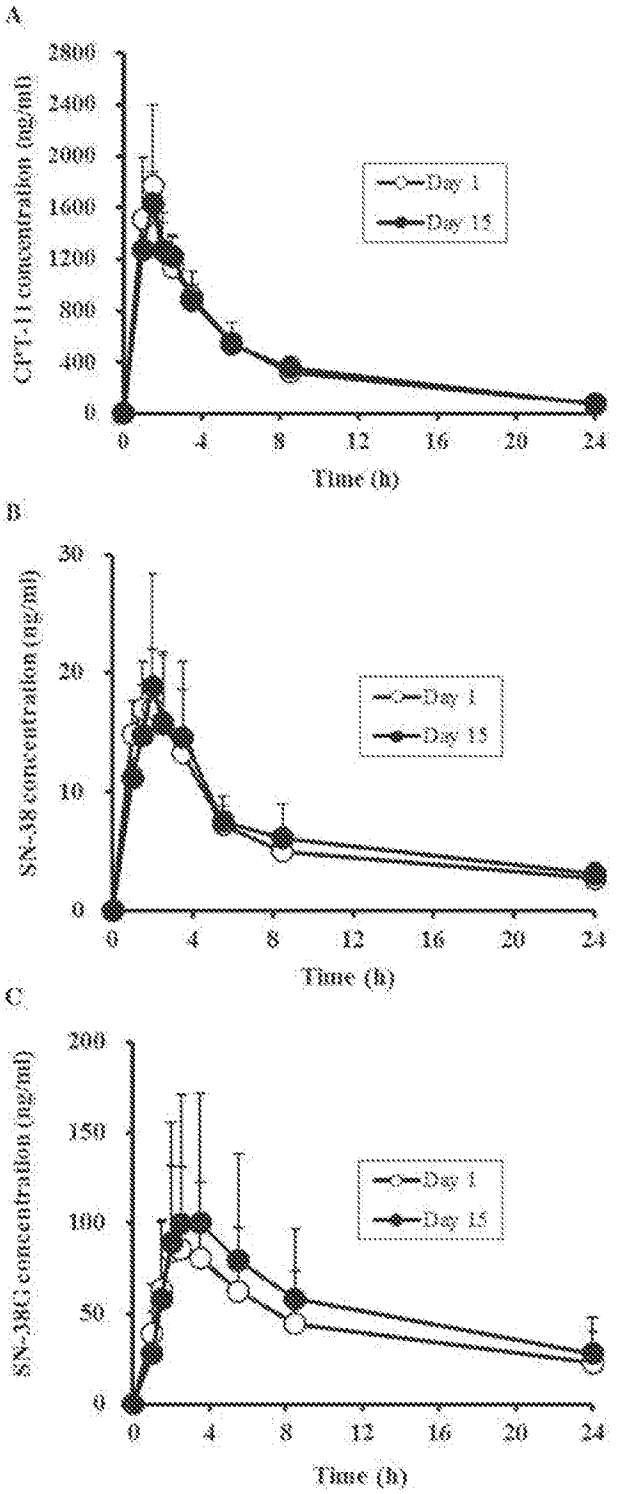

Figure 1. *Mean plasma concentration–time profiles of irinotecan (CPT-11) (A), SN-38 (B), and SN-38G (C) after a 2-h intravenous infusion of CPT-11 on days 1 and 15 in the CPT-11/S-1 (IRIS) treatment group. Day 1: S-1 was orally administered 7 h after starting CPT-11 infusion. Day 15: S-1 was administered twice daily from day 1 and simultaneously administered at the start of CPT-11 infusion on day 15. Each point represents the mean±SD of six patients.*

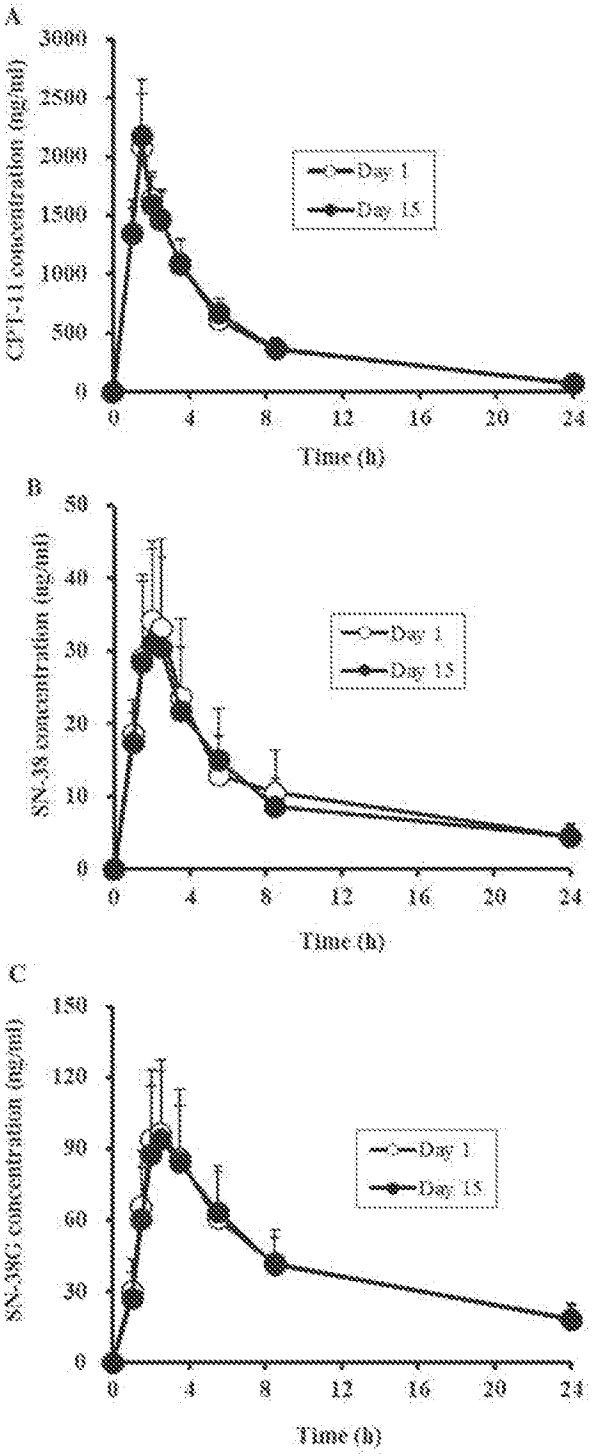

Figure 2. *Mean plasma concentration–time profiles of irinotecan (CPT-11) (A), SN-38 (B), and SN-38G (C) after a 2-h intravenous infusion of CPT-11 on days 1 and 15 in the 5-fluorouracil (FU)/l-leucovorin (LV)/CPT-11 (FOLFIRI) treatment group. The patients received concurrent administration of CPT-11 (150 mg/m^2) and LV (2,400 mg/m^2) over 90 and 120 min, respectively, followed by a bolus injection of 5-FU (400 mg/m^2) and continuous infusion of 5-FU (2,400 mg/m^2) over 46 h on days 1 and 15.*

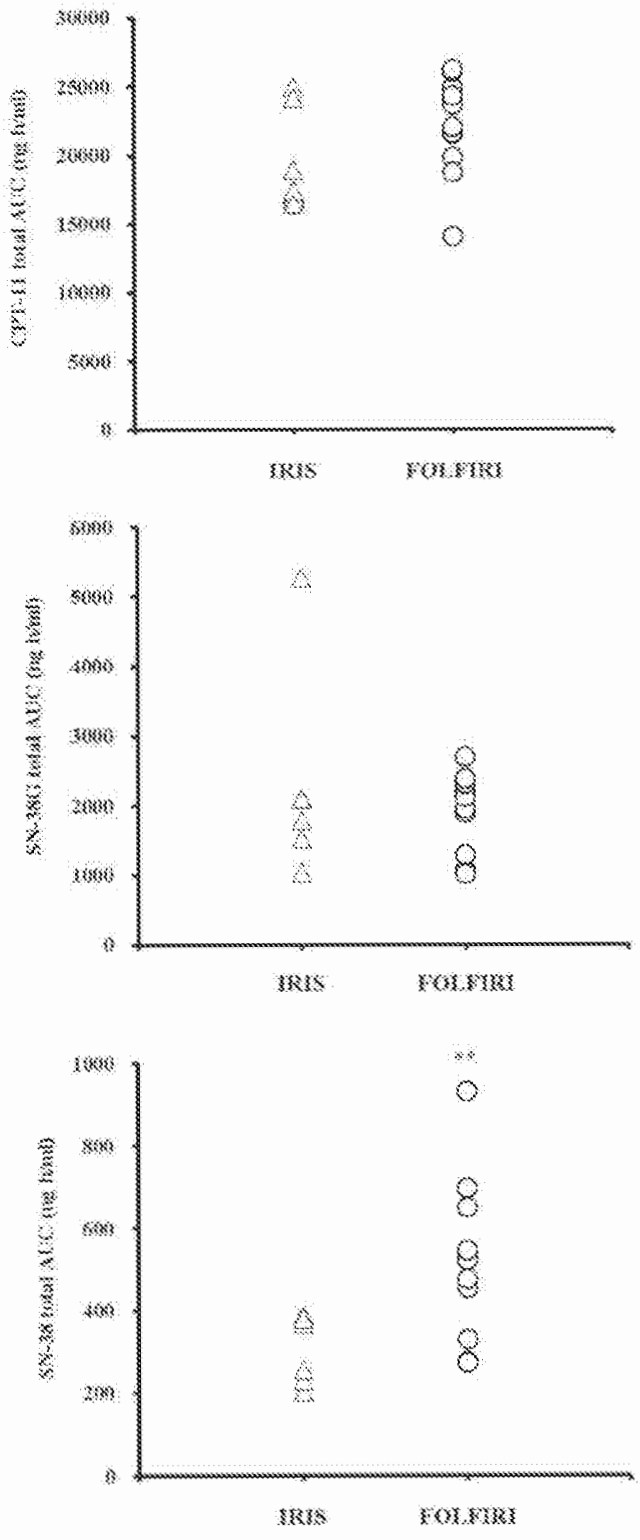

Figure 3. *Comparison of the total area under the curve (AUC) values of irinotecan (CPT-11), SN-38, and SN-38G (sum of the AUCs on days 1 and 15) between CPT-11/S-1 (IRIS) and 5-fluorouracil/l-leucovorin/CPT-11 (FOLFIRI) regimens. **p<0.01, IRIS vs. FOLFIRI.*

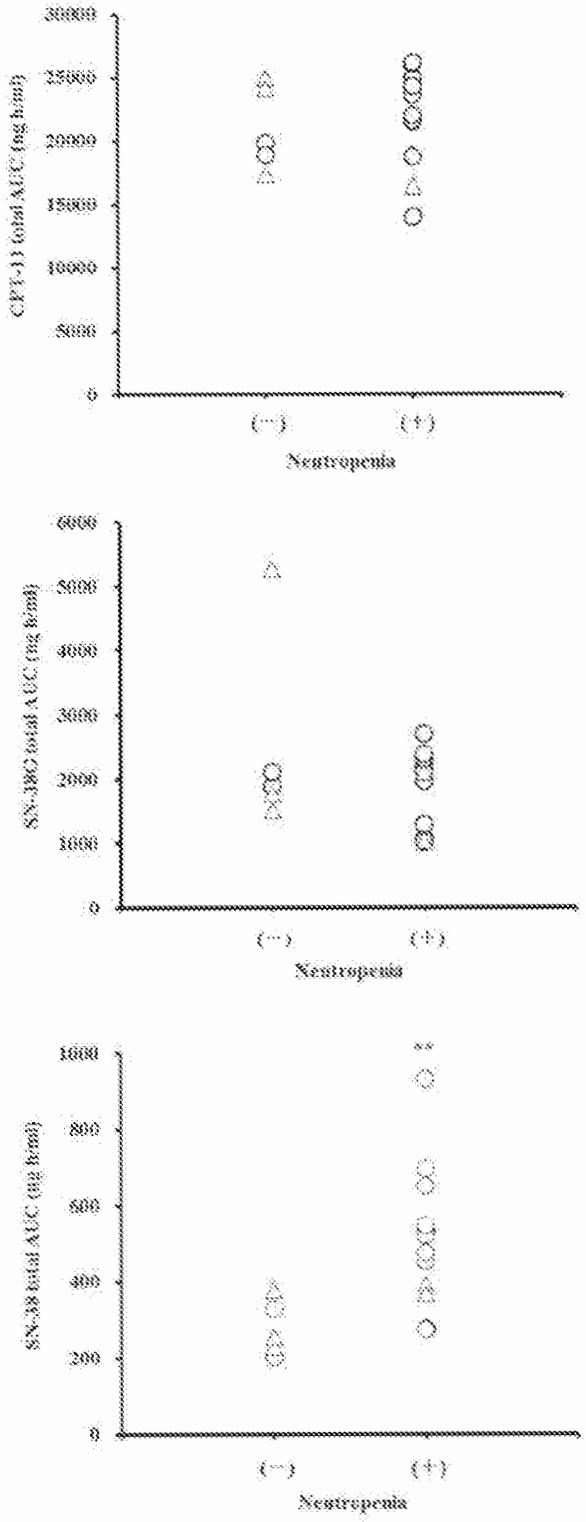

Figure 4. *Correlations between the total area under the curve (AUC) values of irinotecan (CPT-11) (A), SN-38 (B), and SN-38G (C) (sum of the AUCs on days 1 and 15) and the occurrence of neutropenia in patients treated with 5-fluorouracil/l-leucovorin/CPT-11 (FOLFIRI) and CPT-11/S-1 (IRIS) regimens.* *p<0.01, patients without neutropenia (−) vs. with neutropenia (+).*

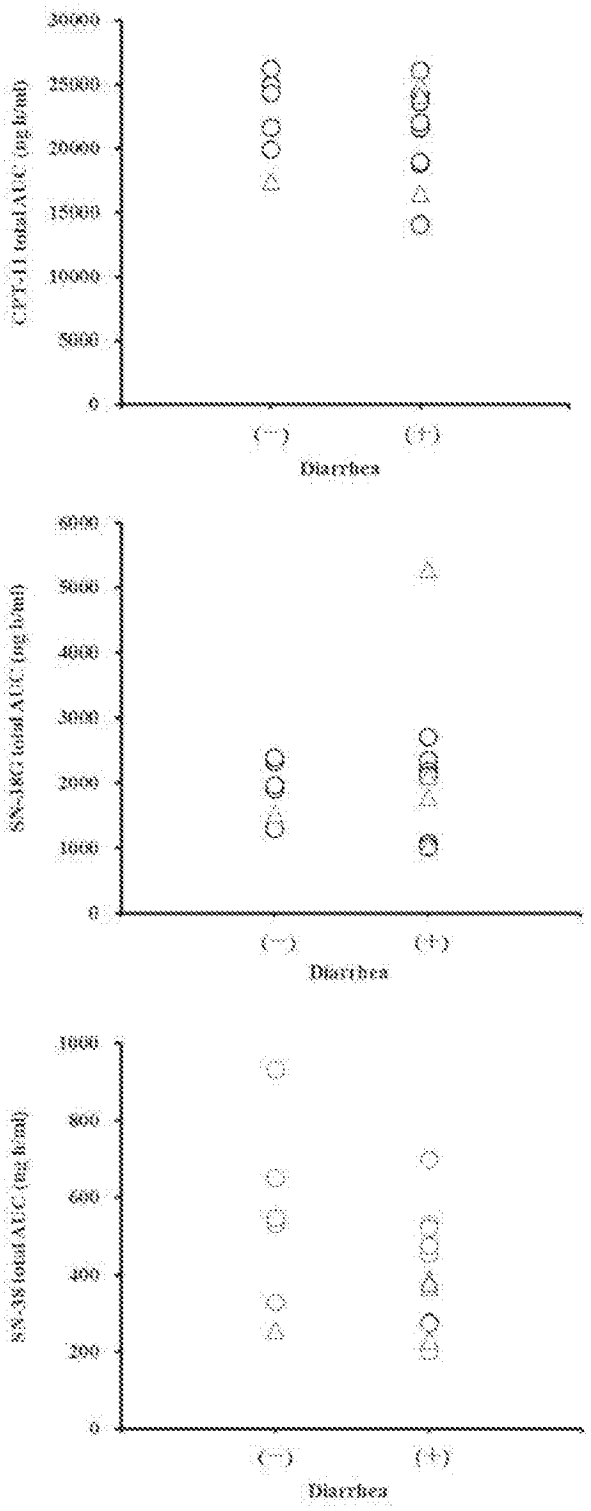

Figure 5. *Correlations between the total area under the curve (AUC) values of irinotecan (CPT-11) (A), SN-38 (B), and SN-38G (C) (sum of AUC on days 1 and 15) and the occurrence of diarrhea in the 5-fluorouracil/l-leucovorin/CPT-11 (FOLFIRI) and CPT-11/S-1 (IRIS) regimens. There were no significant differences in the total AUC values between patients without diarrhea (−) or with diarrhea (+).*

Efficacy and safety. Overall, 17 patients were included in efficacy evaluations, and 18 patients were included in safety evaluations. Partial responses were seen in 3/6 patients in the IRIS group and in 3/11 patients in the FOLFIRI group. Common non-haematological toxicities in the IRIS and FOLFIRI groups included all-grade diarrhea (83.3% *vs.* 66.7%), anorexia (83.3% *vs.* 75.0%), and nausea (50.0% *vs.* 83.3%), with no grade 4 toxicities in either group. Common haematological toxicities in the IRIS and FOLFIRI groups included all-grade neutropenia (33.3% *vs.* 100.0%), anaemia (100.0% *vs.* 66.7%), and thrombocytopenia (66.7% *vs.* 25.0%). The incidence of adverse events in each group was not markedly different from that in the previous report (6).

Pharmacokinetic/pharmacodynamic analyses. Figure 4 shows the total AUC values for CPT-11, SN-38, and SN-38G and the occurrence of neutropenia. The total AUC of SN-38 was significantly higher in patients experiencing neutropenia than in those who did not. However, the total AUC of CPT-11 and SN-38G did not correlate with the occurrence of neutropenia. In contrast, the total AUCs of CPT-11, SN-38, and SN-38G were not correlated with the occurrence of diarrhea (Figure 5).

Discussion

Combination chemotherapies based on infused 5-FU are standard first- and second-line treatments for mCRC. Several clinical trials have assessed the feasibility and safety profile of different combinations of CPT-11 and 5-FU (8, 9). FOLFIRI is a standard first- or second-line regimen for advanced colorectal cancer, with published response rates ranging from 40% to 50%, and is widely used for mCRC (2). In Japan, the non-inferiority of IRIS to FOLFIRI as a second-line chemotherapy for mCRC was verified in the FIRIS study, a phase II/III randomized study (6). Thereafter, in the ESMO Consensus Guidelines for management of patients with colon and rectal cancer, IRIS is listed in the table of the treatment options (22)

The pharmacokinetic profiles of CPT-11 and SN-38 are very complex because of the existence of multiple biotransformation and elimination pathways involving various enzymes (23) and transporters (24-26). Genetic factors, such as uridine diphosphate glucuronyltransferase 1A1 (*UGT1A1*) polymorphisms, also affect their profiles (27-30). Patients treated with CPT-11 occasionally experience severe neutropenia and delayed diarrhea. However, there is marked interpatient variability in the degree of toxicity because of pharmacokinetic variations in CPT-11, SN-38, and SN-38G arising from differential biological backgrounds. Furthermore, pharmacokinetic changes of CPT-11 and its metabolites during the treatment cycle have been reported. For example, Rothenberg *et al.* reported that the C_{max} and AUC_{0-24} of SN-

38 achieved with a fixed weekly dose of CPT-11 monotherapy were lower in week 3 than in week 1 in colorectal cancer patients in a phase II study, which suggests that weekly administration of CPT-11 affects the pharmacokinetics of SN-38 (12). Furthermore, Yokoo *et al.* reported that repeated administration of S-1 reduced the plasma concentration of SN-38 on day 7 (13). Thus, it is particularly important to evaluate the pharmacokinetic profiles of CPT-11, SN-38, and SN-38G during the treatment cycle because they may be related to the occurrence of adverse events.

To assess the pharmacokinetic profiles of CPT-11, SN-38, and SN-38G within the treatment cycles of the IRIS and FOLFIRI regimens, we performed a pharmacokinetic study of CPT-11 on days 1 and 15. We found no significant differences in the pharmacokinetic parameters of CPT-11, SN-38, and SN-38G between days 1 and 15 with either regimen. Rothenberg *et al.* (12) reported that weekly CPT-11 monotherapy resulted in a decrease in the plasma concentration of SN-38, but we found no differences in the pharmacokinetics of CPT-11, SN-38, and SN-38G after repeated administration of CPT-11 as part of the IRIS or FOLFIRI regimens in this study. Moreover, the results observed in the IRIS group suggest that repeated administration of S-1 did not affect the pharmacokinetics of CPT-11, SN-38, or SN-38G. The conversion of CPT-11 to SN-38 is catalysed by a carboxylesterase and leads to a reduction in the AUC of SN-38. It has also been proposed that the 5-FU metabolite, fluorine, inhibits the activity of this enzyme (10). Because S-1 contains CDHP, which inhibits 5-FU degradation, no such inhibition occurs, and the plasma concentrations of CPT-11, SN-38, and SN-38G are unlikely to change after repeated administration of S-1. However, the results of repeated administration in this study differ from those reported by Yokoo *et al.* (13), casting doubt on the validity of this hypothesis.

We also compared the exposure to CPT-11, SN-38, and SN-38G between the IRIS and FOLFIRI regimens. Although there were no significant differences in the total AUC values of CPT-11 and SN-38G between the IRIS and FOLFIRI groups, the exposure to SN-38 was significantly higher in the FOLFIRI group than in the IRIS group. This may be attributed to differences in the dose of CPT-11 (150 mg/m^2 in the FOLFIRI group and 125 mg/m^2 in the IRIS regimen) or to differences in the genetic background of metabolic enzymes (*e.g.*, UGT1A1) between the IRIS and FOLFIRI groups, although we could not confirm the reasons for this in the present study. With regard to adverse events, the incidence of neutropenia tended to be higher in the FOLFIRI group while the incidence of diarrhea tended to be higher in the IRIS group. Moreover, from the results of the pharmacokinetic/pharmacodynamic analysis, patients experiencing neutropenia in this study showed significantly higher exposure to SN-38 (but not to CPT-11 and SN-38G) than those who did not experience neutropenia. These results are consistent with previous reports suggesting that SN-38 exposure is associated with haematological toxicity (31-33). In contrast, despite reports suggesting a correlation between the occurrence of diarrhea and exposure to CPT-11 or SN-38, we found no evidence of any correlation between diarrhea and CPT-11, SN-38, or SN-38G exposure in this study.

The present study has some limitations, and these preclude a definitive conclusion. Firstly, the sample size was too small to evaluate the pharmacokinetic profile. Secondly, there were imbalances in the sample size and sex ratio between the two groups because participation in this additional study was not a stratification factor. Thirdly, *UGT1A1* genetic polymorphisms, which are important in interpreting the pharmacokinetic profile of CPT-11, were not determined in the present study.

Conclusion

We observed no time-dependent changes in the pharmacokinetics of CPT-11, SN-38, and SN-38G during treatment cycles, nor did we observe any clinically important effects of repeated administration of S-1 on the pharmacokinetics of CPT-11 and its metabolites in the IRIS group. However, our data suggest that the greater exposure to SN-38 in the FOLFIRI group was associated with a higher incidence of neutropenia in that group compared with the IRIS group.

Conflicts of Interest

The Authors declare that they have no conflicts of interest to disclose.

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Fluorouracil Plus Racemic Leucovorin Versus Fluorouracil Combined With the Pure *l*-Isomer of Leucovorin for the Treatment of Advanced Colorectal Cancer: A Randomized Phase III Study

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Purpose: **To compare the efficacy and toxicity of fluorouracil (FU) and racemic leucovorin (*d,l*-LV) versus FU combined with the *l*-isomer of leucovorin (*l*-LV) in the treatment of advanced colorectal cancer.**

Patients and Methods: **A total of 248 patients with advanced measurable colorectal cancer previously unexposed to chemotherapy were randomly assigned to treatment with either FU (400 mg/m^2/d by intravenous [IV] infusion for 2 hours) and racemic LV (100 mg/m^2/d by IV bolus injection) given for 5 consecutive days, or the combination of FU and the pure *l*-isomer of LV using the same dose schedule. In both treatment arms, courses were administered every 28 days if toxicity allowed for a total of 6 months, unless evidence of tumor progression was documented earlier.**

Results: **There were no significant differences between the FU/racemic LV and the FU/*l*-LV arm in the overall response rate (25% *v* 32%), duration of response (7.2 *v* 8.0 months), median time to progression or death (6.25 *v* 8.0 months), or median overall survival time (14.5 *v* 15.0 months). Except for minor myeloid toxic effects associated with FU/*l*-LV, there was also no significant difference in terms of adverse reactions. Gastrointestinal symptoms, specifically mucositis and diarrhea, were less frequent and less severe in both treatment arms compared with other trials with FU/racemic LV reported in the literature, which might be because of the prolonged administration of FU used in both arms.**

Conclusion: **The combination of FU/*l*-LV produced response rates, response durations, and survival times similar to those with FU/*d,l*-LV. Biochemical modulation of FU by either pure *l*-LV or racemic LV thus appears to result in equivalent clinical efficacy.**

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COLORECTAL adenocarcinoma is one of the most common solid tumors in the Western societies, and more than 40% of patients will have locoregional recurrences and/or distant metastases at some point during the course of their disease.[1] The mainstay of palliative treatment for advanced colorectal cancer is cytotoxic chemotherapy, which has been shown to prolong survival and improve quality of life in randomized studies.[2,3] The fluorinated pyrimidines, fluorouracil (FU), and its deoxyribose floxuridine are the most active agents, but they have been shown to be more effective when given with biochemical modulators such as leucovorin.[4] However, there is considerable room for improvement in terms of the response rates and tolerability of chemotherapy, and the optimum regimen has yet to be determined.[5,6]

Modulation of FU by leucovorin (5-formyltetrahydrofolate or folinic acid) is caused by the interaction of thymidylate synthase, 5-fluoro-2′-deoxyuridine monophosphate, and 5,10-methylenetetrahydrofolate, which leads to the formation of a stable ternary complex with concomitant enzyme inactivation.[7] The formulation of leucovorin used in preclinical and clinical studies consists of a mixture of equal parts of two diastereomers differing in chirality at the C-6 carbon of the pteridine ring. Only the levorotatory isomer of leucovorin (*l*-LV) is transformed into active folate cofactors. The unnatural isomer (*d*-LV), however, is not inert; investigators have shown that it competes with the active (*l*-) form for uptake by cells.[8,9] It has also been reported that it is an inhibitor and a substrate for folypolyglutamate synthetase,[10] an enzyme that has a critical role in the folate-induced modulation of the fluoropyrimidines.[11] Because after intravenous (IV) administration of racemic LV the *d*-form accumulates in plasma at concentrations highly exceeding those of the *l*-form,[12] the possibility of a deleterious effect of the unnatural isomer on the modulation of FU cannot be ruled out.[8-10] Though tissue culture experiments have suggested that this phenomenon may only be significant with use of extremely high LV concentrations,[13] a recent analysis

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of human tumor biopsies obtained 30 minutes after IV administration of 200 mg/m^2 racemic LV demonstrated that *d*-LV may in fact partially inhibit tissue distribution of the effective *l*-form.[14]

In agreement with pharmacokinetic and in vitro studies of *l*-LV, showing similar effects at half doses,[15] and improved tissue uptake in liver metastases from gastrointestinal malignancies[14] when compared with racemic LV, impressive response rates have been reported in early phase I/II studies in colorectal cancer patients treated with FU plus *l*-LV.[16] However, any advantage over racemic LV must be confirmed in a randomized trial. We report here the results of such a controlled trial in previously untreated patients with advanced colorectal adenocarcinoma. Because previous studies comparing various (ie, low-dose *v* high-dose weekly as well as 5-day bolus) racemic LV/FU regimens have failed to demonstrate a direct relationship between the dose of the biochemical modulator and the therapeutic effectiveness,[17-19] and because the costs of the pure *l*-isomer are substantially higher than that of racemic LV (approximately 2.2-fold in Austria), we decided to compare identical rather than equipotent doses of the two LV preparations. In fact, we wished to determine if use of a double-the-effective dose of LV in combination with FU (*l*-LV arm) would result in any substantial/clinically relevant difference in therapeutic effectiveness or incidence of adverse reactions. The dose and schedule of FU, specifically the extended duration of the infusion used in both treatment arms, was derived from previous clinical studies, suggesting less frequent and severe treatment-associated toxicities.[16,20]

PATIENTS AND METHODS

Eligibility Criteria

Patients were required to have histologically confirmed metastatic and/or recurrent colorectal adenocarcinoma that was not amenable to curative surgical resection. Each patient had to have measurable disease that could be assessed by radiographic measurements, and patients were only eligible when a progression of measurable tumor of at least 25% in size or the appearance of new metastases had been demonstrated within the last 8 weeks. Further eligibility criteria included a World Health Organization (WHO) performance status of 0 to 3, a life expectancy of at least 3 months, and adequate bone marrow (WBC count $>$ 3,500/μL, platelet count $>$ 100,000/μL), renal (serum creatinine concentration $<$ 132 μmol), and hepatic (serum bilirubin level $<$ 34 μmol/L, serum transaminase level $<$ 100 IU/L) function. All patients were previously untreated by chemotherapy, and any kind of radiotherapy must have been stopped at least 8 weeks before study entry. Patients were excluded if they had CNS metastases, presence of osseous metastases as the sole tumor site, serious and/or uncontrolled concurrent medical illness, or a history of other malignancies unless basal carcinoma of the skin or carcinoma in situ of the cervix that was treated adequately. Informed consent was obtained from all patients before randomization, and the study was approved by the Ethical Committee of the University of Vienna.

Randomization Procedures

The participating centers entered patients by telephone at the central study office located at the University of Vienna. After confirming eligibility in agreement with the protocol, patients were stratified according to WHO performance status (score 0 to 1 *v* 2 to 3), presence or absence of liver metastases, and weight loss in the 6 months before study entry. Patients were then randomly assigned to one treatment regimen by the central office. Balance across strata was attained using the method reported by Pocock and Simon.[21]

Treatment Protocol

Patients in both treatment arms received identical dose regimens of FU combined with either racemic LV (Calciumfolinat, Ebewe-Arzneimittel Ges.m.b.H., Unterach, Austria) or *l*-LV (L-Leukovorin, Lederle Arzneimittel-Cyanamid Ges.m.b.H., Vienna, Austria). Racemic LV or *l*-LV was administered at 100 mg/m^2/d by IV bolus injection immediately followed by FU 400 mg/m^2/d administered as a 2-hour infusion.

Chemotherapeutic drugs were given on 5 consecutive days at 4-week intervals for a total of 6 months or until there was evidence of tumor progression. In case of relapse in patients who achieved objective response or stable disease, original therapy was reinstituted for a maximum duration of another six treatment cycles. Treatments were delayed weekly if patients had not recovered from toxicity. In case of WHO grade 3 or 4 toxicity, the FU dosage was reduced by 20% on subsequent courses.

Pretreatment evaluation included a complete medical history, physical examination, routine hematology and biochemistry analysis, chest x-ray, ECG, and computed tomographic (CT) scan of the chest, abdomen, and pelvis.

Toxicity and Response Criteria

Toxicity was evaluated according to WHO criteria.[22] Hematologic parameters were assessed weekly, and all other adverse reactions were evaluated retrospectively before the next cycle. Tumor size was measured by CT scan, x-ray, or any other technique that allows retrospective and independent reassessment every 8 weeks. Objective responses had to be confirmed in one subsequent examination after a 4-week interval. Centers were required to be consistent with respect to the method of assessment for each patient. A complete response (CR) was defined as the disappearance of all detectable disease on two consecutive evaluations 4 weeks apart. A partial response (PR) was defined as a greater than 50% reduction of the summed products of the two greatest diameters of all measurable disease, with no new lesions appearing and none progressing for at least 4 consecutive weeks. No change was defined as a less than 50% reduction and less than 25% increase of measurable tumor lesions lasting for at least 8 weeks. Patients were considered to have progressive disease (PD) if the measurable tumor lesions increased by greater than 25% according to initial staging or if new lesions appeared within the first 2 months of therapy. All CR and PR decisions were reviewed by an independent external panel of oncologists and radiologists blinded to treatment received. Survival was determined from the date of first treatment until death or until the patient

was last examined alive. Time to progression was determined as the interval between the date of first treatment and the date PD was first observed.

Statistical Considerations

The study was designed to detect an improvement in patient survival from 30% to 45% at 1 year, with 120 patients per arm providing at least 90% power (α = 5%). Further end points were objective response, progression-free survival, and toxicity. Pretreatment characteristics, tumor response rates, and treatment toxicities in the two arms were compared using the χ^2 test. Patient survival and progression-free survival were examined with the Kaplan-Meier product-limit method,[23] and treatment arms were compared with the log-rank test.[24]

RESULTS

Between October 1991 and December 1994, a total of 248 patients from eight centers were accrued to the study. Of these patients, 125 were randomized to receive FU/racemic LV and 123 to FU/*l*-LV. Seven patients did not receive the allocated treatment subsequent to randomization because they were found to be ineligible for the following reasons: lack of histologic confirmation, metastatic disease not confirmed on CT (n = 2), noncolonic primary tumor, previous FU treatment (n = 2), and uncontrolled thrombembolism. Thus 241 of 248 patients (92.2%) are included in this analysis. The two groups were well matched for pretreatment characteristics, as listed in Table 1. The median age was 65 years, and the male-to-female ratio was 3:2. The large majority of patients had a WHO performance status of 0 or 1, two thirds had no or only minimal weight loss within 6 months before study entry, and most had moderately differentiated tumors metastatic to the liver. The primary tumor site was colonic in 151 patients and rectal/rectosigmoid in 90. Five patients had been previously treated with radiotherapy, four of whom had rectal primary tumors.

Tumor Response

Response to treatment according to the study arm is listed in Table 2. The overall objective response rates of 25% (FU/racemic LV) and 32% (FU/*l*-LV) were not significantly different (P = .25). Similarly, both median time to response (defined as the time from the start of treatment to the first record of the patient's best response) of 2.7 versus 2.4 months (P = .52) and median durations of overall responses (CRs and PRs) of 7.2 versus 8.0 months (P = .65) suggested only a marginal advantage in favor of the patients treated with FU/*l*-LV. The frequencies of responses by sites of disease did not differ appreciably between the two treatment regimens.

Table 1. Pretreatment Characteristics

Characteristic	FU/LV	FU/*l*-LV	Total
No. of patients			
Entered	125	123	248
Assessable	122	119	241
Sex			
Male	73	66	139
Female	49	53	102
Age (years)			
Median	65	64	65
Range	22-75	26-75	22-75
WHO performance status			
0	45	35	80
1	48	55	103
2	15	17	32
3	14	12	26
Weight loss before study (kg)			
≤ 5	81	79	160
> 5	41	40	81
Location of primary tumor			
Colon	75	76	151
Rectum	47	43	90
Disease-free interval (months)			
< 6	65	60	125
6-12	21	26	47
> 12	36	33	69
Location of metastases			
Liver	78	76	154
Lung	26	28	54
Abdominopelvic mass	51	54	105
Other*	3	5	8
No. of metastatic sites			
Single	87	81	168
Multiple	35	38	73
Histologic grading			
G1	9	8	17
G2	85	85	170
G3	20	15	35
Gx	8	11	19
Number of treatment courses			
Median	6	6	6
Range	1-12	1-13	1-13

*Includes bone, mediastinum, and lymph nodes.

Table 2. Overall Tumor Response

	FU/LV (n = 122)		FU/*l*-LV (n = 119)	
Tumor Response	No.	%	No.	%
CR	4	3	6	5
PR	26	21	32	27
No change	57	47	51	43
PD	27	22	17	14
Not assessable*	8	7	13	11
Overall response	30	25	38	32

*Inadequate follow-up data available to evaluate tumor response.

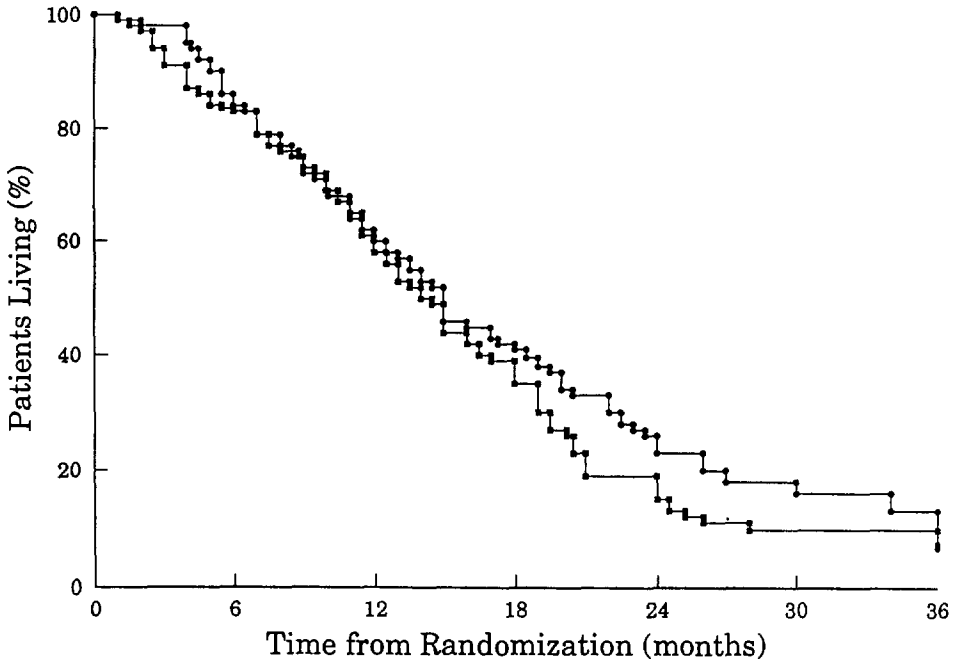

Fig 1. Overall survival for FU/*l*-LV (●---●; n = 119) and FU/racemic LV (■---■; n = 121).

Time to Progression and Survival

At the time of this analysis, less than 2% of the patients are alive without progression. The median time to progression was 6.25 months (95% confidence interval [CI], 5.0 to 8.0) in the FU/racemic LV arm, and 8.0 months (95% CI, 7.0 to 9.0) in the FU/*l*-LV arm, which is of borderline significance (P = .0505).

One hundred eighty-four patients (76.3%) have died. The median follow-up duration for patients still alive is 23 months (range, 17 to 48). Five patients were lost to follow-up for evaluation after 5.5, 7, and 29 months (FU/racemic LV) and after 5.5 and 32 months (FU/*l*-LV), respectively. The median survival time (Fig 1) was not significantly different at 14.5 months (95% CI, 12.0 to 17.0) for patients who received FU/racemic LV versus 15.0 months (95% CI, 12.5 to 18.5) for those who received FU/*l*-LV (P = .28). The 1-year survival rates were 58.3% versus 60.6% (P = .72), and the probability of survival at 2 years was 15.3% versus 23% (P = .16), respectively.

Delivery of Chemotherapy and Toxicity

All eligible patients commenced treatment at full dosage, and 159 (63%; 76 in the FU/racemic LV arm and 83 in the FU/*l*-LV arm) completed the intended 6 months of treatment. Chemotherapy was discontinued prematurely in 82 patients (34%), and there was no significant difference between the two arms in the number who did so (P = .28). The reason for cessation was related to toxicity in 12 patients (15%), including seven in the FU/racemic LV arm and five in the FU/*l*-LV arm (P = 1.0), and because of negative compliance (12 *v* 14) or disease progression in the remainder (27 *v* 17). The percentage of the intended starting dose of FU delivered was only marginally higher in the FU/*l*-LV arm (84% *v* 77%; P = .08). In the FU/racemic LV arm, 22 patients had a dose reduction because of toxicity compared with 16 patients in the FU/*l*-LV arm (P = .38).

Eighty-two (67%) of the 122 patients on study treated with FU/racemic LV reported at least one adverse experience, and 22 (18%) had at least one severe adverse experience. The respective values in the FU/*l*-LV arm were 71 of 119 (60%) and 15 (13%), suggesting no difference between the two arms (P = .23 and P = .29). Hematologic toxicity was commonly noted in both treatment groups (Table 3); leukopenia occurred in 33% (2% grade 3 to 4), granulocytopenia in 30% (4% severe), and anemia in 27%. The median nadir granulocyte counts were 3,323/μL (range, 0 to 12,600) and 3,656/μL (range, 552 to 10,850), and the median nadir platelet counts were 209,000/μL (range, 9,000 to 657,000) and 236,000/μL (range, 69,000 to 745,000) in the FU/racemic LV and FU/*l*-LV arm, respectively. Commonly encountered nonhematologic adverse reactions included nausea/vomiting

Table 3. Treatment-Associated Side Effects

Toxicity	% FU/LV (n = 122) Grade 1	2	3	4	% FU/*l*-LV (n = 119) Grade 1	2	3	4
Leukopenia	23	13	3	2	18	7	—	—
Granulocytopenia	15	16	4	4	14	5	2	—
Thrombocytopenia	6	2	—	—	4	1	—	—
Anemia	27	4	—	—	17	6	—	—
Infection	11	5	—	—	8	3	1	—
Nausea/emesis	17	16	1	—	19	14	3	—
Stomatitis	11	7	6	—	10	7	6	—
Diarrhea	13	10	9	1	10	13	6	1
Alopecia	12	3	—	—	6	4	—	—
Skin	4	1	—	—	2	—	—	—
Peripheral neuropathy	2	—	—	—	2	—	—	—

in 35% (2% grade 3), diarrhea in 32% (7% ≥ grade 3), mucositis in 23% (5% grade 3), and alopecia in 12%. There was significantly greater toxicity experienced by patients in the FU/racemic LV arm only in terms of leukopenia ($P = .01$) and granulocytopenia ($P = .003$). These hematologic side effects were also significantly more likely to be of grade 3 or 4 ($P = .03$). All other differences were not significant at the 5% level. There was only one treatment-related death in a patient who received FU/racemic LV.

DISCUSSION

Numerous phase II and phase III studies of FU given in combination with leucovorin for treatment of advanced colorectal cancer have been conducted.[4,25] These trials, in which the doses of both compounds and their schedules and modalities of administration were varied, produced objective response rates ranging from 15% to 54%. Although randomized trials have established with reasonable certainity that FU/LV-based chemotherapy produces substantial therapeutic gain compared with best supportive care[2,3] or FU alone,[4] until now, it has not been possible to define the regimen that yields optimal antitumor activity. Among several different approaches to refine treatment strategies of fluoropyrimidine biochemical modulation, of current interest seems the levorotatory stereoisomer of leucovorin, presumed to be the active form. Pharmacokinetic and in vitro studies of *l*-LV have shown similar effects at half doses,[15] and improved tumor tissue uptake compared with racemic LV.[14] Encouraging therapeutic results have been reported in recent phase I/II trials in colorectal cancer[16,26] and other malignancies[27,28]; however, any superiority of *l*-LV compared with racemic leucovorin has not been demonstrated yet in a randomized trial.

Together with a preliminary report from the North Central Cancer Treatment Group evaluating equipotent doses of racemic and *l*-LV,[29] this is the first study providing comparative data on the efficacy and toxicity of these two different clinical formulations of leucovorin in combination with FU for the palliative treatment of patients with advanced colorectal cancer. Our results suggest that there are no significant differences between the two regimens in various measures of therapeutic effect, including overall patient survival, progression-free survival, and objective tumor response rate. Except for minor myeloid toxic effects associated with FU plus the levorotatory isomer of leucovorin, there was also no significant difference in terms of treatment-associated side effects. The lower incidence of (severe) leukopenia/granulocytopenia, which had previously been noted in phase I/II studies of FU/*l*-LV[16,28,30] is not clearly understood. Because of the mere absence of cytopenia-related complications in the present study and its rare occurrence in patients treated with FU/racemic leucovorin, this difference might be only of limited clinical significance. It seems noteworthy that in both treatment arms, gastrointestinal symptoms, specifically mucositis and diarrhea, were less frequent and less severe compared with other trials using various dose schedules of FU and racemic LV. The incidence of treatment-associated severe diarrhea and stomatitis reported in the literature ranges up to 23% and 30%, respectively,[31,32] as compared with 8% and 6% in the present series. This phenomenon is most likely to be related to the greater duration of the daily infusions of FU[16,20] used in both arms in the present study, and should be taken into consideration when treating patients with FU/LV to minimize associated toxicity.

In conclusion, this study demonstrated that biochemical modulation of FU by either racemic LV or *l*-LV, the pure active isomer, produced comparable efficacy results in patients with measurable advanced colorectal cancer. Although we cannot exclude that the lack of difference may be attributed to a less-than-optimal dosing of *l*-LV,[33] perfecting the inhibition of thymidylate synthase, in fact, may not be the future to improve therapeutic results in this common malignant disease, neither with further or potentially more effective modulation of FU nor with specific TS inhibitors.[34] Cellular targets, in addition to thymidylate synthase inhibition, will need to be considered to make significant progress in the treatment of advanced colorectal cancer.

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