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#5696

Evaluating determinants for enhanced activity of MM-398/PEP02; a novel nanotherapeutic encapsulation of irinotecan (CPT-11)

Ashish Kalra, Jaeyeon Kim, Milind Chalishazar, Nancy Paz, Stephan Klinz, Daryl Drummond, Dmitri Kirpotin, Victor Moyo, Clet Niyikiza, Jonathan Fitzgerald
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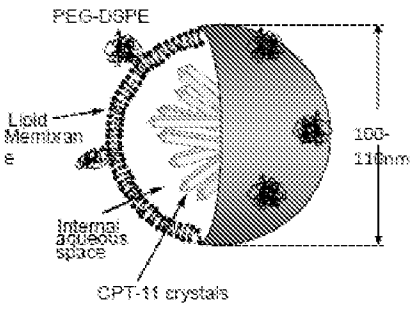
ABSTRACT

MM-398 is a stable nanotherapeutic encapsulation of the pro-drug irinotecan (CPT-11). Irinotecan, a topoisomerase inhibitor is currently being used in clinical practice for treatment of several indications; however, associated toxicities, mainly, neutropenia and gastrointestinal toxicity, have limited its clinical utility. Previously, we have demonstrated that MM-398 treatment resulted in significantly higher intratumoral concentrations of both irinotecan (142-fold) and SN-38 (9-fold), thereby exhibiting enhanced anti-tumor activity compared to free irinotecan in different xenograft models. Multiple phase 1 and 2 studies have established a pharmacokinetic and safety profile. Recent data support continued clinical development for the drug in various indications, including pancreatic, gastric, colorectal and potentially other solid tumors. In order to further understand the enhanced anti-tumor activity of MM-398 we developed a mechanism-based PK model of MM-398 and free irinotecan designed to predict intratumoral levels of SN-38. Based on this model, we evaluated the role of various determinants of response of MM-398. Sensitivity analysis revealed that the local activation of MM-398 was important for obtaining higher SN-38 intratumoral levels compared to free irinotecan. To identify cell types responsible for local activation of MM-398 we investigated cellular liposome phagocytosis ability. *In vitro* studies demonstrated preferential uptake of MM-398 by phagocytic macrophages compared to tumor cells. FACS analysis of tumor samples (from subcutaneous xenografts) highlighted higher uptake of labeled liposomes by CD11b + and F4/80+ cells as compared to tumor cells. We are developing *in vivo* systems using engineered cell lines overexpressing growth factors to recruit macrophages (CSF1) or overexpressing irinotecan conversion enzymes to further investigate the role of macrophages in local tumor conversion of MM-398 and to validate the model prediction that local activation is critical to MM-398 mechanism of action. The extent of tumor vascularization and permeability—were also highlighted in the sensitivity analysis.

To determine the effect of MM-398 on these parameters, we treated mice bearing HT 29 (colorectal cancer) xenografts with a single dose of MM-398 and measured hypoxic markers (CAIX) and microvessel density (CD31) by immunohistochemistry. Tumors treated with MM-398 showed a greater degree of CD31 staining and lower CAIX staining, indicating that MM-398 may be able to affect the tumor microenvironment. We are currently evaluating how the ability of MM-398 to alter the tumor microenvironment affects the activity of other chemotherapeutic agents in combination therapy. These findings could support the use of MM-398 as a combination modality against tumors that may have acquired resistance to traditional chemotherapeutic agents.



MM-398 Background and Pharmacology

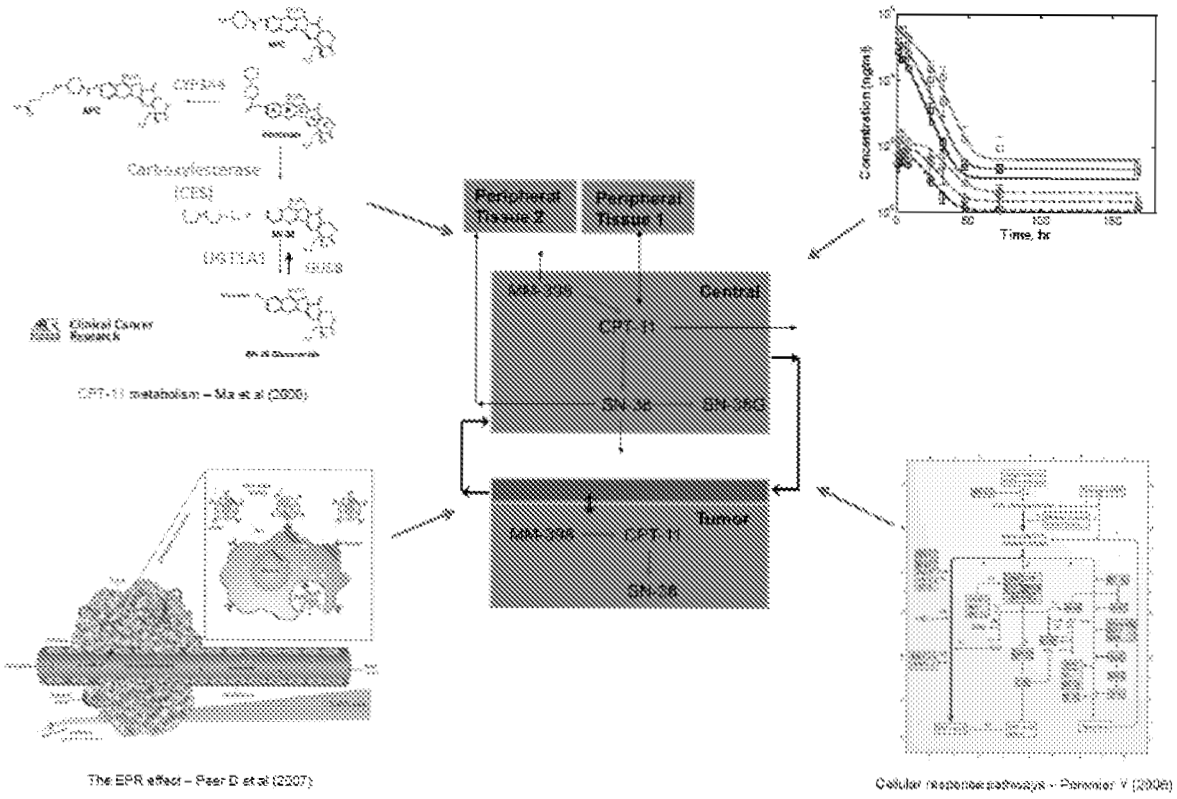
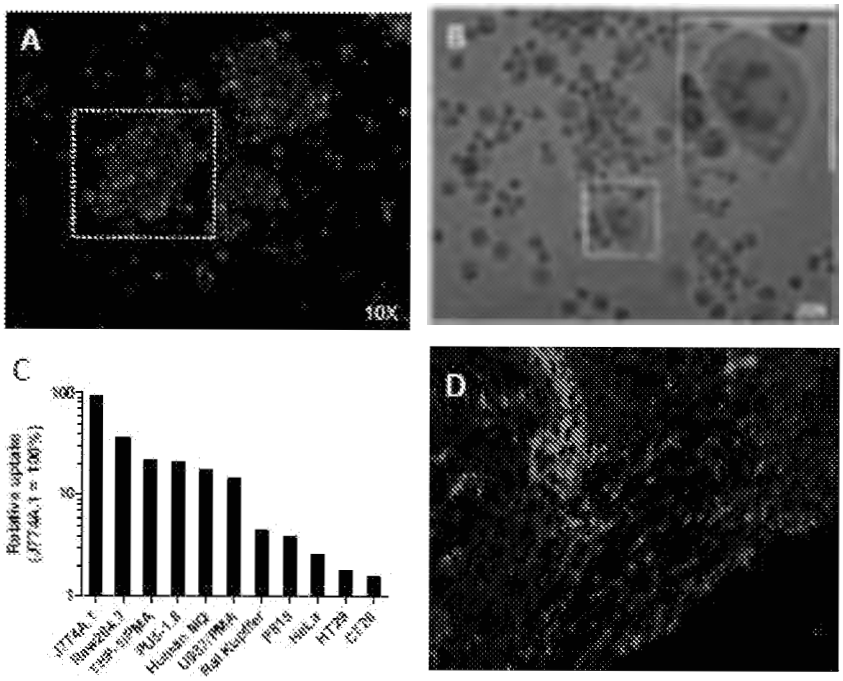


Figure 1. Components to be captured in our mechanism-based PK model include the pathway activities for irinotecan metabolism including pharmacogenomic information, deposition parameters for liposomes and free metabolites as part of the Enhanced Permeability and Retention (EPR) effect, measured PK and 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).

Preferential uptake of liposomes by phagocytic cells (*in vitro* and *in vivo* analysis)

Relative liposome uptake in different cell types



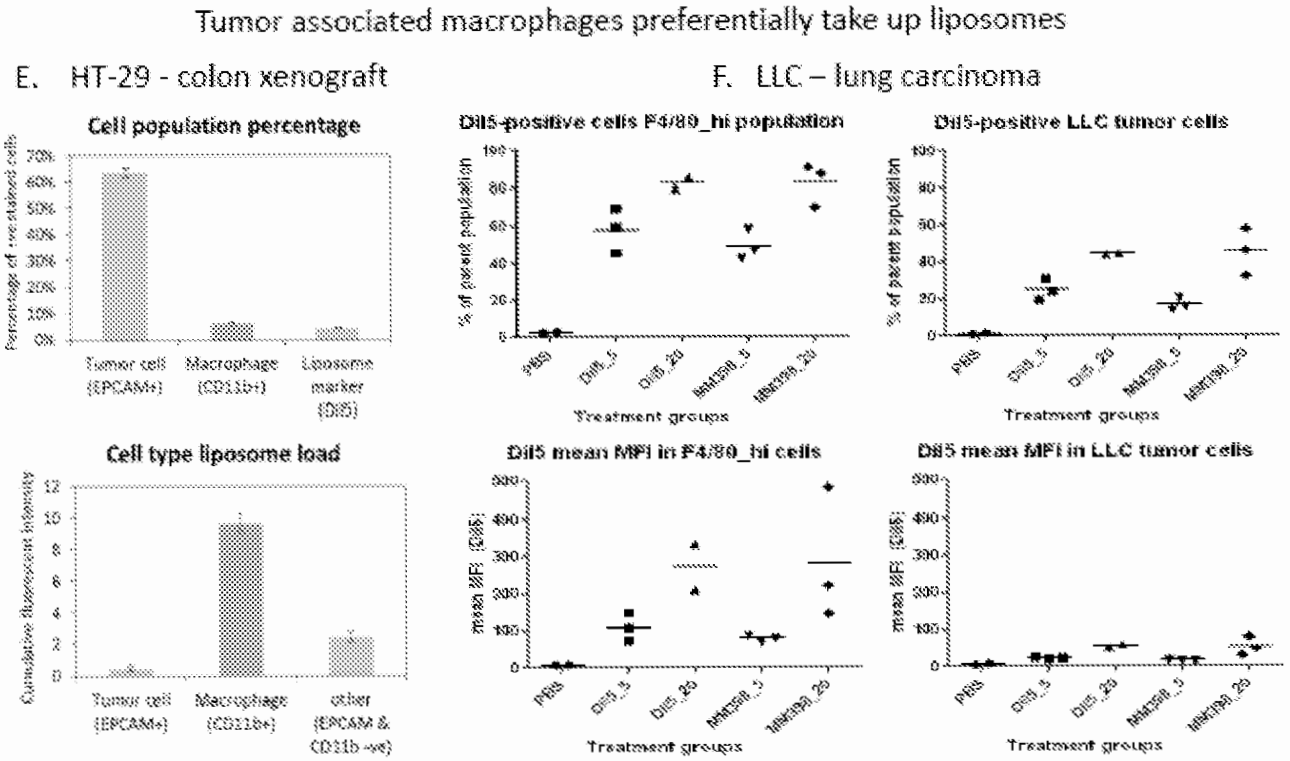
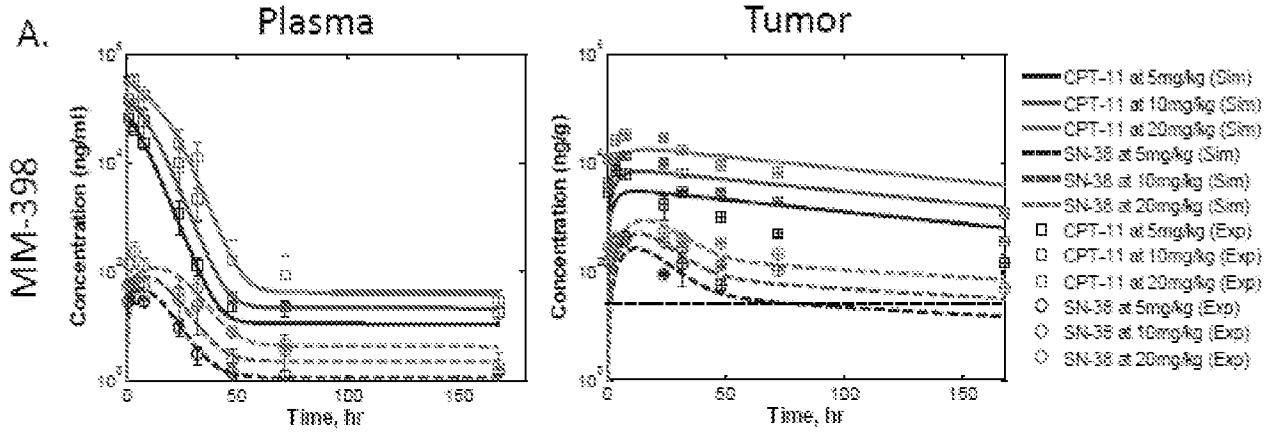
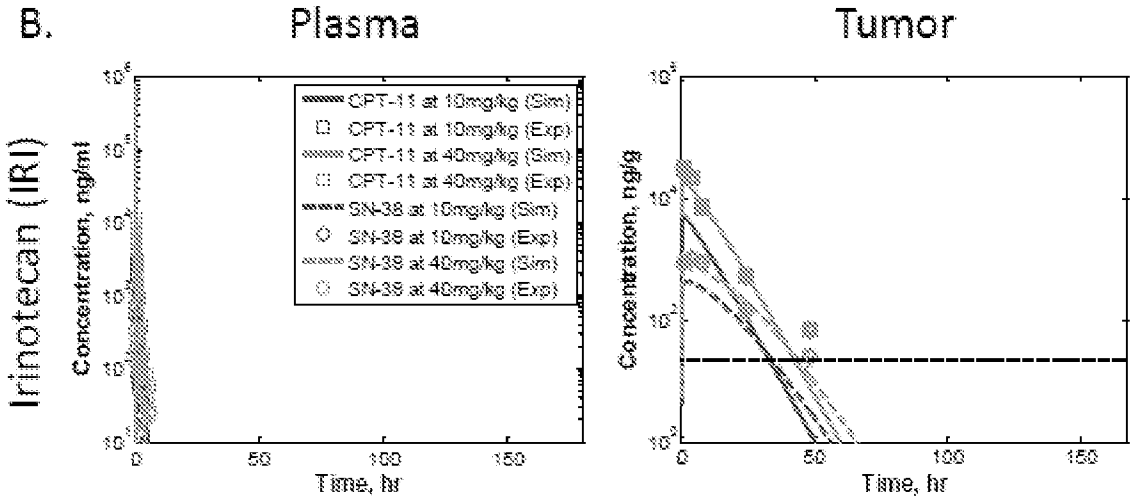
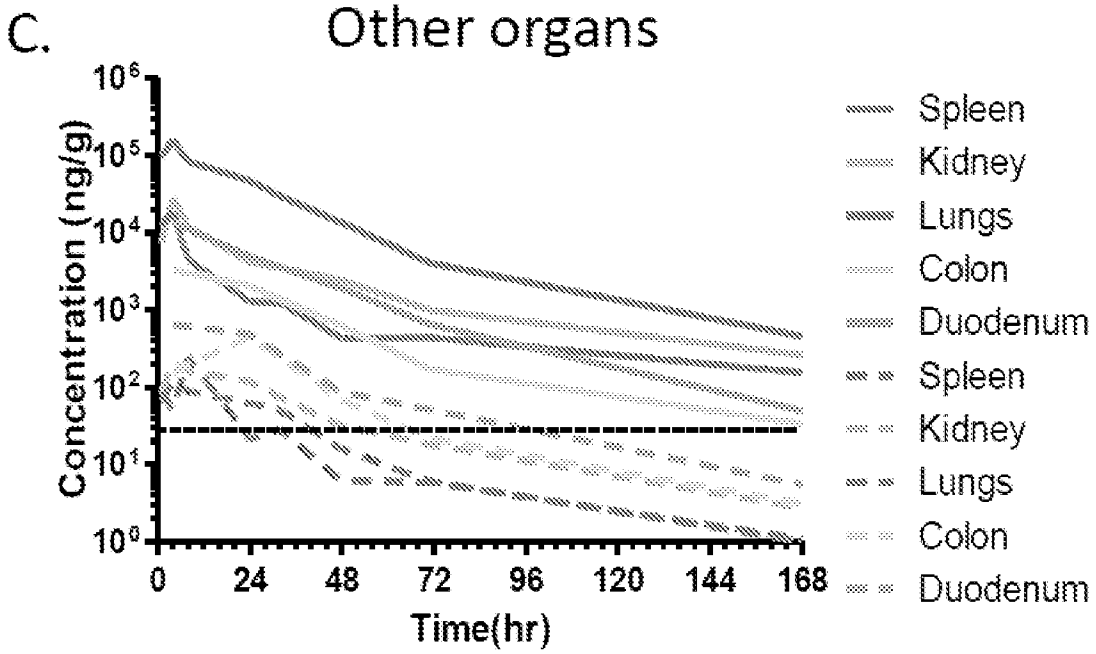


Figure 2. (A) Human monocytic cell line U937 differentiated with PMA (16.2nM) shows uptake of Dil5-labeled liposomes (20µg/ml) after 24h incubation, blue: nuclear staining with Hoechst; red staining: Dil5 liposomes . (B) Higher magnification image showing intracellular accumulation of Dil5 liposomes. (C) FACS analysis to quantify the differential uptake of Dil5 liposomes (20µg/ml, 24h) across multiple cell lines (macrophages and tumor cells). Relatively higher uptake seen in phagocytic cell lines compared to tumor cell lines. (D) *In vivo* uptake and deposition of Dil5 liposomes (20mg/kg, i.v. injection, 24h) seen by fluorescence imaging in HT-29 xenograft (E) *Ex vivo* FACS analysis performed on HT-29 tumors grown in nude mice 24hr after 20mg/kg Dil5-labeled MM-398. (Top) ~63% of the total cells are tumor cells (EPCAM+), ~6% are macrophages (CD11b+) and ~4% cells take up Dil5-labeled liposomes. (Bottom) The highest cumulative fluorescence intensity (frequency x MFI) is observed for the macrophages (CD11b+) when compared to tumors cells. 20% of CD11b+ cells but only 1.5% of EPCAM+ cells are Dil5+, thus demonstrating preferential uptake of liposomes by TAMs. (F) *Ex vivo* FACS analysis performed on murine LLC tumor model 48hr after i.v. injection of either 5mg/kg or 20mg/kg Dil5-labeled liposomes with or without MM-398. (Top L) panel shows that ~80-90% of '(CD11b+ Ly6C- F4/80+ cells) take up liposomes as compared to 40% of tumor cells (Top R). Furthermore, the MFI for TAMs (bottom L) is ~6 fold > tumor cells (bottom R). Addition of MM-398 did not alter the percent of TAMs suggesting that MM-398 does not kill TAMs within 48hr in this tumor model.

Mechanism-based PK modeling: Biodistribution and PK model for MM-398







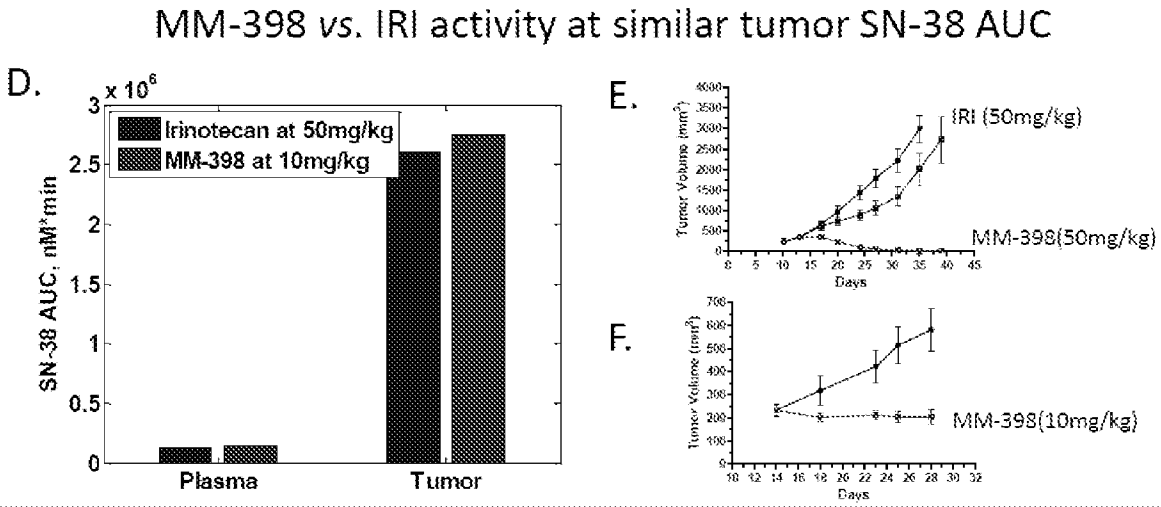
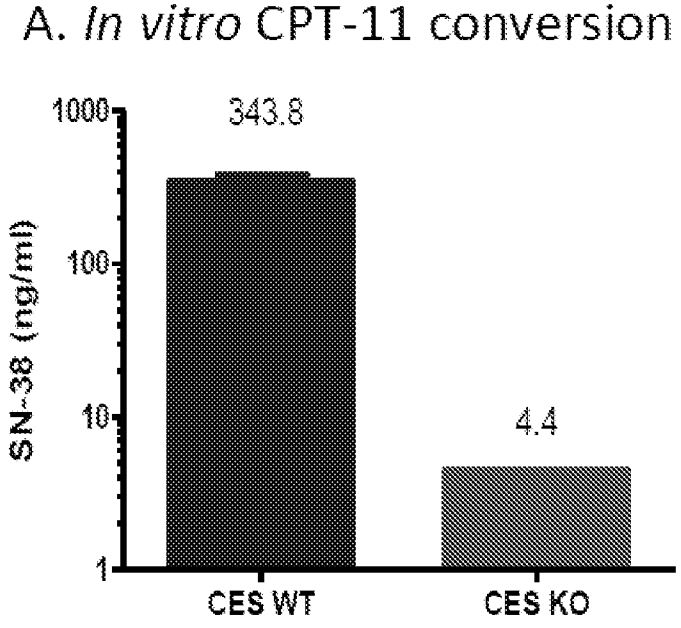
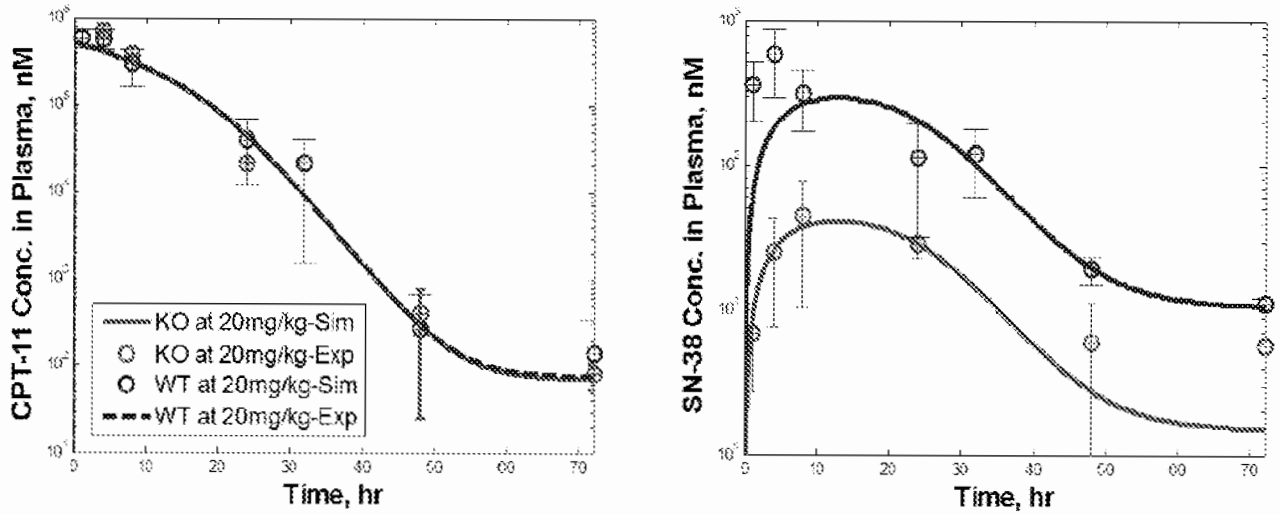


Figure 3. A mechanistic PK model was trained with plasma and tumor CPT-11 and SN-38 data from a *in vivo* HT-29 xenograft biodistribution study. HPLC analysis was used to quantify the plasma and tumor levels of CPT-11 and SN-38 at 1, 4, 8, 24, 36, 48, 72, 168h (symbols: experimental data) following different doses (single i.v. injection) of (A) MM-398 and (B) free irinotecan (IRI). This data set was used to train and develop a PK model (lines: model simulations). (C) CPT-11 (solid lines) and SN-38 (dotted lines) levels observed in other tissues following MM-398 (20mg/kg) dose were much lower compared to tumor levels (dashed lines) (D, E) Comparing tumor activity between MM-398 and free irinotecan at doses with similar plasma and tumor exposure (AUC). (D) PK model simulations show that a lower dose of MM-398 (10mg/kg) can achieve plasma and tumor SN-38 AUC similar to the levels achieved with a much higher free IRI dose (50mg/kg). However, the tumor activity response seen with (E) IRI is much lower when compared to equal exposure dose of (F) MM-398 (10mg/kg), suggesting that the duration for which the SN-38 levels are present in the tumor is more critical (see SN-38 tumor levels in A *vs.* B).

Identifying the role of tumor CES as the rate limiting factor in SN-38 tumor levels



B. CPT-11 and SN-38 serum exposure in CES WT *vs.* KO



C. Effects of serum or tumor CES KO on tumor SN-38 levels

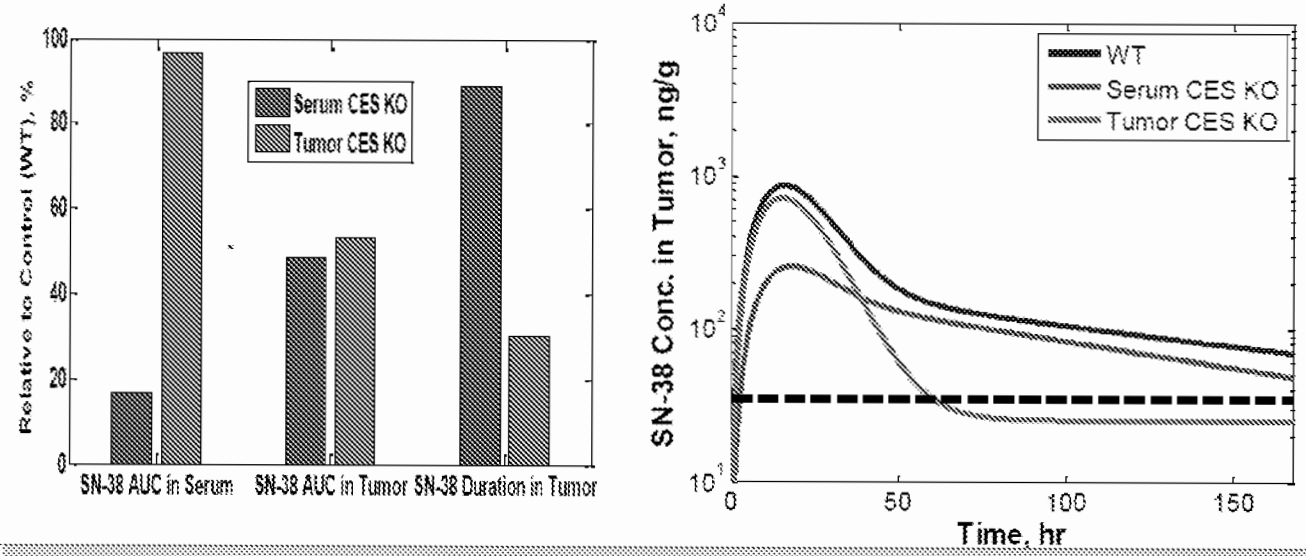


Figure 4. Comparing MM-398 conversion *in vivo* using CES wild-type (WT) and knockout (KO) C57BL/6 mouse strain. (A) *In vitro* CPT-11 conversion assay. Plasma from WT mice had significantly higher ability to form SN-38 as compared to plasma from KO mice when incubated with 5μM CPT-11 for 24h. (B) *In vivo* PK data and simulations in WT *vs.* KO mice. Mice bearing LLC (murine lung carcinoma) were injected i.v. with single dose of MM-398 (20mg/kg) and plasma samples were collected at 1, 4, 8, 24, 48 and 72h. CPT-11 and SN-38 levels in plasma were determined using HPLC (symbols: experimental data; line: model simulations). Plasma profile for CPT-11 was not altered in WT *vs.* KO however the plasma levels of SN-38 lowered ~90 fold in the CES KO mice suggesting significant contribution of plasma CES to CPT-11 systemic conversion in mice. (C) Modeling the effects of CES KO on serum and tumor SN-38 exposure; (Left): Systemic CPT-11 conversion reduced ~80% with serum CES KO with limited effect by tumor CES KO. Serum and tumor CES KO had similar effects on tumor SN-38 levels (50% less compared to WT). However, the tumor CES KO had a more pronounced effect of reducing the SN-38 duration (~70%) within the tumor compared to WT (Right).

Effects of CES conversion activity and drug uptake on MM-398 activity

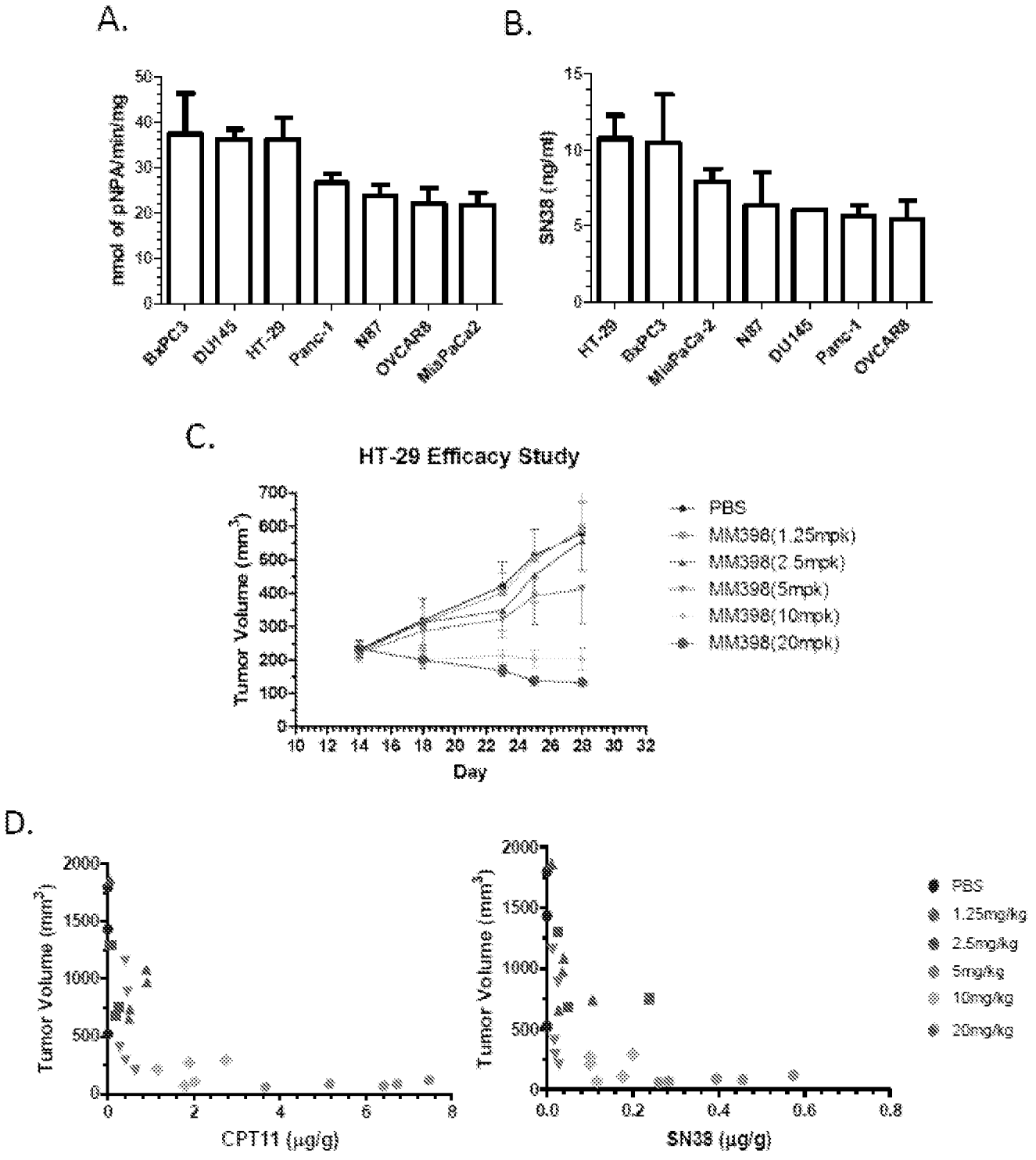
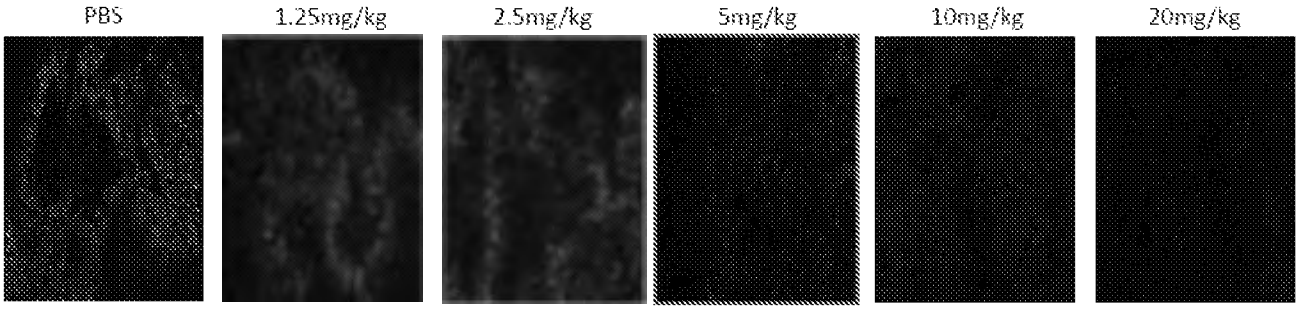
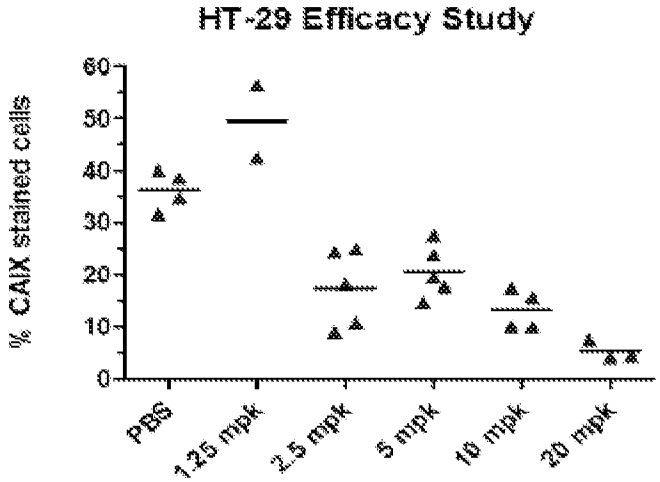


Figure 5. Profiling different xenograft models (tumor lysates) for *in vitro* CES conversion activity using (A) a substrate p-nitrophenolacetate (pNPA) and (B) free CPT-11. Varying levels of conversion (enzyme) activity was detected across xenograft models. (C)) *In vivo* dose-response study in HT-29 xenograft model. Cells were inoculated subcutaneously in SCID mice. Once tumors were well established (~100-200mm^3) MM-398 treatment was initiated. MM-398 was dosed i.v. q7d and tumor volumes were measured twice per week. n=5 mice/group. HT-29 tumors were collected at end of the study for IHC and HPLC analysis. (D) HPLC analysis to quantify the levels of CPT-11 and SN-38 within the tumors. We observed a dose dependent increase in levels of SN-38 and CPT-11 within the tumors which corresponded to higher activity response. This data collectively suggest that levels of local tumor CES (which may effect local drug conversion) and the amount of drug deposition within the tumor may play a critical role in MM-398 efficacy.

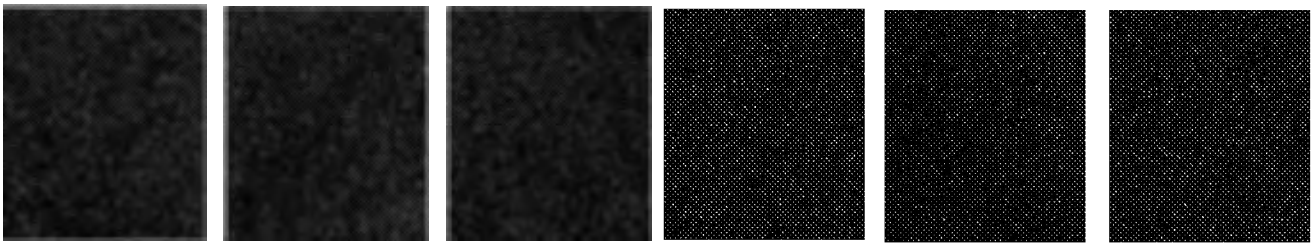
MM-398 effects on tumor microenvironment (hypoxia, proliferation, TAMs, vasculature)

A. MM-398 effect of hypoxia marker CAIX

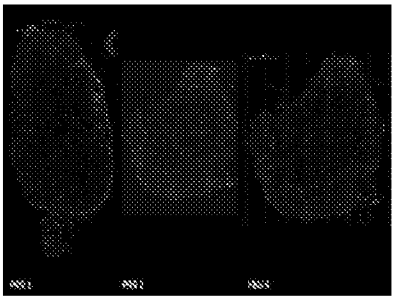


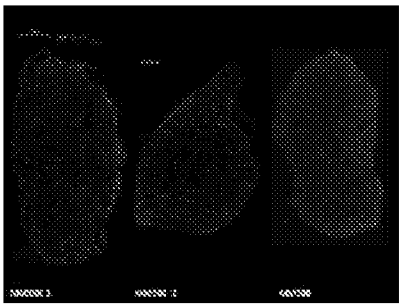


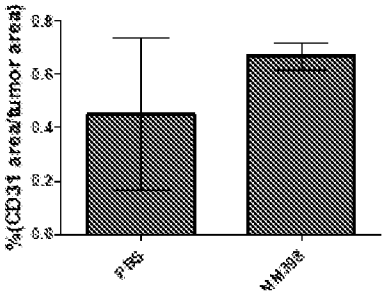
C. MM-398 effect on proliferation marker Ki67



E. MM-398 effect on vasculature marker CD31







D. MM-398 effect on TAMs marker F4/80

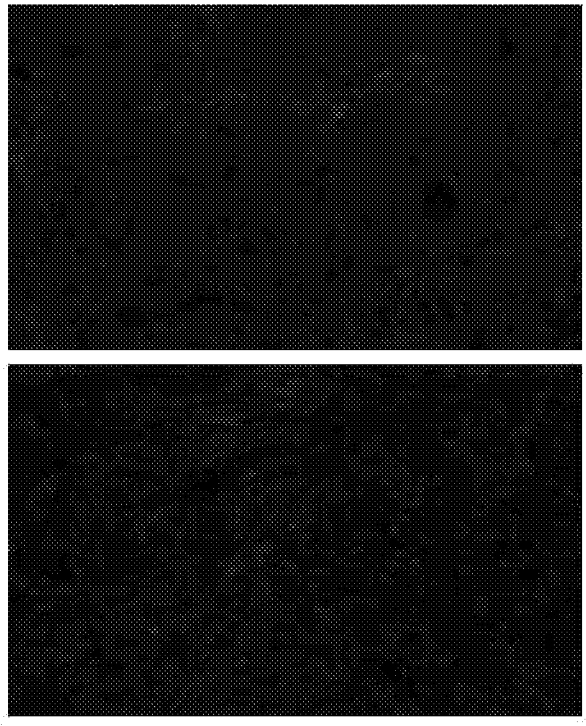


Figure 6. (A) HT-29 tumors were dosed with different concentrations of MM-398 (1.25mg/kg to 20 mg/kg). The tumors were collected at the end of the study. The samples were stained with CAIX as a hypoxia marker and whole-tumor image acquisition was done on an Aperio® ScanScope® FL. (B) The images were quantified with Definiens TissueStudio® software. The quantified data for CAIX staining showed a significant dose-dependent reduction in this tumor hypoxia marker following MM-398 treatment as compared to PBS controls. (C) AsPc-1 tumors treated with MM-398 (20mg/kg, q7d i.v. dose, 3 weeks). The tumors were collected at the end of the study and stained for Ki67 as a marker for proliferating cells. MM-398 treatment reduced the number of Ki67-positive cells compared to untreated tumors. (D) HT-29 tumors were stained for tumor associated macrophages (TAMs) with F4/80 macrophage-specific antibody. MM-398 did not alter the levels of F4/80-positive cells in this tumor model. (E) Image acquisition and quantification of vascular marker CD31 showed no changes in CD31 staining following MM-398 treatment.

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
- Phase 2 monotherapy in 2nd line pancreatic cancer patients; data were presented at the 2011 ASCO Annual Meeting
- Phase 2 monotherapy in 2nd line gastric cancer patients; data were presented at the 2011 ASCO Gastrointestinal Cancers Symposium
- Phase 1 monotherapy in colorectal cancer; data were presented at the 2012 ASCO Gastrointestinal Cancers Symposium

Summary

- Tumor associated macrophages (phagocytic cells) preferentially take up MM-398
- Pharmacokinetic model was developed to predict MM-398 serum and tumor PK profiles. The PK model identifies the critical role of tumor CES levels in the PK profile of MM-398
- Total tumor drug deposition and duration of exposure were identified as critical parameters for observing tumor activity with MM-398
- MM-398 was shown to alter the tumor microenvironment, particularly in reducing hypoxia in tumors.

#5696

Evaluating determinants for enhanced activity of MM-398/PEPO2; a novel nanotherapeutic encapsulation of irinotecan (CPT-11)

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Abstract #5622

The tumor microenvironment modulates the delivery and activation of liposomal encapsulated irinotecan, MM-398

Ashish Kalra, Jaeyeon Kim, Stephan Klinz, Nancy Paz, Joseph Reutt, Jaimini Patel, Daryl Drummond, Dmitri Kirpotin, Victor Moyo, Eliel Bayever, Peter Laivins, Clet Niyikiza, Ulrik Nielsen, Jonathan Fitzgerald
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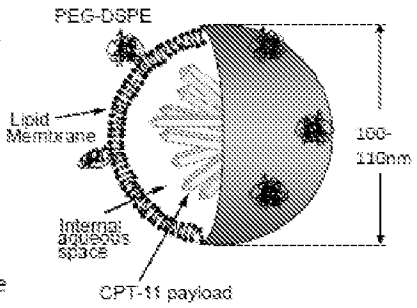
Abstract

MM-398 is a stable nanotherapeutic encapsulation of the pro-drug irinotecan with an extended plasma half-life and higher intratumoral deposition in animal models compared with free irinotecan. MM-398 is currently in multiple clinical trials, including a Phase 3 trial for patients with advanced gemcitabine-resistant pancreatic cancer (NAPOLI-1). By using a systems pharmacology approach, we have previously identified the important determinants for MM-398 activity differentiating it from free rinotecan. One of the critical parameters identified in the model is activity of the tumor carboxylesterase (CES) as an important correlate to the MM-398 preclinical response. Our data also show that tumor associated macrophages (TAMs) are the dominant cell type for phagocytosis of MM-398 in the tumor microenvironment. To validate these findings we used a perturbation strategy involving manipulation of TAMs with the anti-CSF1 antibody 5A1. Depletion of macrophages with 5A1 resulted in a strong size reduction of CD11b+F4/80+ mature macrophage populations in HT29, MC38 and LLC tumor models. Furthermore, other myeloid and non-myeloid cell populations showed tumor model and mouse strain-dependent changes in population size and liposome uptake. The overall impact of 5A1-mediated macrophage depletion was a reduction of the total cellular liposomal load within the tumors. Anti-CSF1 treatment also modulated the systemic clearance of MM-398 due to changes in the macrophage populations in the liver and spleen of the animals. To capture this complex interplay of systemic and local depletion events we have expanded our mechanistically based PK model to include separate tumor macrophage and tumor cell compartments.

The computational model also identified tumor permeability to MM-398 as a rate-limiting factor for drug deposition and *in vivo* activity. To assess the degree of variability in tumor permeability, we measured the levels of irinotecan and SN-38 using HPLC in six different xenograft models following treatment with MM-398. A high degree of variability in irinotecan and SN-38 levels was seen across these models and between tumors from different mice bearing xenografts of the same cell lines. We then investigated the inter-relationship between tumor permeability, macrophage content, CES enzyme activity and SN-38 accumulation by assessing the levels of these markers in multiple human-derived primary xenograft models across different indications. In summary, we demonstrated the impact of TAM modulation on overall tumor levels of irinotecan and SN-38 following treatment with liposomal encapsulated irinotecan, MM-398. We outline differences in permeability of xenografts to MM-398 and the capacity to convert irinotecan into SN-38.



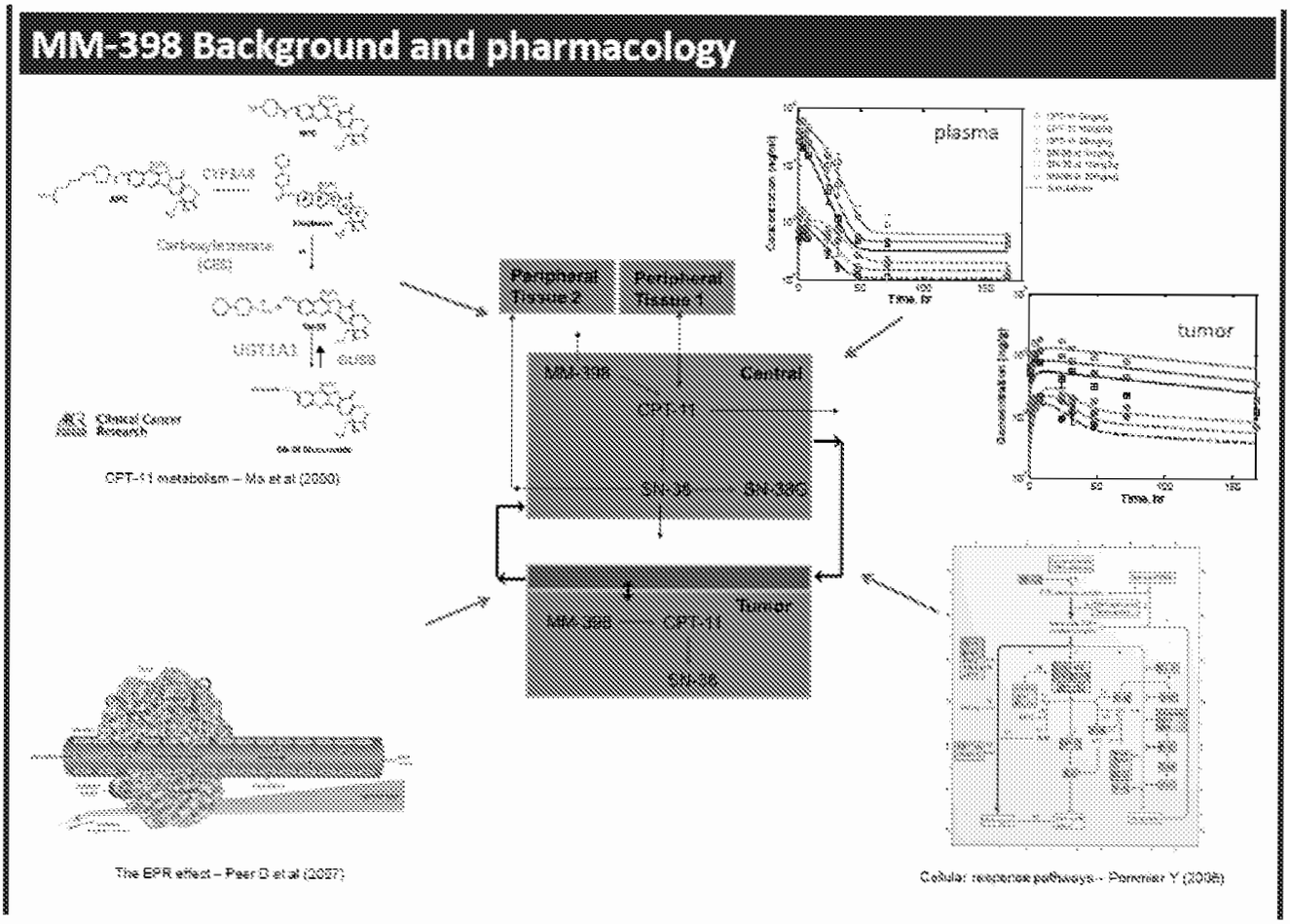
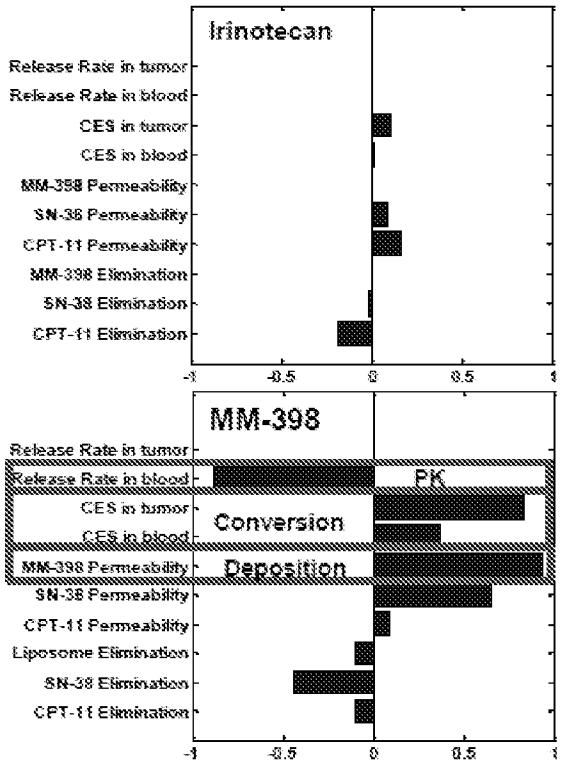


Figure 1. Components to be captured in our mechanism-based PK model include the pathway activities for irinotecan metabolism including pharmacogenomic information, deposition parameters for liposomes and free metabolites as part of the Enhanced Permeability and Retention (EPR) effect, measured PK and 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).

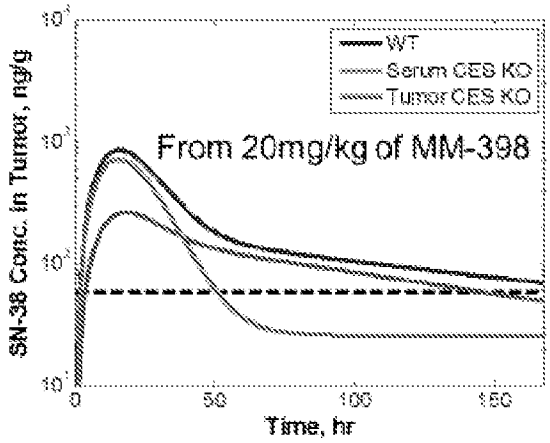
Depletion of tumor associated macrophages impacts the overall tumor uptake of MM-398

A. Modeling parameters important for sustained tumor exposure of SN-38

$$Sensitivity = \frac{\Delta(SN38_{Duration})/SN38_{Duration,0}}{\Delta(p)/p_0}$$

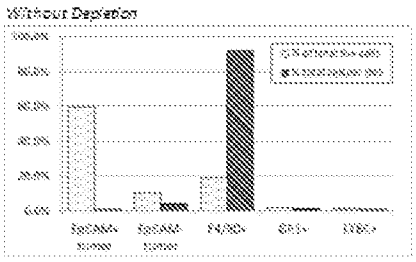


B.

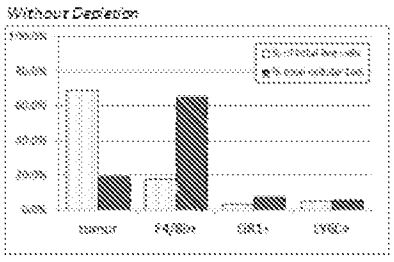


Depletion of macrophages changes fractional distribution of liposomes in tumor cell populations

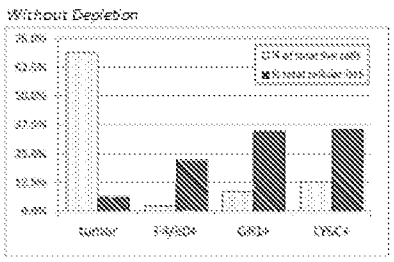
C. *HT-29 tumors*



D. *LLC tumors*



E. *MC-38 tumors*



Systemic effects of MQ depletion change PK behavior of MM-398

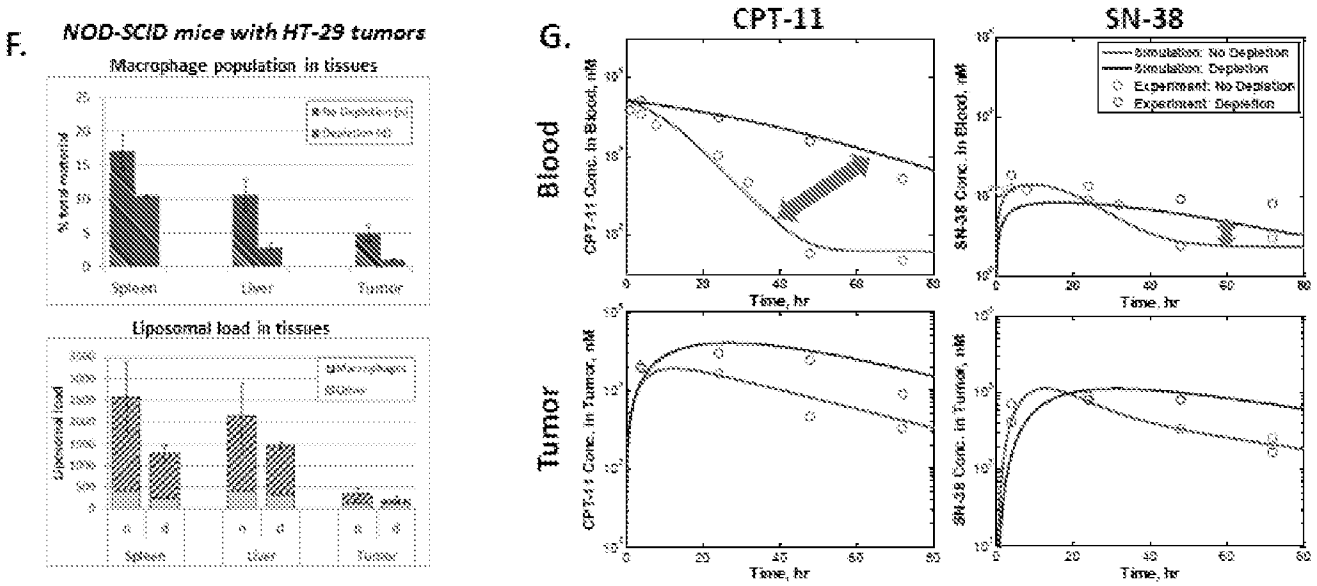
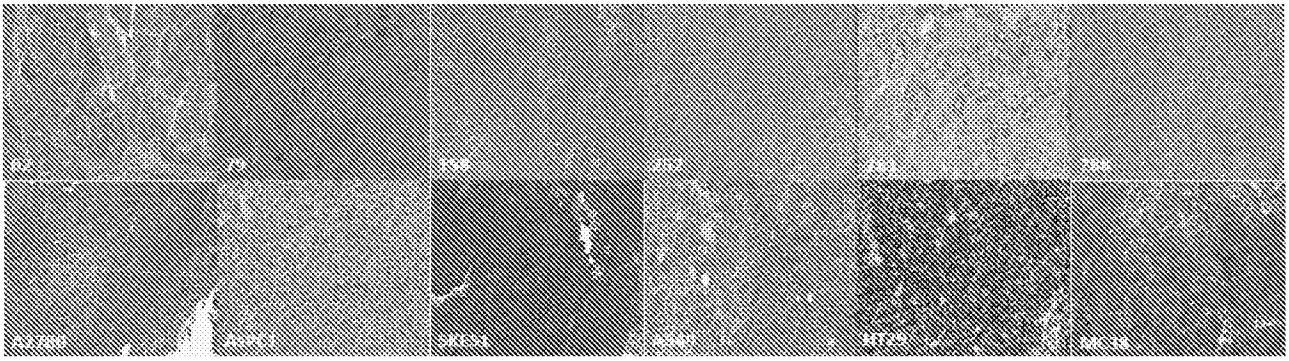


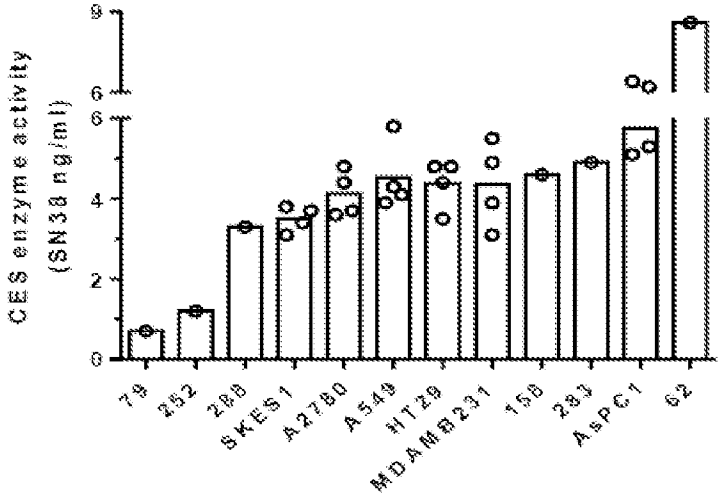
Figure 2. (A) Sensitivity analysis of the MM-398 PK model emphasizes importance of MM-398 PK, liposome deposition and drug conversion for sustained exposure to SN-38. (B) PK modeling supports contribution of tumor-specific conversion of irinotecan to SN-38 for sustained tumor exposure. (C-E) Tumor-bearing animals are depleted of macrophages by injection with anti-CSF1 5A1 antibody (ATCC CRL-2702) prior to i.v. injection of 10mg/kg DiI5-labeled liposomes. *Ex vivo* FACS analyses of live-gated cells performed 24hr after liposome injection on HT-29 colon tumors grown in NOD-SCID mice (C), LLC lung tumors grown in C57Bl6 mice (D) and MC-38 colon tumors grown in C57Bl6 mice (E) are shown with and without macrophage depletion to highlight changes in population sizes and in the fractional distribution of the DiI5 label. Averages from 4 tumors/group are shown. Myeloid cells (CD45+ CD11b+) are classified as mature macrophages (F4/80+), granulocytic (GR1+) and monocytic (LY6C+) cells. Lymphoid cells (CD45+ CD11b-) do not display a significant DiI5-liposomal signal. (F) FACS-based tissue analysis on total events in NOD-SCID mice bearing the HT-29 tumor show that anti-CSF1 5A1 treatment reduces macrophage population sizes in spleen and liver and reduces the overall liposomal load. (G) Analysis of CPT-11 and SN-38 levels by HPLC in plasma and tumor samples of HT-29 tumors show prolonged circulation of encapsulated irinotecan after macrophage depletion. This also increases drug levels present in the tumor tissue. The PK model can fit this data with an assumption of a 70% decrease in clearance in the systemic compartment. This correlates well with depletion levels observed in liver tissue by flow cytometry.

Heterogeneity of markers observed across patient- and cell line-derived xenograft models

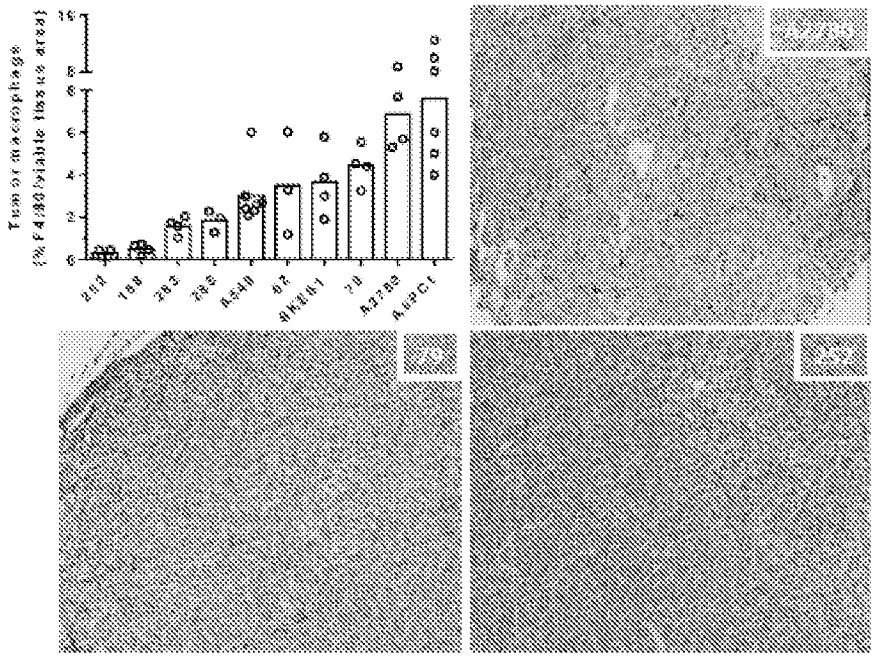
A. Histological differences between patient-derived and cell line-derived xenograft models



B. CES enzyme activity (ex vivo CPT-11 conversion assay)



C. Tumor associated macrophage (TAMs)



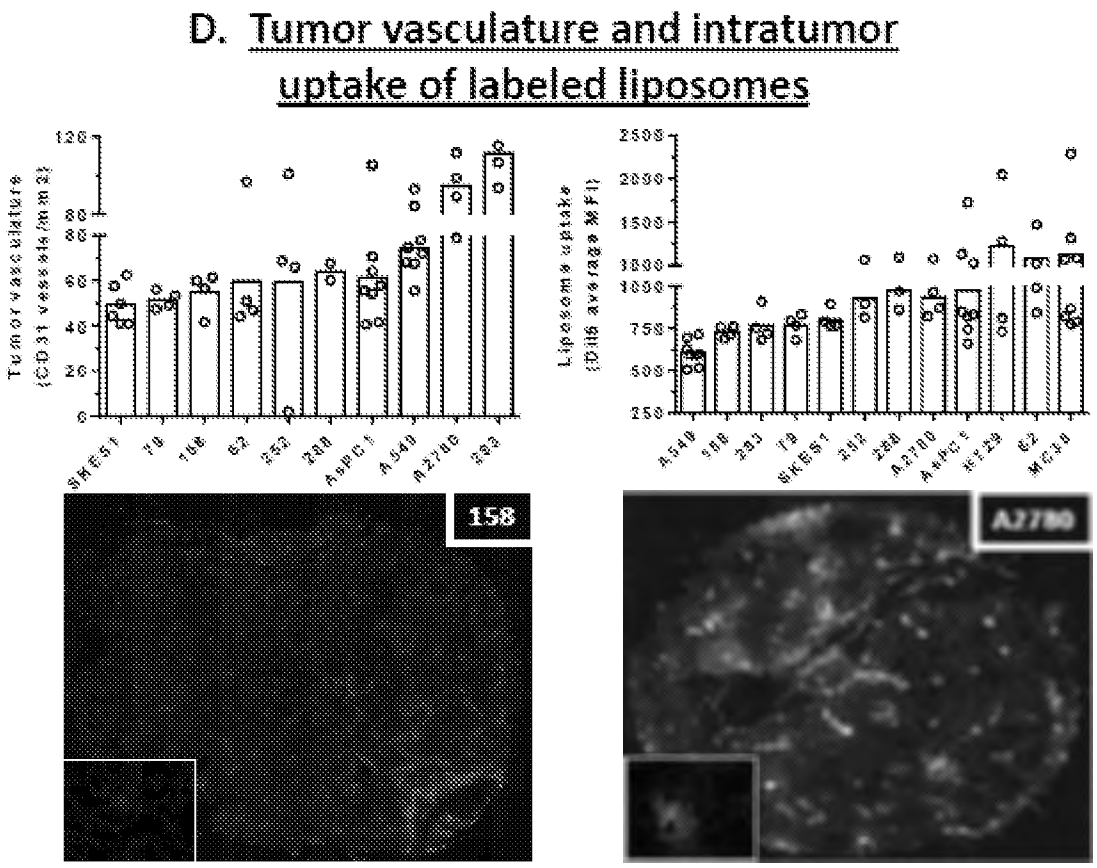
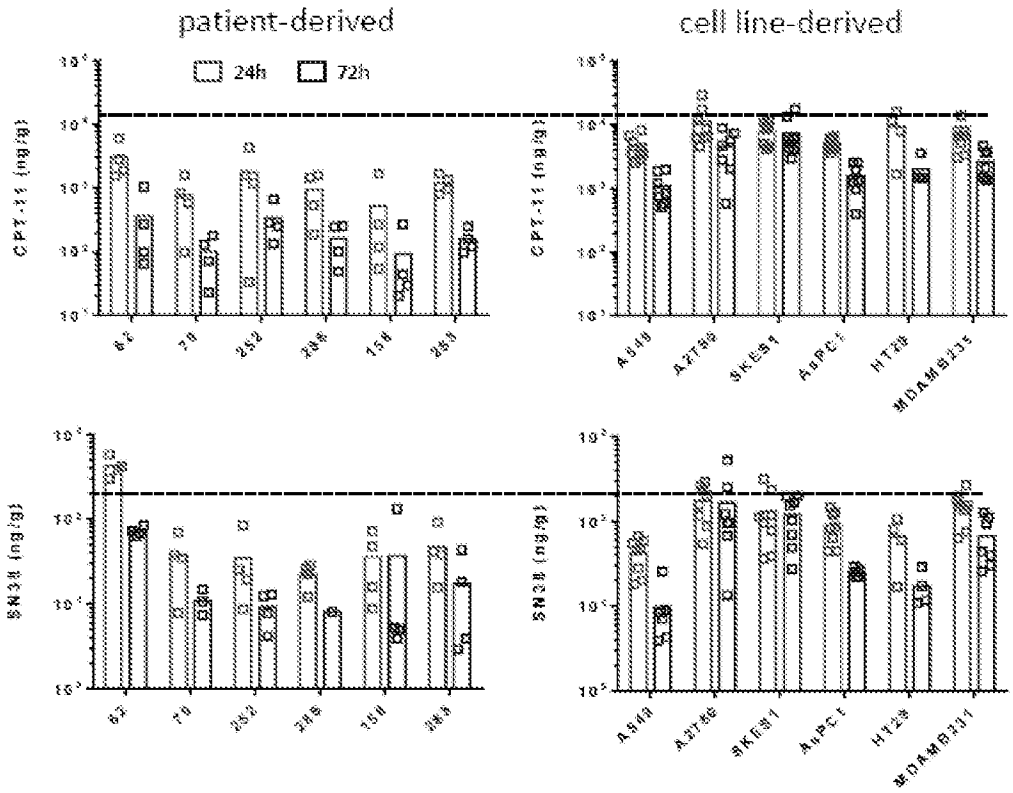


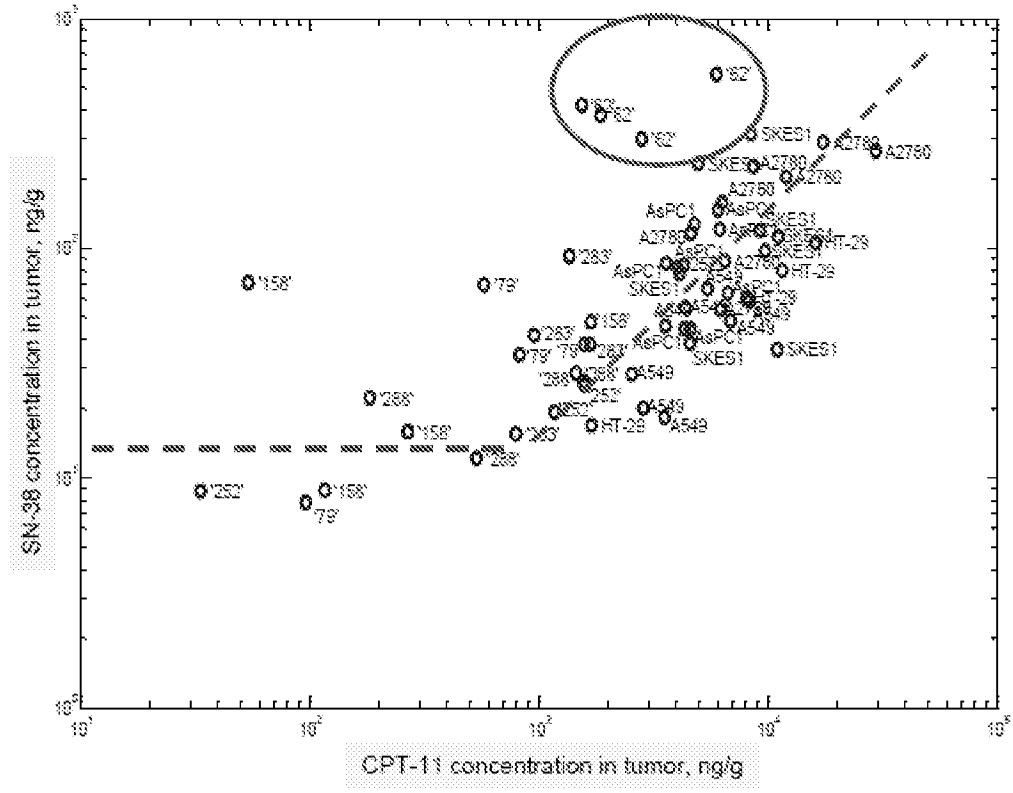
Figure 3. Profiling primary patient and cell line-derived xenograft models for MM-398 deposition and activation markers. (A) H&E staining for 6 patient-derived and 6 cell line xenograft models across a range of indications (pancreatic, ovarian, lung, colon) confirmed most of the models as adenocarcinoma. (B) *In vitro* CES conversion activity was used to measure the ability of tumors to convert the prodrug CPT-11 to active SN-38. CPT-11 (5μM) was incubated with tumor lysates (250μg protein) for 24h at 37C and the metabolites were measured using HPLC. Wide range of CES enzyme activity was observed across the models with model "62" showing highest levels of SN-38 metabolite (~8ng/ml). (C) Tumor-associated macrophages (TAMs) were stained with F4/80, macrophage-specific antibody and blood vessels were stained with anti-CD31 antibody (D). The whole-tumor image acquisition was done on an Aperio® ScanScope® FL and images were quantified with Definiens TissueStudio® software. High degree of variability in TAM levels was observed across the models. A2780 and AsPC1 tumor showed the highest levels of TAM (~7-8%), though there was considerable intra-model variability observed in these models raging from (~4-12%). (D) The mean vessel density (MVD) did not vary across the different models and most of the cell line derived models showed higher uptake of fluorescently labeled (Dil5) liposomes compared to primary models.

Correlations between intratumor MM-398 metabolites and activation/deposition markers

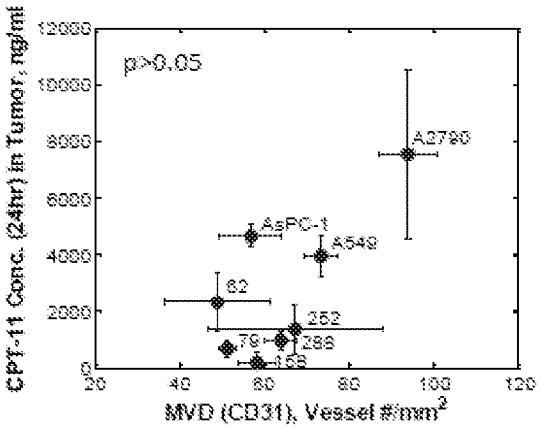
A. Metabolites levels across different tumor models

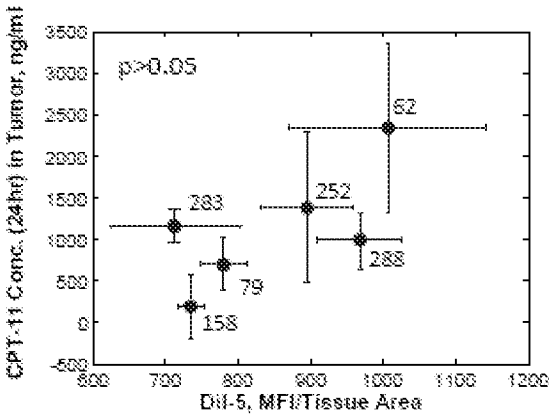


B. Correlation between CPT-11 and SN-38 in tumor

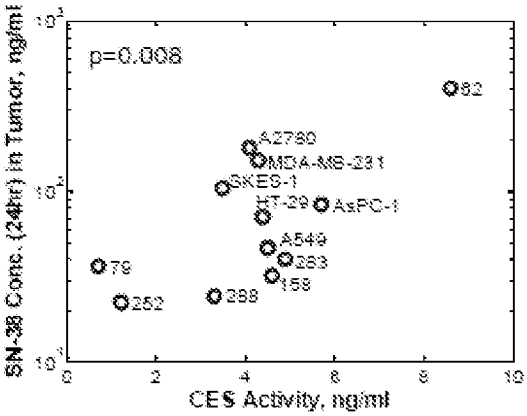


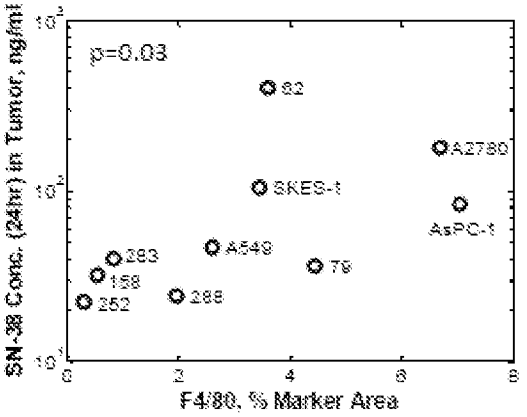
C. Deposition Markers



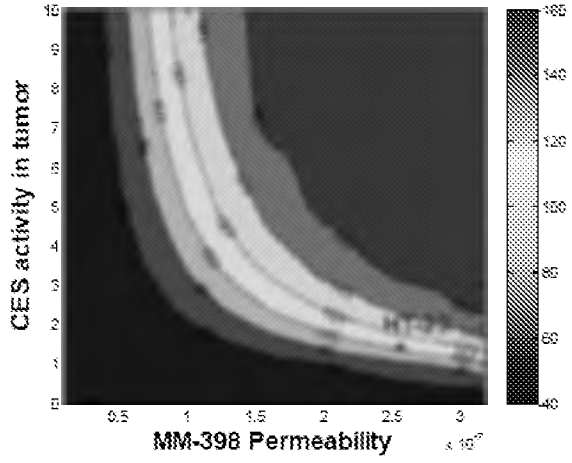


D. Activation Markers





E. Model simulations highlight parameters affecting SN-38 duration



F. Markers affecting the intratumor SN-38 duration

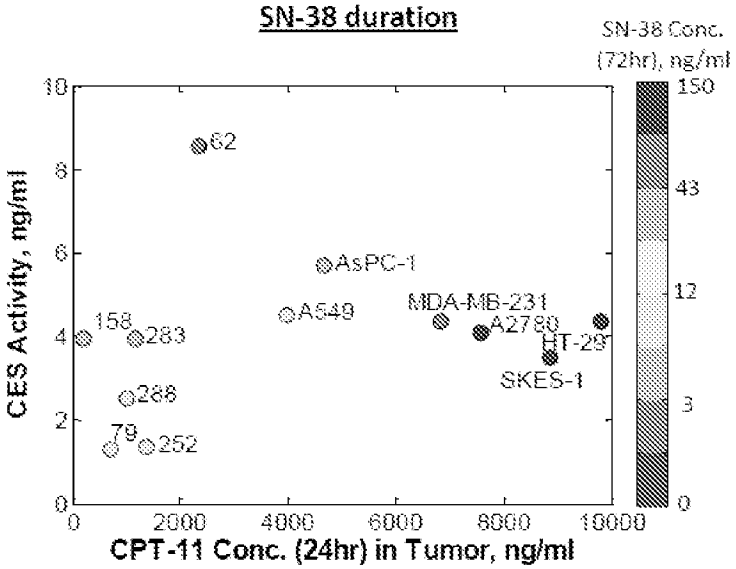


Figure 4. (A) Tumor CPT-11 and SN-38 concentrations from 10mg/kg MM-398 were plotted across different tumor models. Patient-derived xenograft models (shown in left column) showed lower CPT-11 than cell-line derived xenograft models (shown in right column). While most of models showed declining levels of metabolites in 72hr, A2780 and SKES-1 exhibited almost constant levels of metabolites over time. (B) Good correlation between CPT-11 and SN-38 concentrations in tumor (24hr after MM-398) was observed (Spearman's rank correlation r=0.6, p=1e-7) and indicates that SN-38 depends on liposome deposition (CPT-11). However, the model '62', a patient-derived xenograft, suggests that CES activity in tissue could also be an important factor for intratumoral SN-38. (C) DiI-5 signal and CD31 based MVD (mean vessel density) from the untreated tumors were plotted against tumor CPT-11 concentration at 24hr. Either marker did not show any significant correlation with CPT-11 (Spearman's rank correlation p>0.05). (D) CES *in vitro* conversion activity and F4/80 signal from the untreated tumors were compared with tumor SN-38 concentration at 24hr. Both markers showed good correlation with SN-38 (Pearson correlation r=0.72, p=0.008 for CES activity; Spearman's rank correlation r=0.7, p=0.03 for F4/80) suggesting these parameters can modulate the intratumor levels of SN-38. (E) Mechanistic tumor PK model was used to relate tumor CES activity and MM-398 permeability with intratumor SN-38 levels. The model parameters for HT-29 are located in these contour plots, where color code represents the SN-38 duration over threshold in the tumor (Top:120nM, Bottom:60nM). HT-29 can be described as a poor deposition and high activation model. For a more sensitive cell line, lower threshold can be used as shown in the bottom figure. (F) By using CES activity as the activation marker and CPT-11 concentration in tumor (24hr) as deposition marker, all the tumor models (both patient-derived and cell-line derived xenografts) were plotted and color-coded based on their SN-38 concentration in the tumor 72hr after MM-398. We are currently evaluating other potential markers that can be used to validate the model predictions for SN-38 tumor duration.

Current Clinical Activities

- Ongoing Phase 3 study of MM-398 with or without 5-FU and leucovorin, versus 5-FU and leucovorin in metastatic pancreatic cancer patients
- Ongoing Phase 2 combination with 5-FU & Leucovorin in 2nd line colorectal cancer
- Ongoing cross-indication translational study to identify predictive biomarkers

Summary

- Tumor-associated macrophages take up majority of the MM-398 and depleting the macrophages impacts the overall MM-398 tumor uptake.
- High degree of variability in markers (F4/80, CD31, CES levels) was observed across the different tumor models with significant correlation between intratumor CPT-11 versus SN-38 and CPT-11 versus labeled DiI5 liposome levels.
- Further studies (ongoing) will help validate the correlations between MM-398 activation and deposition with SN-38 tumor levels.

The tumor microenvironment modulates the delivery and activation of liposomal encapsulated irinotecan, MM-398
Ashish Kalra, Jaewon Kim, Stephan Klinz, Nancy Paz, Joseph Reutt, Jaimini Patel, Caryl Drummond, Dmitri Kirpotin, Victor Moyo, Eliel Bayever,
Peter Lewins, Chet Nyhuis, Ulrik Nielsen, Jonathan Fitzgerald
Merrimack Pharmaceuticals, Cambridge, MA, USA
Abstract #5622
MERRIMACK

Abstract 2065: Magnetic resonance imaging with an iron oxide nanoparticle demonstrates the preclinical feasibility of predicting intratumoral uptake and activity of MM-398, a nanoliposomal irinotecan (nal-IRI)

Ashish V. Kalra, Joseph Spernyak, Jaeyeon Kim, Arnold Sengooba, Stephan Klinz, Nancy Paz, Jason Cain, Walid Kamoun, Ninfa Straubinger, Yang Qu, Sheryl Trueman, Eliel Ba
DOI: 10.1158/1538-7445.AM2014-2065 Published October 2014

Proceedings: AACR Annual Meeting 2014; April 5-9, 2014, San Diego, CA

Abstract

Sustained intratumoral delivery of cytotoxic agents is a major challenge for effective cancer treatment, and motivated the development of MM-398, a stable nanoliposomal irinotecan (nal-IRI) with an extended plasma half-life and greater tumor deposition than free irinotecan. By using a systems pharmacology approach, we have previously shown that tumor deposition of nal-IRI and the subsequent conversion of irinotecan to the active metabolite, SN-38, by carboxylesterases are important determinants for nal-IRI activity in vivo.

Ferumoxytol (FMX) is a 30nm iron-oxide, super-paramagnetic nanoparticle, known to be taken up by macrophages (as is nal-IRI), and for exhibiting magnetic resonance imaging properties. Since the size of a nanoparticle affects the rate of transcapillary transport significantly, we hypothesized that nal-IRI tumor biodistribution may be predicted by FMX-based MRI (Fe-MRI).

Biodistribution and imaging studies were performed in mice bearing cell-line derived (A2780, HT29, A549) and patient-derived (pancreatic adenocarcinoma) tumor xenografts. The protocol consisted of a baseline MRI scan, i.v. injection of FMX (20mg/kg), and then i.v. injection of fluorescently labeled nal-IRI (10mg/kg) 24hr later. Mice were sacrificed 24hr and 72hr after nal-IRI injection, and irinotecan and SN-38 concentrations were determined in plasma, tumor, and tissues by HPLC analysis.

The presence of FMX did not interfere with nal-IRI PK or biodistribution. Cellular distribution of liposomes within tumors was also not affected by FMX at up to 50mg/kg as measured by flow cytometry. Furthermore, immunohistochemistry showed that both liposomes and FMX were co-localized with tumor-associated macrophages. The drug metabolite measurements from tissue samples showed that the xenograft tumor models display wide ranges of nal-IRI deposition capacity (irinotecan concentrations at 24hr: ~2,104 to 20,098ng/g). A2780 tumors displayed highest concentration of both iron (3.92 µg/ml) and irinotecan (9,466 ng/g) at 72hr after nal-IRI injection, whereas A549 tumors displayed lowest levels of both iron (0.23 µg/ml) and irinotecan (436 ng/g). We observed a correlation between the tumor Fe-MRI signal and intratumoral levels of irinotecan 72hr after nal-IRI injection ($R2=0.9$, $p<0.001$). Furthermore, in vivo activity studies confirmed that xenograft models having higher intratumoral levels of irinotecan and SN-38 at 72hr showed greater tumor growth inhibition.

In summary, preclinical studies demonstrate the potential of utilizing Fe-MRI as a potential diagnostic tool to identify patients with higher tumor permeability. Based on encouraging preclinical data, a pilot study in patients with advanced solid tumors with extensive Fe-MRI scanning and paired tumor biopsies (NCT # 01770353) is being conducted.

Citation Format: Ashish V. Kalra, Joseph Spernyak, Jaeyeon Kim, Arnold Sengooba, Stephan Klinz, Nancy Paz, Jason Cain, Walid Kamoun, Ninfa Straubinger, Yang Qu, Sheryl Trueman, Eliel Bayever, Ulrik Nielsen, Daryl Drummond, Jonathan Fitzgerald, Robert Straubinger. Magnetic resonance imaging with an iron oxide nanoparticle demonstrates the preclinical feasibility of predicting intratumoral uptake and activity of MM-398, a nanoliposomal irinotecan (nal-IRI). [abstract]. In: Proceedings of the 105th Annual Meeting of the American Association for Cancer Research; 2014 Apr 5-9; San Diego, CA. Philadelphia (PA): AACR; Cancer Res 2014;74(19 Suppl):Abstract nr 2065. doi:10.1158/1538-7445.AM2014-2065

Cancer Research

Experimental and Molecular Therapeutics

Abstract 5622: The tumor microenvironment modulates the delivery and activation of liposomal encapsulated irinotecan, MM-398.

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

DOI: 10.1158/1538-7445.AM2013-5622 Published April 2013

Proceedings: AACR 104th Annual Meeting 2013; Apr 6-10, 2013; Washington, DC

Abstract

MM-398 is a stable nanotherapeutic encapsulation of the pro-drug irinotecan with an extended plasma half-life and higher intratumoral deposition in animal models compared with free-irinotecan. MM-398 is currently in multiple clinical trials, including a Phase 3 trial for patients with advanced gemcitabine-resistant pancreatic cancer (NAPOLI-1). By using a systems pharmacology approach, we have previously identified the important determinants for MM-398 activity differentiating it from free irinotecan.

One of the critical parameters identified in the model is activity of the tumor carboxylesterase (CES) as an important correlate to the MM-398 preclinical response. Our data also shows that tumor associated macrophages (TAMs) are the dominant cell type for phagocytosis of MM-398 in the tumor microenvironment. To validate these findings we used a perturbation strategy involving manipulation of TAMs with the anti-CSF1 antibody 5A1. Depletion of macrophages with 5A1 resulted in a strong size reduction of CD11b+F4/80+ mature macrophage populations in HT29, MC38 and LLC tumor models. Furthermore, other myeloid and non-myeloid cell populations showed tumor model and mouse strain-dependent changes in population size and liposome uptake. The overall impact of 5A1-mediated macrophage depletion was a reduction of the total cellular liposomal load within the tumors. Anti-CSF1 treatment also modulated the systemic clearance of MM-398 due to changes in the macrophage populations in the liver and spleen of the animals. To capture this complex interplay of systemic and local depletion events we have expanded our mechanistically-based PK model to include separate tumor macrophage and tumor cell compartments.

The computational model also identified tumor permeability to MM-398 as a rate-limiting factor for drug deposition and in vivo activity. To assess the degree of variability in tumor permeability, we measured the levels of irinotecan and SN-38 using HPLC in six different xenograft models. A high degree of variability in irinotecan and SN-38 levels was seen across these models and between tumors from different mice bearing xenografts of the same cell lines. We then investigated the inter-relationship between tumor permeability, macrophage content, CES enzyme activity and SN-38 accumulation by assessing the levels of these markers in multiple human-derived primary xenograft models across different indications.

In summary, we demonstrated the impact of TAM modulation on overall tumor levels of irinotecan and SN-38 following treatment with liposomally encapsulated irinotecan. We outline differences in permeability of xenografts to MM-398 and the capacity to convert irinotecan into SN-38.

Citation Format: Ashish Kalra, Jaeyeon Kim, Stephan Klinz, Nancy Paz, Joseph Reutt, Daryl Drummond, Dmitri Kirpotin, Victor Moyo, Eliel Bayever, Peter Laivins, Clet Niyikiza, Ulrik Nielsen, Jonathan Fitzgerald. The tumor microenvironment modulates the delivery and activation of liposomal encapsulated irinotecan, MM-398. [abstract]. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2013;73(8 Suppl):Abstract nr 5622. doi:10.1158/1538-7445.AM2013-5622

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CANCER RESEARCH

Experimental and Molecular Therapeutics

Abstract 5696: Evaluating determinants for enhanced activity of MM-398/PEP02; a novel nanotherapeutic encapsulation of irinotecan (CPT-11)

Ashish Kalra, Stephan Klinz, Nancy Paz, Jaeyeon Kim, Milind Chalishazar, Daryl Drummond, Dmitri Kirpotin, Victor Moyo, Clet Niyikiza, and Jonathan Fitzgerald

DOI: 10.1158/1538-7445.AM2012-5696 Published April 2012

Proceedings: AACR 103rd Annual Meeting 2012– Mar 31-Apr 4, 2012; Chicago, IL.

Abstract

MM-398 is a stable nanotherapeutic encapsulation of the pro-drug irinotecan (CPT-11). Irinotecan, a topoisomerase inhibitor is currently being used in clinical practice for treatment of several indications; however, associated toxicities, mainly, neutropenia and gastrointestinal toxicity, have limited its clinical utility. Previously, we have demonstrated that MM-398 treatment resulted in significantly higher intratumor concentrations of both irinotecan (142-fold) and SN-38 (9-fold), thereby exhibiting enhanced anti-tumor activity compared to free irinotecan in different xenograft models. Multiple phase 1 and 2 studies have established a pharmacokinetic and safety profile. Recent data support continued clinical development for the drug in various indications, including pancreatic, gastric, colorectal and potentially other solid tumors. In order to further understand the enhanced anti-tumor activity of MM-398 we developed a mechanism-based PK model of MM-398 and free irinotecan designed to predict intratumor levels of SN-38. Based on this model, we evaluated the role of various determinants of response of MM-398. Sensitivity analysis revealed that the local activation of MM-398 was important for obtaining higher SN-38 intratumor levels compared to free irinotecan. To identify cell types responsible for local activation of MM-398 we investigated cellular liposome phagocytosis ability. In vitro studies demonstrated preferential uptake of MM-398 by phagocytic macrophages compared to tumor cells. FACS analysis of tumor samples (from subcutaneous xenografts) highlighted higher uptake of labeled liposomes by CD11b + and F4/80+ cells as compared to tumor cells. We are developing in vivo systems using engineered cell lines overexpressing growth factors to recruit macrophages (CSF1) or overexpressing irinotecan conversion enzymes to further investigate the role of macrophagesin local tumor conversion of MM-398 and to validate the model prediction that local activation is critical to MM-398 mechanism of action. The extent of tumor vascularization and permeability were also highlighted in the sensitivity analysis. To determine the effect of MM-398 on these parameters, we treated mice bearing HT29 (colorectal cancer) xenografts with a single dose of MM-398 and measured hypoxic markers (CAIX) and microvessel density (CD31) by immunohistochemistry. Tumors treated with MM-398 showed a greater degree of CD31 staining and lower CAIX staining, indicating that MM-398 may be able to affect the tumor microenvironment. We are currently evaluating how the ability of MM-398 to alter the tumor microenvironment affects the activity of other chemotherapeutic agents in combination therapy. These findings could support the use of MM-398 as a combination modality against tumors that may have acquired resistance to traditional chemotherapeutic agents.

Citation Format: {Authors}. {Abstract title} [abstract]. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2012;72(8 Suppl):Abstract nr 5696. doi:1538-7445.AM2012-5696

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April 2012
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HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use KHAPZORY™ safely and effectively. See full prescribing information for KHAPZORY.

KHAPZORY (levoleucovorin) for injection, for intravenous use
Initial U.S. Approval: 1952 (*d,l*-leucovorin)

----------INDICATIONS AND USAGE----------

KHAPZORY is a folate analog indicated for:

- Rescue after high-dose methotrexate therapy in patients with osteosarcoma. (1)
- Diminishing the toxicity associated with overdosage of folic acid antagonists or impaired methotrexate elimination. (1)
- Treatment of patients with metastatic colorectal cancer in combination with fluorouracil. (1)

Limitations of Use

KHAPZORY is not indicated for the treatment of pernicious anemia and megaloblastic anemia secondary to lack of vitamin B12 because of the risk of progression of neurologic manifestations despite hematologic remission. (1)

----------DOSAGE AND ADMINISTRATION----------

- For intravenous administration only. Do not administer intrathecally. (2.1)

Rescue After High-Dose Methotrexate Therapy

- Rescue recommendations are based on a methotrexate dose of 12 grams/m^2 administered by intravenous infusion over 4 hours. Initiate rescue at a dose of 7.5 mg (approximately 5 mg/m^2) every 6 hours, 24 hours after the beginning of the methotrexate infusion. (2.2)
- Continue until the methotrexate level is below 5×10^{-8} M (0.05 micromolar). Adjust dose if necessary based on methotrexate elimination; refer to Full Prescribing Information. (2.2)

Overdosage of Folic Acid Antagonists or Impaired Methotrexate Elimination

- Start as soon as possible after methotrexate overdosage, or within 24 hours of delayed methotrexate elimination. (2.3)
- Administer KHAPZORY 7.5 mg (approximately 5 mg/m^2) intravenously every 6 hours until methotrexate level is less than 5×10^{-8} M (0.05 micromolar). (2.3)

Metastatic Colorectal Cancer in Combination with Fluorouracil

- The following regimens have been used for the treatment of colorectal cancer:
  - KHAPZORY 100 mg/m^2 by intravenous injection over a minimum of 3 minutes, followed by fluorouracil 370 mg/m^2 once daily for 5 consecutive days. (2.4)
  - KHAPZORY 10 mg/m^2 by intravenous injection followed by fluorouracil 425 mg/m^2 once daily for 5 consecutive days. (2.4)
- The above five-day courses may be repeated every 4 weeks for 2 courses, then every 4-5 weeks, if the patient has recovered from toxicity from the prior course. (2.4)
- Do not adjust KHAPZORY dosage for toxicity. (2.4)

----------DOSAGE FORMS AND STRENGTHS----------

For Injection: 175 mg and 300 mg of levoleucovorin lyophilized powder in a single-dose vial for reconstitution. (3)

----------CONTRAINDICATIONS----------

Patients who have had severe hypersensitivity reactions to leucovorin products, folic acid, or folinic acid. (4)

----------WARNINGS AND PRECAUTIONS----------

- Increased gastrointestinal toxicities with fluorouracil: Do not initiate or continue therapy with KHAPZORY and fluorouracil in patients with symptoms of gastrointestinal toxicity until symptoms have resolved. Monitor patients with diarrhea until it has resolved as rapid deterioration leading to death can occur. (5.1,7)
- Drug interaction with trimethoprim-sulfamethoxazole: Increased rates of treatment failure and morbidity with concomitant use of *d,l*-leucovorin with trimethoprim-sulfamethoxazole for Pneumocystis jiroveci pneumonia in patients with HIV. (5.2)

----------ADVERSE REACTIONS----------

- The most common adverse reactions (≥ 20%) in patients receiving high-dose methotrexate therapy with levoleucovorin rescue were stomatitis and vomiting. (6.1)
- The most common adverse reactions (>50%) in patients receiving levoleucovorin in combination with fluorouracil for metastatic colorectal cancer were stomatitis, diarrhea, and nausea. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact Spectrum Pharmaceuticals, Inc. at 1-877-387-4538 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

Revised: 10/2018

FULL PRESCRIBING INFORMATION: CONTENTS*

*Sections or subsections omitted from the full prescribing information are not listed

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FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

KHAPZORY is indicated for:

- rescue after high-dose methotrexate therapy in patients with osteosarcoma.
- diminishing the toxicity associated with overdosage of folic acid antagonists or impaired methotrexate elimination.
- the treatment of patients with metastatic colorectal cancer in combination with fluorouracil.

Limitations of Use

KHAPZORY is not indicated for the treatment of pernicious anemia and megaloblastic anemia secondary to lack of vitamin B_{12} because of the risk of progression of neurologic manifestations despite hematologic remission.

2 DOSAGE AND ADMINISTRATION

2.1 Important Use Information

KHAPZORY is indicated for intravenous administration only. **Do not administer intrathecally**.

2.2 Recommended Dosage for Rescue After High-Dose Methotrexate Therapy

The recommended dosage for KHAPZORY is based on a methotrexate dose of 12 grams/m^2 administered as intravenous infusion over 4 hours in adult and pediatric patients. Twenty-four hours after starting the methotrexate infusion, initiate KHAPZORY at a dose of 7.5 mg (approximately 5 mg/m^2) as an intravenous infusion every 6 hours.

Monitor serum creatinine and methotrexate levels at least once daily. Continue KHAPZORY, hydration, and urinary alkalinization (pH of 7 or greater) until the methotrexate level is below 5×10^{-8} M (0.05 micromolar). Adjust the dose or extend the duration as recommended in Table 1.

Table 1 Recommended Dosage for KHAPZORY based on Serum Methotrexate and Creatinine Levels

Clinical Situation	Laboratory Findings	Recommendation
Normal methotrexate elimination	Serum methotrexate level approximately 10 micromolar at 24 hours after administration, 1 micromolar at 48 hours, and less than 0.2 micromolar at 72 hours.	Administer 7.5 mg by intravenous infusion every 6 hours for 60 hours (10 doses starting at 24 hours after start of methotrexate infusion).
Delayed late methotrexate elimination	Serum methotrexate level remaining above 0.2 micromolar at 72 hours, and more than 0.05 micromolar at 96 hours after administration.	Continue 7.5 mg by intravenous infusion every 6 hours, until methotrexate level is less than 0.05 micromolar.
Delayed early methotrexate elimination and/or evidence of acute renal injury*	Serum methotrexate level of 50 micromolar or more at 24 hours, or 5 micromolar or more at 48 hours after administration, OR 100% or greater increase in serum creatinine level at 24 hours after methotrexate administration (e.g., an increase from 0.5 mg/dL to a level of 1 mg/dL or more).	Administer 75 mg by intravenous infusion every 3 hours until methotrexate level is less than 1 micromolar; then 7.5 mg by intravenous infusion every 3 hours until methotrexate level is less than 0.05 micromolar.

*These patients are likely to develop reversible renal failure. In addition to appropriate KHAPZORY therapy, continuing hydration and urinary alkalinization, and monitoring of fluid and electrolyte status, until the serum methotrexate level has fallen to below 0.05 micromolar and the renal failure has resolved.

Impaired Methotrexate Elimination or Renal Impairment

Decreased methotrexate elimination or renal impairment which are clinically important but less severe than the abnormalities described in Table 1 can occur following methotrexate administration. If toxicity associated with methotrexate are observed, in subsequent courses extend KHAPZORY rescue for an additional 24 hours (total of 14 doses over 84 hours).

Third-Space Fluid Collection and Other Causes of Delayed Methotrexate Elimination

Accumulation in a third space fluid collection (i.e., ascites, pleural effusion), renal insufficiency, or inadequate hydration can delay methotrexate elimination. Under such circumstances, higher doses of KHAPZORY or prolonged administration may be indicated.

2.3 Recommended Dosage for Overdosage of Folic Acid Antagonists or Impaired Methotrexate Elimination

Start KHAPZORY in adult and pediatric patients as soon as possible after an overdosage of methotrexate or within 24 hours of methotrexate administration when methotrexate elimination is impaired. As the time interval between methotrexate administration and KHAPZORY increases, the effectiveness of KHAPZORY to diminish methotrexate toxicity may decrease. Administer KHAPZORY 7.5 mg (approximately 5 mg/m^2) as an intravenous infusion every 6 hours until the serum methotrexate level is less than 5×10^{-8} M (0.05 micromolar).

Monitor serum creatinine and methotrexate levels at least every 24 hours. Increase the dose of KHAPZORY to 50 mg/m^2 intravenously every 3 hours until the methotrexate level is less than 5×10^{-8} M for the following:

- if the serum creatinine at 24-hours increases 50% or more compared to baseline
- if the methotrexate level at 24-hours is greater than 5×10^{-6} M
- if the methotrexate level at 48-hours is greater than 9×10^{-7} M

Continue concomitant hydration (3 L per day) and urinary alkalinization with sodium bicarbonate. Adjust the bicarbonate dose to maintain urine pH at 7 or greater.

2.4 Dosage in Combination with Fluorouracil for Metastatic Colorectal Cancer

The following regimens have been used for the treatment of colorectal cancer:

- KHAPZORY at 100 mg/m^2 by intravenous injection over a minimum of 3 minutes, followed by fluorouracil at 370 mg/m^2, once daily for 5 consecutive days
- KHAPZORY at 10 mg/m^2 by intravenous injection, followed by fluorouracil at 425 mg/m^2, once daily for 5 consecutive days

This five-day course may be repeated every 4 weeks for 2 courses, then every 4-5 weeks, if the patient has recovered from toxicity from the prior course. Do not adjust KHAPZORY dosage for toxicity.

Refer to fluorouracil prescribing information for information on fluorouracil dosage and dosage modifications for adverse reactions.

2.5 Preparation

Reconstitute the 175 mg and 300 mg vial contents with 3.6 mL and 6.2 mL of 0.9% Sodium Chloride Injection, USP, respectively to obtain a clear, colorless to yellowish solution (resultant concentration 50 mg per mL levoleucovorin). Reconstitution with a sodium chloride solution with preservatives (e.g., benzyl alcohol) has not been studied. Do not store reconstituted solution for more than 12 hours at room temperature. Protect from light.

Dilute reconstituted solution immediately (if possible), to concentrations of 0.5 mg/mL to 5 mg/mL in 0.9% Sodium Chloride Injection, USP or 5% Dextrose Injection, USP. Do not store diluted reconstituted solution for more than 12 hours at room temperature. Protect from light.

Visually inspect parenteral drug products for particulate matter and discoloration prior to administration. Discard if particulate matter or discoloration is observed.

3 DOSAGE FORMS AND STRENGTHS

For Injection: 175 mg and 300 mg of levoleucovorin as a sterile, white to yellowish lyophilized powder in a single-dose vial for reconstitution.

4 CONTRAINDICATIONS

KHAPZORY is contraindicated in patients who have had severe hypersensitivity to leucovorin products, folic acid, or folinic acid *[see Adverse Reactions (6.2)]*.

5 WARNINGS AND PRECAUTIONS

5.1 Increased Gastrointestinal Toxicities with Fluorouracil

Leucovorin products increase the toxicities of fluorouracil *[see Drug Interactions (7)]*. Gastrointestinal toxicities, including stomatitis and diarrhea, occur more commonly and may be of greater severity and of prolonged duration. Deaths from severe enterocolitis, diarrhea, and dehydration have occurred in elderly patients receiving weekly *d,l*-leucovorin and fluorouracil. Do not initiate or continue therapy with KHAPZORY and fluorouracil in patients with symptoms of gastrointestinal toxicity until those symptoms have resolved. Monitor patients with diarrhea until it has resolved as rapid deterioration leading to death can occur.

5.2 Drug Interaction with Trimethoprim-Sulfamethoxazole

Concomitant use of *d,l*-leucovorin with trimethoprim-sulfamethoxazole for the acute treatment of Pneumocystis jiroveci pneumonia in patients with HIV infection increased treatment failure and morbidity.

6 ADVERSE REACTIONS

The following serious adverse reactions are described elsewhere in the labeling:

- Increased gastrointestinal toxicities with fluorouracil *[see Warnings and Precautions (5.1)]*
- Drug-interaction with trimethoprim-sulfamethoxazole *[see Warnings and Precautions (5.2)]*

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

High-Dose Methotrexate Therapy

Table 2 presents the frequency of adverse reactions which occurred during the administration of 58 courses of high-dose methotrexate 12 grams/m^2 followed by levoleucovorin rescue, for osteosarcoma, in 16 patients, ages 6-21 years. Most patients received levoleucovorin 7.5 mg every 6 hours for 60 hours or longer, beginning 24 hours after completion of methotrexate administration.

Table 2 Adverse Reactions with High-Dose Methotrexate Therapy

Adverse Reaction	Levoleucovorin n=16 (%)	
	All Grades	Grade 3-4
Gastrointestinal		
Stomatitis	38	6
Vomiting	38	0
Nausea	19	0
Diarrhea	6	0
Dyspepsia	6	0
Typhlitis	6	6
Respiratory		
Dyspnea	6	0
Skin and Appendages		
Dermatitis	6	0
Other		
Confusion	6	0
Neuropathy	6	0
Renal function abnormal	6	0
Taste perversion	6	0

Combination with Fluorouracil in Colorectal Cancer

Table 3 presents the frequency of adverse reactions which occurred in 2 arms of a randomized trial conducted by the North Central Cancer Treatment Group (NCCTG) in patients with metastatic colorectal cancer. The trial failed to show superior overall survival with fluorouracil + levoleucovorin compared to fluorouracil + *d,l*-leucovorin. Patients were randomized to fluorouracil 370 mg/m^2 intravenously and levoleucovorin 100 mg/m^2 intravenously, both daily for 5 days, or to fluorouracil 370 mg/m^2 intravenously and *d,l*-leucovorin 200 mg/m^2 intravenously, both daily for 5 days. Treatment was repeated week 4 and week 8, then every 5 weeks until disease progression or unacceptable toxicity.

Table 3 Adverse Reactions Occurring in ≥ 10% of Patients in Either Arm

Adverse Reaction	Levoleucovorin/ fluorouracil n=318 (%)		*d,l*-Leucovorin/ fluorouracil n=307 (%)	
	Grade 1-4	Grade 3-4	Grade 1-4	Grade 3-4
Gastrointestinal Disorders				
Stomatitis	72	12	72	14
Diarrhea	70	19	65	17
Nausea	62	8	61	8
Vomiting	40	5	37	6
Abdominal Pain*	14	3	19	3
General Disorders				
Asthenia/Fatigue/Malaise	29	5	32	11
Metabolism and Nutrition				
Anorexia/Decreased Appetite	24	4	25	2
Skin Disorders				
Dermatitis	29	1	28	1
Alopecia	26	0.3	28	1

*Includes abdominal pain, upper abdominal pain, lower abdominal pain, and abdominal tenderness

6.2 Postmarketing Experience

The following adverse reactions were identified during post approval use of levoleucovorin. Because these adverse reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to drug exposure. The following have been reported:

- *Respiratory*: dyspnea
- *Dermatologic*: pruritus, rash
- *Other Clinical Events*: temperature change, rigors, allergic reactions

7 DRUG INTERACTIONS

Effect of leucovorin products on fluorouracil

Leucovorin products increase the toxicity of fluorouracil *[see Warnings and Precautions (5.1)]*.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

There are limited data with levoleucovorin use in pregnant women. Animal reproduction studies have not been conducted with levoleucovorin.

Levoleucovorin is administered in combination with methotrexate or fluorouracil, which can cause embryo-fetal harm. Refer to methotrexate and fluorouracil prescribing information for additional information.

In the U.S. general population, the estimated background risk of major birth defects and miscarriage in clinically recognized pregnancies is 2-4% and 15-20%, respectively.

8.2 Lactation

Risk Summary

There are no data on the presence of levoleucovorin in human milk or its effects on the breastfed infant or on milk production.

Levoleucovorin is administered in combination with methotrexate or fluorouracil. Refer to methotrexate and fluorouracil prescribing information for additional information.

8.4 Pediatric Use

The safety and effectiveness of KHAPZORY have been established in pediatric patients for rescue after high-dose methotrexate therapy in osteosarcoma and diminishing the toxicity associated with overdosage of folic acid antagonists or impaired methotrexate elimination. Use of levoleucovorin in pediatric patients is supported by open-label clinical trial data in 16 pediatric patients 6 years of age and older, with additional supporting evidence from literature *[see Clinical Studies (14.1)]*.

The safety and effectiveness of KHAPZORY have not been established for the treatment of pediatric patients with advanced metastatic colorectal cancer.

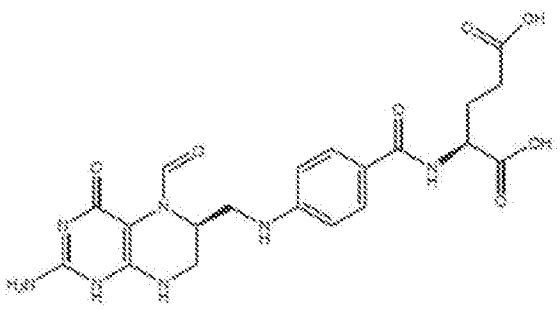
8.5 Geriatric Use

Clinical studies of levoleucovorin in the treatment of osteosarcoma did not include patients aged 65 years and over to determine whether they respond differently from younger patients.

In the NCCTG clinical trial of levoleucovorin in combination with fluorouracil in the treatment of metastatic colorectal cancer, adverse reactions were consistent with fluorouracil related toxicity and were similar for patients age 65 years and older and patients younger than 65 *[see Clinical Studies (14.2)]*.

11 DESCRIPTION

KHAPZORY is a folate analog and the pharmacologically active levo-isomer of *d,l*-leucovorin. The chemical name is (2S)-2-[[4-[[(6S)-2-amino-5-formyl-4-oxo-1,6,7,8-tetrahydropteridin-6- yl] methylamino] benzoyl] amino] pentanedioate. The molecular formula is $C_{20}H_{23}N_7O_7$ and the molecular weight is 473.45. The chemical structure is:



- Levoleucovorin is a slightly hygroscopic, crystalline, yellow powder which is soluble in water when pH is at or above 8.

KHAPZORY 175 mg is a sterile lyophilized powder consisting of 175 mg levoleucovorin, 29.6 mg sodium hydroxide, and 105 mg mannitol in each vial. Additional sodium hydroxide and/or hydrochloric acid may be used to adjust the pH during manufacture. It is intended for intravenous administration after reconstitution with 3.6 mL of sterile 0.9% Sodium Chloride Injection, USP [*See Dosage and Administration (2.5)*].

KHAPZORY 300 mg is a sterile lyophilized powder consisting of 300 mg levoleucovorin, 50.7 mg sodium hydroxide, and 180 mg mannitol in each vial. Additional sodium hydroxide and/or hydrochloric acid may be used to adjust the pH during manufacture. It is intended for intravenous administration after reconstitution with 6.2 mL of sterile 0.9% Sodium Chloride Injection, USP [*See Dosage and Administration (2.5)*].

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

High-dose methotrexate therapy

Levoleucovorin is the pharmacologically active isomer of 5-formyl tetrahydrofolic acid (THF). Levoleucovorin does not require reduction by dihydrofolate reductase to participate in reactions utilizing folates as a source of "one-carbon" moieties. Administration of levoleucovorin counteracts the therapeutic and toxic effects of folic acid antagonists such as methotrexate, which act by inhibiting dihydrofolate reductase.

Combination with Fluorouracil in Colorectal Cancer

Levoleucovorin enhances the therapeutic and toxic effects of fluorouracil. Fluorouracil is metabolized to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), which binds to and inhibits thymidylate synthase (an enzyme important in DNA repair and replication). Levoleucovorin is converted to another reduced folate, 5,10-methylenetetrahydrofolate, which then acts to stabilize the binding of FdUMP to thymidylate synthase, thereby enhancing the inhibition of thymidylate synthase.

12.3 Pharmacokinetics

Distribution

The pharmacokinetics of levoleucovorin after intravenous injection of a 15 mg dose was studied in healthy subjects. The mean maximum serum total tetrahydrofolate (total-THF) concentration was 1722 ng/mL (CV 39%) and the mean maximum serum (6S)-5-methyl-5,6,7,8-tetrahydrofolate concentration was 275 ng/mL (CV 18%) observed around 0.9 hours post injection.

Elimination

The mean terminal half-life was 5.1 hours for total-THF and 6.8 hours for (6S)-5-methyl-5,6,7,8-tetrahydrofolate.

Drug Interaction Studies

A published cross study comparison showed that the mean dose-normalized steady-state plasma concentrations for both levoleucovorin and 5-methyl-THF were comparable whether fluorouracil (370 mg/m^2/day IV bolus) was administered in combination with levoleucovorin (250 mg/m^2 and 1000 mg/m^2 as a continuous IV infusion for 5.5 days, N=9) or in combination with *d,l*-leucovorin (500 mg/m^2 as a continuous IV infusion for 5.5 days, N=6).

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No studies have been conducted to evaluate the potential of levoleucovorin for carcinogenesis, mutagenesis and impairment of fertility.

14 CLINICAL STUDIES

14.1 Rescue after High-Dose Methotrexate Therapy in Patients with Osteosarcoma

The efficacy of levoleucovorin rescue following high-dose methotrexate were evaluated in 16 patients, ages 6-21 years, who received 58 courses of chemotherapy for osteogenic sarcoma. High-dose methotrexate was one component of several different combination chemotherapy regimens evaluated across several trials. Methotrexate 12 g/m^2 IV over 4 hours was administered to 13 patients, who received levoleucovorin 7.5 mg every 6 hours for 60 hours or longer beginning 24 hours after completion of methotrexate. Three patients received methotrexate 12.5 g/m^2 IV over 6 hours, followed by levoleucovorin 7.5 mg every 3 hours for 18 doses beginning 12 hours after completion of methotrexate. The mean number of levoleucovorin doses per course was 18.2 and the mean total dose per course was 350 mg. The efficacy of levoleucovorin rescue following high-dose methotrexate was based on adverse reaction profile *[see Adverse Reactions (6.1)]*.

14.2 Metastatic Colorectal Cancer

In a randomized clinical study conducted by the Mayo Clinic and the North Central Cancer Treatment Group (Mayo/NCCTG) in patients with metastatic colorectal cancer comparing *d,l*-leucovorin (LV) 200 mg/m^2 and fluorouracil 370 mg/m^2 versus LV 20 mg/m^2 and fluorouracil 425 mg/m^2 versus fluorouracil 500 mg/m^2, with all drugs administered by intravenous infusion daily for 5 days every 28 to 35 days, response rates were 26% (p=0.04 versus fluorouracil alone), 43% (p=0.001 versus fluorouracil alone), and 10%, respectively. Respective median survival times were 12.2 months (p=0.037), 12 months (p=0.050), and 7.7 months. The low dose LV regimen was associated with a statistically significant improvement in weight gain of more than 5%, relief of symptoms, and improvement in performance status. The high dose LV regimen was associated with a statistically significant improvement in performance status and trended toward improvement in weight gain and in relief of symptoms but these were not statistically significant.

In a second Mayo/NCCTG randomized clinical study the fluorouracil alone arm was replaced by sequentially administered methotrexate (MTX), fluorouracil, and LV. Response rates with LV 200 mg/m^2 and fluorouracil 370 mg/m^2 versus LV 20 mg/m^2 and fluorouracil 425 mg/m^2 versus sequential MTX and fluorouracil and LV were 31% (p≤0.01), 42% (p≤0.01), and 14%, respectively. Respective median survival times were 12.7 months (p≤0.04), 12.7 months (p≤0.01), and 8.4 months. There was no statistically significant difference in weight gain of more than 5% or in improvement in performance status between the treatment arms.

A randomized controlled trial conducted by the NCCTG in patients with metastatic colorectal cancer failed to show superiority of a regimen of fluorouracil + levoleucovorin to fluorouracil + *d,l*-leucovorin in overall survival. Patients were randomized to fluorouracil 370 mg/m^2 intravenously and levoleucovorin 100 mg/m^2 intravenously, both daily for 5 days, or to fluorouracil 370 mg/m^2 intravenously and *d,l*-leucovorin 200 mg/m^2 intravenously, both daily for 5 days. Treatment was repeated week 4 and week 8, then every 5 weeks until disease progression or unacceptable toxicity.

16 HOW SUPPLIED/STORAGE AND HANDLING

KHAPZORY (levoleucovorin) for injection is a sterile, preservative-free, white to yellowish lyophilized powder in a single-dose vial. It is available as:

175 mg vial – NDC 68152-112-01.

300 mg vial – NDC 68152-114-01.

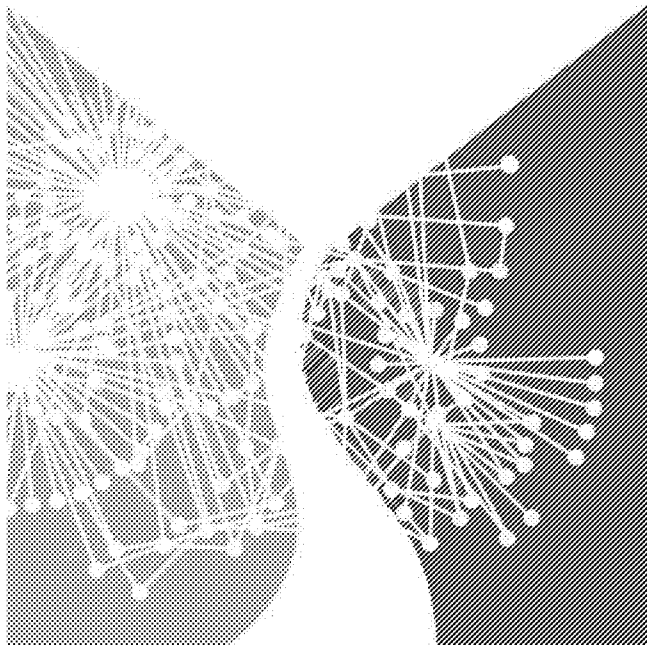
Store at 20°C to 25°C (68°F to 77°F); excursions permitted between 15°C and 30°C (59°F and 86°F) [see USP Controlled Room Temperature]. Store vial in original carton until contents are used. Protect solutions from light.

Distributed by:
Spectrum Pharmaceuticals, Inc.
Irvine, CA 92618

KHAPZORY is a trademark of Spectrum Pharmaceuticals, Inc.

Efficient prioritization of potential diagnostic biomarkers using a systems pharmacology approach:

Case study of MM-398 (Irinotecan sucrosofate liposome injection)



Jaeyeon Kim, Ph.D.

Senior Scientist

Merrimack Pharmaceuticals Inc.

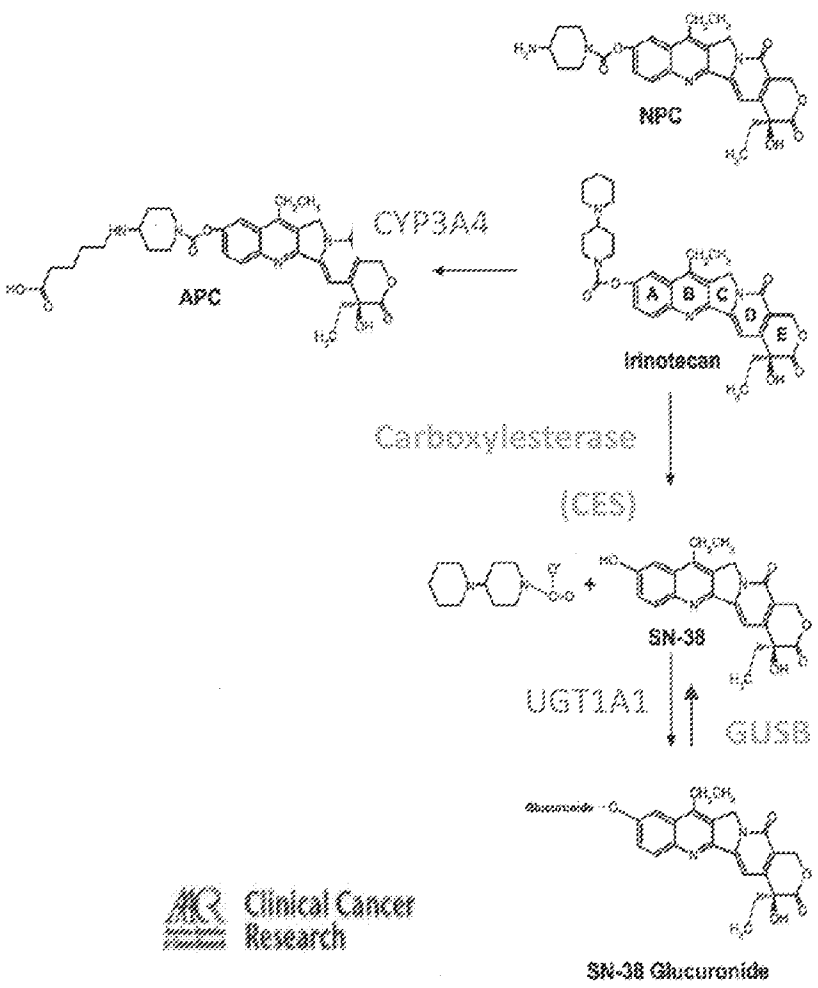
Oct 31 2013

Outline

- Background
  - Limitation of free irinotecan in clinic
  - MM-398 to realize true potential of free irinotecan
- Differentiating MM-398 from free irinotecan
  - Identifying pharmacological determinant for *in vivo* activity
- Translating preclinical observations to clinic
  - Dissecting out mouse-specific artifact
- Developing strategy for diagnostic biomarkers
  - Using model sensitivity analysis to identify biomarkers that modulate *in vivo* activity

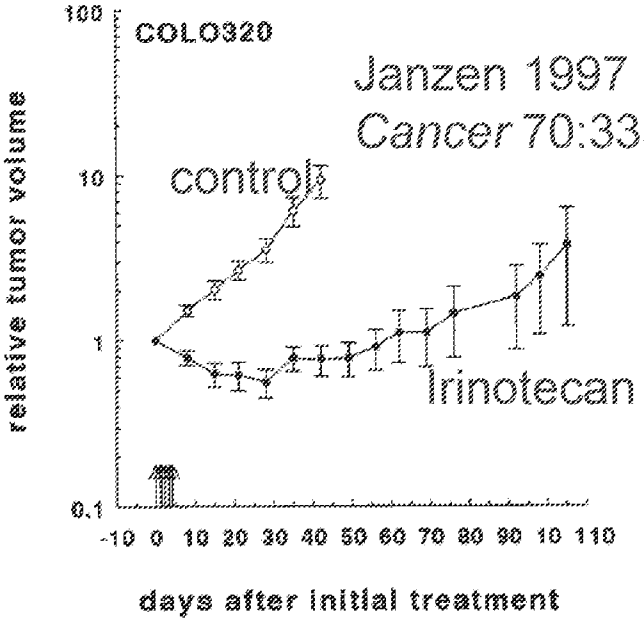
Revisiting history: Irinotecan drug development

Activation of Irinotecan (IRI)



Clinical Cancer Research

Ma M K et al. Clin Cancer Res 2000;6:813-819



Irinotecan (Camptosar®) received 1st approval in 2L CRC in 1998

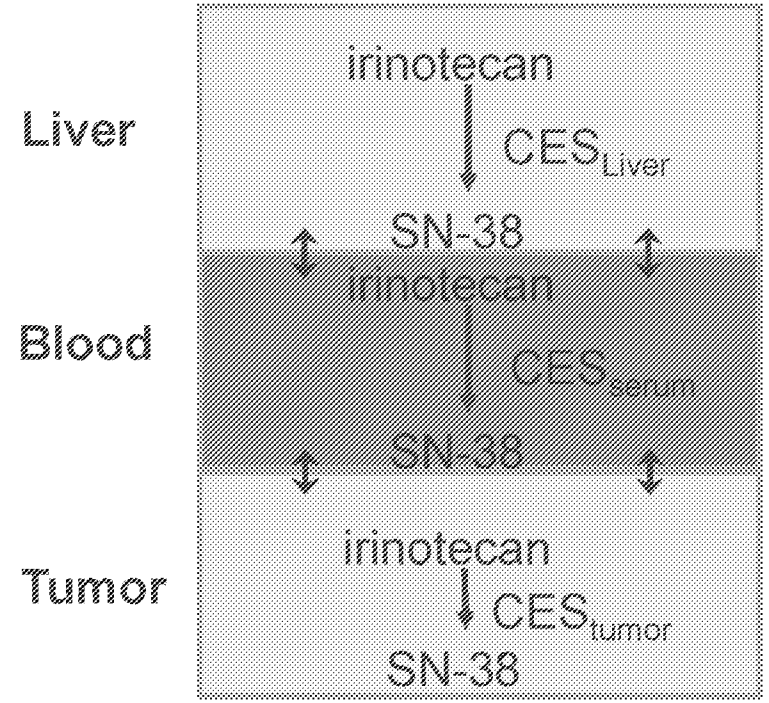
"CPT-11 [irinotecan] appears to be one of the most exciting new drugs to reach clinical development in the past decade, and its seemingly wide spectrum of clinical activity suggests that it may have a substantial impact on the treatment of many of the most common epithelial malignancies"
Rothenberg 1996 Seminars in Oncology 23:21

Irinotecan approved only in CRC: Failure of translating preclinical activity to human?

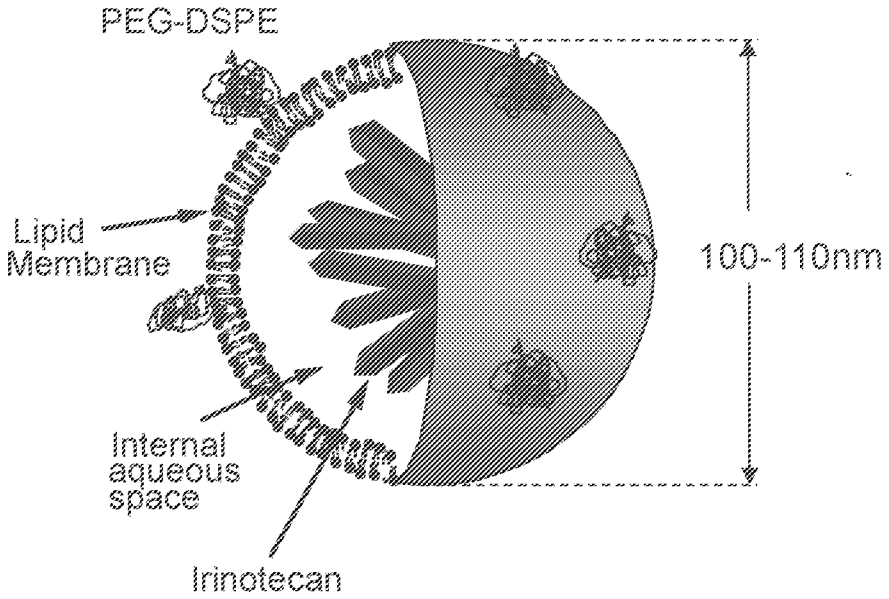
- Activity by free irinotecan could be due to the artifacts inherent to *in vitro* and *in vivo* model systems

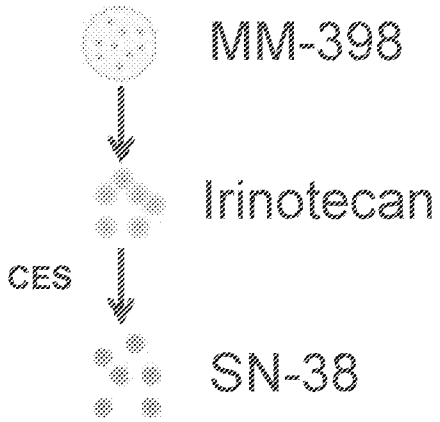
- *in vitro* cell culture model:
  - Short plasma half-life of irinotecan cannot warrant enough exposure used in *in vitro* system

- *in vivo* xenograft mouse model:
  - Systemic activation is enhanced in mice due to mouse-specific CES in serum
    - Plasma SN-38 levels in mice are ~10 fold higher than in human



MM-398, irinotecan sucrosofate liposome injection: To realize true potential of irinotecan as a cancer drug





Extended circulation

- Low Pegylation & size sustain circulation half-life
- Liposomal encapsulation reduces systemic conversion of prodrug irinotecan

Enhanced tumor accumulation

- Passive accumulation in tumor due to EPR effect to achieve high drug levels

Local cytotoxic activation

- Effective packaging of irinotecan with ~70,000 molecules/liposome
- After conversion to SN-38 binds to TOPO1 and induces DNA damage

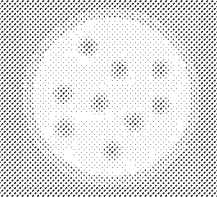
Currently in Phase 1/2/3 trials

Questions to be answered by the model

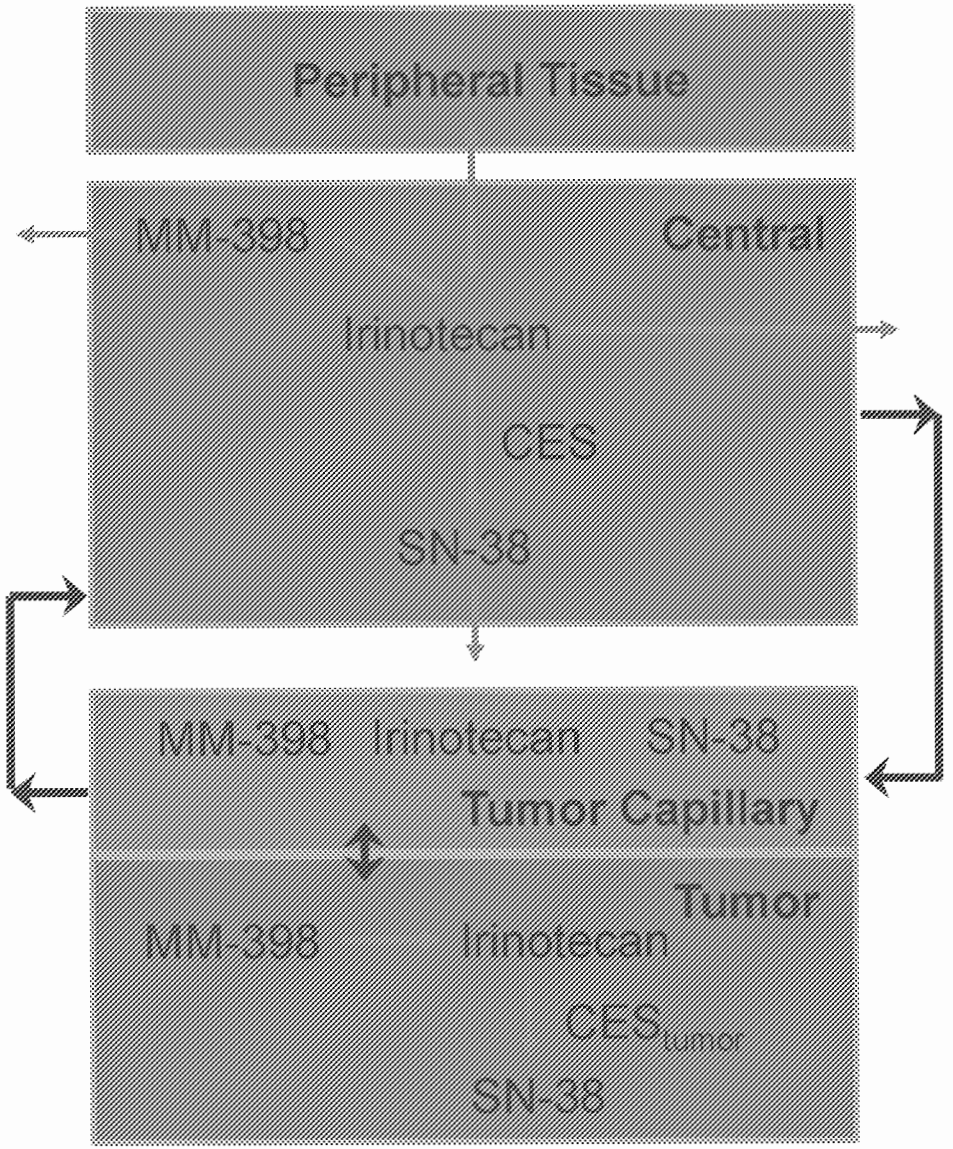
Differentiation

MM-398 Irinotecan

Can the model identify any factor that drives the differential *in vivo* activity?

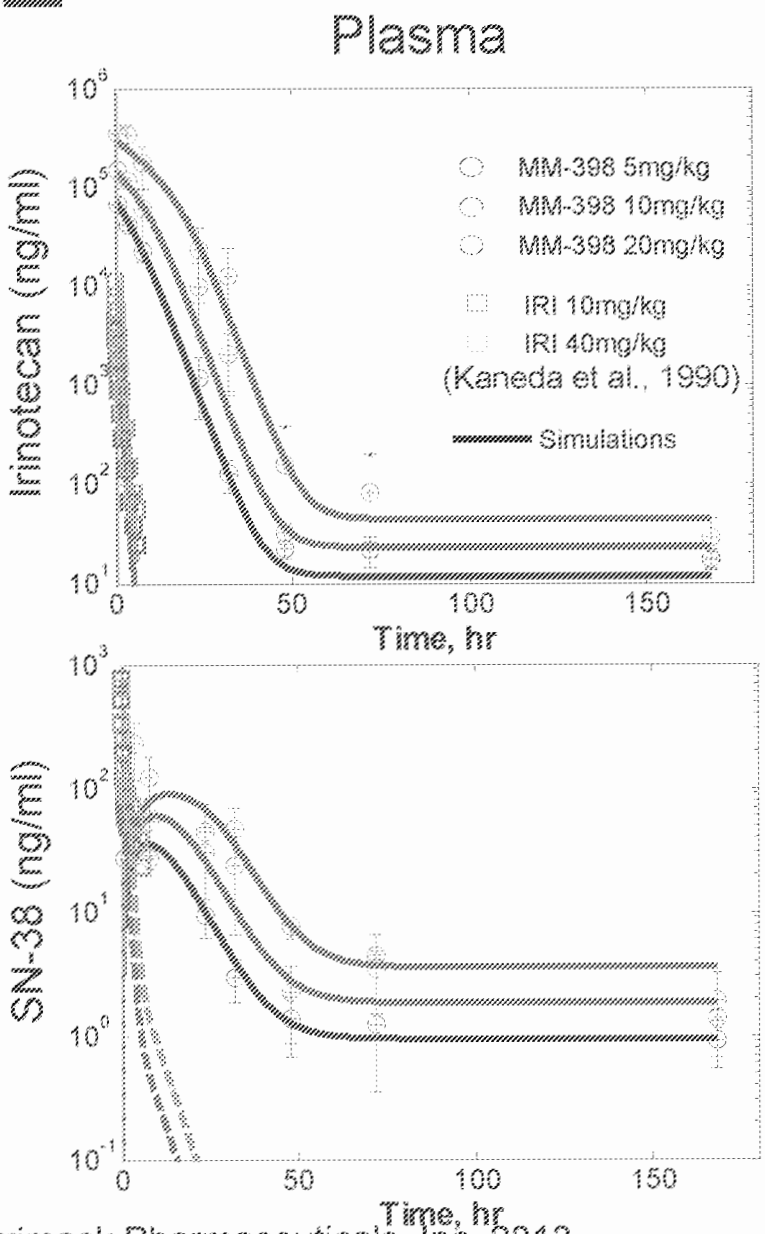


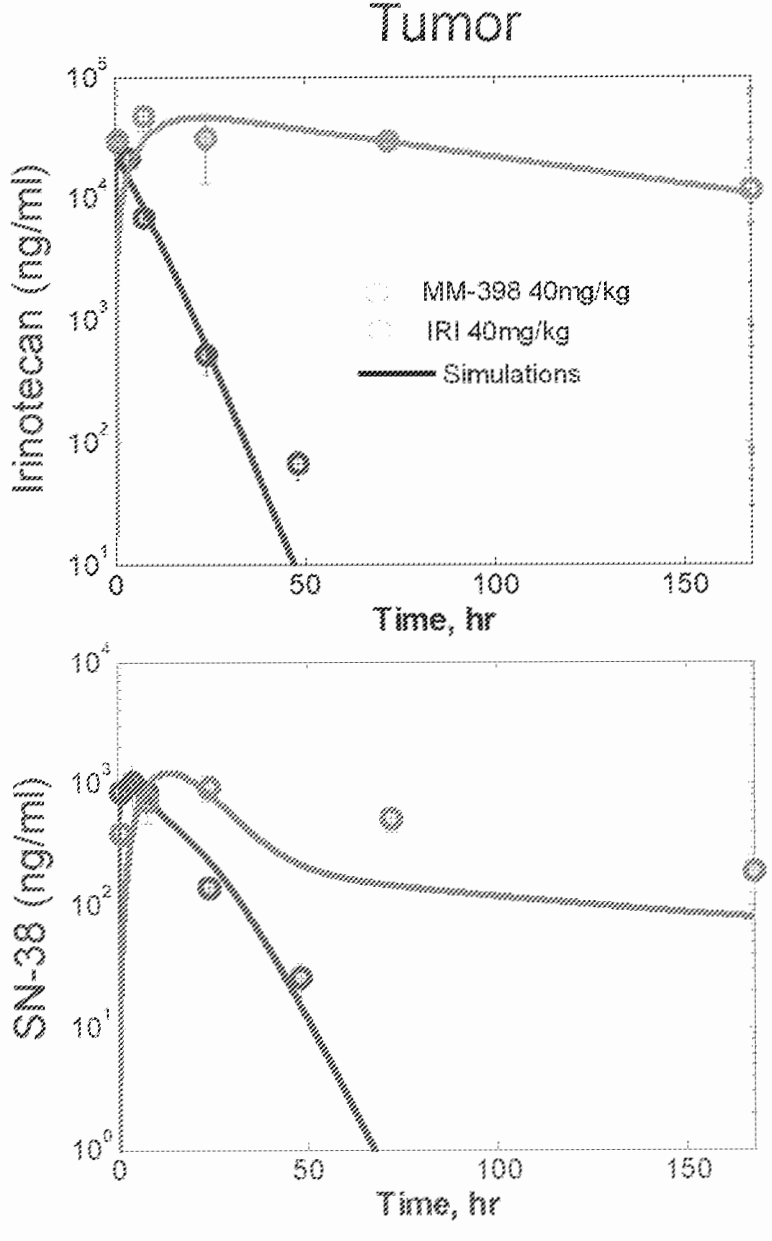
Developing and training mechanistic tumor PK model for MM-398 and free irinotecan



- Model incorporates processes related to PK, free drug release, drug metabolism and tissue deposition
- Model was developed by using SimBiology® toolbox in MATLAB ®
- Particle swarm optimization (PSO) method - a global optimization algorithm - was used to estimate model parameters

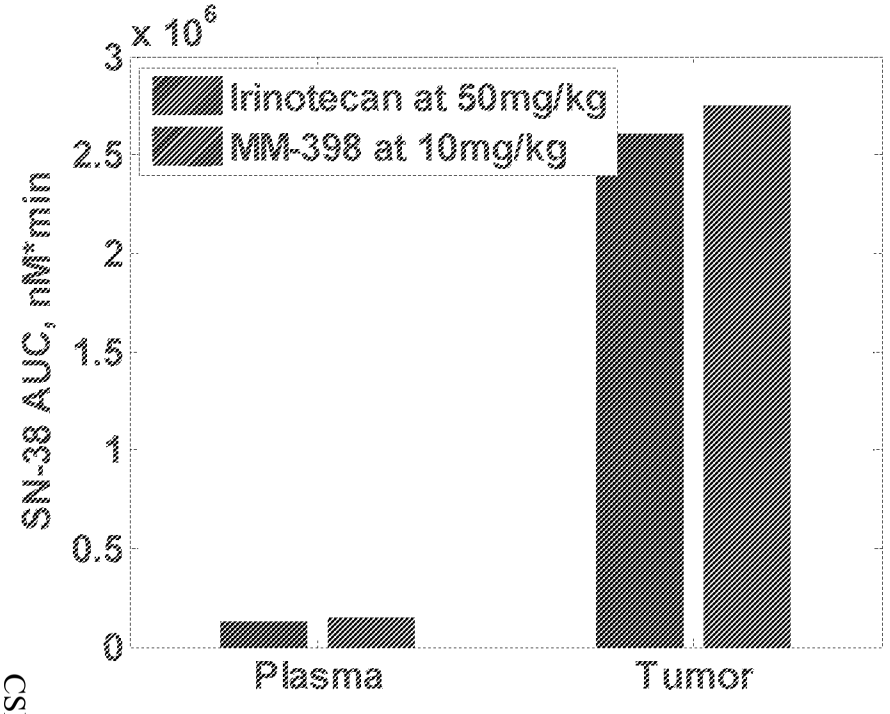
Mechanistic mouse PK model describes plasma and tumor exposure of MM-398, Irinotecan (IRI) and SN-38



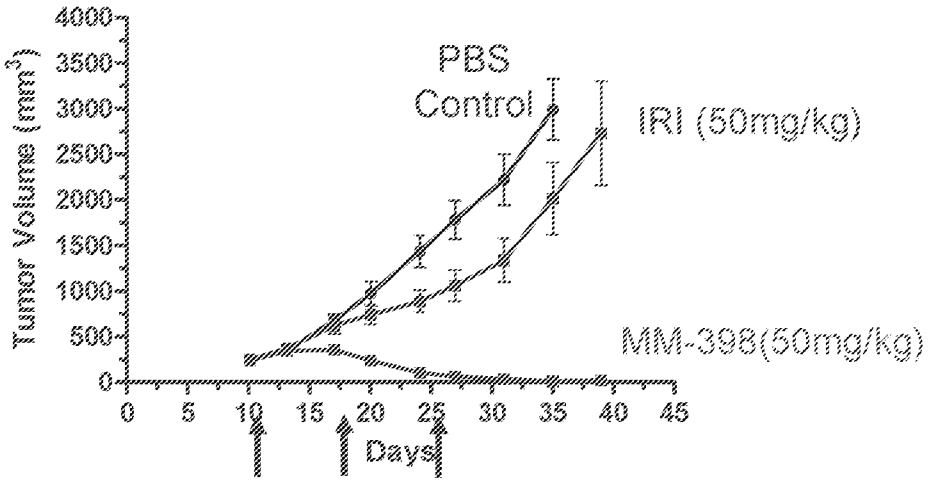


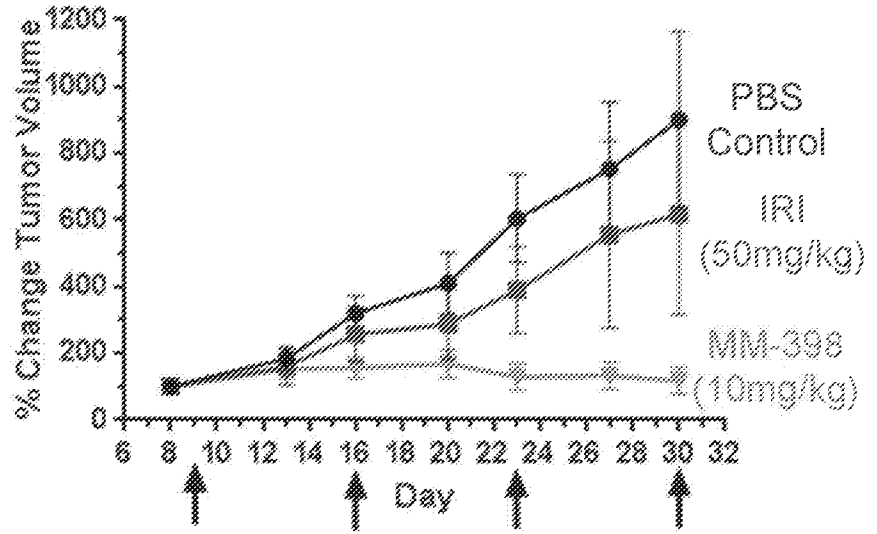
Doses of MM-398 and IRI with equal exposure show different tumor growth inhibition

PK model simulation



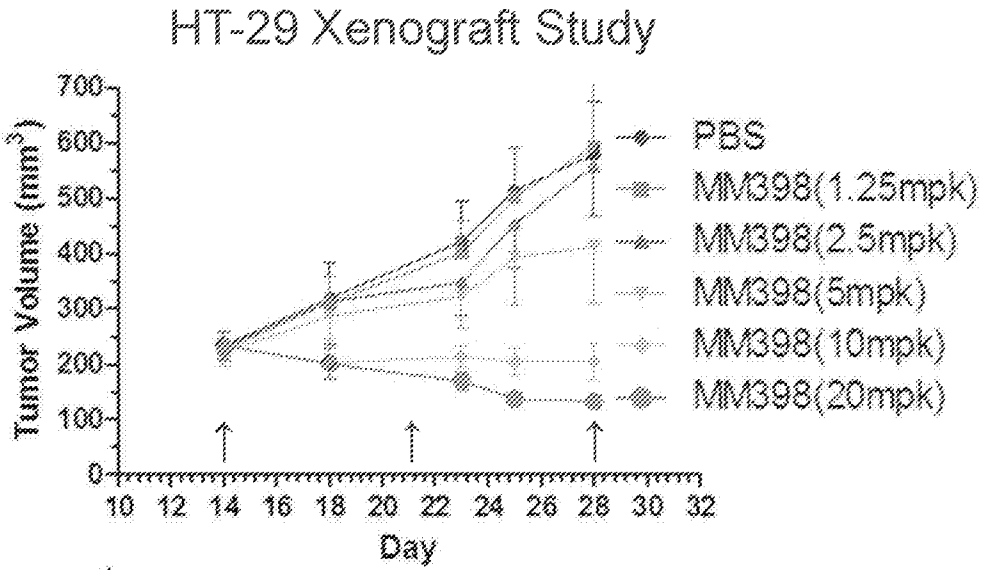
in vivo activity in HT-29 xenograft

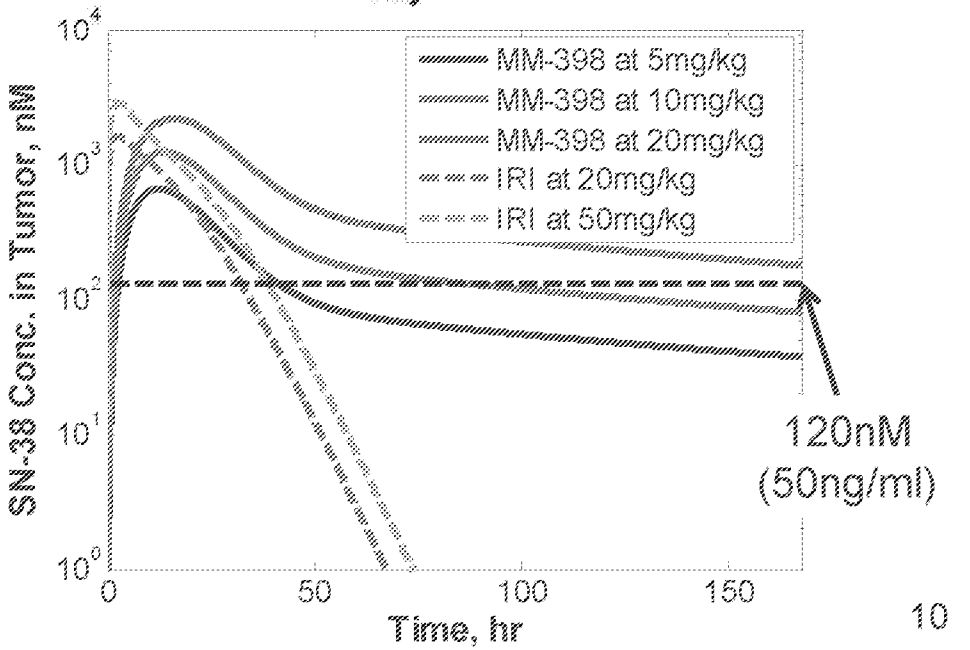




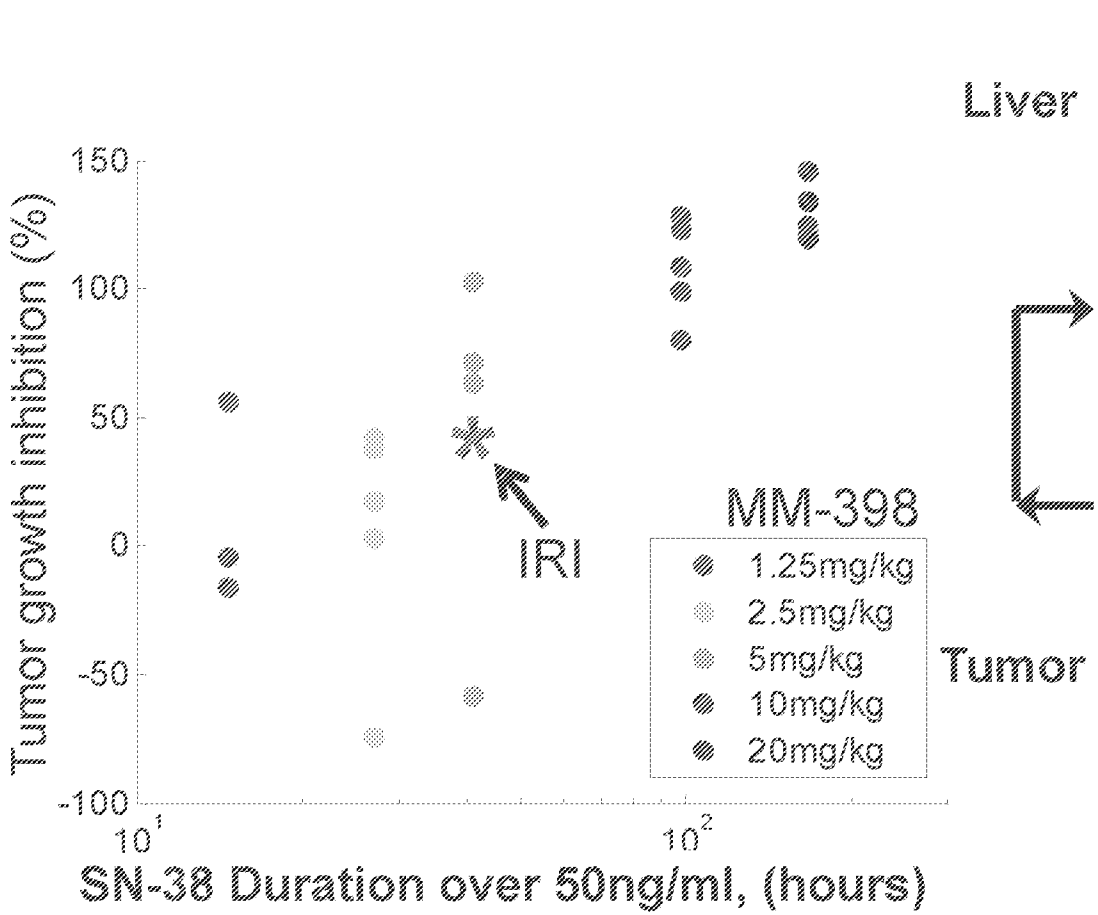
SN-38 duration over threshold differentiates MM-398 from irinotecan

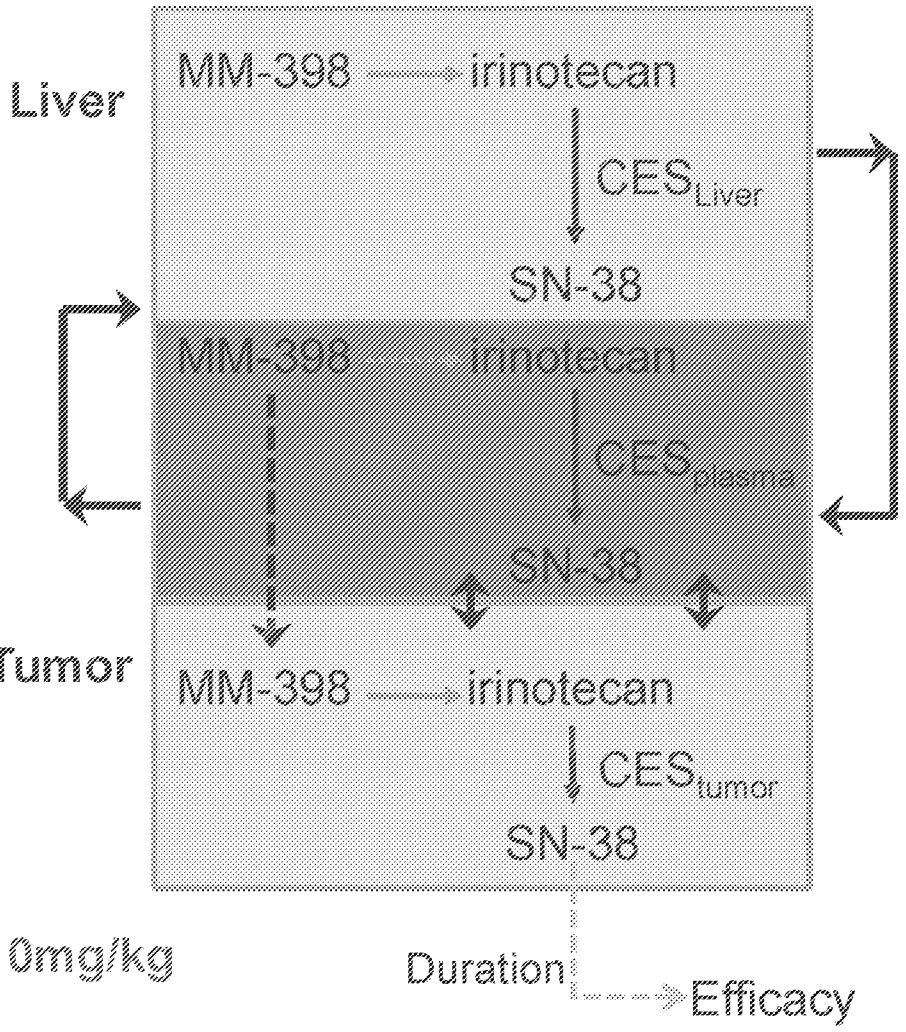
- Dose titration for MM-398 in HT29 tumor model suggests threshold effect for *in vivo* activity
- Duration of SN-38 levels above this threshold translates "sustained deposition and activation" into a model parameter predictive for in *vivo* activity





SN-38 duration over threshold can describe exposure-response relationship for both irinotecan and MM-398



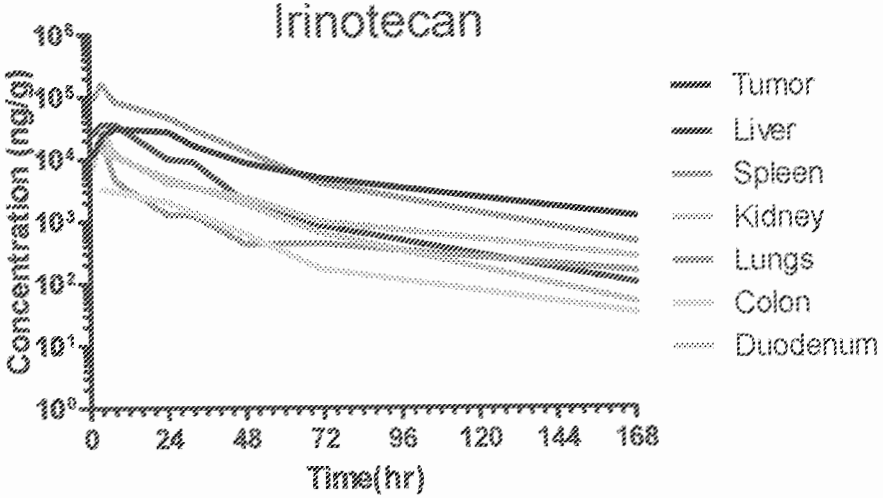


*: IRI (AUC) at 50mg/kg = MM-398 (AUC) at 10mg/kg

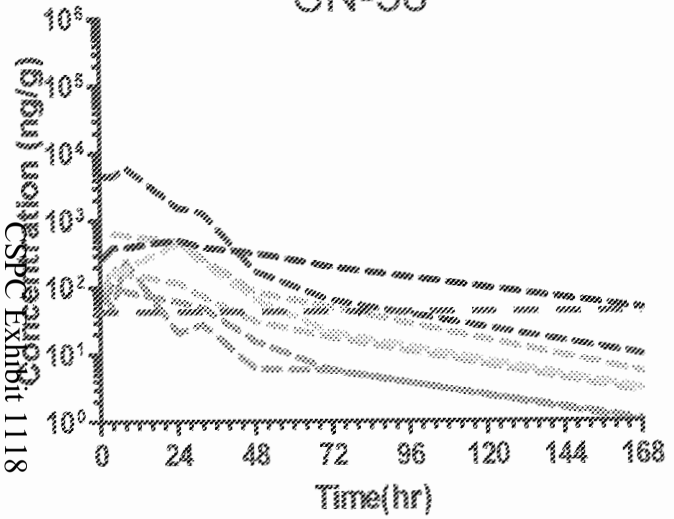
SN-38 duration from MM-398 is more pronounced in tumor than in normal tissues

Following 20mg/kg MM-398 infusion

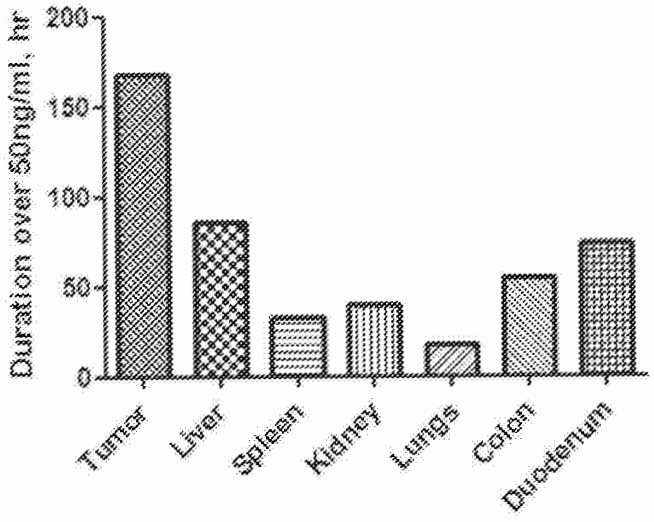
Irinotecan



SN-38



- Nanoparticles accumulate and are retained longer in tumor since lymphatic drainage is impaired
- Relatively longer duration in tumor could ensure the therapeutic windows

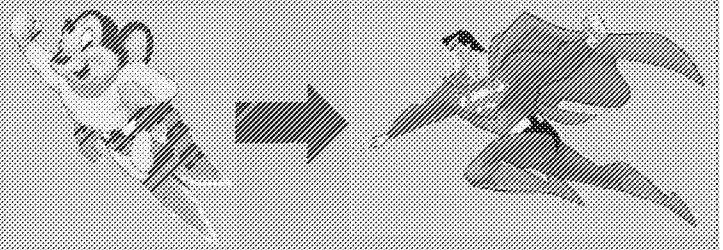


Take home message #1

- Duration of SN-38 in tumor, not SN-38 AUC, was more predictive of *in vivo* activity in mice bearing HT-29 xenografts
  - Despite the use of same active metabolite (i.e, SN-38), MM-398 delivers better *in vivo* activity realizing true potential of irinotecan
  - SN-38 duration was not responsive to increasing dose levels of free irinotecan
- Enhanced SN-38 duration is more pronounced in tumor compared to normal tissues

Translation

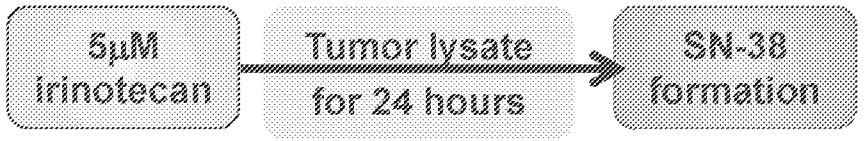
Can the model dissect out the effect of mouse-specific artifact on model predictions?

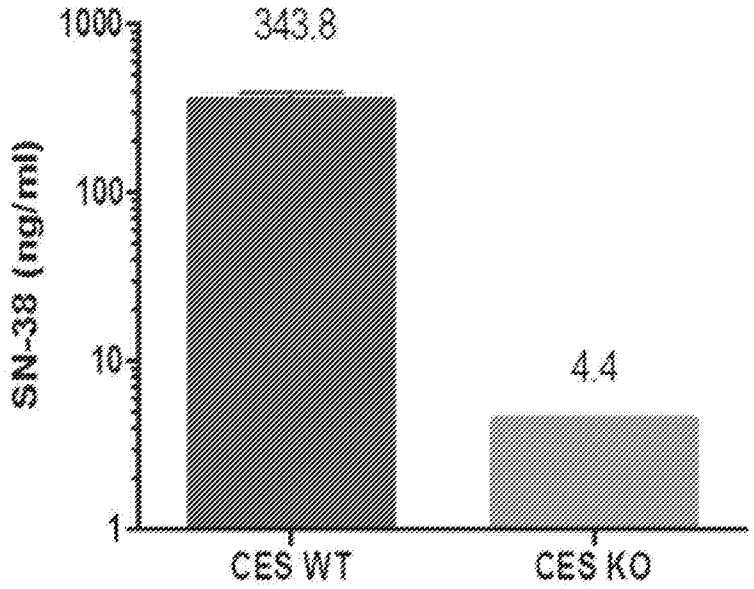


Mouse, not human serum contains irinotecan converting activity: *How does this affect MM-398 tumor exposure?*

- Murine plasma is major source of CES-mediated SN-38 formation from irinotecan (Kaneda et al., 1990)
- Rate is **~10x** that of liver
- Responsible enzyme is Es1/Ces1c, which lacks ER-retention signal and is secreted from liver
- *This enzyme is not present in humans*
- CES KO Mouse models:
  - CES1c$^{-/-}$ in C57Bl6
  - Es1^{e} in SCID (Knockdown)

***In vitro* CES activity**

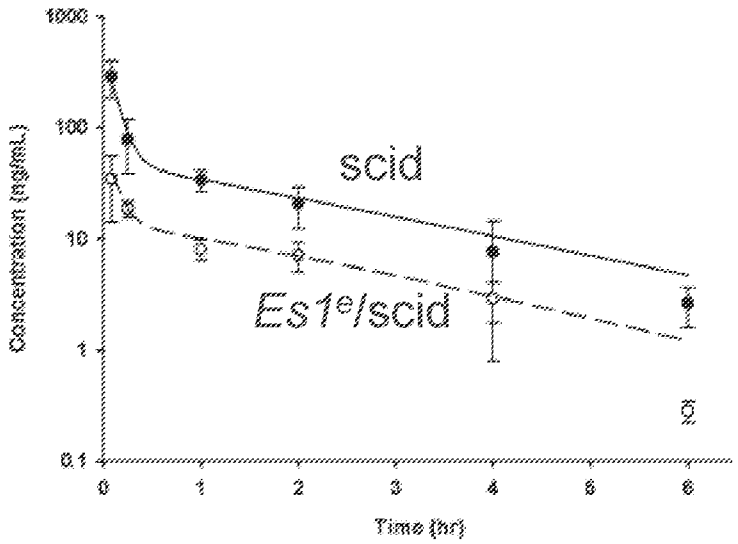


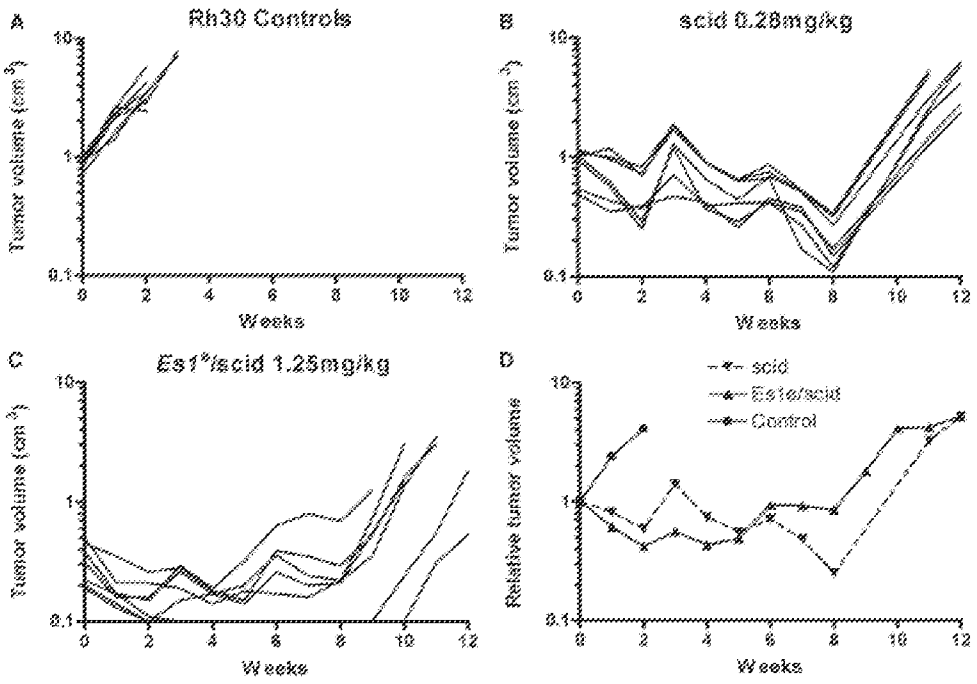


Reducing systemic activation of irinotecan similarly reduces potency

- Es1^{e} mouse strain, deficient in plasma carboxylesterases

SN-38 exposure after irinotecan administration



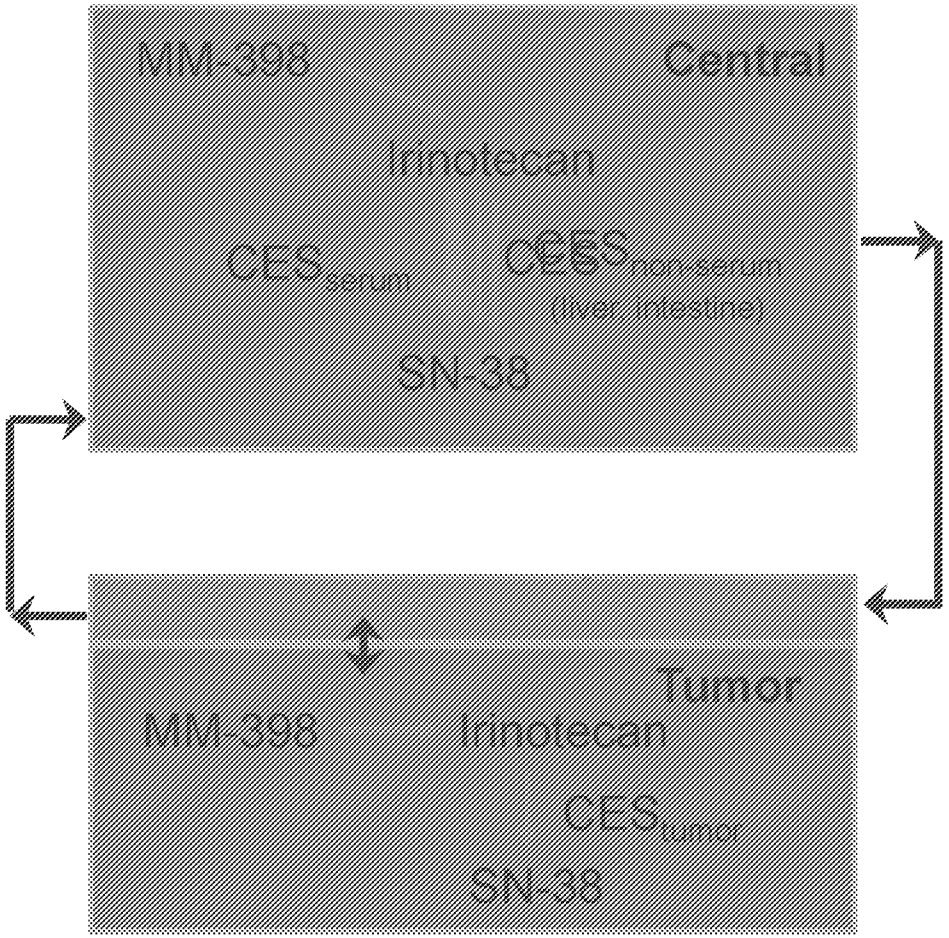


SN-38 exposure
$scid_{AUC}$ ~ 4X $Es1^{e}/scid_{AUC}$

Comparable efficacy in *Es1^{e}*/scid mice with 4X dose used in WT scid mice

Morton et al 2005 Cancer Chemotherapy Pharmacology 56:629

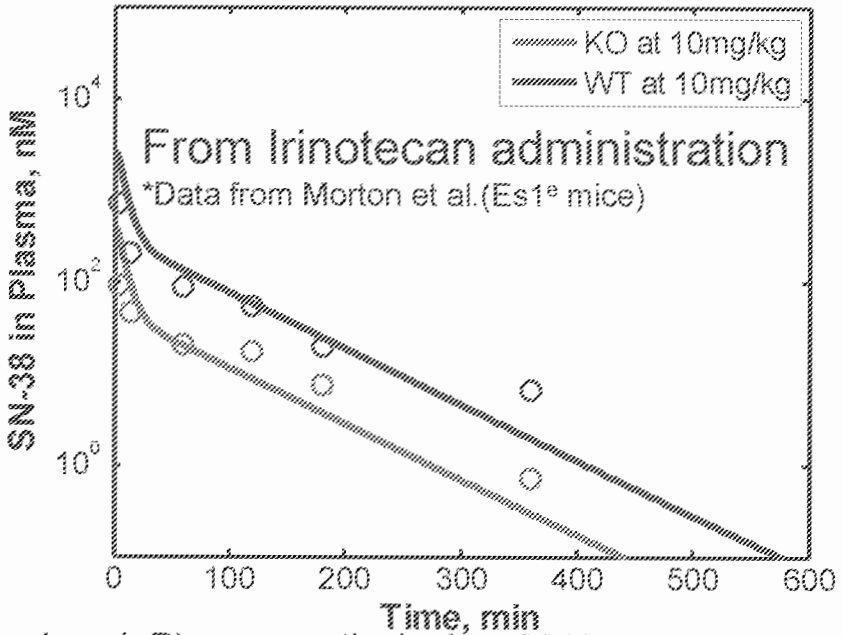
Irinotecan conversions to SN-38 are catalyzed by multiple CESs

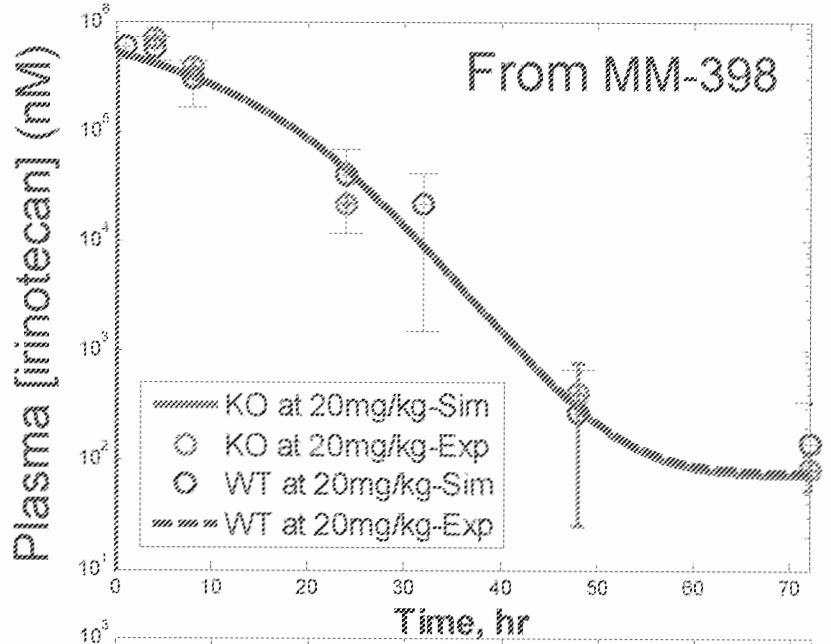


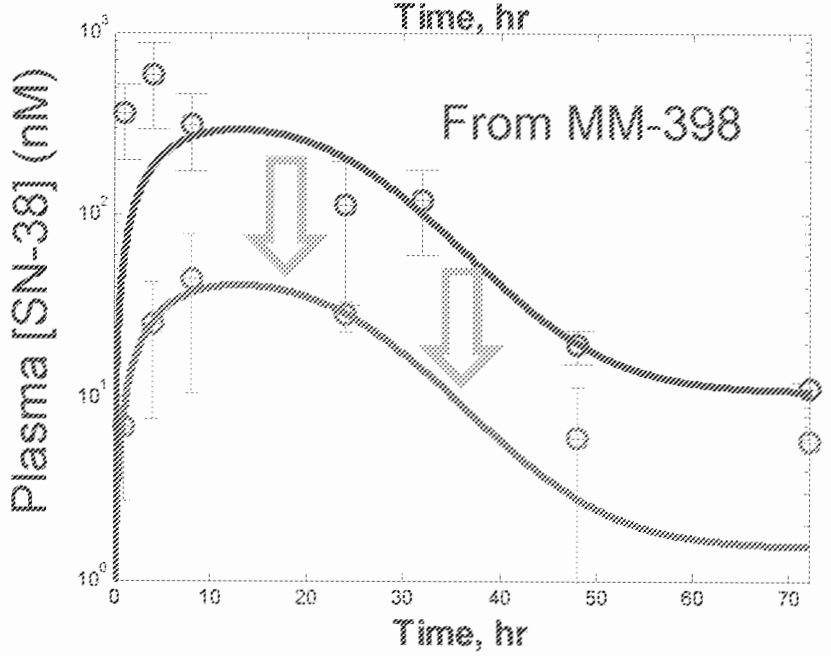
- Mouse has a unique CES in its serum (CES_{serum}) making it difficult to translate the observation from mouse to human
- PK data in CES1c KO mice (no CES_{serum}) could be used to retain the model quantifying the contribution of CES_{serum} to systemic conversion
- With the retrained model, *in silico* KO of either serum CES or tumor CES would provide useful insights without repeating experiments

Updated model identifies the contribution of serum CES to the systemic conversion of irinotecan

- Irinotecan PK from MM-398 is not affected
- SN-38 PK from MM-398 is affected
- KO mice PK data allowed the estimation of serum specific CES activity (CES1c)
  - Plasma driven activity is responsible for ~86% of total systemic conversion
  - Assuming the same contribution, the free irinotecan mouse PK model was able to predict Es1^e mice SN-38 PK from MM-398

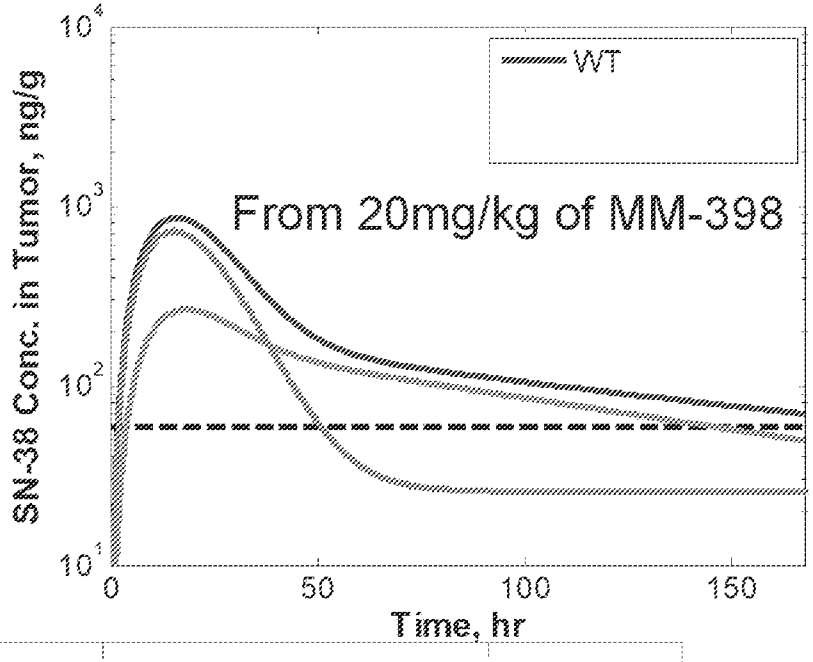


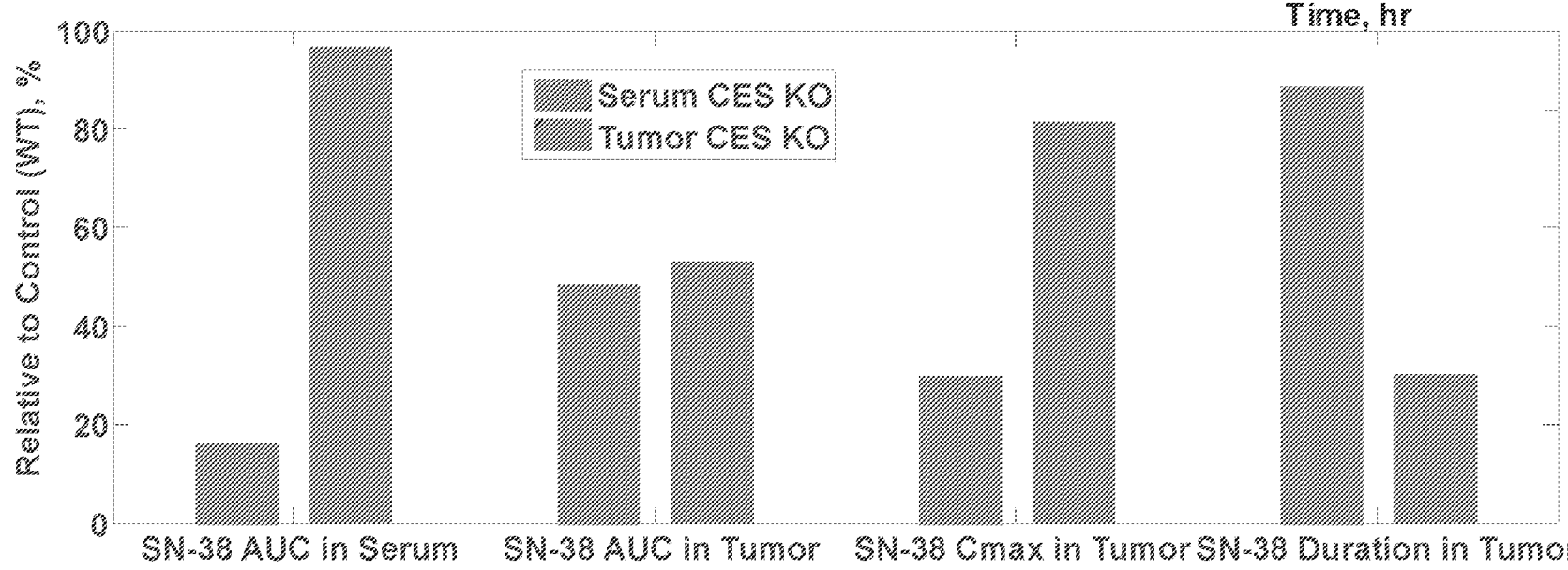




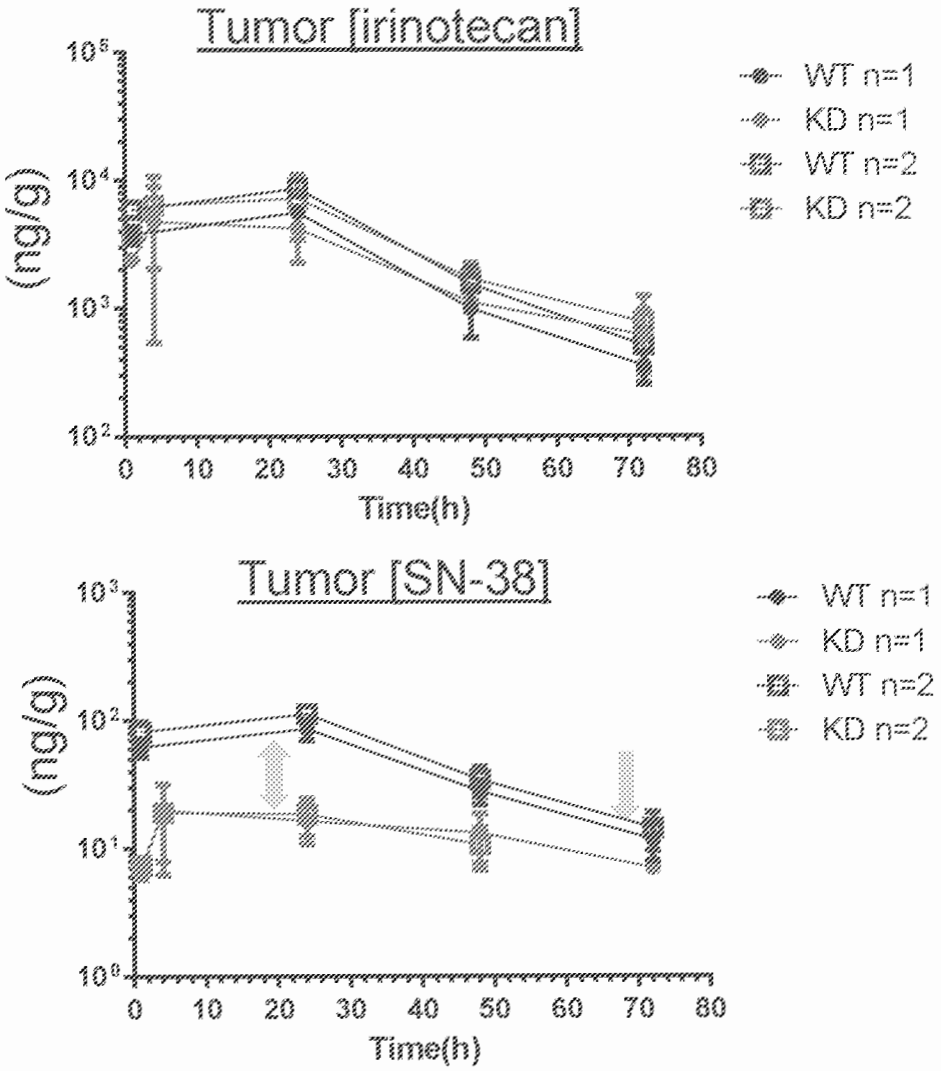
CES in tumor, not CES in serum is responsible for the longer duration of SN-38 from MM-398

- Knocking out either serum CES or tumor CES decreases SN-38 AUC in tumor by 50%
- Serum CES mainly affects Cmax, whereas tumor CES affects duration
- KO or KD of serum CES may not be an important issue to translate observations in mice to humans

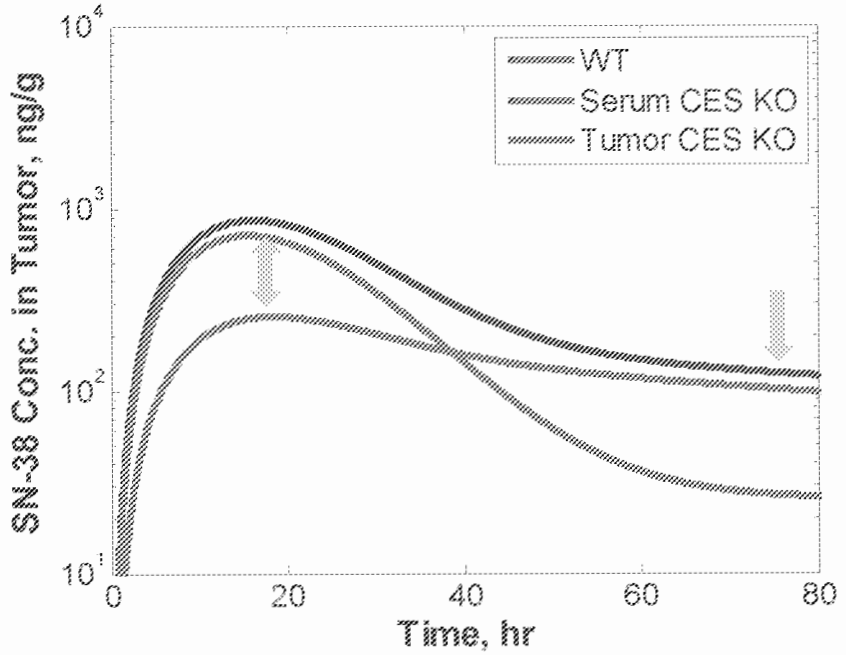




Tumor SN-38 data confirms the model prediction on the effect of serum CES KO



Confirmatory efficacy study in WT versus *Es1*^{e} mice ongoing at University of Buffalo (Prof. Robert Straubinger)

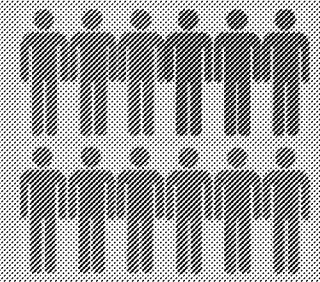


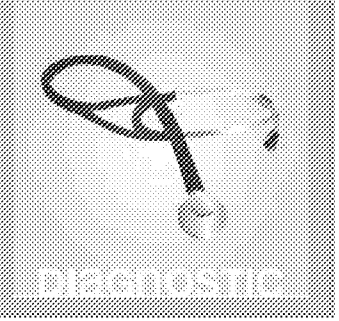
Take home message #2

- Tumor CES is more responsible for intratumoral SN-38 duration
- Mouse-specific serum esterase has minimal effect on SN-38 duration in tumor
- Serum CES may not be an important issue to translate observations in mice to humans

Stratification

Can the model identify potential diagnostic parameters for MM-398 *in vivo* activity?

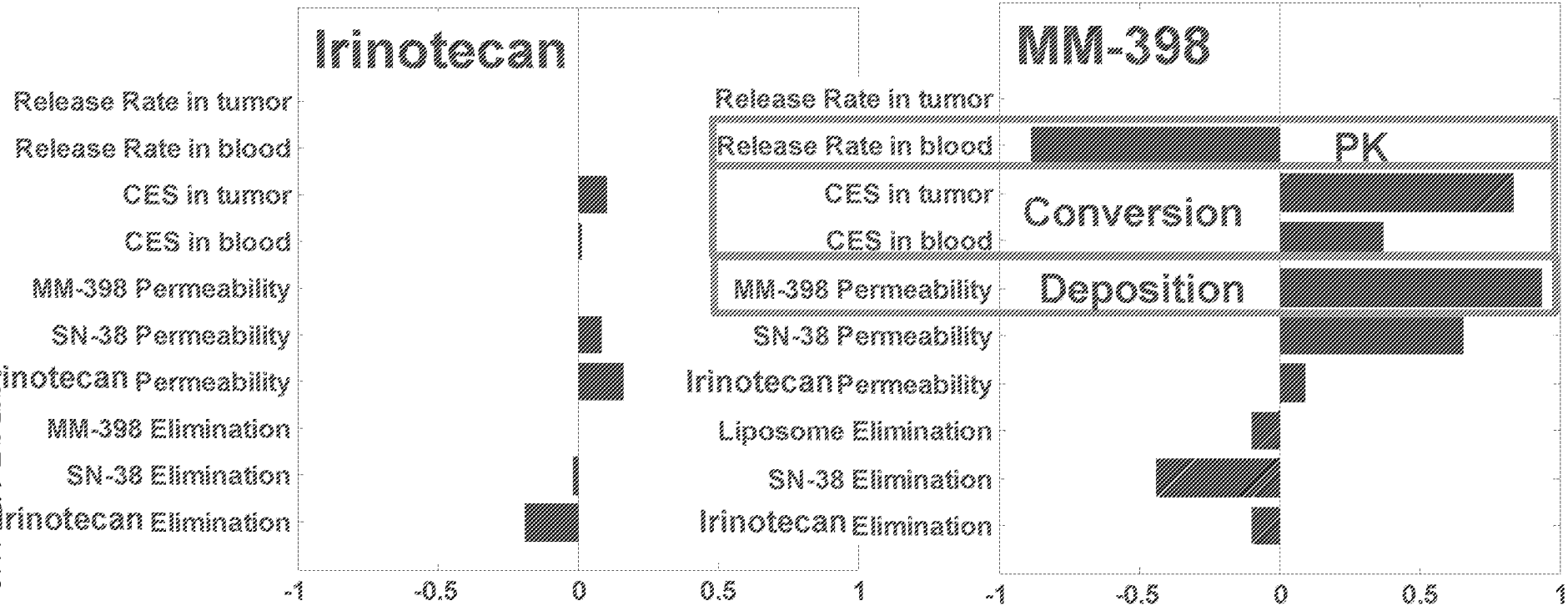




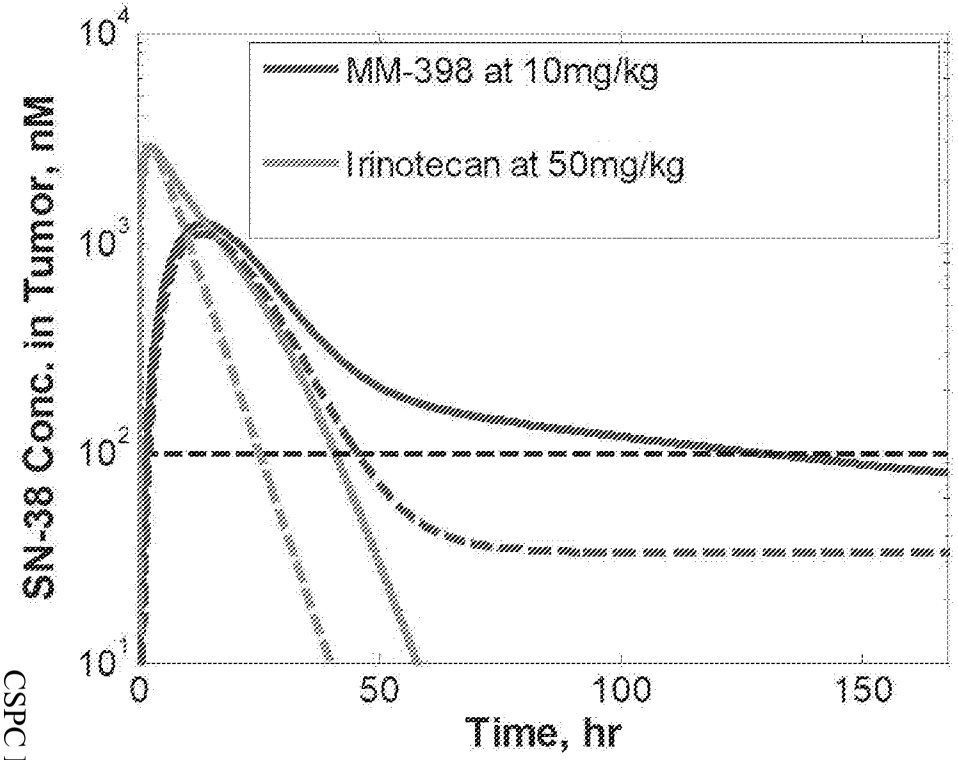
Sensitivity analysis identifies important determinants for MM-398 activity

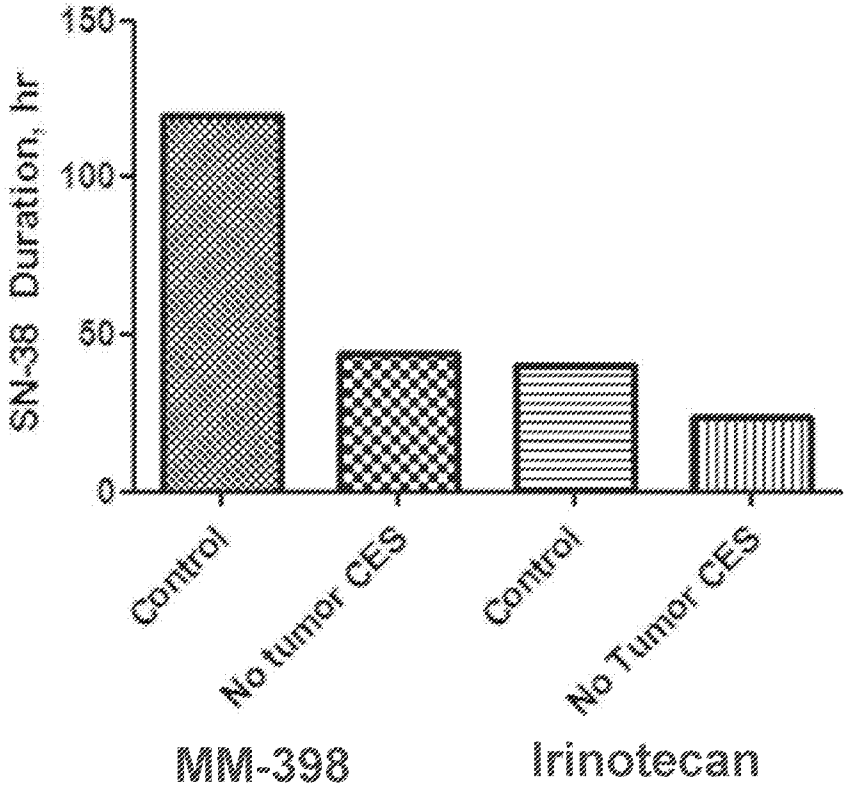
- Sensitivity was evaluated by using SN-38 duration in tumor
- Parameters for irinotecan are not sensitive to SN-38 duration in tumor
- MM-398 PK, deposition and conversion are important for MM-398 activity

$$Sensitivity = \frac{\Delta(SN38_{Duration})/SN38_{Duration,0}}{\Delta(p)/p_0}$$



Local conversion of irinotecan into intratumoral SN-38 is more crucial for *in vivo* activity of MM-398 than irinotecan

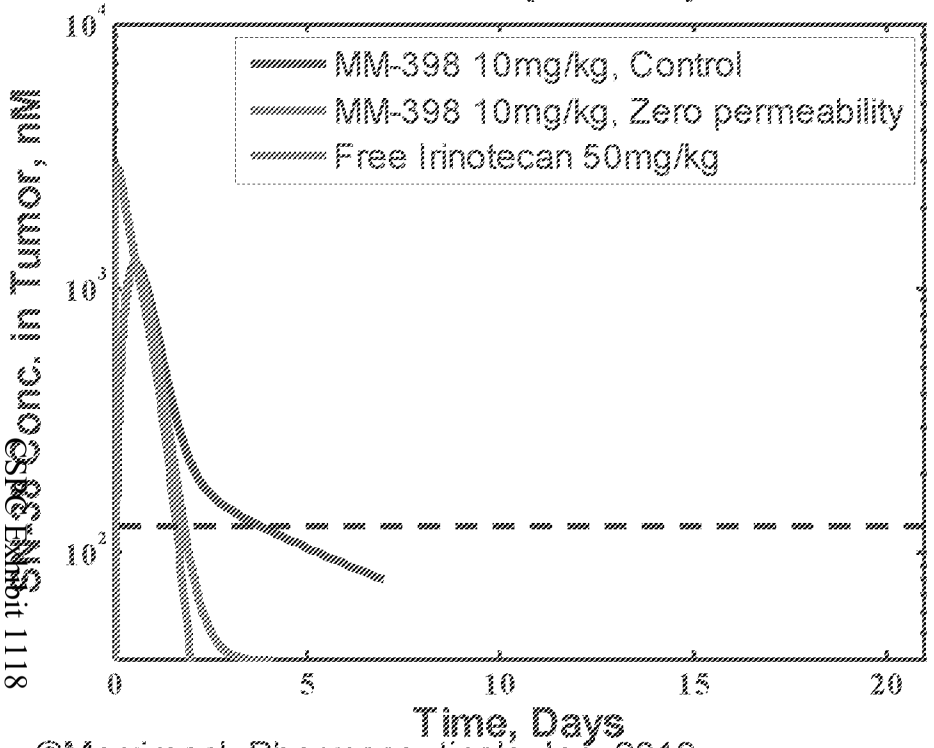




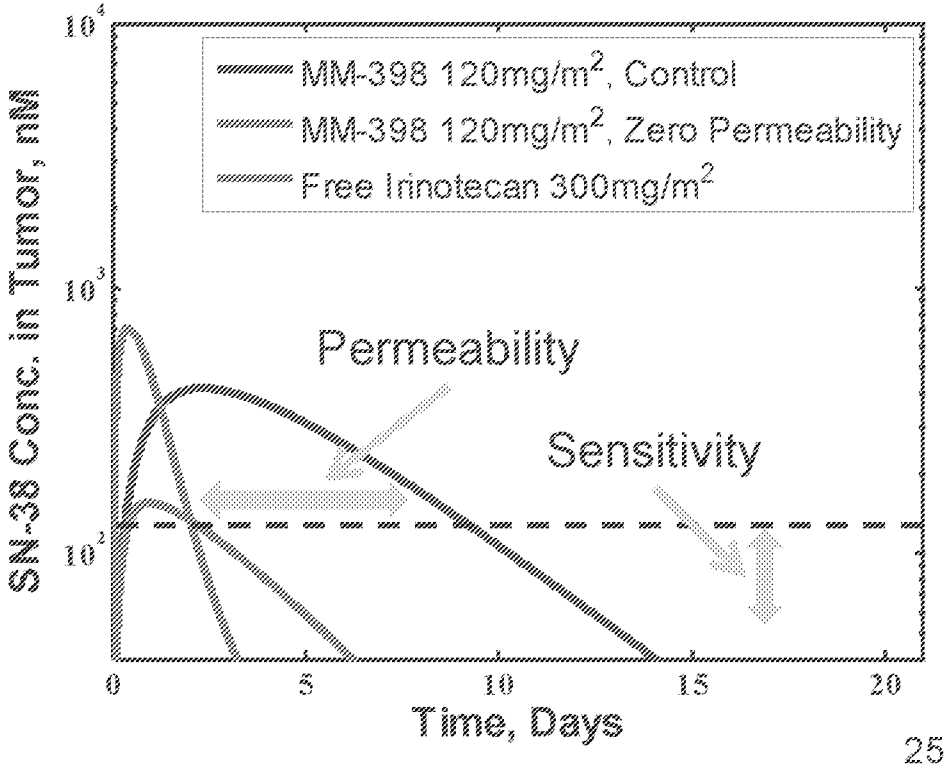
Effect of MM-398 Permeability on Tumor SN-38

- Without MM-398 deposition into tumor (zero permeability), SN-38 duration from MM-398 becomes closer toward that of free irinotecan
- Levels of permeability and tumor sensitivity to SN-38 (Threshold) determine the actual SN-38 duration

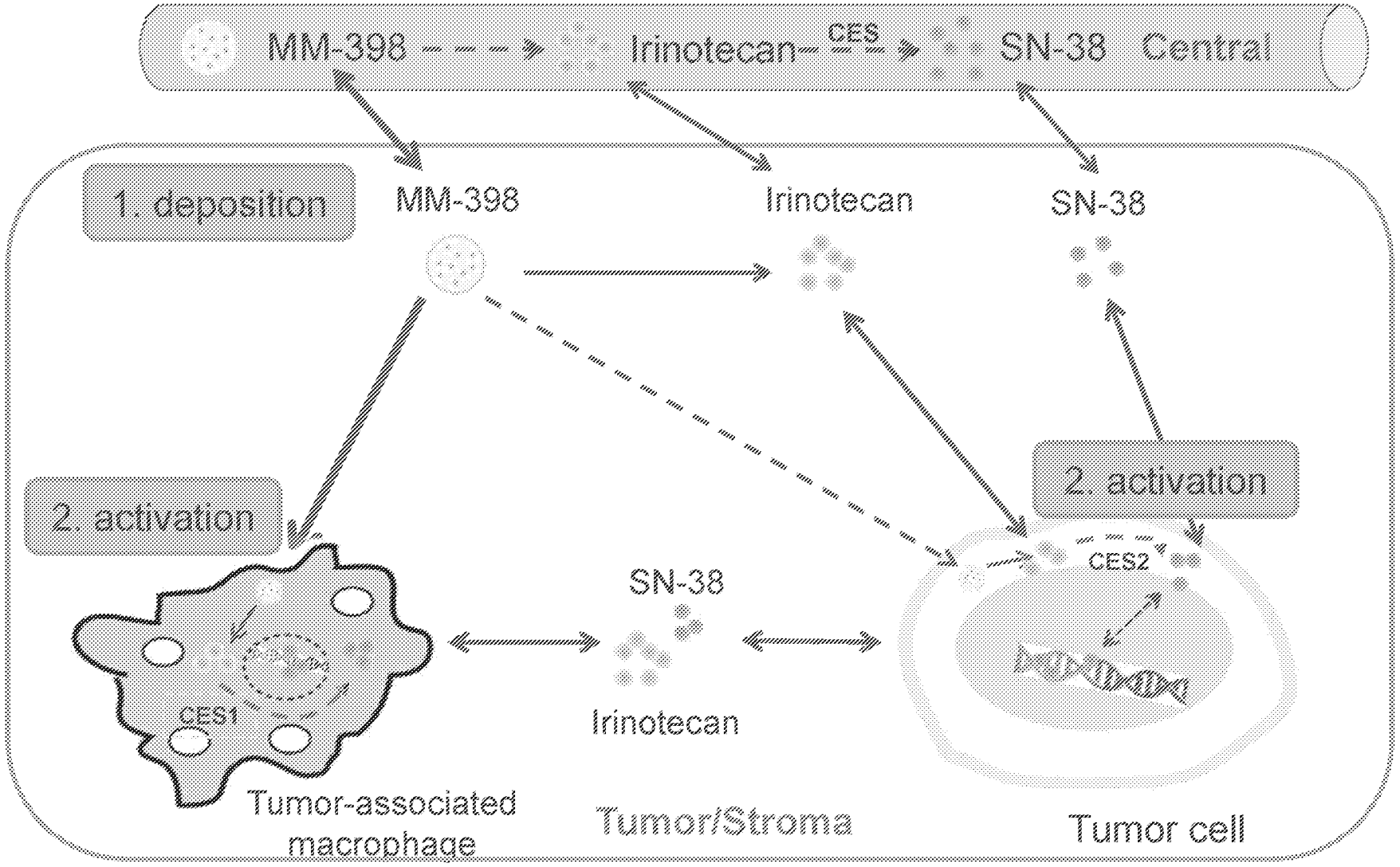
Mouse (Q1W)



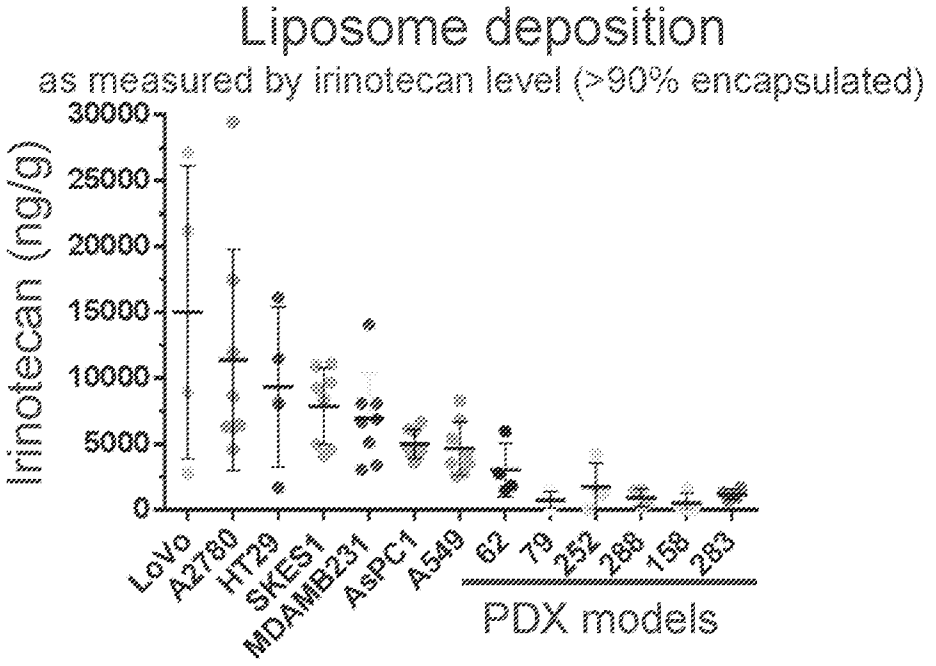
Human (Q3W)

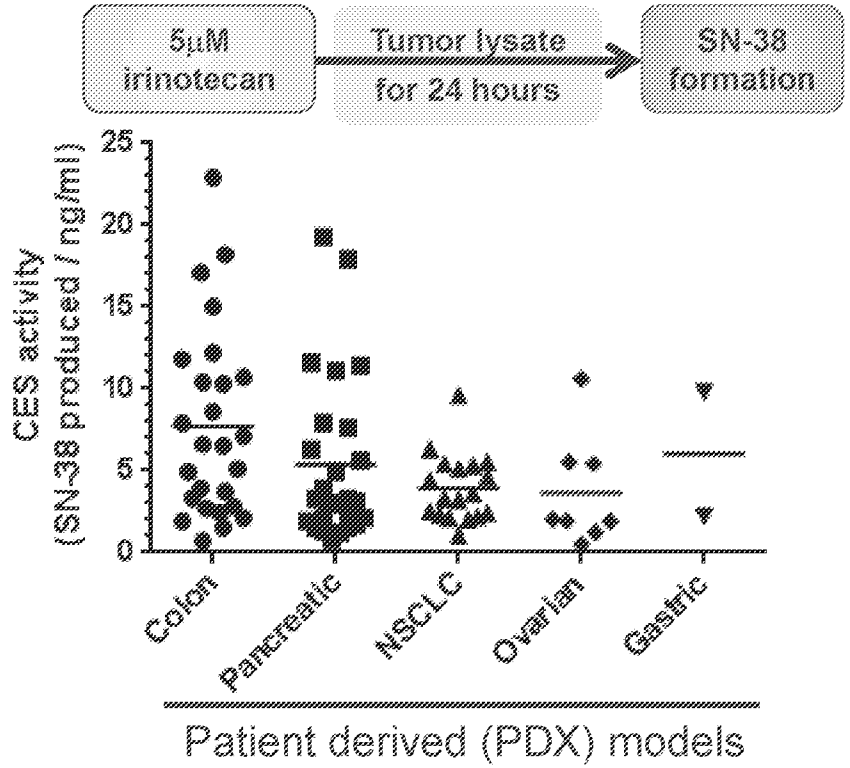


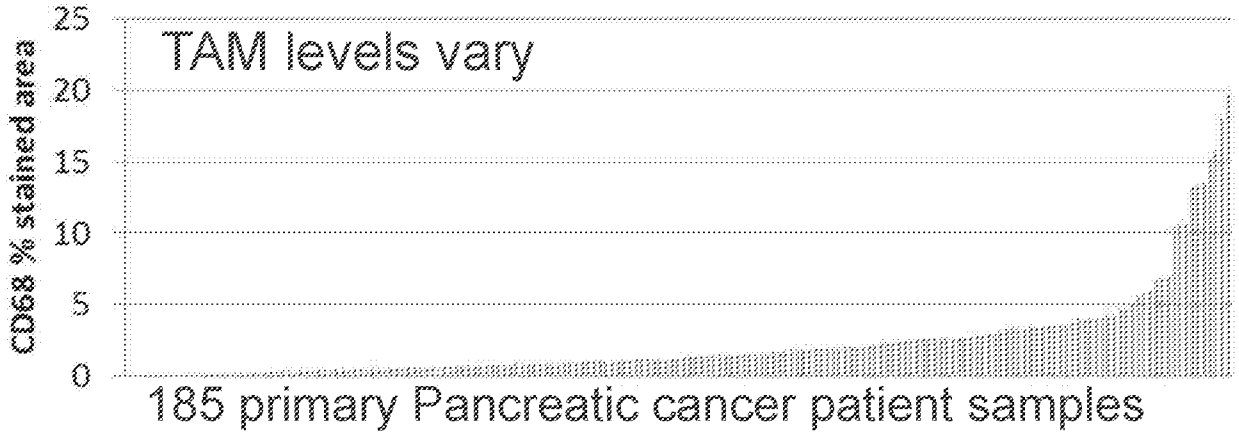
Pharmacological system in which MM-398 operates



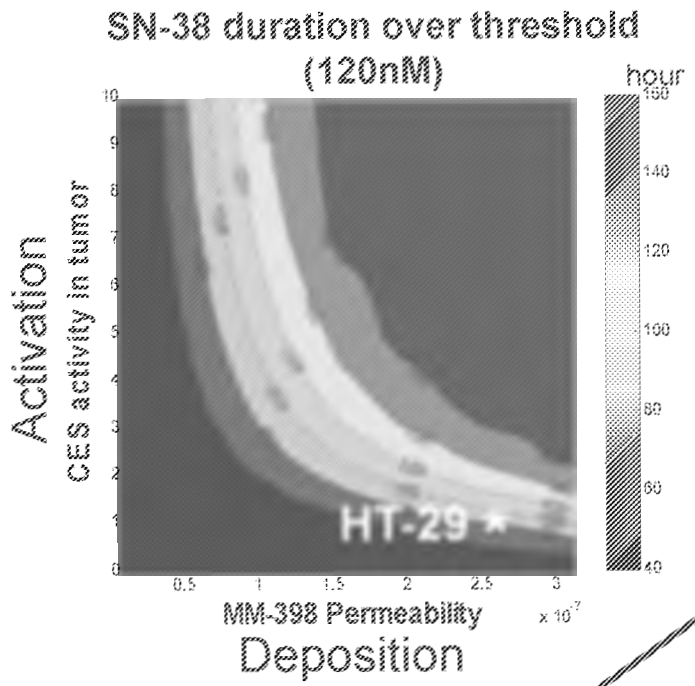
Factors affecting delivery of SN-38 vary considerably in patients and preclinical models

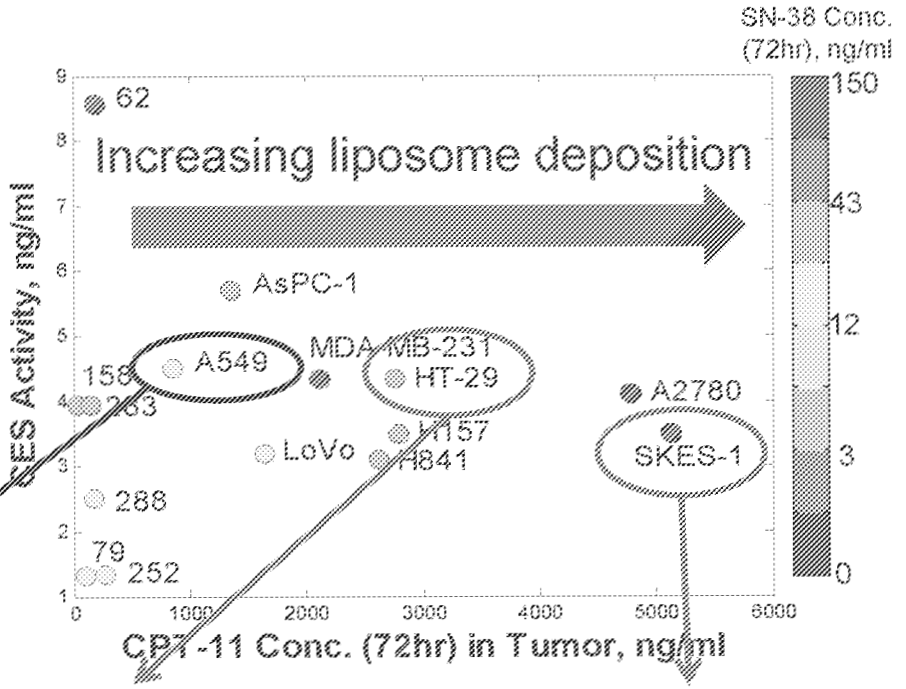


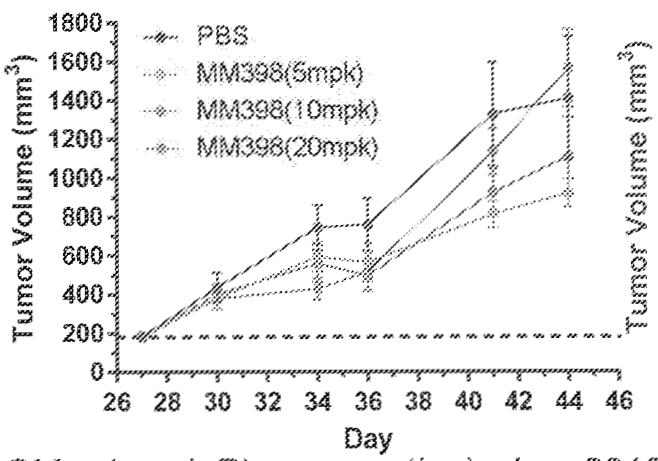


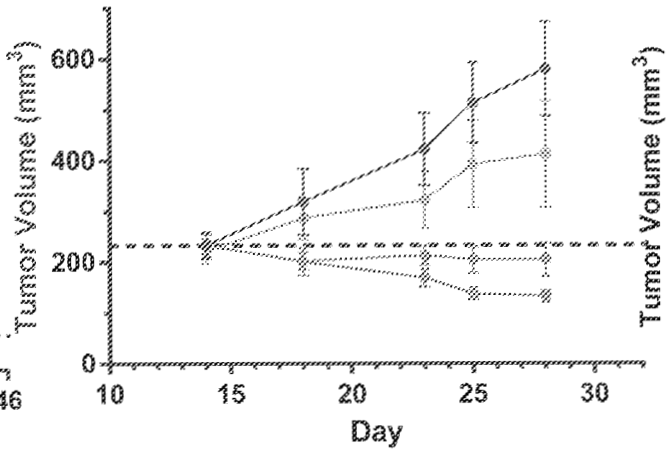


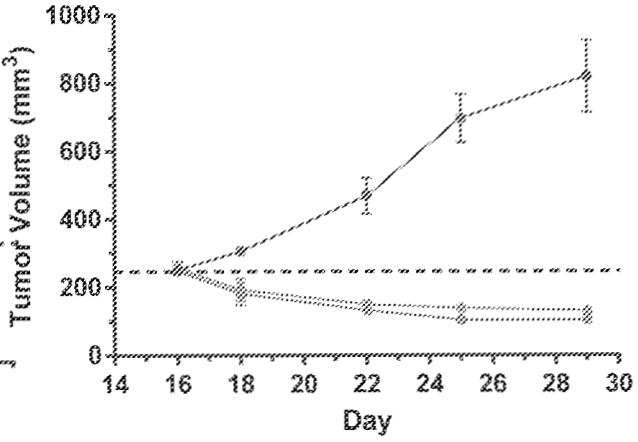
Experimental data confirms the simulated effect of local activation and deposition on SN-38 duration





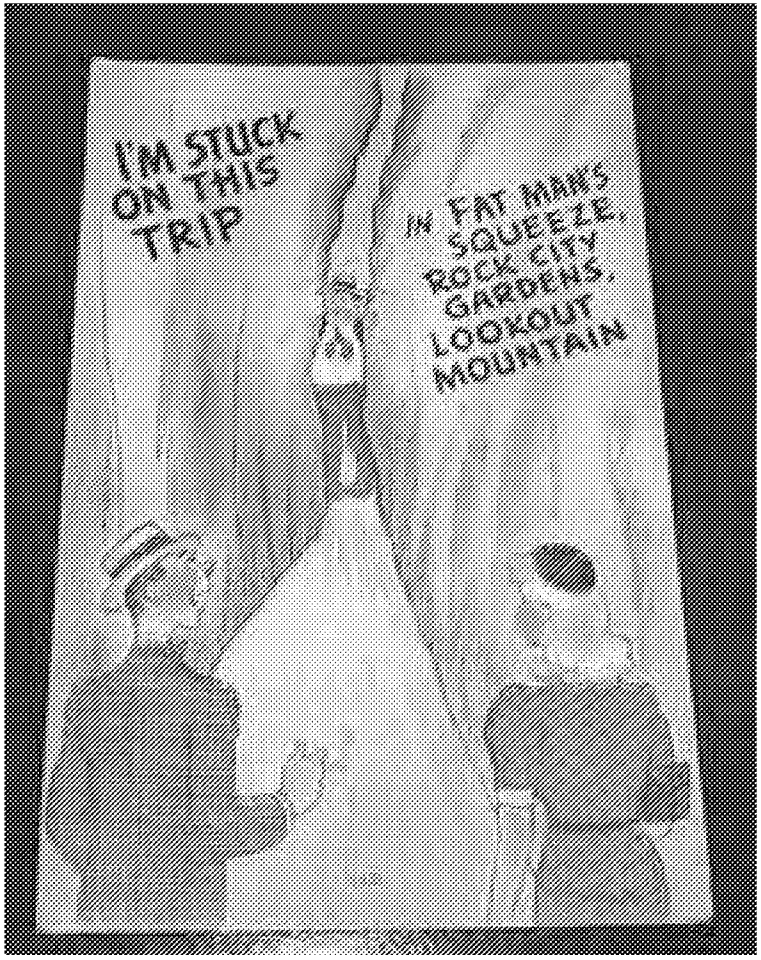


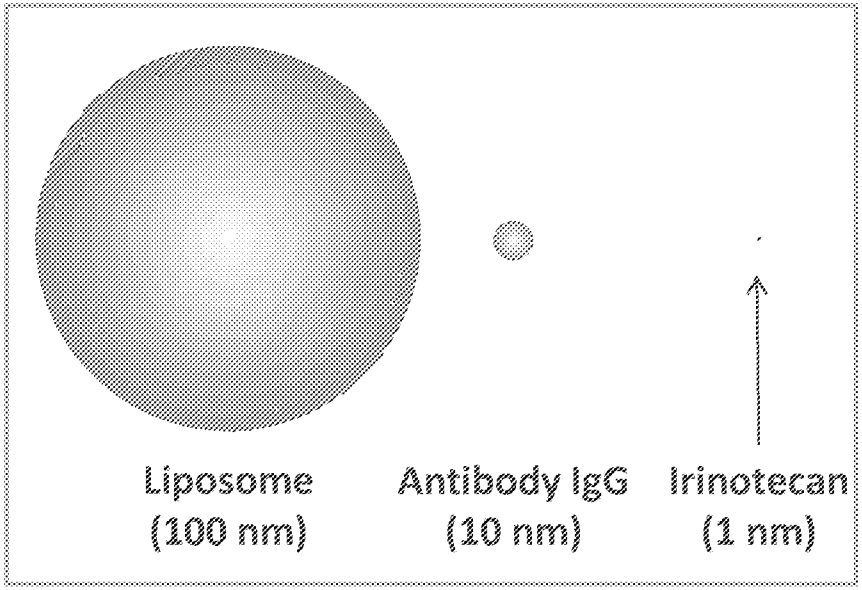




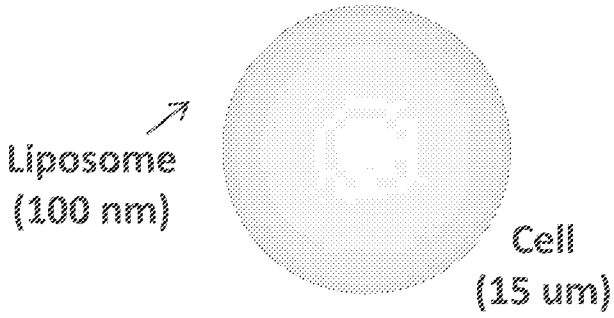
Size matters. Nanoparticles are big!

- MM-398 permeability was the most sensitive parameter



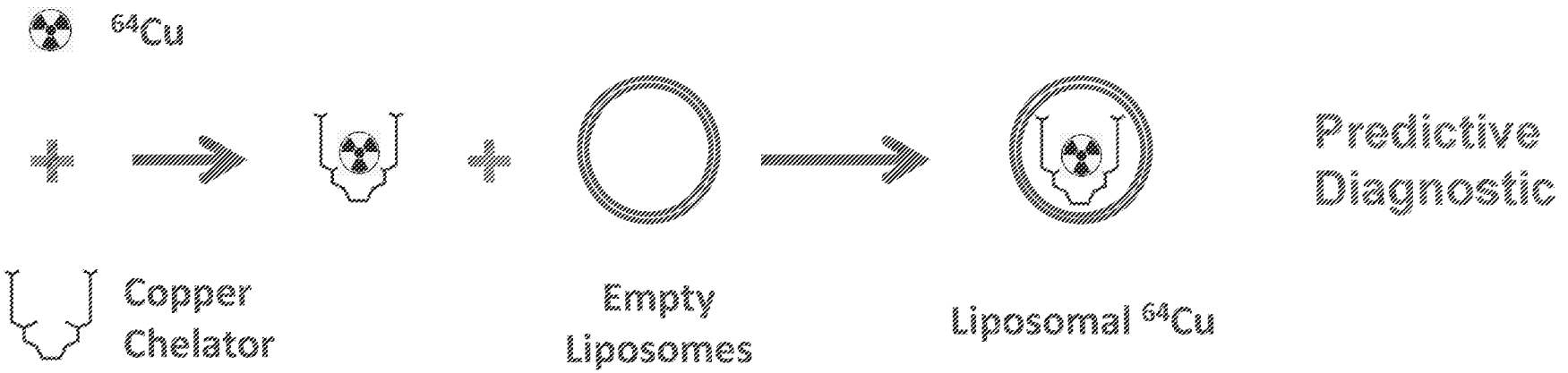


(drawn to scale)

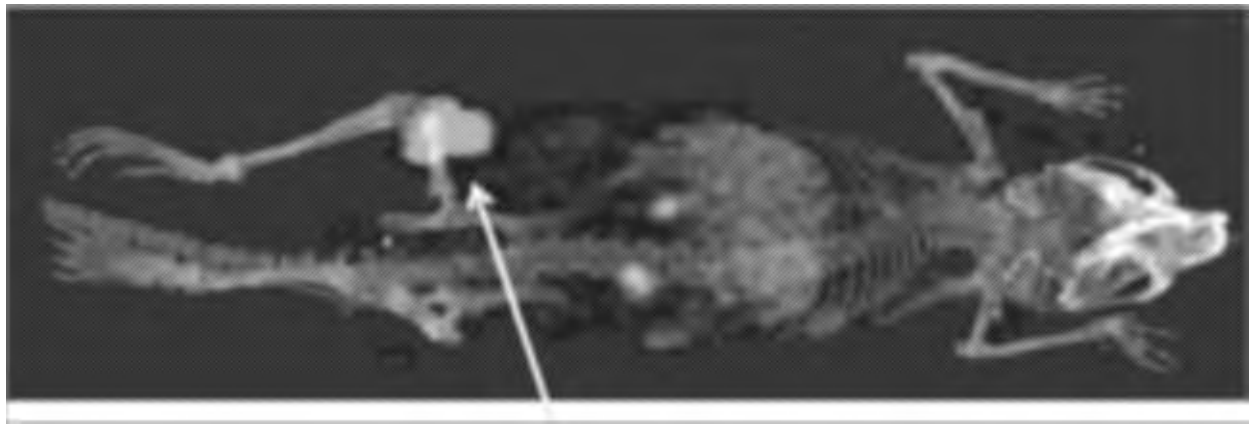


(drawn to scale)

Size & PK-matched drug delivery PET imaging diagnostic



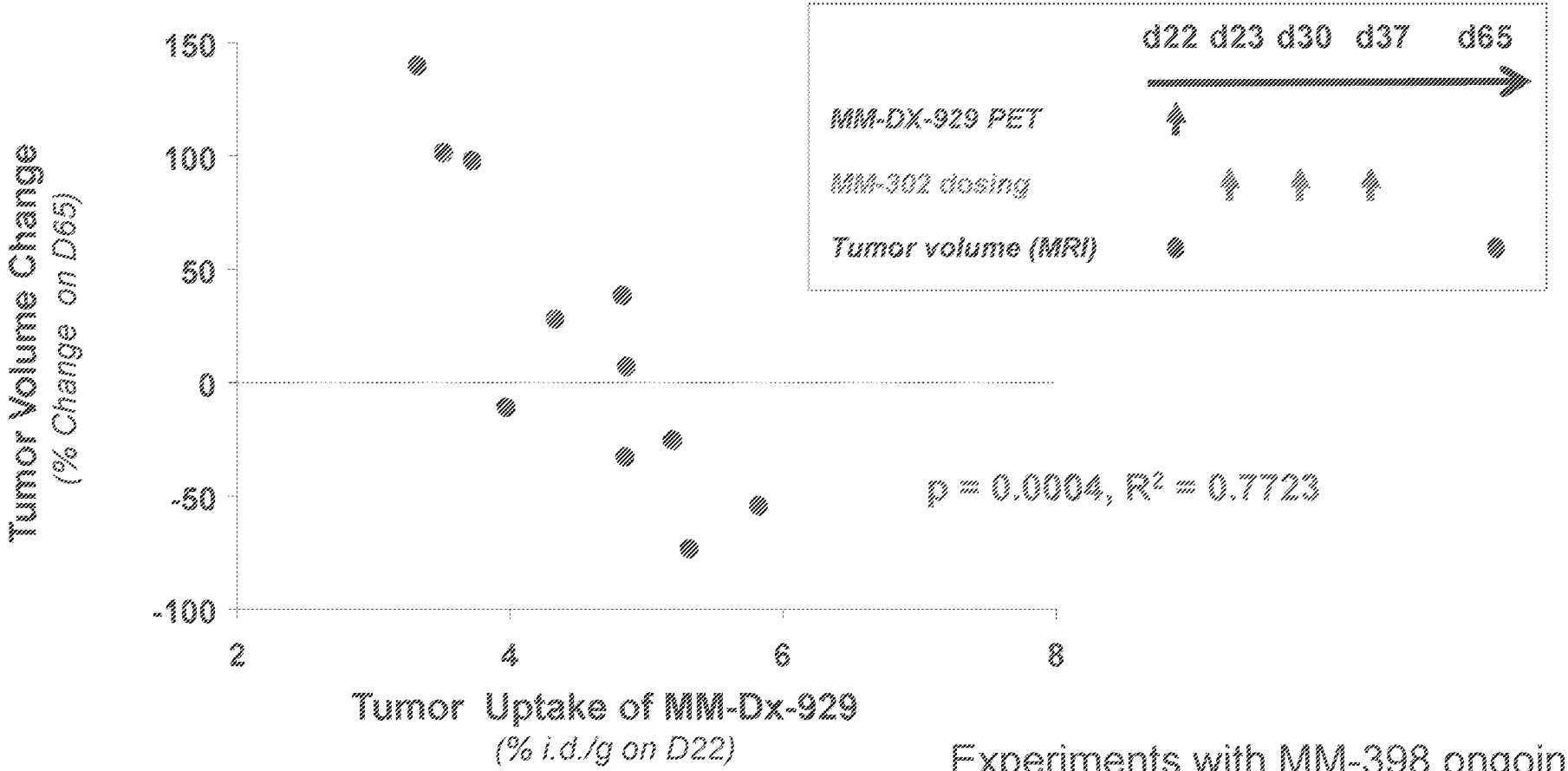
- Same PK as therapeutic liposomes
- ^{64}Cu t1/2 ~ 12hours, suitable for measuring peak liposome deposition at 24 hours



MM-Dx-929 deposition in xenograft model

Using MM-Dx-929 to predict response to HER2 targeted liposomal doxorubicin (MM-302)

Correlation of MM-302 Tumor Response with MM-Dx-929 Deposition in BT-474 Model

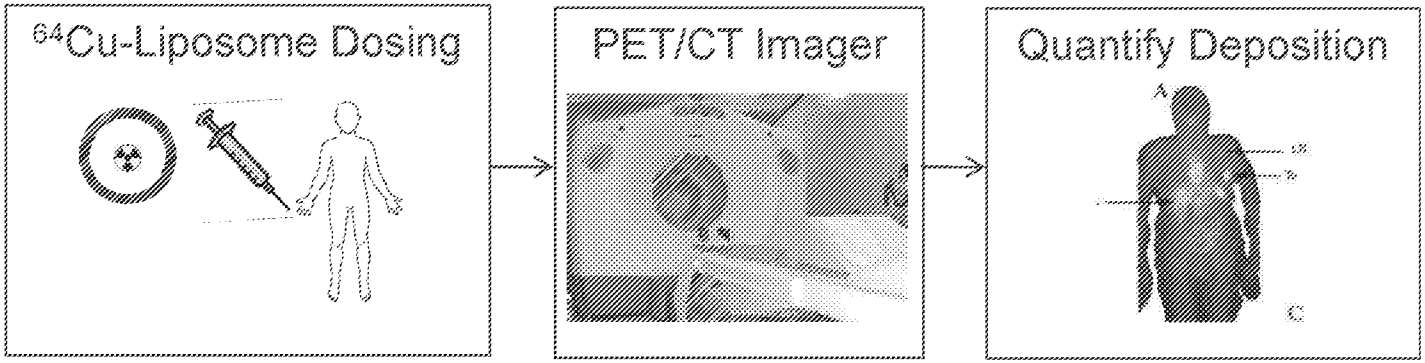


Experiments with MM-398 ongoing

MM-Dx-929: A liposomal PET imaging agent

HYPOTHESIS: Variable tumor deposition of nanoparticle therapeutics can be predicted with non-invasive imaging technology and used to guide treatment for patients

MM-Dx-929 ⇨ **Measure** ⇨ **Assess** ⇨ **Treatment Decision**



Predict Patients Who Won't Respond

Expect to enter clinical trials with liposomal therapeutics in 2014

Take home message #3

- Sensitivity analysis on the model parameters identified MM-398 deposition and intratumoral conversion as critical parameters for *in vivo* activity
- Profiling cell-line and patient derived xenograft models confirmed the model predictions and allowed us to construct a Dx map for MM-398 *in vivo* activity
- Further development and testing of imaging and tissue based biomarkers in clinical studies is warranted

Acknowledgements

MM-398 team

- Jonathan Fitzgerald
- Ashish Kalra
- Stephan Klinz
- Jason Cain
- Nancy Paz
- Arnold Sengooba
- Aurelie Bouzelmat
- Eliel Bayever

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- Bob Straubinger

MM-DX-929 team

- Bart Hendriks
- Helen Lee
- Daniel Gaddy
- Thomas Wickham

Merrimack Pharmaceuticals

- Daryl Drummond
- Dmitri Kirpotin
- Ulrik Nielsen

10. Efficient prioritization of potential diagnostic biomarkers using a systems pharmacology approach: Case study of MM-398, an irinotecan sucrosofate liposome injection

Jaeyeon Kim

Merrimack Pharmaceuticals Inc., Cambridge, MA, USA

Background: MM-398 is a stable nanotherapeutic encapsulation of irinotecan designed to improve drug delivery to the tumor and increase local drug activation compared to free-irinotecan. Extended circulation through reduced clearance rate, high tumor deposition due to the enhanced permeability and retention (EPR) effect, tumor associated macrophages (TAMs) and the irinotecan conversion enzymes all could contribute to these properties.

Aim: To computationally identify and experimentally confirm potentially predictive biomarkers for MM-398

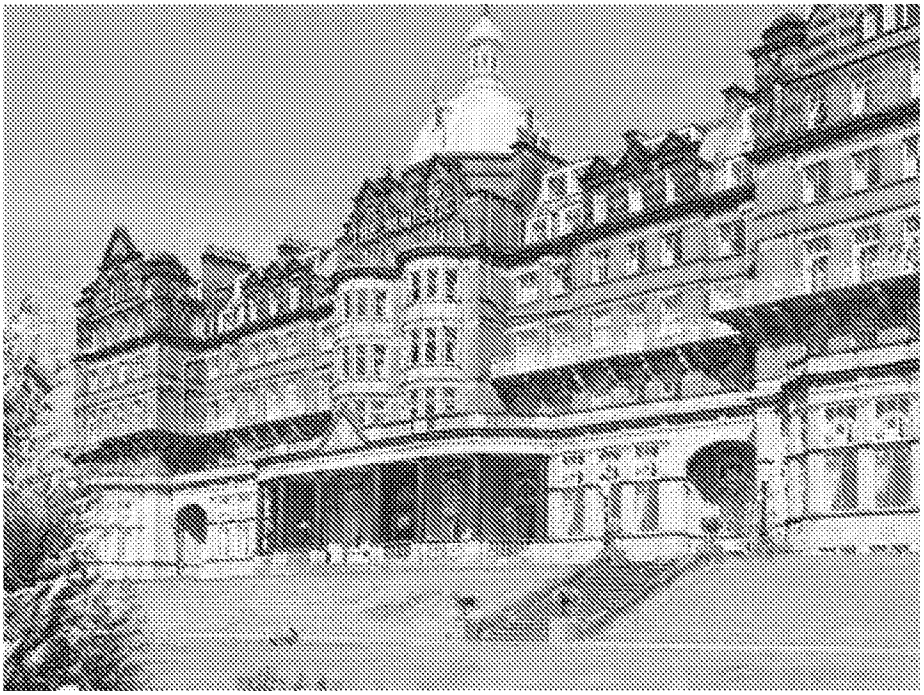
Methods: A mechanistic pharmacokinetic (PK) model for MM-398 and free irinotecan has been developed, to predict both plasma and intratumoral levels of irinotecan and SN-38. The model incorporates processes related to PK of drugs, metabolic pathways and tumor deposition. Particle swarm optimization, a global parameter estimation method, was used to train the model with PK and biodistribution data in mice bearing HT-29 xenografts, which were administered intravenously with varying doses of MM-398 or free irinotecan. Sensitivity analysis on model parameters was performed to identify important determinants for SN-38 duration in tumor, which were then tested for their use as preclinical predictive biomarkers for MM-398 activity in mice. In order to validate the model prediction, various cell line derived and patient-derived xenograft models were profiled for tumor metabolite levels following MM-398 injection and other immunohistochemistry markers from untreated tumor samples.

Results: Model simulations predicted that MM-398 resulted in equivalent SN-38 exposure (area under curve, AUC) in tumor at 5-fold lower dose than free irinotecan. However, an *in vivo* activity study showed that a 15-fold lower dose of MM-398 had equal growth inhibition of HT-29 xenograft compared to free irinotecan, revealing the limit of relating simple AUC based exposure to *in vivo* tumor response. While intratumoral SN-38 exposure from free irinotecan was limited to the first 48 hours after dosing, MM-398 maintained high levels of SN-38 throughout the weeklong time window studied. Further analysis of the exposure-response identified that the duration of intratumoral SN-38 levels above a threshold (i.e., two times the *in vitro* IC50 value for HT-29) was more predictive of *in vivo* activity. Sensitivity analysis on the model parameters identified that MM-398 permeability in tumor (deposition) and the activity of carboxylesterase in tumor (activation) are the most important determinants for *in vivo* activity of MM-398. By using preclinical markers for deposition and activation, various xenograft tumor models were classified for their ability to have sustained intratumoral SN-38 levels, which was then confirmed by the *in vivo* activity study on a subset of tumor models.

Conclusions: We applied a systems pharmacology approach to identify the important determinants for MM-398 *in vivo* activity. The model-based analysis provided an efficient way of pre-clinically identifying and testing the potentially predictive biomarkers.

Pharmacokinetics (U.K.) UK

Welcome to the Pharmacokinetics UK 2013 Meeting



Wednesday 30th October – Friday 1st November

The Majestic Hotel,
Ripon Road,
Harrogate,
North Yorkshire, HG1 2HU

Programme and Abstract Book

Title: Systems pharmacology modeling identifies unique parameters that drive tumor SN38 levels for liposomal irinotecan (MM-398) compared to irinotecan

Authors: Jaeyeon Kim*, Ashish Kalra, Milind Chalishazar, Stephan Klinz, Nancy Paz, Jason, Cain, Daryl Drummond, Bart Hendriks, Eliel Bayever, Ulrik Nielsen, Jonathan Fitzgerald

Institutions: Merrimack Pharmaceuticals Inc., Cambridge, MA, USA

MM-398 is a stable nanotherapeutic encapsulation of the pro-drug irinotecan designed to improve drug delivery to the tumor and increase the dependence on local drug activation compared to free-irinotecan. Extended circulation through reduced clearance rate, high tumor deposition due to the enhance permeability and retention (EPR) effect, tumor associated macrophages (TAMs) and the irinotecan conversion enzymes all could contribute to these properties. We sought to better understand how MM-398, a relatively large (100nm) liposomal nanotherapeutic, can treat a variety of solid tumors by determining the relative roles of systemic vs. local tumor activation of irinotecan in contributing to the activity of MM-398. Using a systems pharmacology approach, we developed a mechanistic pharmacokinetic model of MM-398 and free-irinotecan to predict both plasma and intratumoral levels of irinotecan and SN-38. The model incorporates processes related to pharmacokinetics of drugs, metabolic pathways and tumor deposition. The particle swarm optimization, a global parameter estimation method was used to train the model with pharmacokinetic and biodistribution data in mice bearing HT-29 xenografts. Analysis of the exposure-response identified that the duration of intratumoral SN-38 levels above the threshold (i.e., two times the *in vitro* IC50 value for HT-29) was more predictive of *in vivo* activity. We investigated the role of each CES enzyme activity on the SN-38 exposure in plasma and tumor. The model simulations emphasize the importance of locally activating irinotecan to SN-38 by tumor CES as the main driver for longer duration of SN-38 levels in tumor, which in turn correlates with *in vivo* activity. In conclusion, we applied a systems pharmacology approach to identify the importance of tumor CES for MM-398 activity. Liposomal encapsulation of irinotecan dramatically alters the pharmacokinetic profile of SN-38 in the tumor, as well as *in vivo* activity by extending the duration of SN-38 levels above the response-threshold.

Systems pharmacology modeling identifies unique parameters that drive tumor SN38 levels for liposomal irinotecan (MM-398) compared to irinotecan

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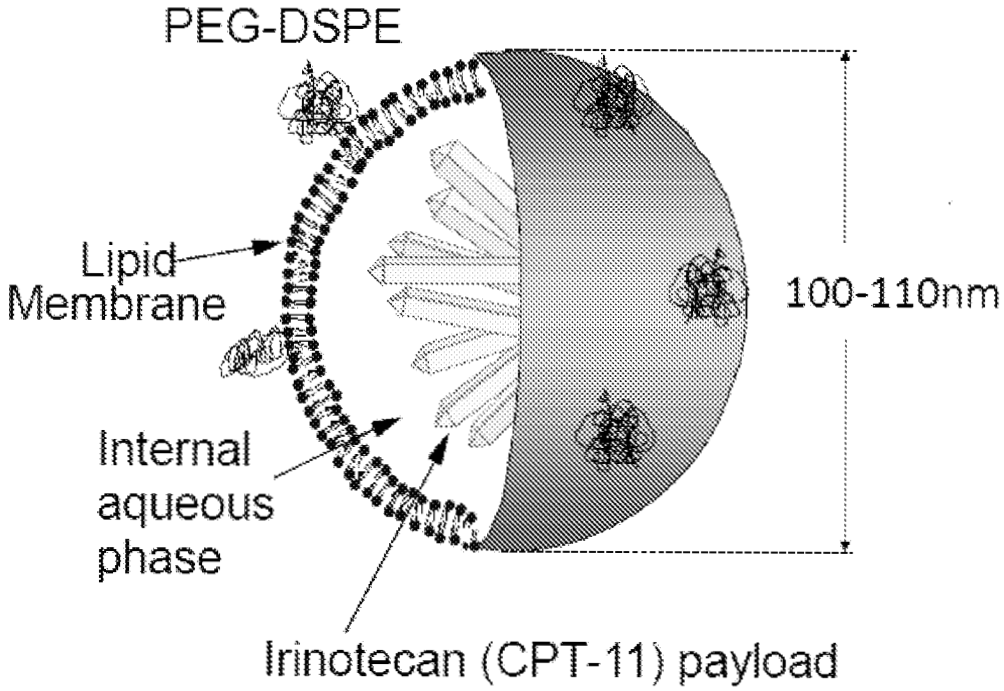
ABSTRACT

MM-398 is a stable nanotherapeutic encapsulation of the pro-drug irinotecan designed to improve drug delivery to the tumor and increase the dependence on local drug activation compared to free irinotecan. Extended circulation through reduced clearance rate, high tumor deposition due to the enhance permeability and retention (EPR) effect, tumor associated macrophages (TAMs) and the irinotecan conversion enzymes all could contribute to these properties. We sought to better understand how MM-398, a relatively large (100nm) liposomal nanotherapeutic, can potentially treat a variety of solid tumors by determining the relative roles of systemic vs. local tumor activation of irinotecan in contributing to the activity of MM-398. Using a systems pharmacology approach, we developed a mechanistic pharmacokinetic model of MM-398 and free-irinotecan to predict both plasma and intratumoral levels of irinotecan and SN-38. The model incorporates processes related to pharmacokinetics of drugs, metabolic pathways and tumor deposition. The particle swarm optimization, a global parameter estimation method, was used to train the model with pharmacokinetic and biodistribution data in mice bearing HT-29 xenografts. Analysis of the exposure-response identified that the duration of intratumoral SN-38 levels above the threshold (i.e. two times the in vitro IC50 value for HT-29) was more predictive of in vivo activity. We investigated the role of each CES enzyme activity on the SN-38 exposure in plasma and tumor. The model simulations emphasize the importance of locally activating irinotecan to SN-38 by tumor CES as the main driver for longer duration of SN-38 levels in tumor, which in turn correlates with in vivo activity. In conclusion, we applied a systems pharmacology approach to identify the importance of tumor CES for MM-398 activity. Liposomal encapsulation of irinotecan appears to alter the pharmacokinetic profile of SN-38, as well as in vivo activity by extending the duration of SN-38 levels above the response-threshold.

MM-398 background

Low-pegylated non-targeted liposomal irinotecan

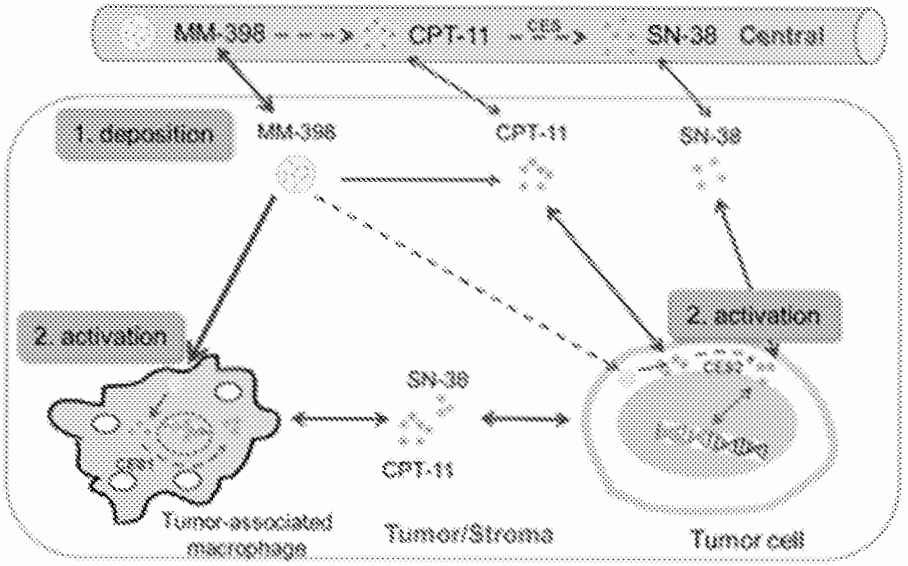


Current Clinical Trials

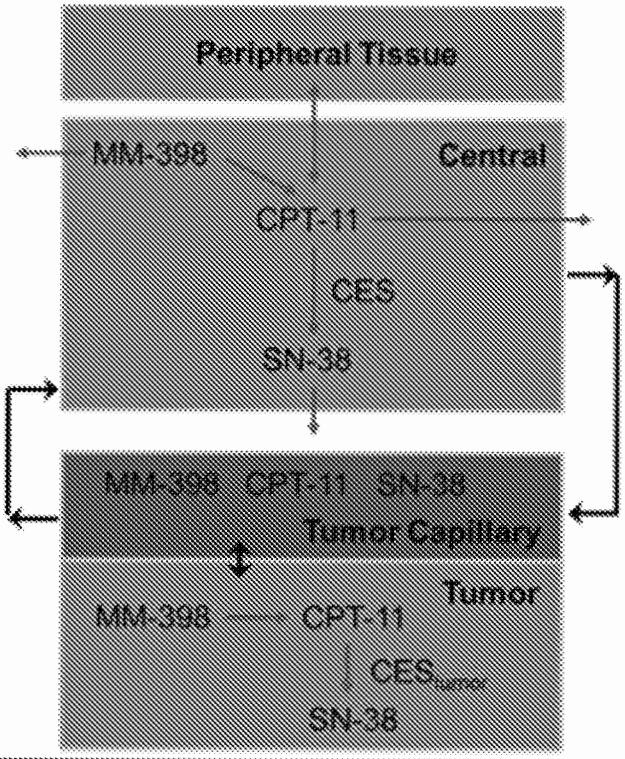
- Ongoing Phase 3 monotherapy and 5-FU/LV combination therapy in 2nd line pancreatic cancer patients (NAPOLI-1)
- Ongoing Phase 2 combination with 5-FU/LV and Avastin in 2nd line colorectal cancer

Systems pharmacology approach to identify MOA for MM-398

- Extended half-life with "passive" accumulation in tumor to achieve high drug levels
- Effective packaging of irinotecan: ~70,000 molecules/liposome
- Drug release in tumor into interstitial space or intracellular after endocytosis
- Irinotecan prodrug is converted to the active metabolite, SN-38 by carboxylesterase (CES) to induce DNA damage response

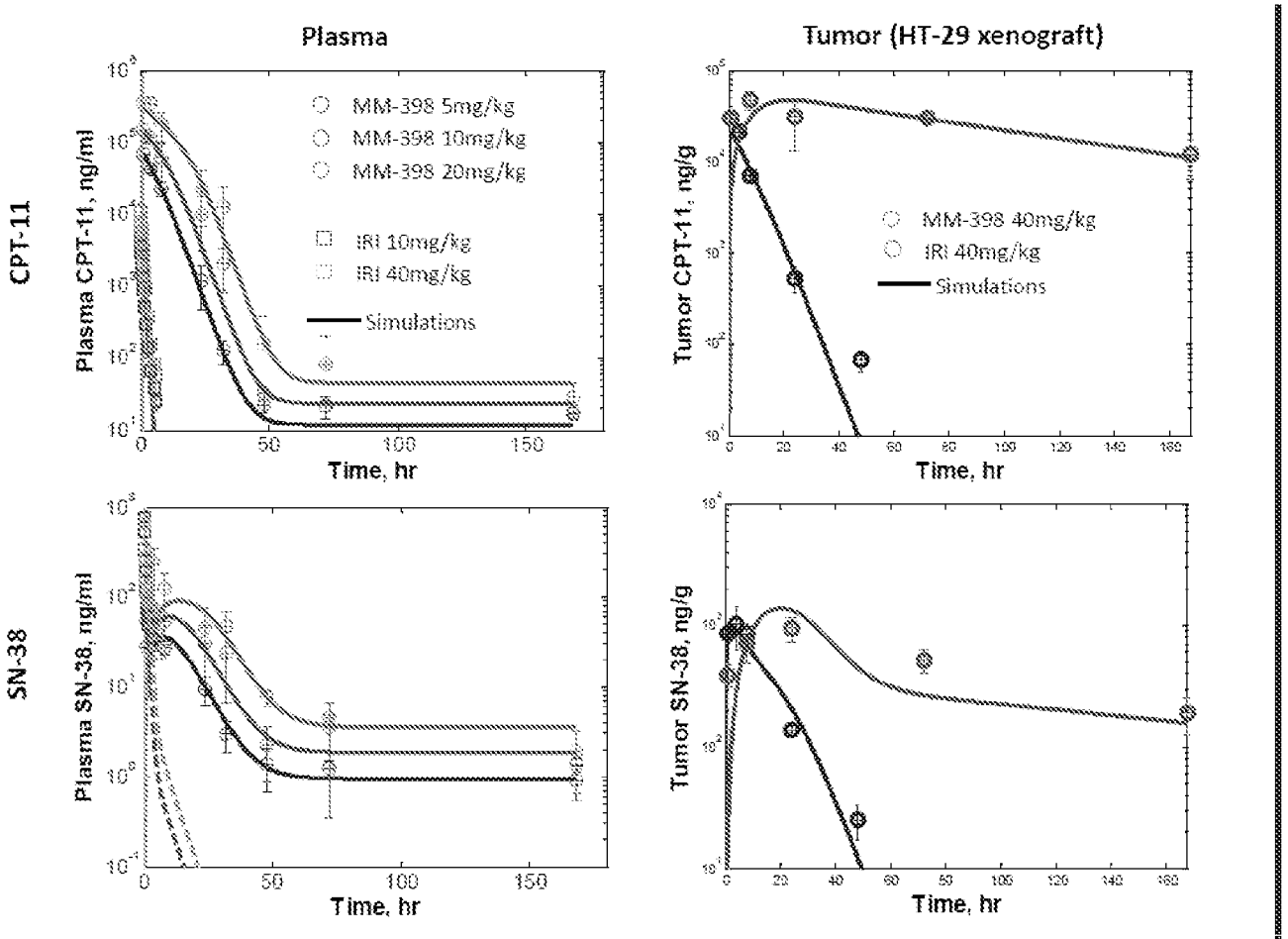


- Mechanistic PK model incorporates PK in plasma, metabolic pathways of ironotecan and biodistribution of metabolites in tumor

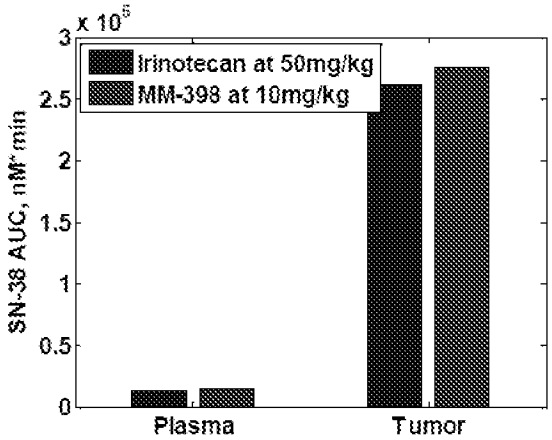


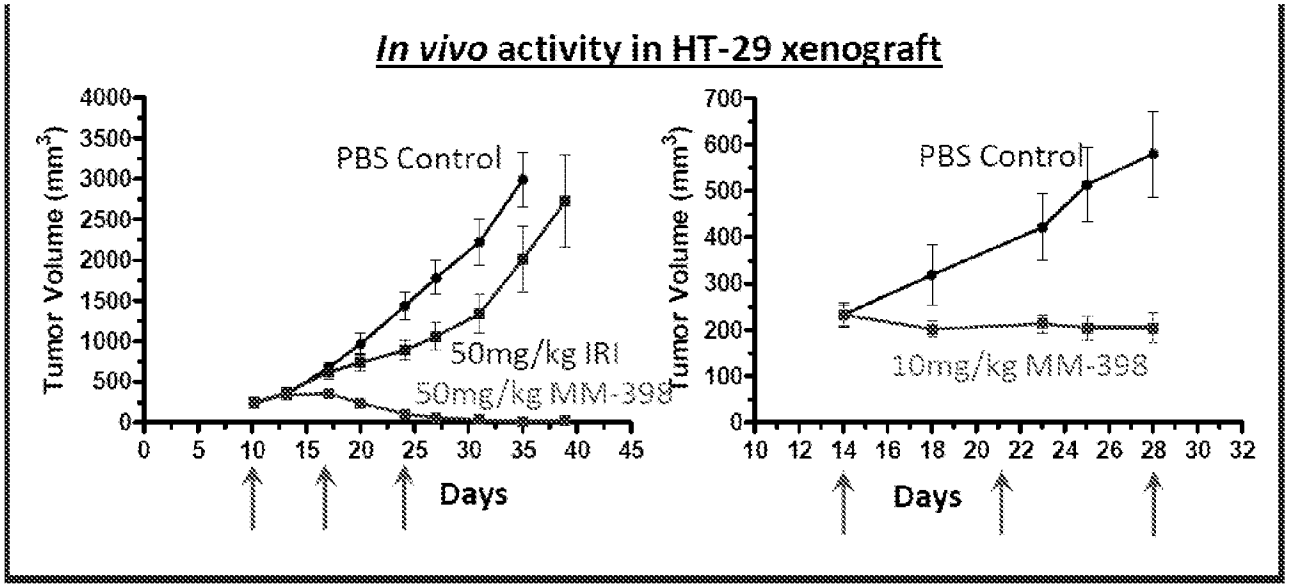
Mechanistic PK model identifies doses of MM-398 and irinotecan (IRI) with equal exposure but not equal activity in mice

- **Mechanistic mouse PK model was trained to describe plasma and tumor exposure of CPT-11 and SN-38 from MM-398 and free irinotecan**
- **Model was developed by using SimBiology® toolbox in MATLAB®**
- **Particle swarm optimization was used to estimate the model parameters**



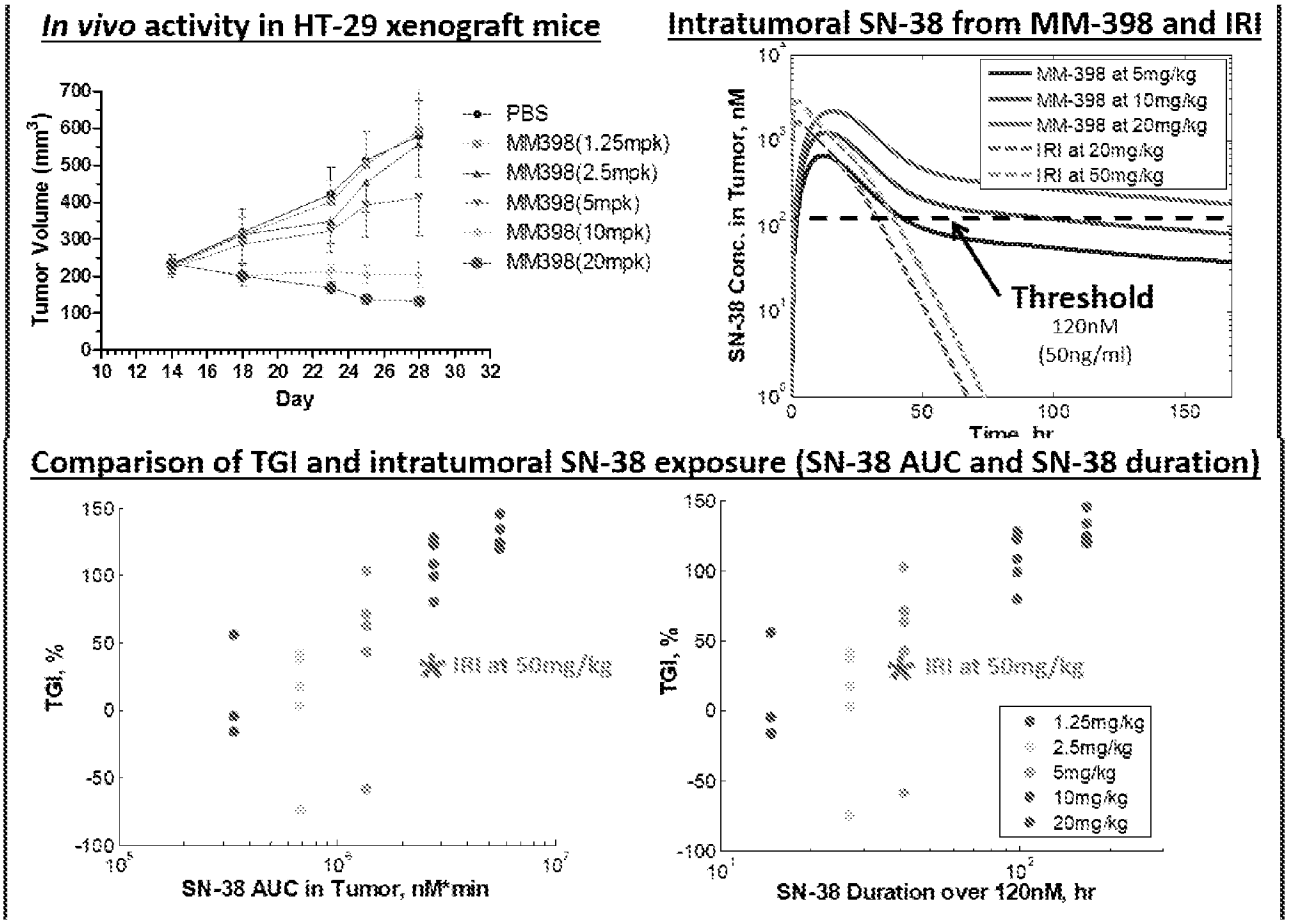
- **PK model identified equal SN-38 exposure (AUC) doses of MM-398 and free irinotecan in plasma and tumor: 10mg/kg MM-398 = 50mg/kg IRI (Irinotecan)**
- **MM-398 showed better tumor growth inhibition in HT-29 xenograft model than IRI with the equal SN-38 exposure dose**



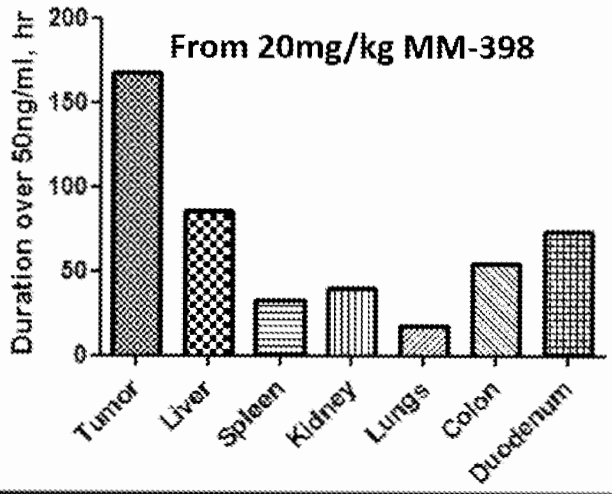


Duration above threshold differentiates MM-398 from irinotecan

- ***In vivo*** **activity data in HT-29 xenografts model suggests that intratumoral SN-38 duration above threshold, not SN-38 AUC correlates with tumor growth inhibition (TGI) from MM-398 and free irinotecan**



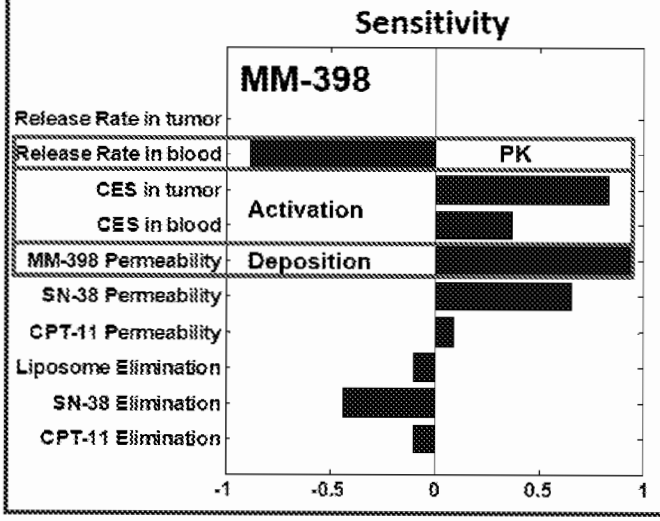
- **SN-38 duration is significantly higher in tumor than in other normal tissues**
- **MM-398 deposition and SN-38 C_{max} are higher in liver, where SN-38 is cleared faster than tumor**

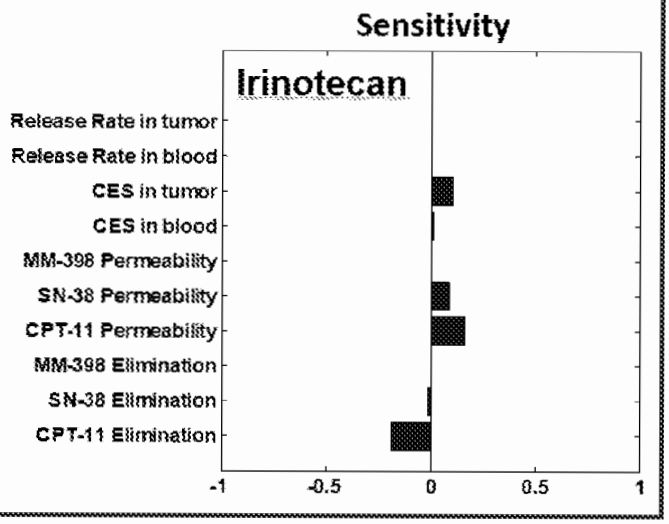


Sensitivity analysis reveals critical parameters for SN-38 duration in tumor

- **Sensitivity was evaluated by using SN-38 duration in tumor as an output variable**
- **Parameters for irinotecan are not sensitive to SN-38 duration in tumor**
- **MM-398 PK, deposition and conversion are important for SN-38 duration**

$$Sensitivity = \frac{\Delta(SN38_{Duration})/SN38_{Duration,0}}{\Delta(p)/p_0}$$

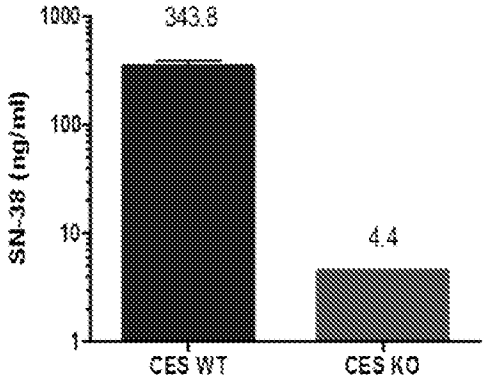




Mechanistic PK model identifies the relative importance of mouse-specific serum CES

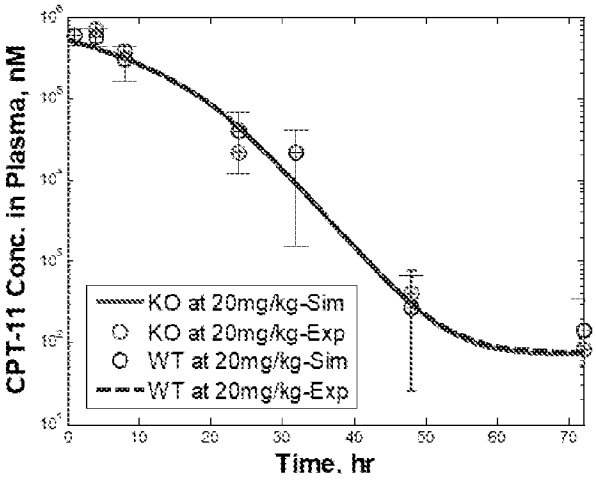
- Mouse has a unique CES in its serum (CESserum) making it difficult to translate the observation from mouse to human
- PK data in CES1c KO mice (no CESserum) could be used to retain the model quantifying the contribution of CESserum to systemic conversion

Serum conversion activity in mice

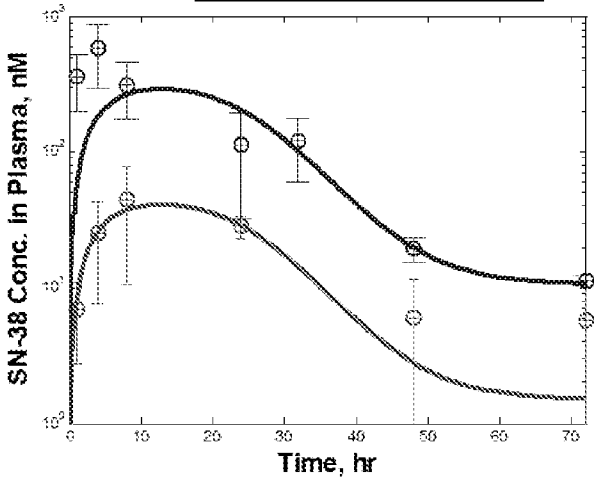


- CPT-11 PK from MM-398 is not affected by CES1c in mice
- KO mice PK data allowed the estimation of serum specific CES activity

CPT-11 PK from MM-398

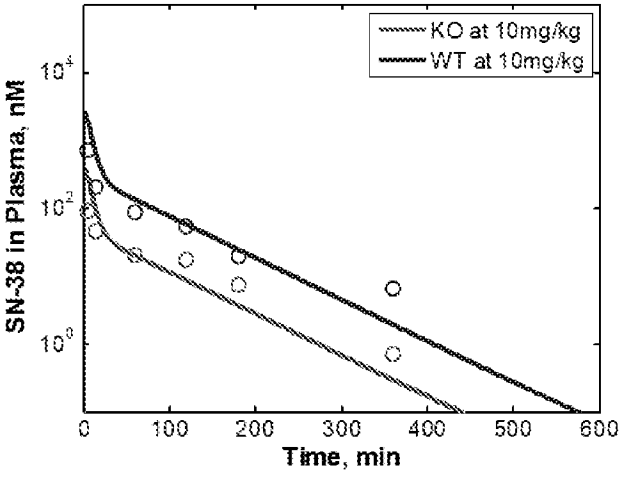


SN-38 PK from MM-398



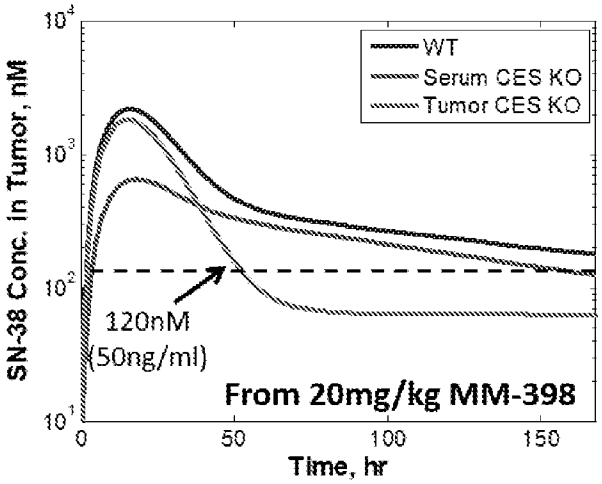
- Plasma-driven activity was responsible for ~86% of total systemic conversion
- Assuming the same contribution, the free CPT-11 mouse PK model was able to predict SN-38 PK in Es1^{e} mice (KD of CES1c)

SN-38 PK from free irinotecan

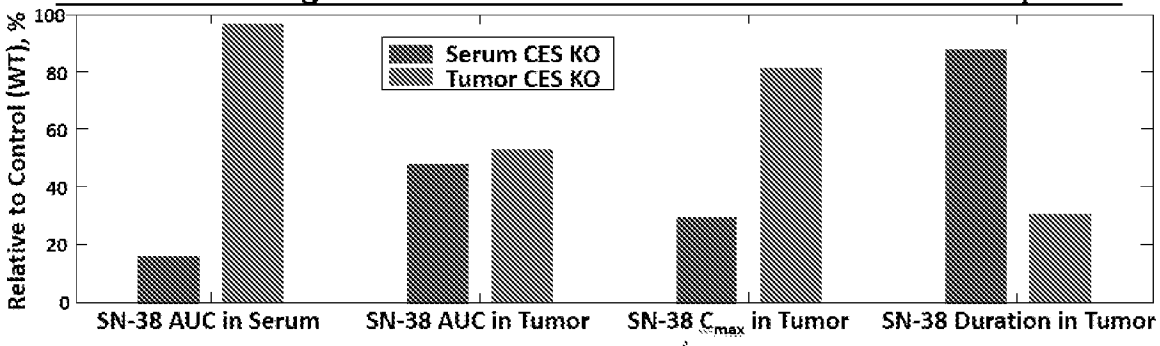


Tumor CES is responsible for longer duration of SN-38 in tumor even in the mouse system with high serum CES

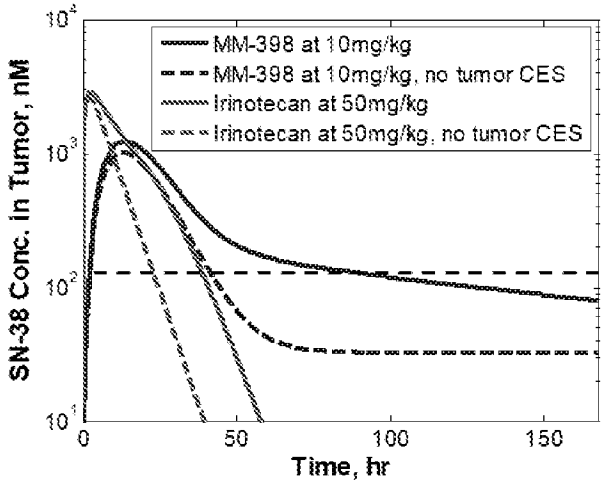
- Knocking out either serum CES or tumor CES decreases SN38 AUC in tumor by 50%
- Serum CES mainly affects SN-38 C_{max}, whereas tumor CES affects SN-38 duration
- Validation experiments have been designed and ongoing



Effect of knocking out either serum CES or tumor CES on SN-38 exposure

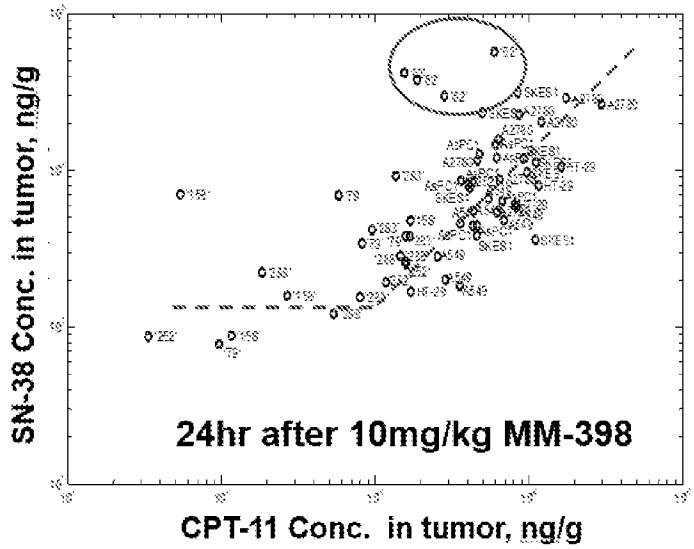


- Local conversion of irinotecan into intratumoral SN-38 is more crucial for *in vivo* activity of MM-398 than irinotecan

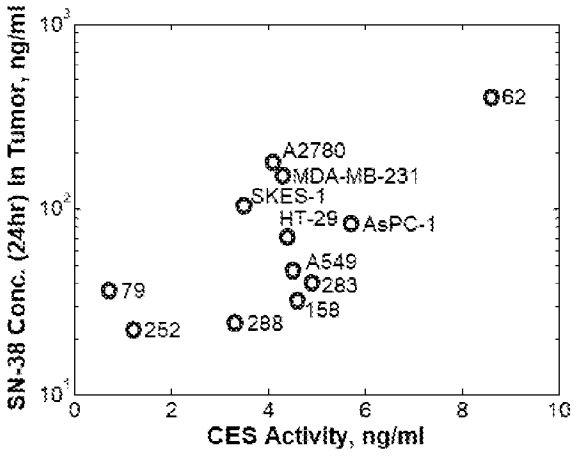


Deposition and activation markers can be used to predict intratumoral SN-38 duration

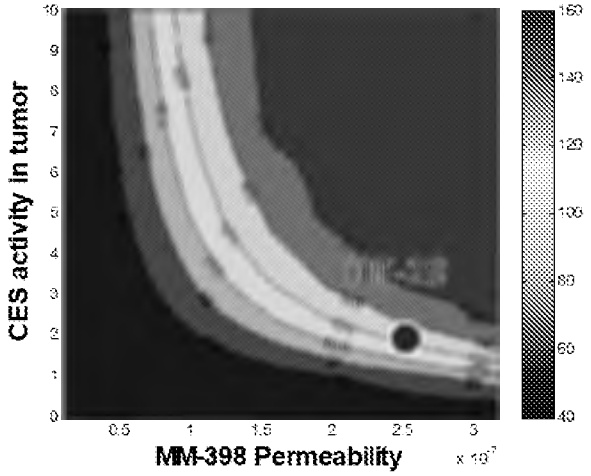
Tumor CPT-11 vs. SN-38 in various xenograft models



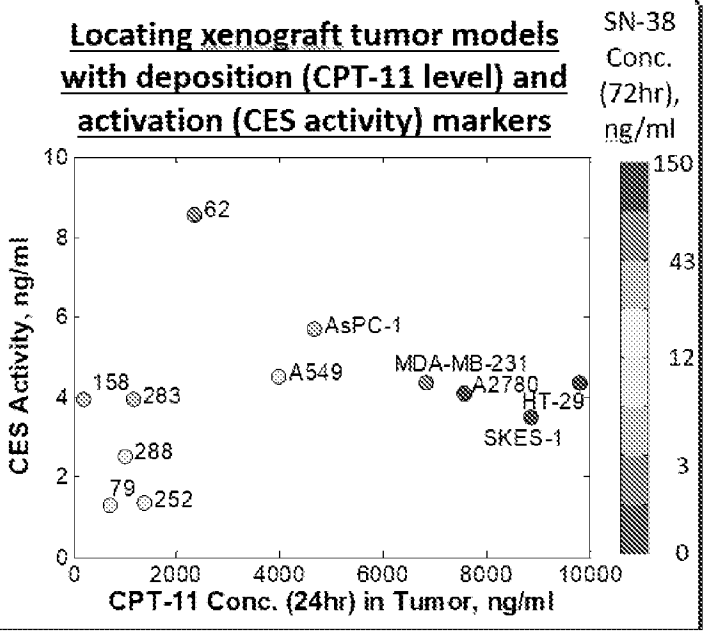
CES activity vs. SN-38 in Tumor



Simulated effect of CES and permeability on SN-38 duration (Color code: SN-38 duration, hr)



Locating xenograft tumor models with deposition (CPT-11 level) and activation (CES activity) markers



Summary

- **SN-38 duration over threshold in tumor accurately predicts the *in vivo* activity from both MM-398 and IRI**
- **Model predicted that the local conversion of irinotecan is the main driver for extended duration of SN-38 in tumor from MM-398**
- **Sensitivity analysis identified tumor CES activity and MM-398 permeability as critical parameters for SN-38 duration in tumor**
- **Drug deposition and tumor CES activity correlated with increased tumor SN-38 levels at the later time point (72hr), a surrogate for SN-38 duration**

Systems pharmacology modeling identifies unique parameters that drive tumor SN38 levels for liposomal irinotecan (MM-398) compared to irinotecan

Jaeyeon Kim*, Ashish Kalra, Milind Chalishazar, Stephan Klinz, Nancy Paz, Jason Cain, Daryl Drummond, Bart Hendriks, Eliel Bayever, Ulrik Nielsen, Jonathan Fitzgerald
Merrimack Pharmaceuticals, Cambridge, MA, USA

MERRIMACK PHARMACEUTICALS

ABSTRACT

MM-398 background

Systems pharmacology approach to identify MOA for MM-398

Mechanistic PK model identifies drivers of MM-398 and irinotecan (IRI) with equal exposure but vast tumor toxicity differences

Duration above threshold differentiates MM-398 from irinotecan

Sensitivity analysis reveals critical parameters for SN-38 duration in tumor

Mechanistic PK model identifies the relative importance of mouse-specific serum CES

Tumor CES is responsible for longer duration of SN-38 in tumor even in the mouse system with high serum CES

Depletion and activation markers can be used to predict tumor SN-38 duration

Summary

Cancer Research

Signaling Pathways

Abstract A6: Sustained intratumoral activation of MM-398 results in superior activity over irinotecan demonstrated by using a systems pharmacology approach

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

DOI: 10.1158/1538-7445.CSB12-A6 Published July 2012

Article | Info & Metrics

Proceedings: AACR Special Conference on Chemical Systems Biology: Assembling and Interrogating Computational Models of the Cancer Cell by Chemical Perturbations--Jun 27-30, 2012; Boston, MA

Abstract

MM-398 is a stable nanotherapeutic encapsulation of the prodrug irinotecan with an extended plasma half-life and higher intratumoral deposition compared with free-irinotecan. MM-398 is currently in multiple clinical trials, including a phase 3 trial for patients with advanced gemcitabine-resistant pancreatic cancer (NAPOLI-1). Pancreatic cancer has been described as being notoriously difficult to treat, potentially due to inadequate drug penetration through the dense stroma, or because the hypoxic tumor microenvironment suppresses cytotoxic activity. We sought to better understand how MM-398, a relatively large (100nm) liposomal nanotherapeutic, could potentially treat pancreatic cancer by determining the relative roles of systemic vs. local tumor activation of irinotecan in contributing to the activity of MM-398.

Using a systems pharmacology approach, we developed a mechanistic pharmacokinetic (PK) model of MM-398 and free-irinotecan to predict both plasma and intratumoral levels of irinotecan and SN-38. The model was trained with PK and biodistribution data from mice bearing HT-29 xenografts, which were administered intravenously with varying doses of MM-398 or free-irinotecan. Model simulations predicted that MM-398 resulted in equivalent SN-38 exposure (area under curve, AUC) in tumor at a fivefold lower dose than free-irinotecan. However, an in vivo animal activity study showed that 15-fold lower dose of MM-398 was sufficient to yield equal growth inhibition of HT-29 xenografts, which reveals the limit of relating simple AUC-based exposure to in vivo tumor response. While intratumoral SN-38 exposure

from free-irinotecan was limited to the first 48 hours after dosing, MM-398 maintained high levels of SN-38 throughout the week-long time window. Further analysis of the exposure-response identified that the duration of intratumoral SN-38 levels above the threshold was a valid predictive marker for xenograft tumor response.

Identifying the source of intratumoral SN38 is confounded by the fact that the mouse species has an additional carboxylesterase (CES) that can convert irinotecan to SN-38 in serum. The serum SN-38/irinotecan ratio in mice is tenfold higher than that observed in humans. In order to translate this preclinical observation into the clinic, it is critical to identify the role of mouse-specific serum CES on intratumoral SN-38 exposure. Thus, we performed a PK study with knockout mice lacking the Ces1c gene, which encodes serum CES, and then retrained our mechanistic PK model. Serum SN-38 levels in the Ces1c knockout mice were measurably decreased by ˜85% in the central compartment. In contrast, simulating the effect of knock-out of either serum CES or tumor CES, predicts that the duration of intratumoral residence of SN-38 is significantly affected by tumor CES, rather than serum CES. This suggests that local activation to SN-38 by tumor CES as the main driver for SN-38 tumor residence, which in turn drives response.

In summary, we applied a systems pharmacology approach to identify the importance of tumor CES (local SN-38 generation) as one of the determinants of MM-398 response. Liposomal encapsulation of irinotecan dramatically alters the pharmacokinetic profile of SN-38 in the tumor, as well as tumor response, by maintaining SN-38 levels above the response threshold. Local, sustained activity of this active irinotecan metabolite could result in prolonged cytotoxic and tumor microenvironment modifications with beneficial effects on treatment of pancreatic cancer and other solid tumors.

Citation Format: Jaeyeon Kim, Eliel Bayever, Peter Laivins, Clet Niyikiza, Ulrik Nielsen, Jonathan Fitzgerald, Ashish Kalra, Milind Chalishazar, Stephan Klinz, Nancy Paz, Bart Hendriks, Daryl Drummond, Dmitri Kirpotin, Victor Moyo. Sustained intratumoral activation of MM-398 results in superior activity over irinotecan demonstrated by using a systems pharmacology approach [abstract]. In: Proceedings of the AACR Special Conference on Chemical Systems Biology: Assembling and Interrogating Computational Models of the Cancer Cell by Chemical Perturbations; 2012 Jun 27-30; Boston, MA. Philadelphia (PA): AACR; Cancer Res 2012;72(13 Suppl):Abstract nr A6.

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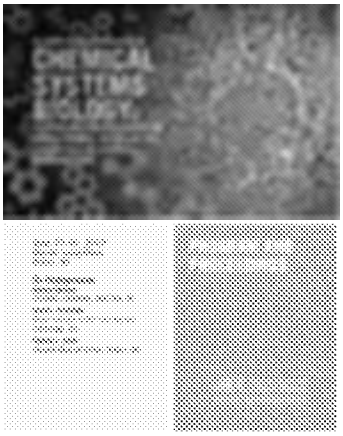

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CHAPTER SEVEN

Building and Characterizing Antibody-Targeted Lipidic Nanotherapeutics

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Abstract

Immunoliposomes provide a complementary, and in many instances advantageous, drug delivery strategy to antibody-drug conjugates. Their high carrying capacity of 20,000–150,000 drug molecules/liposome, allows for the use of a significantly broader range of moderate-to-high potency small molecule drugs when compared to the comparably few subnanomolar potency maytansinoid- and auristatin-based immunoconjugates. The multivalent display of 5–100 antibody fragments/liposome results in an avidity effect that can make use of even moderate affinity antibodies, as well as a cross-linking of cell surface receptors to induce the internalization required for intracellular drug release and subsequent activity. The underlying liposomal drug must be effectively engineered for long circulating pharmacokinetics and stable *in vivo* drug retention in order to allow for the drug to be efficiently delivered to the target tissue and take advantage of the site-specific bioavailability provided for by the targeting arm. In this chapter, we describe the rationale for engineering stable immunoliposome-based therapeutics, methods required for preparation of immunoliposomes, as well as for their physicochemical and *in vivo* characterization.

1. Introduction

A liposome is a vesicle having one or more lipid bilayers enclosing an aqueous interior. As used herein, "immunoliposome" means a liposome that has antibody molecules displayed on its outer surface. Among various uses of immunoliposomes, the focus of this chapter is the use of immunoliposomes for the targeted delivery of pharmaceuticals. Immunoliposomes targeted to cancer-overexpressing cell surface epitopes such as ErbB2 (Kirpotin *et al.*, 2006; Nielsen *et al.*, 2002; Park *et al.*, 2002), EGFR (Mamot *et al.*, 2005), GD2 (Pagnan *et al.*, 2000; Raffaghello *et al.*, 2003), and CD19 (Sapra and Allen, 2002) have been shown to improve the efficacy of encapsulated therapeutics. The improved efficacy for vascularly accessible targets, such as in hematologic cancers or on the surface of angiogenic blood vessels supporting the tumors results in part from an altered deposition and increased accumulation at the target. However, for solid tumors, we have shown that gross distribution of immunoliposomes to the tumor is not significantly affected by molecular targeting (Kirpotin *et al.*, 2006), and thus is limited by the extravasation of liposomes across the vasculature and into the tumor. The improved efficacy in solid tumors likely results from the differential microdistribution of immunoliposomes within the tumor, including internalization into cancer cells (Kirpotin *et al.*, 2006) where they can be processed by intracellular enzymes. Indeed, there have now been multiple studies demonstrating the requirement for internalization on improved anticancer

activity (Kirpotin *et al.*, 2006; Noble *et al.*, 2004; Park *et al.*, 2002; Sapra and Allen, 2002; Sugano *et al.*, 2000).

Antibody-targeted lipidic nanocarriers, such as immunoliposomes, offer multiple levels of targeting and significant flexibility in the specific delivery of entrapped therapeutic agents. This flexibility is essential in adapting the immunoliposome for use with a specific drug, different routes of administration, or specific application. The various components or characteristics of the lipidic nanocarrier that can be modified are shown in Fig. 7.1. The lipid matrix forms the membrane barrier that protects the entrapped drug from premature interaction with the biological milieu, allowing the drug to take on the pharmacokinetic and distribution characteristics of the carrier when properly designed. The lipid composition can vary in membrane fluidity at physiological temperature, surface charge density, cholesterol content, and degree of pegylation. The relative importance and complex interdependence of these various components is the subject of multiple reviews (Allen *et al.*, 2006; Drummond *et al.*, 1999, 2008) and will not be discussed further here.

Gradient-based drug-loading methods for weakly basic small molecule drugs (Drummond *et al.*, 2006, 2008; Fenske and Cullis, 2005; Haran *et al.*, 1993) and condensation with lipophilic amines of complex nucleic acid-based therapeutics (Hayes *et al.*, 2006; Pagnan *et al.*, 2000) can provide for highly stabilized lipidic nanocarrier formulations that resist premature release of the entrapped drug while in the general circulation, an important property for immunotargeted formulations. Unlike small molecule immunoconjugates where the drug is covalently conjugated directly to the targeting antibody, in immunoliposomes, the antibody-targeting ligand and therapeutic are associated indirectly through the carrier (Fig. 7.1; Noble *et al.*, 2004). Thus, drug retention in the circulation is critical to ensure that the drug is able to take advantage of molecular targeting upon reaching the tumor. We will describe in this chapter an exemplary method for encapsulating and stabilizing some more challenging weakly basic small molecule drugs.

Antibody-targeting ligands provide the specificity for increasing delivery to target tissue either at a tissue or cellular level. Significant engineering must be applied to ensure target specificity, lack of immunogenicity, accessibility, and conjugate and particle uniformity (Noble *et al.*, 2004; Sapra *et al.*, 2005). The ability to induce internalization is a key attribute of antibody-targeting ligands used in the construction of immunoliposomes (Kirpotin *et al.*, 1997, 2006; Sapra and Allen, 2002). Internalization provides access to intracellular enzymes and acidic pH that can be used to release the entrapped therapeutic from the confines of the carrier, thus improving bioavailability. Some of the methods for selecting internalizing antibodies, conjugating them to lipopolymer anchors, and incorporating them into liposomes will be described in this chapter.

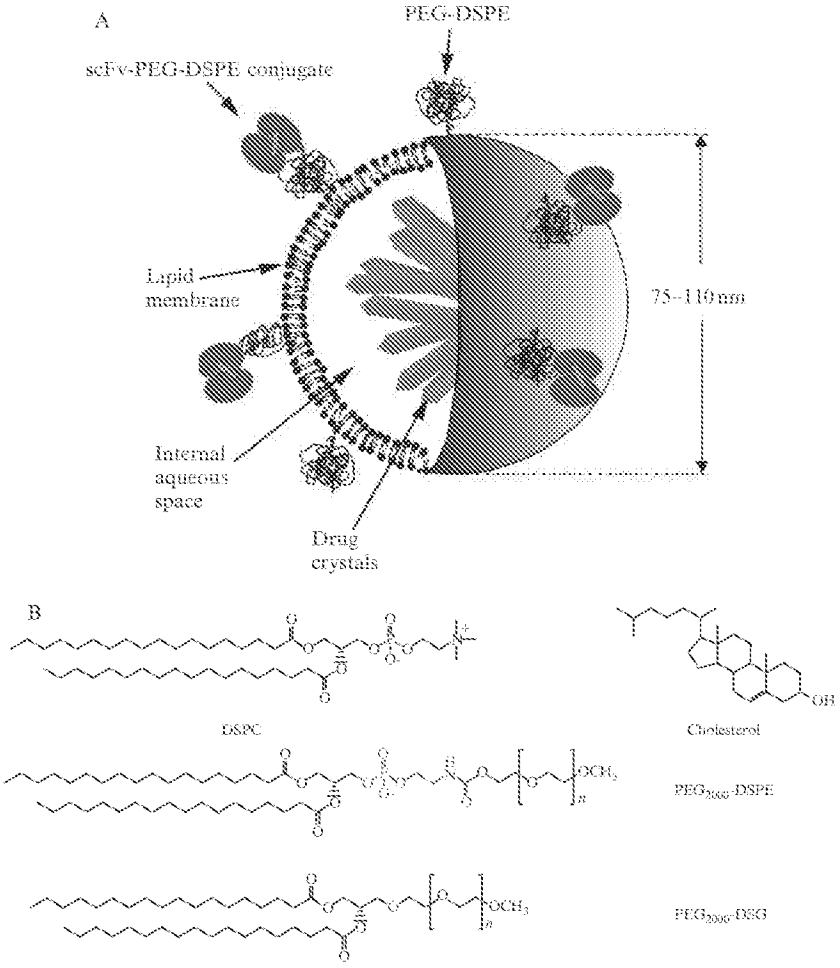

Figure 7.1 (A) Suggested design of a therapeutic immunoliposome. The lipids form a vesicle 75–120 nm in diameter, having a single bilayer membrane that encloses internal aqueous space. The drug is stably encapsulated within the internal space in a precipitated form. The membrane surfaces are grafted with the hydrophilic polymer polyethylene glycol (PEG) by inclusion of a lipopolymer PEG-DSPE. Some PEG chains are conjugated to the antigen-binding antibody fragment (e.g., scFv) at their distal termini. (B) Structures of the lipid components: DSPC, distearoylphosphatidylcholine; Chol, cholesterol; PEG_{2000}-DSPE, poly(ethylene glycol)-derivatized phosphatidylethanolamine ($n = 45$); PEG_{2000}-DSG, poly(ethylene glycol)-derivatized 1,2-distearoylglycerol ($n = 45$). Conjugation to distearoylglycerol through an ether bond results in a neutral charge at the membrane interface, whereas conjugation through ethanolamine results in a negatively charged phosphate at the interface.

2. Preparation of Immunoliposomes

There are a series of steps required in the preparation of immunoliposomes. In our preferred process, the underlying highly stable liposomal drug formulation is prepared independently from an antibody-lipopolymer conjugate, followed by incorporation of the conjugate into the liposomal drug to form the final immunoliposome. The initial step in producing stable liposomes is the preparation of the intracellular drug-loading aid to facilitate the loading and subsequent *in vivo* stabilization of the drug. Liposomes are then formed through a combination of lipid hydration, sizing, and gradient generation by removal of the external drug-loading aid. The drug is encapsulated at high efficiency, typically >95%, utilizing an electrochemical gradient. Internalizing and highly selective antibodies are identified using high throughput screening methods and subsequently engineered to have a single C-terminal cysteine. Antibody conjugates manufactured separately are prepared through reaction of the reactive thiol on this C-terminal cysteine with a maleimide-terminated lipopolymer. The resulting conjugate is finally inserted into the outer monolayer of the liposomes, converting a previously inert liposomal drug into an active immunoliposome. The detailed steps involved in the preparation of an exemplary liposomal drug are given below.

2.1. Drug-loading aid preparation

A variety of salts can be used to aid remote loading of drugs into liposomes. The method of preparation for each salt solution depends on commercial availability. Many of the salts that are commonly used for remote-loading such as manganese sulfate, ammonium sulfate, ammonium citrate, and citric acid are commercially available and can be prepared by simply dissolving in water. If the loading agent is not available with the desired counterion, but is supplied in the acidic form, a solution can be formed and simply titrated with the corresponding amine. Often the agent is supplied with an alternate counterion such as Na^+, K^+, Ca^{2+}, or Mg^{2+} due to ease of preparation or stability concerns of acids; this is common with poly- or oligoanionic compounds. Our lab regularly uses sucrose octasulfate, which is typically supplied as the sodium salt, as the intraliposomal trapping agent. A weak binding di- or tri-alkylammonium salt serves as the final drug-loading aid, and is formed through a combination of ion-exchange chromatography (IEX) and subsequent titration with the appropriate amine (Fig. 7.2). An ion exchange column (e.g., Dowex 50Wx8-200, Dow Chemical Co.) is used to acidify the sucrose octasulfate. The column is first conditioned by treatment

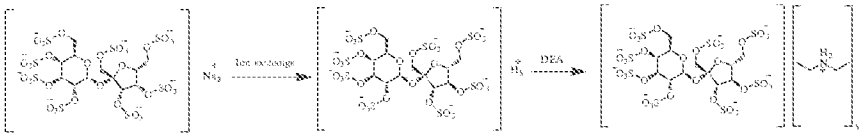

Figure 7.2 Production of diethylammonium sucrose octasulfate. Sodium sucrose octasulfate is brought into free acid form by ion exchange on Dowex 50Wx8-200 resin in hydrogen form; sucrose octasulfuric acid is neutralized with neat diethylamine to pH 6–7.

with 2 vols. of 1 N NaOH, followed by 2 vols. of ddH_2O, and finally 3 vols. of 3 N HCl. In order to avoid excessive dilution, the sodium salt of sucrose octasulfate is added to the column at maximum solubility, and elution is monitored using an inline conductivity meter to allow for batching of high concentration sucrose octasulfate fractions.

The acidic form of the agent should be quickly titrated with the appropriate weakly basic amine as degradation is often associated with extended time at low pH. Upon titration, the charge of the anionic loading agent is now countered with the positive charge of a protonated amine. The basic amines our group prefers are di- or trialkyl-substituted amines, including triethylamine, diethylamine, and 2-diethylaminoethanol. The resulting solution should be characterized to ensure complete exchange of the initial cationic species; the use of an ion-specific electrode is a relatively straightforward technique. Additional pharmaceutically acceptable substituted ammonium salts are reviewed in Stahl and Wermuth (2002). Regardless of the method used for preparing the loading agent, the pH for the solution should be adjusted near 6.5 to minimize hydrolysis of the liposomal lipids during the process of liposome formation. A variety of factors should determine the concentration of the drug-loading agent. The drug-loading capacity is generally proportional to the concentration of the drug-loading agent. However, as the osmolality increases with concentration, care must be taken to avoid creating an excessive osmotic gradient across the liposome bilayer that can result in rupture of the liposome during drug loading or in the presence of plasma (Mui *et al.*, 1993, 1994).

2.2. Lipid hydration and liposome sizing using extrusion

The formation of liposomes can be accomplished through a range of methods. One commonality in preparing liposomes is the hydration of the lipids in an aqueous solution. The lipids can be hydrated from a solid or from an organic solution. Hydration of the lipids from a solid is more thorough if the mixture is repeatedly frozen and thawed which forces water in between the lipid membranes. If the lipids are hydrated from a solid, and more than one lipid is used, the solid lipids can be initially dissolved in an organic

solvent (e.g., chloroform/methanol, 9:1, vol:vol) to ensure proper mixing of the different lipids, followed by solvent removal through rotary evaporation and lyophilization. Ethanol injection involves hydration by mixing an ethanol solution of lipids with the aqueous hydrating buffer. Most lipids will require heating the ethanol to completely dissolve the lipids, in which case the aqueous buffer should be heated to the same temperature before mixing. Our lab prefers to use the ethanol injection method for hydrating lipids as this method eliminates the need for toxic solvents, and results in the formation of large (~500 nm) multilamellar vesicles rather than significantly larger multilamellar structures.

The formation of small (50–150 nm) unilamellar vesicles (liposomes) requires additional processing of the hydrated lipid sheets or large multilamellar vesicles to reach the appropriate size. Careful control over particle size is a critical parameter for immunoliposomes, as it affects clearance rates from the circulation, extravasation rates into solid tumors and efflux rates from the same tumors, drug encapsulation and retention, and filterability required for terminal sterilization. The two most common procedures for sizing liposomes are sonication and extrusion through defined pore membranes, such as polycarbonate membranes manufactured using track-etching technology (Nuclepore(R), Whatman, USA). Unlike common depth filters, the membrane-spanning channels in polycarbonate track-etched filters are round, smooth-walled, and of well-defined, uniform diameter (Fig. 7.3). Generally the liposomes are formed by extrusion through polycarbonate membranes having a pore size of 80–100 nm with a total of 10–13 passes through the pores, with the average liposome diameter and polydispersity being reduced with each pass. The solution can either be passed through single membrane multiple times, or through a stack of membranes using a lower number of passes. Stacked membranes will typically require more pressure to complete the extrusion process. The temperature of the extruded solution should be maintained above the phase transition of the particular lipid mixture comprising the liposomes, which for many lipid compositions requires a heat-jacketed extruder at 60–70 °C.

2.3. Gradient generation and drug encapsulation

Active loading of the drug into the liposomes requires removal of the loading aid from the liposome exterior in order to generate a gradient across the liposome membrane (Fig. 7.4). The extraliposomal loading agent can be removed using size-exclusion chromatography (SEC), dialysis, IEX, or a combination of these approaches. SEC and dialysis techniques take advantage of the considerable size difference between the liposomes and the loading agents. Size discriminating separation methods are less effective with large polymeric drug-loading agents and the utility of ion exchange is more pronounced. Sucrose octasulfate, having eight negative charges and

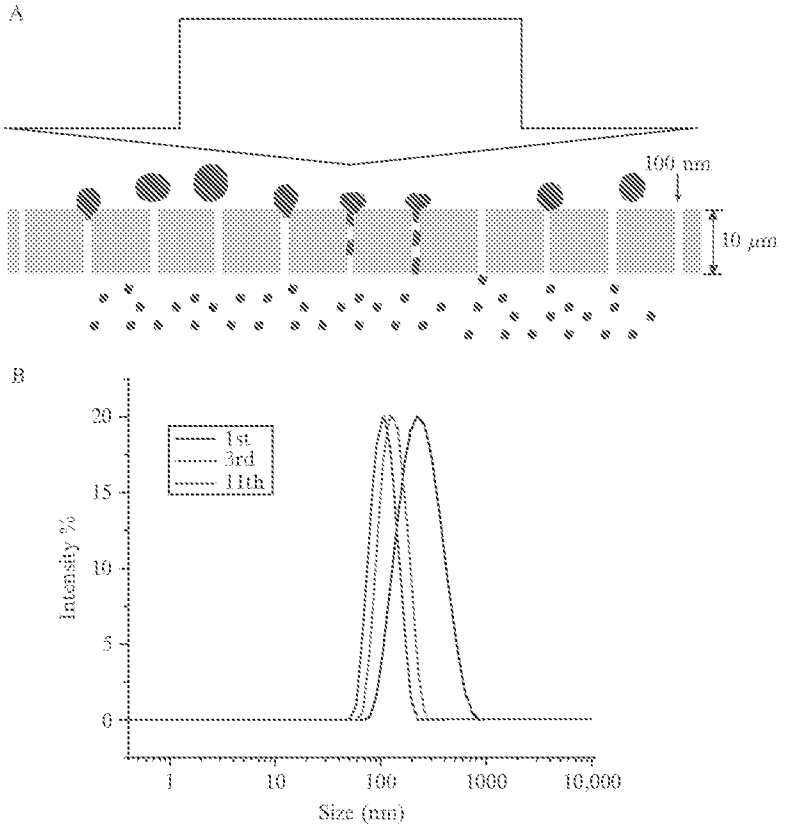

Figure 7.3 Forming unilamellar liposomes of the defined size by extrusion through a defined pore (track-etched) membrane filter. (A) Schematic representation of the extrusion process. Multilamellar liposomes (top) are fragmented into smaller, unilamellar vesicles (bottom) by forcing them through the pores under pressure. (B) Uniformity of size is reached by repeated passages of the liposomes through the membrane. Scattering intensity-based size distribution of the liposomes obtained after the passage number shown at the legend.

a larger hydrodynamic radius than sulfate, presents some challenge for separation by size exclusion or dialysis separation when compared to more common salts such as ammonium sulfate or citric acid. We typically use a Sepharose CL-2B or CL-4B column when employing SEC and a 500-kDa molecular weight cutoff pore membrane for diafiltration. Diafiltration, particularly hollow fiber tangential flow filtration (TFF), is very practical for larger scale separations. The solution used for replacing the loading agent should contain both an isotonicity agent (sucrose, dextrose, saline) and an appropriate buffer. The final osmolality (300–600 mmol/kg for most applications) of the

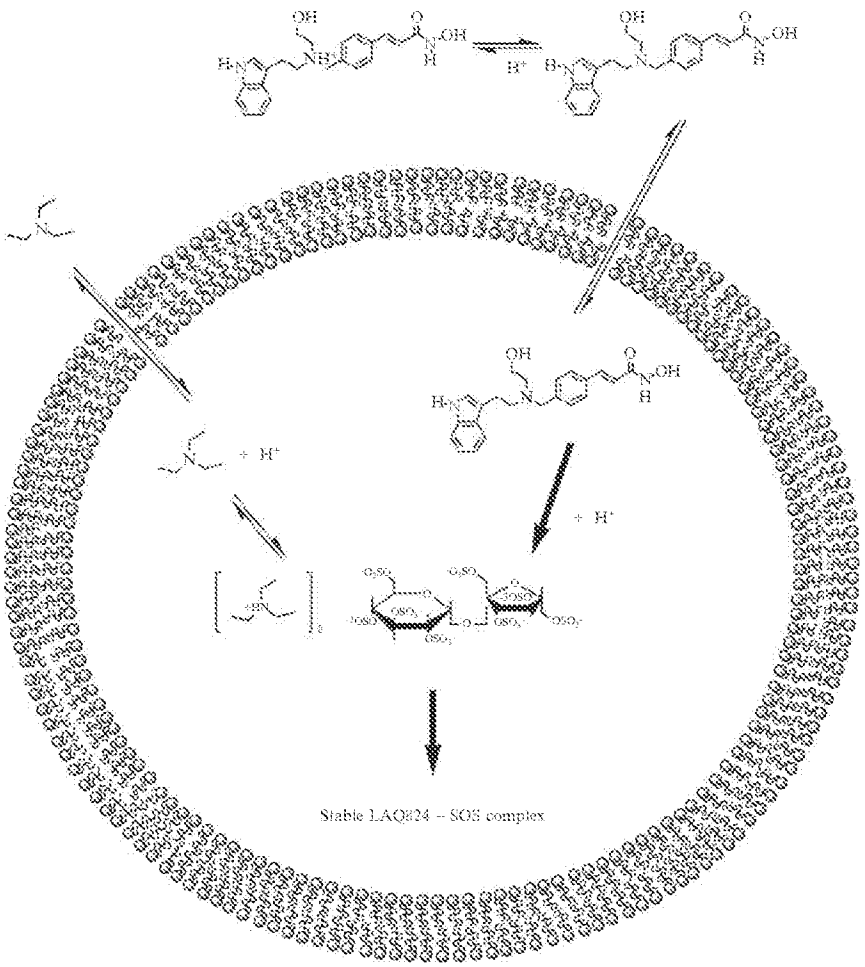

Figure 7.4 Stable loading of a weakly basic substance into the liposome assisted by a small molecule polyanion with high charge density. A weakly basic HDAC inhibitor, LAQ824, in its neutral form crosses the liposome membrane, becomes protonated, and tightly binds to sucrose octasulfate anion (SOS^{8-}) forming a poorly soluble salt deposited as a gel or a precipitate within the liposome. Deprotonated triethylammonium (triethylamine) is membrane-permeable and leaves the liposome maintaining the balance of charge.

extraliposomal solution should be in a range that will not cause the liposomes to burst when the temperature is raised above the lipids' phase transition temperature during drug loading. The solubility of the drug being loaded should be considered when choosing the isotonicity agent, as some drugs will have low solubility in ionic salts. The pH of the liposome solution depends upon the optimum for loading each specific drug.

Drug loading is accomplished by introducing the free unencapsulated drug to the liposomes at temperature above the phase transition temperature of the lipids. The drug and lipid solutions can be heated before or after mixing depending on the particular situation. The loading time, temperature, pH, and drug to lipid ratio (drug payload) are all factors that can be optimized in an attempt to determine the most efficient drug-loading protocol. Our lab has determined that general conditions including a pH between 5.0 and 7.0, time of 30 min, and temperature of 60–65 °C results in efficient loading for most of the more common liposomal cancer agents including camptothecins, anthracyclines, vinca alkaloids, and a weakly basic HDAC inhibitor (Drummond *et al.*, 2005b, 2009, 2010; Mamot *et al.*, 2005; Noble *et al.*, 2009). Cooling the solution will stop the loading process. Similar to the drug-loading agent, removal of the unencapsulated drug from the liposomes can be accomplished using SEC, dialysis, IEX, or a combination of these approaches. Highly efficient drug loading can mitigate the requirement for removal of the unencapsulated drug.

2.4. Antibody selection

There are multiple requirements of the antibody component, including specificity, lack of immunogenicity, binding affinity, and ability to induce internalization (Noble *et al.*, 2004; Sapra and Allen, 2003). We have focused on antibody fragments selected from fully human antibody libraries to eliminate Fc-receptor-mediated liposome clearance resulting from conjugation of full IgG molecules (Harding *et al.*, 1997; Noble *et al.*, 2004) and the generation of an immune response that would compromise multiple administrations if using nonhuman antibodies. Phage- or yeast-display libraries have been used to screen for specific, tight binding, and highly internalizing antibodies (Becerril *et al.*, 1999; Noble *et al.*, 2004; Poul *et al.*, 2000; Zhou and Marks, 2009; Zhou *et al.*, 2010). Selection directly on cell lines has allowed us to screen antibodies directly for their ability to induce internalization. In this screening protocol, the library is first depleted on control cells to deplete the library of nonspecific binders. The depleted library is then incubated with cancer cells overexpressing the receptor of interest, followed by washing to remove nonbinders, incubation at 37 °C to allow for receptor-mediated endocytosis, and stripping of cell surface-bound phage from the surface using multiple low pH (i.e., 2.5) glycine washes. The cells are then trypsinized and lysed using 100 m*M* triethylamine (with subsequent neutralization using 0.33 *M* tris) to recover the internalizing phage, and finally amplified for additional rounds of selection. Typically three rounds of selection are performed.

Antibody panels that were previously assembled based on selection for binding can also later be screened for internalization using a CHElated Ligand-induced Internalization Assay (CHELIA) that detects the amount

of immunoliposomes on the cell surface and/or inside the cell (Fig. 7.5; Nielsen *et al.*, 2006). This assay has the advantage of measuring the internalization potential of antibodies in the format (i.e., immunoliposomes) that they are too eventually to be used in, as well as use with nonpurified and low concentrations of antibodies, and avoids the necessity for preparing stable immunoconjugates of a large panel of prospective antibodies. In this method, hexahistidine (His_6)-tagged antibodies are conjugated to fluorescent liposomes through a Ni(II)-activated nitrilotriacetic (NTA) lipid (Huang *et al.*, 2006, 2009; Nielsen *et al.*, 2006). These liposomes are then

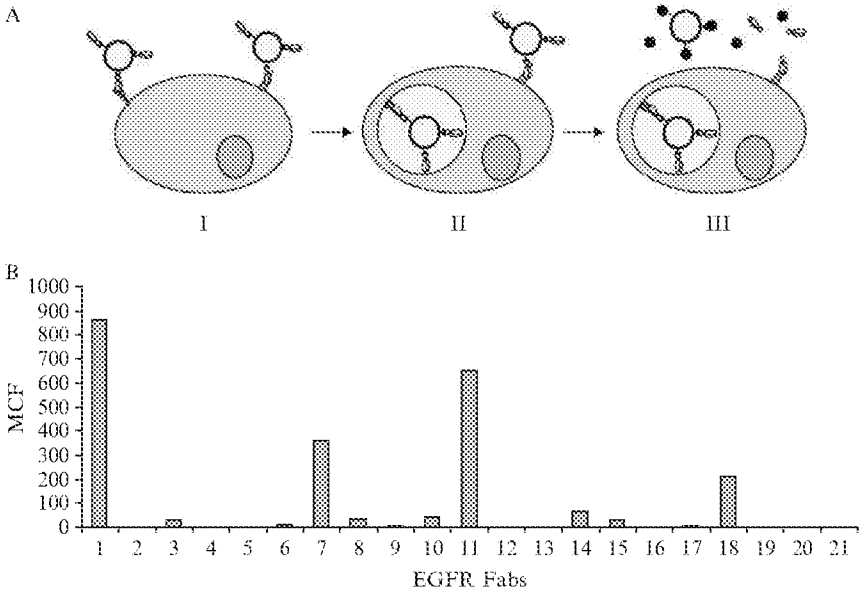

Figure 7.5 (A) Chelated liposome-antibody (CHELIA) assay for liposome-internalizing antibody ligands. I. Fluorescently labeled Ni-NTA-immunoliposomes, liposomes bearing antibodies attached via His-tag, are panned on the live cells and allowed to adsorb on the antigens on the cell surface. II. The cells are incubated at 37 °C to allow internalization of the liposomes linked to internalizable antibodies: at this point, the total uptake of the liposomes (surface-bound and internalized) can be quantified, for example, by flow cytometry. III. The cells are treated with imidazole or EDTA to dissociate His-tag—Ni-NTA bond of the extracellular immunoliposomes, removing them from the cells. Now the flow cytometry assay will quantify only the cells with internalized liposomes. (B) Using CHELIA assay to screen anti-EGFR Fabs according to their quality as targeting ligands for EGFR-targeted immunoliposomes. Twenty-one clone of His-tagged Fabs were produced by the display library selection for the binding to EGFR ECD and panned over A431 cells in the presence of the fluorescently labeled PEGylated liposomes containing Ni-NTA-PEG-DSPE (1 mol% of the phospholipid). Relative cellular uptake of the liposomes across different clones was assessed by flow cytometry. MFS—mean cell fluorescence (in arbitrary units).

added to cells and allowed to internalize for 2–4 h, after which cell-associated fluorescence is measured either before or after disassociation of cell surface-bound antibodies with an imidazole buffer to disrupt the His_6–NTA bond. The fluorescence of the pre-imidazole wash provides a measurement of total cell-associated liposomes, and post-imidazole wash, a measurement of internalized liposomes. Care should be taken in not overinterpreting the results. Because the screening is typically completed at one or two concentrations of antibodies, there is the chance that the optimum antibody density on the liposome surface is not utilized during the screening for many of the antibodies. Thus, this initial screen is simply used as a quick screen to identify potential hits that can be further studied as covalent conjugates. An example of this is shown in Fig. 7.5B where a panel of 21 antibodies was screened using the CHELIA protocol, with four hits being identified for further characterization. We also typically perform this screening on a series of four cell lines overexpressing the receptor and one to two cell lines with low levels of the receptor to help ensure that the selected antibodies have broad applicability and specificity.

We have previously used monovalent NTA-derivatized lipids (Nielsen *et al.*, 2006) but have recently moved to the use of novel trivalent-NTA lipids developed by Huang *et al.* (2006, 2009) due to the three orders of magnitude higher equilibrium dissociation constant (K_d) for His_6 and mono-NTA when compared to the tris-NTA lipid (10 μ*M* vs. 10 n*M*). The lipophilic fluorescent dye DiIC18(5)-DS is incorporated into the membrane of the liposomes at a concentration of 0.3 mol% and has excitation and emission wavelengths of 650 and 670 nm, respectively. The tris-NTA lipid is incorporated at a concentration of 0.5 mol%. A typical lipid composition for the CHELIA-detectable liposomes would also include hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and PEG-DSPE in a 3:2:0.015, mol:mol:mol ratio.

For the screening protocol, a solution containing the NTA liposomes (50 μ*M* PL), 10–50 μg Fab′/ml, and 50 μ*M* $NiSO_4$ is freshly prepared. Four cell lines known to overexpress the receptor of interest and two control cell lines are trypsinized, counted, and aliquoted at a concentration of 1×10^5 cells/well and 100 μl in polypropylene V-bottom-shaped 96-well plates. The cells are pelleted by centrifugation at 1200 rpm for 5 min and at 4 °C, and the media subsequently removed by aspiration and replaced with 100 μl of the above immunoliposomes. Control Ni^{2+}–NTA liposomes without antibody are also added as a control. The plates are then incubated on a shaker at 37 °C and 5% CO_2 for 2–4 h. For one set of cells, the unassociated material is removed by washing with nondisassociating phosphate-buffered saline and the cell-associated fluorescence determined by flow cytometry using the FL4 channel (FACSCalibur, Becton Dickinson, USA). For a second set of cells, the cells are washed with 250 m*M* imidazole buffer post liposome incubation and the cell-associated fluorescence determined

by flow cytometry as a measure of the total cell-internalized immunoliposomes. For screens where material is limited, only internalized samples can be measured (Fig. 7.5B). However, measuring both total and internalized provides a more detailed understanding of both the extent and efficiency of internalization.

2.5. Antibody fragment generation and conjugation

The bond between the liposome and the antibody molecule is the crucial element of the immunoliposome design. In order to be effective as a vehicle for antibody-targeted delivery of its payload, the liposome-antibody bond must fulfill a number of requirements: (1) Stability over the period of time from making the immunoliposome and its interaction with the target cell or tissue, including stability during storage in the vial and while *in vivo* in the circulation, (2) compatibility with the liposome payload, (3) minimal effects on the antigen-binding properties of the antibody on the liposome, including the effects from any polymer coating, (4) minimal effects on the pharmacokinetics and non-target-related pharmacodynamics, (5) Ease, control, and reproducibility of the antibody-liposome conjugation process, including lot-to-lot consistency, and industrial scalability, and (6) the potential for multiple antibodies/specificities on the same particle.

One particular strategy seems to have met the above criteria well and was adopted by the authors. This strategy, outlined in Fig. 7.1A, also known as "micellar insertion," involves the following steps: First, producing the antibody molecule that has a unique conjugation site for attachment of the linker group that will link the antibody to the surface of the liposome. For example, one very convenient conjugation site is created by a thiol group of cysteine residue exposed at the C-terminus of the antibody polypeptide chain. It may be engineered into the protein sequence by recombinant methods, or proteolytically generated. The antibody in this context is any polypeptide carrying antibody-binding domain(s) of an immunoglobulin molecule, such as Fab′, Fv, or scFv. While we focus on the antibody ligands, it is clear that any protein ligand can be used. The second step is producing an amphiphilic linker molecule that includes a hydrophobic domain, such as a lipid moiety that includes one or more hydrocarbon chains or a sterol, and a hydrophilic spacer arm attached to the hydrophobic domain, typically a flexible, hydrated polymer, such as poly (ethylene glycol) (PEG). On the terminus, contralateral to the hydrophobic domain, the spacer arm bears a functional group reactive to the conjugation site on the protein molecule.

A third step includes forming a conjugate between the antibody protein and the linker molecule in an aqueous solution. Due to self-association of the hydrophobic domains and relatively large size of the hydrophilic portions of its molecule, the linker–antibody conjugate remains in solution in the form of micelles and can be purified from the unconjugated protein at this time.

Finally, incubating the liposome with the micellar antibody–linker conjugate, during which step the conjugate molecules leave the micelles and their hydrophobic domains merge into the outer leaflet of the liposome bilayer, forming a link between the liposome and the antibody. Optionally, the residual micellar material can then be separated from the antibody-conjugated liposomes. Each of these steps is considered in more detail below.

2.6. Engineering antibody molecules having C-terminal cysteine

Nonimmunogenic short flexible C-terminal conjugation sequences can be engineered into the antibody fragments. The most convenient way of introducing this feature is expression of a recombinant protein with a single C-terminal cysteine. While pepsin digestion of the whole IgG molecule is an established method for generation of Fab′ fragments having free thiols at the hinge region, we have found that the presence of a CPPC hinge sequence and/or extra free Cys (e.g., in the case of human IgG1) makes proteolytically generated Fab′ prone to forming stable intramolecular disulfide bridges at the hinge and otherwise to attachment of more than one linker chain, both leading to low yields of the liposome-linked antibody. In addition, we have removed Myc-His_6 sequences typically found in the C-terminus of many antibody libraries in order to reduce nonspecific immunogenicity and an observed rapid clearance observed in immunoliposome constructs prepared from them. For scFv antibody fragments, we have used a preferred sequence of Gly4Cys (Nellis *et al.*, 2005a), while for Fab's we have typically employed the sequence THTCAA in the C-terminus (Carter *et al.*, 1992).

2.7. Thiol-reactive lipopolymer linkers

The linker molecule generally contains a hydrophilic polymer chain, such as PEG, terminally linked to a lipid domain (phospholipid or sterol), and bears a thiol-reactive functional group at the terminus contralateral to the lipid domain (Fig. 7.6A). The linkers comprising PEG spacers of various size, phosphatidylethanolamine (PE) lipid anchors of various hydrocarbon chain length, and terminal maleimide or iodoacetate groups are currently commercially available from Avanti Polar Lipids (Alabama, USA) and NOF Corporation (Japan). The PE-based lipopolymer linkers introduce an additional negative charge due to the phosphate residue in the lipid head group, and contain fatty acid ester bonds are modestly susceptible to hydrolysis leading to single-chain lipid (lysolipid) anchors that make the conjugates more prone to leaving the liposome bilayer. To avoid these disadvantages, thiol-reactive lipopolymers with nonionizable and non-ester lipid moieties, such as distearylamine, have been reported (Drummond *et al.*, 2005a). DSPE-PEG-based linkers have advantage due to the availability and

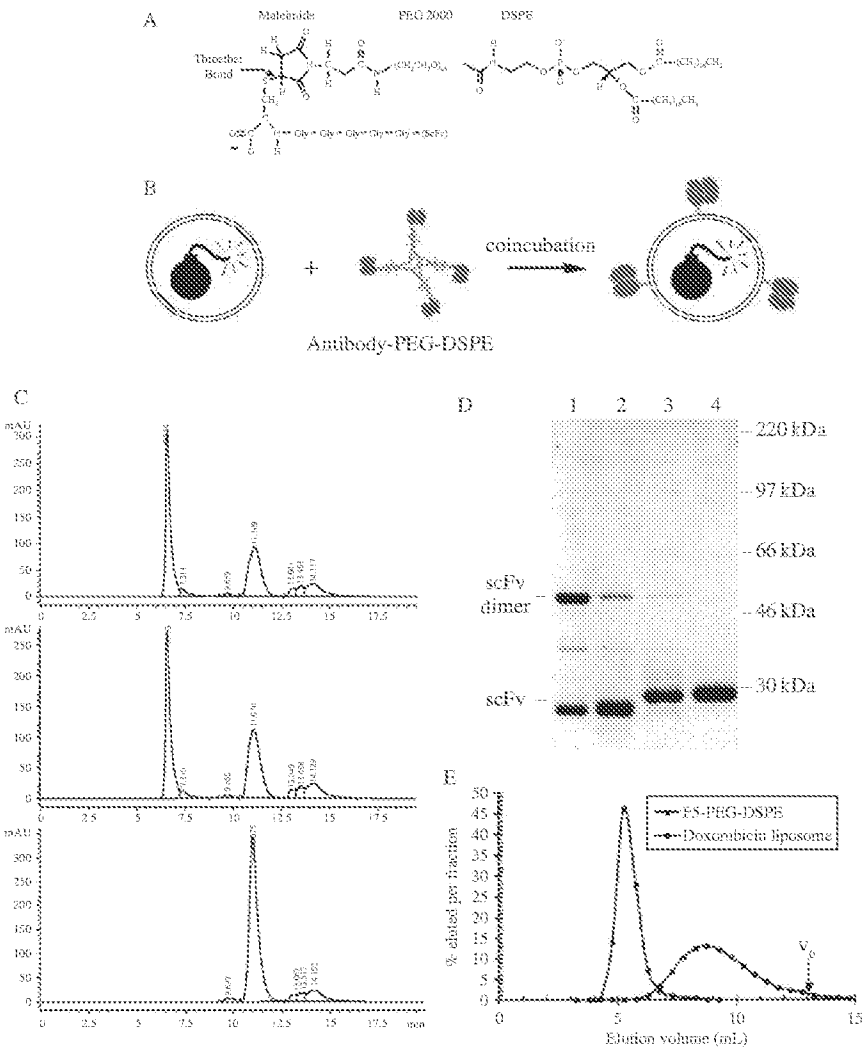

Figure 7.6 (A) Chemical structure of the antibody-lipopolymer conjugate. Anti-HER2 single-chain Fv antibody fragment (F5) with appended C-terminal GGGC sequence (F5-SH) is conjugated through the thiol group to maleimide-terminated PEG-DSPE linker molecule (Nellis *et al.*, 2005a). (B) Schematic representation of the "micellar insertion" strategy for the antibody-liposome conjugation. A drug-loaded liposome (I) (in this case, PEGylated unilamellar vesicle) is incubated with the micelles of the antibody-lipopolymer linker conjugate (II) whereby the hydrophobic domains of the conjugate merge into the lipid bilayer, producing an immunoliposome (III). (C) Monitoring of the antibody-lipopolymer conjugation reaction by SEC. A recombinant human IgG1 Fab fragment with the C-terminal THTCAA sequence (Fab-SH) was expressed in *Escherichia coli*, purified from the periplasmic extract by protein A resin capture, reduced with 15 m*M* cysteine and incubated with the micellar solution of

positive clinical experience with PEG-DSPE conjugates as liposome constituents. The antibody-lipopolymer conjugate is retained in the liposome bilayer as a result of the hydrophobic interactions between the lipid domain and the bilayer membrane. PEG-PE molecules with shorter acyl chain lengths than stearic acid have been shown to more rapidly disassociate from the liposomes *in vivo* and thus should be avoided (Parr *et al.*, 1994).

2.8. Preparing the protein for conjugation

The next step is to prepare the scFv-Cys or Fab-Cys antibody fragment for conjugation with the lipopolymer linker. The conjugation requires the presence of free reactive thiol group at the C-terminal Cys-containing sequence. Using bacterial expression system, the secretion of the terminally cysteinated antibody fragment into the culture medium frequently lead to the irreversible blockade of the terminal Cys by an unknown component. Periplasmic expression is reported to preserve the reactivity of C-terminal Cys more effectively (Carter *et al.*, 1992). In our experience, scFv-Cys and Fab-Cys isolated from the bacterial periplasm, as well as scFv-Cys produced in the mammalian system (CHO cells), after affinity isolation step (e.g., protein A resin) come out as a mixture of disulfide-linked dimers and monomers with the terminal Cys partially blocked (Fig. 7.6D, lane 2). Reduction of the protein with a thiol compound results in the dissociation of the dimer and partial removal of the

maleimido-PEG_{45}-DSPE linker (protein/linker molar ratio 1:4, mol:mol) at pH 6.0 and ambient temperature. At indicated times, 0.05-ml aliquots of the reaction mixture were quenched with cysteine (0.5 m*M* final) and chromatographed on the TSK Gel Super SW3000 4.6 × 30 column in 0.45 *M* NaCl, 0.01*M* phosphate buffer pH 7.1 at 0.35 ml/min with UV detection at 280 nm. Incubation times: bottom panel, zero time; middle panel, 1 h; top panel, 2 h. Notice the appearance of a micellar protein eluted at 6.5 min and the gradual decrease of the free Fab-SH eluted at 10.7–11.1 min. (D) SDS-PAGE analysis of the antibody-lipopolymer conjugate preparation. F5-SH scFv was isolated from the *E. coli* periplasmic extract using protein A chromatography (lane 1), reduced by passage through a Reduce-Imm column (Pierce) at pH 6.0 (lane 2), incubated with maleimido-PEG-DSPE micelles in an aqueous buffer (lane 3), the resulting conjugate was incubated with commercial PEGylated doxorubicin liposomes (Doxil, Alza Corporation) at 30–40 scFv/liposome, the liposomes were separated from the unconjugated proteins and micelles by Sepharose CL-4B chromatography (see E), solubilized in the SDS sample buffer, and applied on the gel (lane 4). The gel is stained with Coomassie Blue R. Notice the disappearance of the F5 dimer band after the reduction step, and the shift of ~3 kD between the unconjugated protein (26 kDa, lanes 1,2) and the F5-PEG-DSPE conjugate (lanes 3,4; Nielsen *et al.*, 2002). (E) Separation of immunoliposomes from unbound micellar antibody-lipopolymer conjugate using Sepharose CL-4B chromatography. Doxil liposomes incubated with F5-PEG-DSPE conjugate (30 min, 60 °C) were applied on a Sepharose CL-4B column (bed volume 15 ml) and eluted with PBS. The liposomes were quantified by doxorubicin absorbance at 485 nm, the micellar and/or free protein was quantified by absorbance at 280 nm. The presence of liposome-associated scFv was confirmed by SDS-PAGE (D; Nielsen *et al.*, 2002).

blocking group. The choices of the reducing compound and the reduction conditions are governed by the specificity toward the terminal cysteine while keeping intramolecular disulfide bridges intact. Hydrophilic thiols, such as 2-mercaptoethylamine, cysteine, or dithioerythritol, are best suited for the task due to their tendency to avoid hydrophobic pockets that harbor intramolecular disulfides. Strong reducing agents such as dithiothreitol (DTT) or tris-carboxymethylphosphine tend to overreduce the protein. The protein is incubated with 10–20 m*M* reducing agent at pH < 7, and the excess of the reducing agent is thoroughly removed, for example, by SEC on Sephadex G-25 or similar carrier. Alternatively, the reducing agent removal step can be combined with the protein purification step, for example, by IEX. Fabs and scFvs often have high isoelectric points (pI 8.0–9.0) and therefore can be conveniently purified on a cation exchanger, such as SP Sepharose (GE Healthcare). The reducing agent, especially of the net zero ionic charge (DTE, cysteine), usually appears in the eluate at lower ionic strength than does the protein, making is unnecessary to remove it in advance of the IEX (Nellis *et al.*, 2005a). During the IEX step, it is important to maintain the pH of elution buffers sufficiently low (pH 5.5–6.5), to remove the dissolved oxygen (e.g., by bubbling of argon or nitrogen), and to include 1–5 m*M* chelator (EDTA) to prevent heavy metal-catalyzed thiol oxidation. The reactive thiol group content of the reduced protein is usually about 0.7–0.8/ molecule. The higher thiol content is often associated with partial reduction of the intramolecular disulfides and should be avoided.

2.9. Antibody-lipopolymer conjugation

The purified and reduced antibody fragment can be directly conjugated to the liposomes bearing thiol-reactive groups. In our preferred method, the antibody fragment is first conjugated to a thiol-reactive, micelle-forming lipopolymer and then incubated with the liposomes to effect insertion of the antibody-lipopolymer into the outer leaflet of the liposome bilayer. The reaction between maleimide-terminated PEG-DSPE linker (mal-PEG-DSPE; Fig. 7.6A) and scFv-Cys or Fab-Cys is carried out in aqueous solution at pH about 6.0; it takes only a few hours and proceeds to completion. The reaction proceeds to completion even at a stoichiometric ratio between the available protein thiols and linker maleimides. However, we usually add an excess of mal-PEG-DSPE (protein/linker molar ratio of 1:4) to act as a "spacer" that prevents instability of the micelle due to the excluded volume of the conjugated protein chains. At the end of the reaction, the excess maleimide groups are quenched by a small amount of cysteine or mercaptoethanol. Quenching is especially important if the conjugate is later transferred into a medium at pH > 6, since the excess maleimide groups have the tendency to react, albeit slowly, with the side chains of lysine, leading to gradual accumulation of polyconjugated species that have reduced ability to insert

into the liposome bilayer. If excess mal-PEG-DSPE or the quenching agent have negative effects on the protein or the liposome, PEG-DSPE molecule without the terminal maleimide can be substituted for excess mal-PEG-DSPE (i.e., 1 mol part of mal-PEG-DSPE and 3 mol parts of PEG-DSPE for 1 mol part of the protein) at the expense of slightly lower yield and longer incubation time. Although it is not necessary to remove unconjugated protein prior to the subsequent membrane insertion step (Nielsen *et al.*, 2002), it is useful to purify the conjugate for the purposes of storage and better characterization. Due to the large size of the lipopolymer micelles (equivalent molecular weight 850 kDa; Nellis *et al.*, 2005a), SEC is a convenient way to do so (Fig. 7.6C). SEC is also convenient for monitoring the reaction by the amount of protein (OD 280) appearing in the first-eluted, micellar fraction (Fig. 7.6E). The antibody-lipopolymer conjugate can be concentrated and/or purified using ultrafiltration on the membrane with 300-kDa molecular weight cutoff, in a stirred cell or, at a larger scale, using a hollow fiber cartridge. In order to maintain stability of the conjugate against aggregation in solution, it is useful to maintain at least 1 m*M* of a polycarboxylic acid buffer, such as citrate. The purified conjugate can be stored frozen at −70 °C for many months without deterioration. Alternatively, the purified conjugate can be lyophilized from the solution containing citrate buffer and a stabilizer, such as 10% sucrose, stored, refrigerated, and reconstituted in distilled water.

The course of protein reduction, conjugation, and purification of the conjugate can be monitored using nonreducing SDS-PAGE with the regular protein Coomassie or Sypro Ruby stain (Fig. 7.6D). Compared to the unconjugated protein, the conjugate has a small but distinct shift due to the addition of a 2.9 kDa linker. Protein species having more than one conjugated species appear as a ladder of yet slower moving bands and usually are the sign of the low-quality preparation. Excess lipopolymer appears on the gel as a faint band near the tracking dye front. Despite the micellar nature of the conjugate in solution, as long as the protein concentration is low (<1 mg/ml), it is possible to quantify the protein using UV spectrophotometry at 280 nm and the same extinction value as for the free protein. The effect of light scattering by micelles on the UV spectrum manifests as the reduced OD280/OD260 ratio (1.60–1.85 vs. 1.90–2.00 for the free protein). As mentioned above, it is important to include sufficient amount of citrate (1–10 m*M*) in all dilution buffers to prevent aggregation.

2.10. "Insertion" of the antibody-lipopolymer conjugate into the liposome

Simple coincubation of the preformed liposome and the antibody-lipopolymer conjugate in an aqueous medium at elevated temperatures results in the transfer of the conjugate molecules into the lipid bilayer via anchoring of the lipopolymer hydrophobic domain in the hydrophobic

inner portion of the bilayer. The process usually is remarkably efficient, resulting in the capture of at least 70% of the conjugate onto the liposome membrane, and typically over 80%, with little dependence on the antibody/lipid on the range of 5–100 proteins/liposome. If the liposome size is close to 100 nm (which corresponds to the average of 80,000 phospholipid molecules/liposome), the density of liposome-conjugated antibody can be quite accurately defined by the antibody/phospholipid molar ratio in the coincubation mixture. If the inner space of the liposome is loaded with the drug, such as doxorubicin, the drug should be retained during the insertion process. Addition of a mixture of several antibody–lipopolymer conjugates having the same linker molecules results in the liposome bearing the combination of antibodies essentially at the same ratio as in the conjugate mixture. The rate of antibody–lipopolymer transfer from the micelle onto the bilayer depends on the aggregate state of the bilayer lipids. Liquid-crystalline bilayers (above T_m of the liposome lipid) incorporate the conjugate faster than gel-state bilayers (below T_m); however, even in the gel state, the transfer of the antibody–lipopolymer onto the liposome is efficient and not overly slow. To initiate conjugate transfer, the liposomes and the conjugate are mixed in any liposome- and antibody-compatible aqueous buffer containing ≥ 1 m*M* polycarboxylate (citrate) to maintain stability of the conjugate micelles. If the denaturation temperature of the antibody so permits, the conjugate–liposome mixture is quickly heated to the temperature above the lipid T_m, and the incubation continues for 20–30 min with slow agitation. Then the mixture is quickly chilled down to ambient temperature or below. For less thermostable antibodies, overnight incubation at 37 °C is effective.

The amount of liposome-linked protein can be quantified using SDS-PAGE essentially the same way as for the conjugate itself (Fig.7.6D). Premade gradient gels (4–15% for Fab, 10–20% for scFv, e.g., Ready Gel Tris–HCl from Bio-Rad) are suitable for this purpose. Liposome lipids, complexed with SDS, move close to the tracking dye front. The antibody conjugate band is quantified by densitometry of the gel stained with colloidal Coomassie (Bio-Safe(R) Coomassie G-250 protein stain, Bio-Rad) according to the manufacturer. The protein standards (made from the conjugate solution of the known protein concentration) are run concurrently. Typically, the band staining is linear to the amount of protein in the range of 50–500 ng/lane.

3. Physicochemical Characterization of Immunoliposomes

There are a number of physicochemical parameters which play a critical role in determining the effectiveness of an immunoliposome. These include particle size, drug encapsulation rate, lipid stability, drug

degradation, antibody association rate, and immunoreactivity. As described above, particle size affects the rate of clearance from the circulation, stability of encapsulation, and rate of tumor uptake of immunoliposomal drugs.

3.1. Particle size

The particle size for immunoliposomes is measured (1) as an in-process control to ensure the completion of the extrusion step, (2) following drug loading and antibody conjugation to ensure that antibody conjugation did not result in undesirable interliposomal cross-linking and thus the formation of aggregates, and (3) during storage. Dynamic light scattering, also sometimes referred to as photon correlation spectroscopy, is typically used to monitor the particle size of these submicron immunoliposomes (Ostrowsky, 1993). The original Coulter or Nicomp instruments primarily measured light scattering at a 90° angle. However, modern instruments such as the Malvern Zetasizer can measure light scattering at higher angles (e.g., 173°), thus allowing measurement of liposomes in undiluted samples and over a greater dynamic range. The *z*-average particle diameter and polydispersity index (PI) are calculated using the cumulants method by the instrument's built-in software. The desired average diameter can vary depending on the formulation and application, but is generally in the range of 80–120 nm. For most liposomal drugs a PI of 0.2 or less is preferable.

3.2. Drug encapsulation efficiency

The efficiency of drug encapsulation can be measured in two parameters, the percentage of encapsulated drug and the ratio of drug-to-lipid matrix. We typically calculate both parameters following measurement of a pre- and post-column-purified liposome sample for drug and phospholipid content. The specific chromatograph employed for separating the free and encapsulated drugs may vary depending on the active drug entrapped. However, for many small molecule drugs, we utilize 10 ml PD-10 columns packed with Sephadex G-75 resin and eluted with buffered saline. When phosphorous buffers and trapping solutions are omitted during liposome preparation, then liposomal phospholipid can be determined indirectly by measuring total inorganic phosphorus following acid digestion with sulfuric acid (Bartlett, 1959). The drug is generally extracted with an acidic methanol solution, and subsequently quantified by HPLC with UV or fluorescence detection. The entrapment efficiency is calculated as follows: entrapment efficiency (%) = $100 \times [\text{drug/lipid}]_{post}/[\text{drug/lipid}]_{pre}$, where pre and post refer to the respective ratios pre and post purification on the Sephadex G-75 column.

3.3. Drug stability

Drug degradation products can be observed in liposome formulations due in part to the fact that the electrochemical gradient employed to load the drug can result in extremes of pH, and the fact that liposomes concentrate drugs at exceedingly high concentrations putting them in close proximity to another and increasing the propensity for dimerization reactions. It is thus, helpful to do early stress testing of the free drugs at extremes of pH as well as accelerated stability studies of the actual liposomal formulations to help identify potential degradation products early in the formulation optimization process. Drug impurities and degradation products are typically detected using HPLC through two runs at an order of magnitude difference in concentration to accurately detect both the parent drug, as well as most minor metabolites. The specifics of the HPLC method vary depending on the specific drug formulated. However, often solvents optimized for dissolution of the corresponding free drugs require modification in order to solubilize the high lipid concentrations typically present in immunoliposome formulation.

3.4. Lipid stability

Analysis of individual lipids will reveal degradation products generated during the liposome formulation process or subsequent storage. In aqueous environment, double-chain phospholipids are known to degrade slowly. The major degradation product in phosphatidylcholine is lysophosphatidylcholine, resulting from simple hydrolysis at the sn-2 ester position A robust method involves the use of a normal phase HPLC method to separate diacylphosphatidylcholine and lysophosphatidylcholine with either evaporative light scattering detection (ELSD) or charged aerosol detection (CAD). We use a YMC PVA-Sil column with 5 μm particle size, 120 Å pore size, 2.0 × 250 mm column size (Waters Corporation) and the separation is performed in a gradient fashion using hexanes, isopropyl alcohol (IPA), and water. The portion of each starts as 57% hexanes: 40% IPA: 3% water and increases C to 52% hexanes: 40% IPA: 8% water over 20 min, followed by a 20 min equilibration back to the starting mobile phase ratio. We prefer a CAD detector due to the increased sensitivity and dynamic range. For the ESA Corona Plus CAD, the detection range is set to 200 pA and the nebulizer temperature to 26 °C. The input gas (N_2) pressure is set at 35 psi and the gas flow rate regulated automatically. Typical retention times are 18.0 min for HSPC and 25.4 min for lysoPC. The 50 μl sample in hexanes:IPA (3:2, vol:vol) is injected on at both 500 and 5 μ*M* total phospholipid to capture the PC and lysoPC at concentrations well within the linear range of the detection method. Typically, lysoPC is seen in the range of 0.1–5 mol% in well-controlled liposomes.

3.5. Antibody association rate

An accurate determination of the amount of targeting antibodies or fragments attached to each liposome is required to evaluate the formulation process and assess influence of antibody density on cellular binding and internalization efficiency. Quantitation of the antibody fragment is complicated by the complex nature of the immunoliposome, which contains, drug, loading agent, a variety of lipids, and antibody fragments which are conjugated to lipids. SDS-PAGE has proven to be valuable, as the technique isolates each analyte by molecular weight. The antibody-lipid conjugate has the highest molecular weight of all the individual components which make up immunoliposomes which simplifies resolution from the mixture. Solubilization of the immunoliposome components by heating in detergent enables proper separation. Treatment of the liposomes with commonly used SDS-PAGE sample buffers produces good results; however, for immunoliposomes with low protein content (that require larger lipid burden/sample), it is useful to double the amount of the sample buffer. Premanufactured gels (10–20% polyacryamide, e.g., Ready Gel, Bio-Rad) are suitable for both scFv and Fab quantification. The lipids in the form of SDS complexes migrate close to the gel front. The conjugate can be quantified after regular Coomassie staining and densitometry of the protein band by comparison of the concurrently run antibody standards in the range of 100–500 ng/lane (for 1 mm gels). Fluorescent stains commonly used for protein detection on gels allows for detection in the 10 ng range (Nellis *et al.*, 2005b). If the antibody is added to the liposomes as a micellar lipid conjugate, care must be taken to ensure complete removal of the uninserted antibody-lipid conjugate because there is no way to distinguish it from that which is associated with the liposome.

4. *In Vitro* and *In Vivo* Characterization of Immunoliposomal Therapeutics

4.1. Optimization of antibody density on liposomal carriers

The antibody density can be varied on the liposome surface by simply mixing liposomes with varying concentrations of antibody–drug conjugates. We typically vary the density between 5 and 100 copies/liposome using the micellar insertion method outlined above. The liposomes are then purified using Sepharose CL-4B gel chromatography followed by measuring the phospholipid content by standard phosphorous assay (Bartlett, 1959) and the antibody by densitometric analysis of fluorescently stained proteins in an SDS gel (see above). This quality control of the immunoliposome formulations is important to correct for differences in conjugate insertion efficiencies

between preparations. The liposomes used contain 0.3 mol% of DiIC18(5)-DS and can thus be used to monitor cell uptake using flow cytometry. Between 10 and 12 cell lines containing various receptor densities (typically over two orders of magnitude) are incubated with either a nontargeted liposome control or an immunoliposome containing increasing concentrations of conjugate (5, 10, 15, 30, 60, and 90 Fab′ or scFv′/liposome) at 37 °C for 4 h. Cells are then washed using PBS and analyzed by flow cytometry using the Cy5 channel. The amount of total cell-associated fluorescence is plotted as a function of antibody density and evaluated over the entire panel of cell lines to determine the optimum density with respect to both specificity and overall cell uptake (Fig. 7.7).

4.2. *In vivo* pharmacokinetic and biodistribution studies

After selection of the appropriate liposome, drug and targeting strategy *in vitro*, (discussed earlier) pharmacokinetic and disposition studies in mice or rats are a useful predictive tool to see how the liposome formulation would perform in an *in vivo* setting. In order to benefit from the EPR effect, the liposome formulation must be sufficiently stable in circulation to be able to

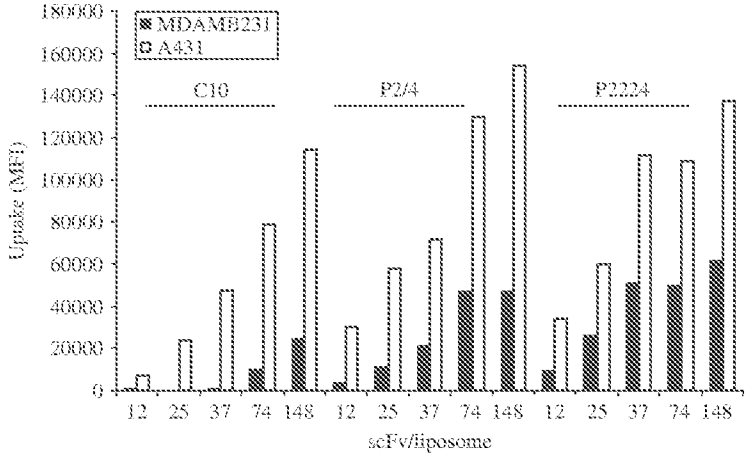

Figure 7.7 Effect of the grafted antibody density in the uptake of immunoliposomes by the cells with the high or medium surface abundance of the antigen. Fluorescently labeled anti-EGFR immunoliposomes of the identical size and various numbers of scFv copies per liposome were prepared from anti-EGFR scFv with low (C10, K_d = 264 n*M*), medium (P2/4, K_d = 15 n*M*), and high (P2224, K_d = 0.9 n*M*) affinity. The immunoliposomes were incubated at identical concentrations with the high EGFR (A431) or medium EGFR (MDA-MB-231) cells, and the amount of immunoliposome uptake by the cells was assessed by flow cytometry. MFI—mean cell fluorescence intensity (in arbitrary units).

accumulate in the tumor. Many factors contribute to the pharmacokinetic and biodistribution behavior of liposomal formulations. Lipid composition, surface charge, size, degree of pegylation, dose, dosing schedule, route of administration, encapsulated drug, and targeting ligand can all affect the clearance rates of the carrier and ultimately affect the ability of the immunoliposome to deliver drug in a site-specific manner (Drummond *et al.*, 2008).

To maximize the information garnered from such studies, it is important to measure drug and liposome-associated lipid in the collected plasma and tissue samples, in order to follow the clearance and distribution of both the liposomal carrier as well as the encapsulated drug. A nonexchangeable lipid marker, [^{3}H]cholesteryl hexadecyl ether (Fig. 7.8A) is used to follow the liposomal carrier. In this way, individual PK parameters of the drug and carrier can be measured, but also their relative concentrations can give information pertaining to the leakage of the drug from the carrier. This is important, for example, in order to discriminate between a fast leaking formulation and a fast clearing formulation, that is, changes in the initial [drug]/[lipid] levels indicate drug leakage. Drug release rates from liposomes can be characterized by their half-life of release times ($T_{1/2}$), and are calculated using the exponential constant (λ), from a single exponential Wt to the plot of drug/phospholipid ratio versus post injection time [$N(t) = N_0 e^{-\lambda t}$]. $N(t)$ is the drug-to-PL ratio at time t and $N(0)$ is the same ratio at time 0 (Drummond *et al.*, 2009; Noble *et al.*, 2009). We typically screen multiple formulations *in vivo* in PK studies to aid in the engineering of a liposomal drug with optimum drug release and clearance rates. For simplicity, early studies focused on formulation stability are completed in the absence of targeting ligand. However, it is also critical once a stable formulation is identified to repeat the measurement of clearance and *in vivo* drug release using the targeted formulation to ensure that the addition of the targeting ligand has not disrupted the membrane and increased drug leak rates or increased clearance via either specific or nonspecific mechanisms. Finally, we compared optimized targeted and nontargeted liposomes in full biodistribution studies, looking at the effect of targeting on drug deposition in tumor, organs of the mononuclear phagocyte system (MPS; i.e., liver and spleen), organs of potential sites of toxicity, or organs where distribution may occur based on target receptor expression levels. Again, measuring changes in drug-to-lipid ratios provides an approximation of drug release rates in various tissues.

In the case of hydrophilic drugs, one can indirectly measure the drug concentration by assuming that the clearance rate of the unencapsulated drug is sufficiently faster than the clearance rate of the liposomal drug (Drummond *et al.*, 2006). Direct measurement of the *in vivo* encapsulated drug concentration can also be obtained by purifying the liposomes from plasma components using gel chromatography or solid phase extraction methods (Gabizon *et al.*, 1994; Zamboni *et al.*, 2007). Typically, HPLC (Noble *et al.*, 2009; Taggar *et al.*, 2006) or photometric (Charrois and Allen, 2004) analysis of plasma or tissue extracts

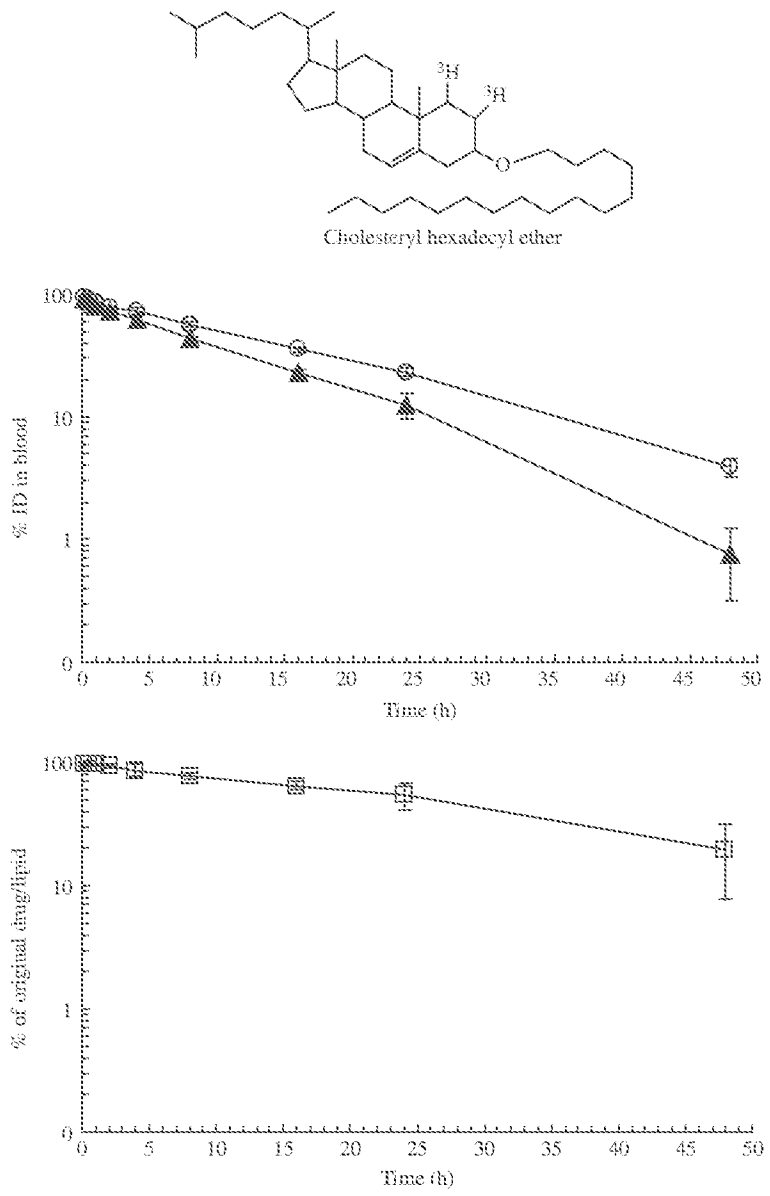

Figure 7.8 Determinations of pharmacokinetics and *in vivo* drug release of a liposomal drug. (A) Chemical structure of the nonexchangeable lipid marker, [^{3}H]cholesteryl hexadecyl ether. (B) Monitoring of topotecan (triangles) and lipid marker (circles) in the blood of an animal following the administration of a drug-loaded immunoliposome. (C) Kinetic curve of the *in vivo* drug release derived from the data of Panel B.

can be used to measure the drug concentrations. For measuring liposome concentrations in plasma or tissue extracts, the authors typically use a non-exchangeable radiolabeled lipid marker ([^{3}H]-CHE) followed by scintillation counting. Techniques such as ELISA (Park *et al.*, 2002) or gamma counting of ^{125}I-labeled antibody may be used to determine the biodistribution of the targeting ligand in the plasma or tissues. Premature dissociation of the ligand from the liposome surface will lessen the ability of the carrier to target cells.

REFERENCES

Allen, T. M., Cheng, W. W., Hare, J. I., and Laginha, K. M. (2006). Pharmacokinetics and pharmacodynamics of lipidic nano-particles in cancer. *Anticancer Agents Med. Chem.* **6,** 513–523.

Bartlett, G. R. (1959). Phosphorous assay in column chromatography. *J. Biol. Chem.* **234,** 466–468.

Becerril, B., Poul, M. A., and Marks, J. D. (1999). Toward selection of internalizing antibodies from phage libraries. *Biochem. Biophys. Res. Commun.* **255,** 386–393.

Carter, P., Kelley, R. F., Rodrigues, M. L., Snedecor, B., Covarrubias, M., Velligan, M. D., Wong, W. L., Rowland, A. M., Kotts, C. E., Carver, M. E., *et al.* (1992). High level Escherichia coli expression and production of a bivalent humanized antibody fragment. *Biotechnology (N. Y.)* **10,** 163–167.

Charrois, G. J., and Allen, T. M. (2004). Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of pegylated liposomal doxorubicin formulations in murine breast cancer. *Biochim. Biophys. Acta.* **1663,** 167–177.

Drummond, D. C., Meyer, O., Hong, K., Kirpotin, D. B., and Papahadjopoulos, D. (1999). Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol. Rev.* **51,** 691–743.

Drummond, D. C., Kirpotin, D. B., and Hong, K. (2005a). Liposomes Useful for Drug Delivery. (United States).

Drummond, D. C., Marx, C., Guo, Z., Scott, G., Noble, C., Wang, D., Pallavicini, M., Kirpotin, D. B., and Benz, C. C. (2005b). Enhanced pharmacodynamic and antitumor properties of a histone deacetylase inhibitor encapsulated in liposomes or ErbB2-targeted immunoliposomes. *Clin. Cancer Res.* **11,** 3392–3401.

Drummond, D. C., Noble, C. O., Guo, Z., Hong, K., Park, J. W., and Kirpotin, D. B. (2006). Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. *Cancer Res.* **66,** 3271–3277.

Drummond, D. C., Noble, C. O., Hayes, M. E., Park, J. W., and Kirpotin, D. B. (2008). Pharmacokinetics and in vivo drug release rates in liposomal nanocarrier development. *J. Pharm. Sci.* **97,** 4696–4740.

Drummond, D. C., Noble, C. O., Guo, Z., Hayes, M. E., Park, J. W., Ou, C. J., Tseng, Y. L., Hong, K., and Kirpotin, D. B. (2009). Improved pharmacokinetics and efficacy of a highly stable nanoliposomal vinorelbine. *J. Pharmacol. Exp. Ther.* **328,** 321–330.

Drummond, D. C., Noble, C. O., Guo, Z., Hayes, M. E., Connolly-Ingram, C., Gabriel, B. S., Hann, B., Liu, B., Park, J. W., Hong, K., Benz, C. C., Marks, J. D., *et al.* (2010). Development of a highly stable and targetable nanoliposomal formulation of topotecan. *J. Control. Release* **141,** 13–21.

Fenske, D. B., and Cullis, P. R. (2005). Entrapment of small molecules and nucleic acid-based drugs in liposomes. *Methods Enzymol.* **391,** 7–40.

Gabizon, A., Catane, R., Uziely, B., Kaufman, B., and Safra, T. (1994). Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res.* **54,** 987–992.

Haran, G., Cohen, R., Bar, L. K., and Barenholz, Y. (1993). Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphiphathic weak bases. *Biochim. Biophys. Acta.* **1151,** 201–215.

Harding, J. A., Engbers, C. M., Newman, M. S., Goldstein, N. I., and Zalipsky, S. (1997). Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)-grafted immunoliposomes. *Biochim. Biophys. Acta.* **1327,** 181–192.

Hayes, M. E., Drummond, D. C., Kirpotin, D. B., Zheng, W. W., Noble, C. O., Park, J. W., Marks, J. D., Benz, C. C., and Hong, K. (2006). Genospheres: Self-assembling nucleic acid-lipid nanoparticles suitable for targeted gene delivery. *Gene Ther.* **13,** 646–651.

Huang, Z., Park, J. L., Watson, D. S., Hwang, P., and Szoka, F. C., Jr. (2006). Facile synthesis of multivalent nitrilotriacetic acid (NTA) and NTA conjugates for analytical and drug delivery applications. *Bioconjug. Chem.* **17,** 1592–1600.

Huang, Z., Hwang, P., Watson, D. S., Cao, L., and Szoka, F. C., Jr. (2009). Tris–nitrilotriacetic acids of submanomolar affinity toward hexahistidine tagged molecules. *Bioconjug. Chem.* **20,** 1667–1672.

Kirpotin, D., Park, J. W., Hong, K., Zalipsky, S., Li, W.-L., Carter, P., Benz, C. C., and Papahadjopoulos, D. (1997). Sterically stabilized anti-HER2 immunoliposomes: Design and targeting to human breast cancer cells *in vitro*. *Biochemistry* **36,** 66–75.

Kirpotin, D. B., Drummond, D. C., Shao, Y., Shalaby, M. R., Hong, K., Nielsen, U. B., Marks, J. D., Benz, C. C., and Park, J. W. (2006). Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Res.* **66,** 6732–6740.

Mamot, C., Drummond, D. C., Noble, C. O., Kallab, V., Guo, Z., Hong, K., Kirpotin, D. B., and Park, J. W. (2005). Epidermal growth factor receptor-targeted immunoliposomes significantly enhance the efficacy of multiple anticancer drugs in vivo. *Cancer Res.* **65,** 11631–11638.

Mui, B. L.-S., Cullis, P. R., Evans, E. A., and Madden, T. D. (1993). Osmotic properties of large unilamellar vesicles prepared by extrusion. *Biophys. J.* **64,** 443–453.

Mui, B. L.-S., Cullis, P. R., Pritchard, P. H., and Madden, T. D. (1994). Influence of plasma on the osmotic sensitivity of large unilamellar vesicles prepared by extrusion. *J. Biol. Chem.* **269,** 7364–7370.

Nellis, D. F., Ekstrom, D. L., Kirpotin, D. B., Zhu, J., Andersson, R., Broadt, T. L., Ouellette, T. F., Perkins, S. C., Roach, J. M., Drummond, D. C., Hong, K., Marks, J. D., *et al.* (2005a). Preclinical manufacture of an anti-HER2 scFv-PEG-DSPE, liposome-inserting conjugate. 1. Gram-scale production and purification. *Biotechnol. Prog.* **21,** 205–220.

Nellis, D. F., Giardina, S. L., Janini, G. M., Shenoy, S. R., Marks, J. D., Tsai, R., Drummond, D. C., Hong, K., Park, J. W., Ouellette, T. F., Perkins, S. C., and Kirpotin, D. B. (2005b). Preclinical manufacture of anti-HER2 liposome-inserting, scFv-PEG-lipid conjugate. 2. Conjugate micelle identity, purity, stability, and potency analysis. *Biotechnol. Prog.* **21,** 221–232.

Nielsen, U. B., Kirpotin, D. B., Pickering, E. M., Hong, K., Park, J. W., Refaat Shalaby, M., Shao, Y., Benz, C. C., and Marks, J. D. (2002). Therapeutic efficacy of anti-ErbB2 immunoliposomes targeted by a phage antibody selected for cellular endocytosis. *Biochim. Biophys. Acta.* **1591,** 109–118.

Nielsen, U. B., Kirpotin, D. B., Pickering, E. M., Drummond, D. C., and Marks, J. D. (2006). A novel assay for monitoring internalization of nanocarrier coupled antibodies. *BMC Immunol.* **7,** 24.

Noble, C. O., Kirpotin, D. B., Hayes, M. E., Mamot, C., Hong, K., Park, J. W., Benz, C. C., Marks, J. D., and Drummond, D. C. (2004). Development of ligand-targeted liposomes for cancer therapy. *Expert Opin. Ther. Targets* **8,** 335–353.

Noble, C. O., Guo, Z., Hayes, M. E., Marks, J. D., Park, J. W., Benz, C. C., Kirpotin, D. B., and Drummond, D. C. (2009). Characterization of highly stable liposomal and immunoliposomal formulations of vincristine and vinblastine. *Cancer Chemother. Pharmacol.* **64,** 741–751.

Ostrowsky, N. (1993). Liposome size measurements by photon correlation spectroscopy. *Chem. Phys. Lipids* **64,** 45–56.

Pagnan, G., Stuart, D., Pastorino, F., Raffaghello, L., Montaldo, P., Allen, T., Caabretta, B., and Ponzoni, B. (2000). Delivery of c-myb antisense oligodeoxynucleotides to human neuroblastoma cells vis disialoganghoside GD2-targeted immunoliposomes: Antitumor effects. *J. Natl. Cancer Inst.* **92,** 253–261.

Park, J. W., Hong, K., Kirpotin, D. B., Colbern, G., Shalaby, R., Baselga, J., Shao, Y., Nielsen, U. B., Marks, J. D., Moore, D., Papahadjopoulos, D., and Benz, C. C. (2002). Anti-HER2 immunoliposomes: Enhanced efficacy attributable to targeted delivery. *Clin. Cancer Res.* **8,** 1172–1181.

Parr, M. J., Ansell, S. M., Choi, L. S., and Cullis, P. R. (1994). Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. *Biochim. Biophys. Acta.* **1195,** 21–30.

Poul, M. A., Becerril, B., Nielsen, U. B., Monsson, P., and Marks, J. D. (2000). Selection of tumor-specific internalizing human antibodies from phage libraries. *J. Mol. Biol.* **301,** 1149–1161.

Raffaghello, L., Pagnan, G., Pastorino, F., Cosmo, E., Brignole, C., Marimpietri, D., Montaldo, P. G., Ganbini, C., Allen, T. M., Bogenmann, E., and Ponzoni, M. (2003). In vitro and in vivo antitumor activity of liposomal Fenretinide targeted to human neuroblastoma. *Int. J. Cancer* **104,** 559–567.

Sapra, P., and Allen, T. M. (2002). Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs. *Cancer Res.* **62,** 7190–7194.

Sapra, P., and Allen, T. M. (2003). Ligand-targeted liposomal anticancer drugs. *Prog. Lipid Res.* **42,** 439–462.

Sapra, P., Tyagi, P., and Allen, T. M. (2005). Ligand-targeted liposomes for cancer treatment. *Curr. Drug Deliv.* **2,** 369–381.

Stahl, P. H., and Wermuth, C. G. (eds.). (2002). Monographs on acids and bases. In "Handbook of Pharmaceutical Salts: Properties, Selection, and Use." Wiley-VCH, Weiheim, 2002, pp. 265–327.

Sugano, M., Egilmez, N. K., Yokota, S. J., Chen, F. A., Harding, J., Huang, S. K., and Bankert, R. B. (2000). Antibody targeting of doxorubicin-loaded liposomes suppresses the growth and metastatic spread of established human lung tumor xenografts in severe combined immunodeficient mice. *Cancer Res.* **60,** 6942–6949.

Taggar, A. S., Alnajim, J., Anantha, M., Thomas, A., Webb, M., Ramsay, E., and Bally, M. B. (2006). Copper-topotecan complexation mediates drug accumulation into liposomes. *J. Control. Release* **114,** 78–88.

Zamboni, W. C., Strychor, S., Joseph, E., Walsh, D. R., Zamboni, B. A., Parise, R. A., Tonda, M. E., Yu, N. Y., Engbers, C., and Eiseman, J. L. (2007). Plasma, tumor, and tissue disposition of STEALTH liposomal CKD-602 (S-CKD602) and nonliposomal CKD-602 in mice bearing A375 human melanoma xenografts. *Clin. Cancer Res.* **13,** 7217–7223.

Zhou, Y., and Marks, J. D. (2009). Identification of target and function specific antibodies for effective drug delivery. *Methods Mol. Biol.* **525,** 145–160, xv.

Zhou, Y., Zou, H., Zhang, S., and Marks, J. D. (2010). Internalizing cancer antibodies from phage libraries selected on tumor cells and yeast-displayed tumor antigens. *J. Mol. Biol.* **404,** 88–99.

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TARGETING OF LIPOSOMES TO SOLID TUMORS: THE CASE OF STERICALLY STABILIZED ANTI-HER2 IMMUNOLIPOSOMES

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ABSTRACT

Novel therapies for cancer call for a carrier capable of intracellular delivery of systemically administered drugs to cancer cells in solid tumors. Such carrier, sterically stabilized immunoliposomes specific to the cells expressing HER2 protooncogene (anti-HER2 SSL), was designed by conjugating Fab' fragments of a recombinant humanized anti-HER2 MAb to the distal termini of poly(ethylene glycol) chains on the surface of unilamellar liposomes (size 90-100 nm) of phosphatidylcholine, cholesterol, and poly(ethylene glycol)-derivatized phosphatidylethanolamine. Anti-HER2 SSL avidly and specifically bound to cultured HER2-overexpressing cancer cells (8,000-23,000 vesicles per cell) and became endocytosed (k_e=0.022-0.033 $min.^{-1}$) via the coated pit pathway. Anti-HER2 SSL showed prolonged circulation lifetime in rats (blood MRT approx. 24 hours) and significantly increased antitumor activity of encapsulated doxorubicin against HER2-overexpressing human breast cancer xenografts in nude mice. Although the accumulation of anti-HER2 SSL in HER2-overexpressing tumor xenografts was not increased over that of non-targeted SSL, microscopic examination revealed abundance of anti-HER2 SSL in the interstitial spaces, as well as within the cytoplasm of cancer cells, while identical liposomes lacking

anti-HER2 Fab' were located predominantly within tumor-resident macrophages. Anti-HER2 SSL, a targeted vehicle capable of *in vivo* intracellular delivery of substances to HER2-overexpressing solid cancers, enhances the potential for tumor targeting and opens new avenues for better treatment of cancer.

INTRODUCTION

The use of liposomes as vehicles for targeted delivery of drugs to diseased tissues has already been discussed (1). There is convincing experimental evidence for specific association of the cells exposing a characteristic ligand with liposomes bearing recognition molecules, such as antibodies or antigen-binding antibody fragments (2-5). Specific association with the target potentially leads to better therapeutic efficacy and less toxicity of liposome-encapsulated pharmaceuticals (2,3). This is especially important in cytotoxic chemotherapy of cancer, where therapeutic indices are narrow and systemic toxicity is high. Introduction of liposome designs which avoid early clearance by mononuclear phagocytic system (MPS), have longer circulation times and increased likelihood for a liposome drug to reach its intended destination in the body (6-10) attracted new attention to the targeting of liposomes. Grafting of hydrophilic, flexible chains of poly(ethylene glycol) on the liposome surface (sterically stabilized or Stealth® liposomes) has proved to be among the most successful methods for achieving long-circulating properties (8-10). Targeting of sterically stabilized liposomes containing phospholipids with poly(ethylene glycol)-modified head groups (PEG-DSPE) was at first effected by attachment of antibodies, via a hydrophobic anchor, in close proximity to the liposome surface (11). Such liposomes had long-circulating properties, but their interaction with the target was reduced presumably by the same mechanism which contributed to the MPS avoidance (12,13). More recently, target-specific molecules (antibodies, sugars, or peptides) were attached to the distal termini of liposome-grafted poly(ethylene glycol) chains, leading to a targeted sterically stabilized liposome with fully preserved target-binding capability (14-16).

The potential of this approach will be illustrated here by our studies with sterically stabilized liposomes targeted to cancer cells overexpressing the HER2 (c-ErbB-2, *neu*) oncoprotein. Our evidence indicate that sterically stabilized anti-HER2 immunoliposomes (anti-HER2 SSL) are not only capable of target-specific

binding and internalization by cancer cells in culture (17,18), but are also able to cross the vascular barrier and to be internalized by target cancer cells in solid tumor xenografts following intravenous injection (19). Anti-HER2 SSL carrying the anticancer drug doxorubicin are more efficient than their non-targeted counterparts in suppressing the growth of HER2-overexpressing tumor xenografts (20,21). Inclusion of a cationic lipid in the composition of anti-HER2 SSL results in the vehicle for specific delivery of nucleic acids into HER2-overexpressing cancer cells (19,20,22).

RATIONALE FOR HER2 ONCOPROTEIN AS A TARGET MOLECULE

The HER2 (c-erbB-2, neu) protooncogene encodes a 185 KDa (1255 amino acids) receptor tyrosine kinase ($p185^{HER2}$, ErbB-2, or HER2) which belongs to the family of receptor tyrosine kinases including also the products of epidermal growth factor (EGFR), HER3 (erbB-3), and HER4 (erbB-4) genes (23,24). Studies have shown overexpression of HER2 in a variety of malignancies, including cancers of the breast (25,26), ovary (27), endometrium (28) , lung (non-small cell) (29) , stomach (30,31) , pancreas (32), bladder (33) , and prostate (34). Especially high incidence of HER2 overexpression (up to 50%) was found in breast ductal carcinoma in situ (DCIS), particularly in the lesions having high risk of recurrence (35,36). HER2 oncoprotein appears to be an important mediator of tumor growth directly contributing to tumor onset and progression, and conferring an especially aggressive malignant phenotype (23).

There is a number of advantages of HER2 as a recognition marker for targeted delivery of anticancer agents. HER2 is a readily accessible cell surface protein with substantial levels of overexpression (10^5-10^6 copies/cell) in various malignancies (37) . In normal adult tissues, HER2 occurs only in certain epithelial types, and at very low levels (38). HER2-overexpression is relatively homogenous within primary tumors, and is maintained at metastatic sites, suggesting continuous requirement for high levels of HER2 throughout the malignant process (39), while many other tumor-associated antigens show variable expression patterns within the tumor tissue and/or in the course of tumor progression. Last, but not least, is the fact that HER2 activation is accompanied by its internalization into the cell, which may occur upon interaction with an agonistic antibody (40-42). Therefore, ligands targeted to HER2 by means of such antibodies would have a better chance to enter the cell rather than stay attached to the cell surface.

There has been considerable effort to achieve antitumor effect by blocking the function of this receptor protein. A variety of monoclonal antibodies (40-43) as well as phage-display library generated single-chain Fv's (44,45) reactive with the extracellular portion of HER2 have been reported, offering a vast palette of target-specific molecules suitable for liposome attachment. The variants with the highest antiproliferative effect have also demonstrated the highest rate of cell internalization (41) . One such antibody, muMAb4D5, is highly reactive toward HER2 (43) and inhibitory for the growth of HER2 overexpressing tumor cells *in vitro* (37) and in animal models (46). This antibody was engineered into a fully humanized version, rhuMAbHER2, to reduce the potential for immunogenicity (47), and entered clinical trials showing antitumor responses (12% response rate) in patients with metastatic HER2-overexpressing breast cancer (48).

ANTI-HER2 IMMUNOLIPOSOME DESIGN

Antibody-targeted pharmaceutical liposomes for the treatment of solid tumors should satisfy a number of requirements aimed at maximum targeting effect of systemically administered immunoliposomes (Table 1). Antigen binding sites of the liposome-conjugated antibody must be accessible for unperturbed interaction with antigens on the surface of target cells. To ensure that immunoliposomes will reach their target cells, the rate of MPS clearance or other "non-productive" elimination of blood-borne immunoliposomes must be minimized in comparison with the rate of extravasation in the tumor. Since the liposome-conjugated antibody is a foreign protein likely to elicit host immune response, their immunogenicity must be minimized. Immunoliposomes must allow efficient loading and retention of a selected anticancer drug. And finally, the drug and antibody incorporation must be stable enough to permit liposomal entry into the tumor tissue without the loss of either of these agents.

Immunoliposome design in our studies was developed to maximally satisfy these requirements. Lipid composition of anti-HER2 immunoliposomes was based on hydrogenated soy phosphatidylcholine (T_m=54 °C) so that at body temperature the liposome bilayer maintained the "solid" (gel) state. Cholesterol (40 mol.%) was included to increase bilayer stability in the presence of plasma (49). To reduce MPS clearance rate, the liposomes contained up to 5.7 mol.% of

TABLE 1.

Design of Immunoliposomes for the Therapy of Solid Tumors (20).

Component	Considerations for Optimal Design	
Target Antigen	*Expression:*	Highly and homogeneously overexpressed in target tissue.
	Function:	Vital to tumor progression, so that down-modulation does not occur or is associated with therapeutic benefit.
	Antigen Shedding :	Limited, to avoid binding to soluble antigen and accelerated clearance.
Antibody	*Affinity:*	High enough to ensure binding at low liposome concentrations.
		Low enough to avoid "binding barrier" effect
	Immunogenicity:	Humanized MAb, to remove murine sequences. Use fragments without Fc portion.
	Internalization:	Efficiently endocytosed by target cells.
	Biological Activity:	Intrinsic antitumor activity may enhance antitumor effect.
	Production:	Easy and economical scale-up, e.g. by efficient bacterial expression system
		Stable during storage.
Linkage	*Stability:*	Covalent attachment to hydrophobic anchor, stable in blood.
	Attachment Site:	Away from the binding site, to ensure correct orientation of antibody molecule.
		Well-defined, to ensure reproducibility and uniformity of coupling.
		Avoids steric hindrance (e.g. from PEG) of MAb binding and internalization.
	Chemical Nature of the Linker:	Non-toxic. Non-immunogenic. Avoids opsonization.
		Does not affect drug loading and membrane stability.
		Excess linker may be quenched to avoid non-specific coupling to biomolecules.
		Good availability, economical manufacturing process.
Liposome	*Stability:*	Stable as intact construct *in vivo.*
	Pharmacokinetics:	Long circulating.
	Tumor Penetration:	Capable of extravasation in tumors.
		Smaller size improves penetration into tumor tissue.
Drug	*Encapsulation:*	Efficient, high capacity (e.g. by remote loading).
		Encapsulated drug storage-stable and resists leakage.
	Bystander Effect:	Drug affects tumor cells not directly targeted (bystander cells)
	Interaction with Target Cells:	Effective against target cell population.
		Cytotoxicity enhanced by binding of MAb.

DSPE modified with methoxypoly(ethylene glycol) with molecular mass 1,900 (7). To assist extravasation, the liposomes were of uniform, small size (90-110 nm) achieved by extrusion of hydrated lipid suspension (multilamellar vesicles) through track-etched polycarbonate membranes (pore sizes 100 and 50 nm) after several cycles of freezing and thawing (50,52).

Elimination of immunogenic effects of the Fc portion which is associated with increased MPS clearance through specific recognition by phagocytic cells carrying Fc receptor, was achieved by using Fab' fragments instead of intact anti-HER2 MAb. The use of Fab' fragments also allowed better way of conjugation to the liposome through unique thiol groups in the hinge region (51,52) providing predictable and correct orientation of the antigen-binding sites. The binding affinity of liposome-conjugated Fab' was not compromised in comparison with the binding of the whole antibody (see below) probably because the presence of multiple Fab' fragments on the liposome restored the multicenter interaction characteristic for the whole antibody which carries two antigen- binding sites (18). Anti-HER2 Fab' fragments were produced as a recombinant protein derived from a humanized anti-HER2 MAb (rhuMAbHER2) developed by Genentech, Inc. (South San Francisco, CA, USA) for the therapy of cancers overexpressing HER2 (47,48). This antibody was itself derived from a murine MAb muMAb4D5, which induces endocytosis of HER2 receptors upon binding to their extracellular domains (43). The use of Fab' antibody fragments with humanized sequence further reduces the risk of immune reaction in human patients; recombinant technology provided stable, reproducible and more economical source of this protein, and the ability to induce internalization upon binding to the target antigen was favorable for intracellular delivery of liposome-associated pharmaceuticals.

Attachment of anti-HER2 Fab' to sterically stabilized liposomes was effected by using two linkers both containing a hydrophobic anchor (DSPE or DPPE), and a thiol-reactive maleimide function. The use of aromatic maleimides, such as maleimidophenylbutyric acid derivatives (MPB-PE) (51,53) was avoided because of their reported ability to cause leakage of encapsulated drugs from liposomes (54) Linkers of the first type consisted of DPPE or DSPE with their amino groups acylated by hydroxysuccinimide esters of 4-(N-maleimido)methylcyclohexyl-carboxylic acid (MMC-PE) or β-(N-maleimido)propionic acid (MP-PE) (17). These linkers positioned the conjugated Fab' fragment "in parallel" with the surface-attached PEG chains. The second type

of linkers additionally included PEG chain (M_r=2,000) between DSPE and the maleimido group (MMC or MP) (Fig. 1) (18,52). Linkers of this type positioned Fab' fragments "in series" with surface-attached PEG, i.e. outside of the PEG "cloud" surrounding the liposome. The second type of conjugation was essential for preservation of the target-binding and also for internalization of the anti-HER2 liposomes, as discussed below.

Conjugations of Fab' were performed by incubation of SSL containing 1.2 mol.% of appropriate linker with anti-HER2 Fab' (approx. 30 μg/μmol of liposomal phospholipid) for 2 hours to overnight at 4 °C and pH 7.2-7.4. Under these conditions, the protein was covalently attached to liposomes, and co-eluted with liposomes in the void volume fraction during gel-exclusion chromatography on Sepharose 4B. More than 90% of Fab' bearing free thiol groups were coupled to the liposomes containing MP-PEG-DSPE as a conjugation linker, resulting in 50-60 Fab'/liposome. Coupling of Fab' fragments to MMC-PE-containing liposomes was quite efficient (40% yield) even at PEG densities associated with the "brush" regime, i.e. at PEG(M_r=2,000)-DSPE >5 mol.% of the liposome phospholipid, or, in our case, >3.3 mol.% or total lipid (55), which means that. Fab' molecules still were able to reach the liposome bilayer. Therefore, penetrability of the PEG layer for protein molecules may be higher than previously predicted (12,56).

Doxorubicin was the anticancer drug of choice for this study because it has been well characterized in a similar, non-targeted sterically stabilized liposome system, Doxil® (57-59). Drug loading (0.09-0.15 mg of doxorubicin per μmol of liposomal phospholipid) was performed prior to the conjugation of anti-HER2 Fab' by the ammonium sulfate gradient method (60) at the temperature above T_m of the bilayer.

INTERACTION WITH CANCER CELLS *IN VITRO*

In vitro binding specificity of anti-HER2 SSL was studied in cultures of human breast carcinoma cells with high (SK-BR-3, 10^6 HER2/cell) or low (MCF-7, 10^4 HER2/cell) expression of HER2 (37) using confocal microscopy and spectrofluorometry of liposome-associated fluorescent markers. Liposomes of HSPC, cholesterol, 2.6 mol.% of methoxyPEG-DSPE, 1.2 mol.% of MMC-PEG-DSPE, and 0.2 mol.% of a fluorescent lipid marker N- lissamine-rhodamine B-

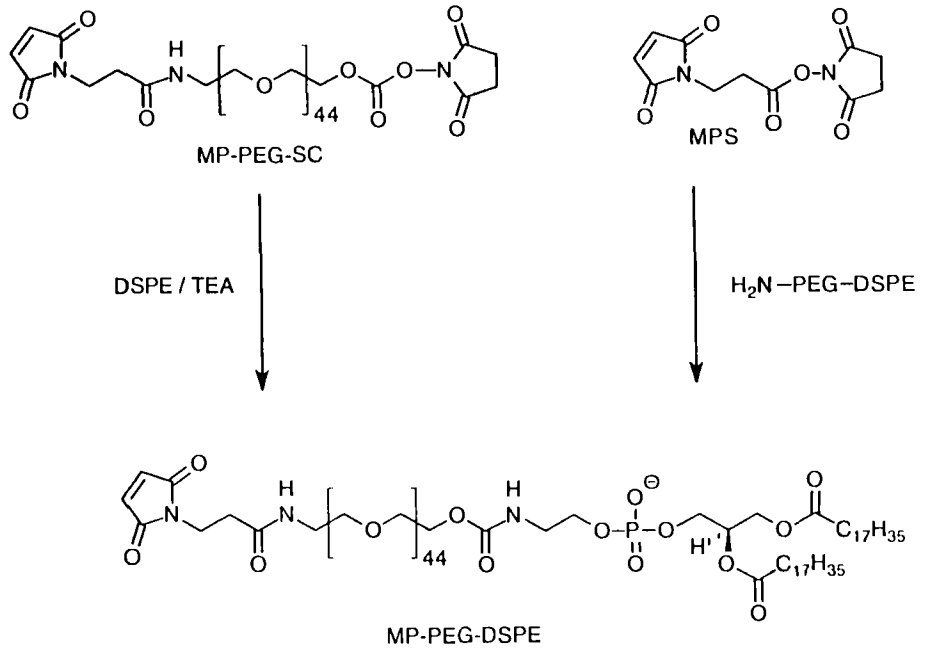

FIG. 1. Synthesis of maleimido-terminated PEG-DSPE derivatives (17).

dihexadecanoyl phosphatidylethanolamine (Rh-PE) were prepared and conjugated to anti-HER2 Fab' as described above. The liposomes were incubated with cells at 37 °C in the presence of fluorescein-labeled (FITC) transferrin, which served as a marker for coated pit endocytosis. The liposomes were at first observed on the surface of SKBR-3 cells, and further entered the cells and became co-localized with FITC-transferrin, as it was evidenced by yellow color produced by overlapping green (FITC) and red (Rh-PE) fluorescences. Under the same experimental conditions, "target-negative" MCF-7 cells displayed only green punctate fluorescence of endocytosed FITC-transferrin, but not of the lipid label; similar results were obtained with SKBR-3 cells incubated with Rh-PE labeled SSL lacking conjugated anti-HER2 Fab' (17). These results confirmed the ability of anti-HER2 SSL to specifically bind to target cells and undergo endocytosis. Endocytosis of anti-HER2 liposomes without PEG-DSPE by SKBR-3 cells was also shown by electron microscopy using liposomes labeled with colloidal gold (18).

Cell binding and endocytosis were quantitatively assessed using liposomes with encapsulated pH-sensitive probe 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS, pyranine). Fluorescence excitation spectrum of HPTS undergoes rapid changes in response to the liposome entry into acidic environment of endosomes and lysosomes, but it also has a pH-independent isosbestic point for accurate quantitation of the probe (61). For faster equilibration of protons across the liposome bilayer, in these studies we substituted 1-palmitoyl-2-oleoyl-phosphatidyl-choline (POPC) for HPTS, since POPC bilayers are in more more proton-permeable liquid crystalline state rather than gel state at ambient conditions. Upon incubation with HER2-overexpressing SKBR-3 cells, HPTS-loaded anti-HER2 SSL became rapidly associated with cells in a neutral compartment (cell surface), followed by acidification of the liposome environment indicating endocytosis. The amount of cell-associated anti-HER2 SSL reached a plateau after 3-4 hours of incubation, with more than 80% of liposomes endocytosed. At saturating concentrations, the uptake of anti-HER2 SSL by SKBR-3 cells, estimated from the fluorescence at HPTS isosbestic point, was in the range of 8,000-25,000 vesicles /cell, while the uptake of liposomes by "target-negative" MCF-7 cells was essentially undetectable (<100 vesicles/cell) (17).

Conjugation of Fab' via hinge thiol group and maleimide-activated hydrophobic linker (51) was a high yield reaction without any loss of antigen-binding activity; however, the relative position of liposome-conjugated Fab' and PEG was crucial for maintaining high uptake of anti-HER2 SSL by target cells. During incubation of SKBR-3 cells at a constant concentration of liposomes (25 μM of liposome phospholipid) in the cell growth medium, the increasing PEG-DSPE content inhibited the uptake of anti-HER2 SSL prepared with MMC-DSPE linker, while no such inhibition occurred when anti-HER2 Fab' were attached via MP-PEG-DSPE linker (Fig. 2). Similar effect of the placement of a targeting antibody on the uptake of immuno-SSL by target cells/tissues was reported earlier (16,62) in studies utilizing whole antibodies rather than Fab' fragments. Since the cellular uptake of anti-HER2 liposomes includes steps of binding and subsequent endocytosis, we have studied the effect of PEG-DSPE and Fab' placement on each of these steps separately. Liposome-cell binding was characterized by dissociation constants (K_d) estimated from the amounts of cell-associated anti-HER2 liposomes after incubation of SKBR-3 cells at various liposome concentrations

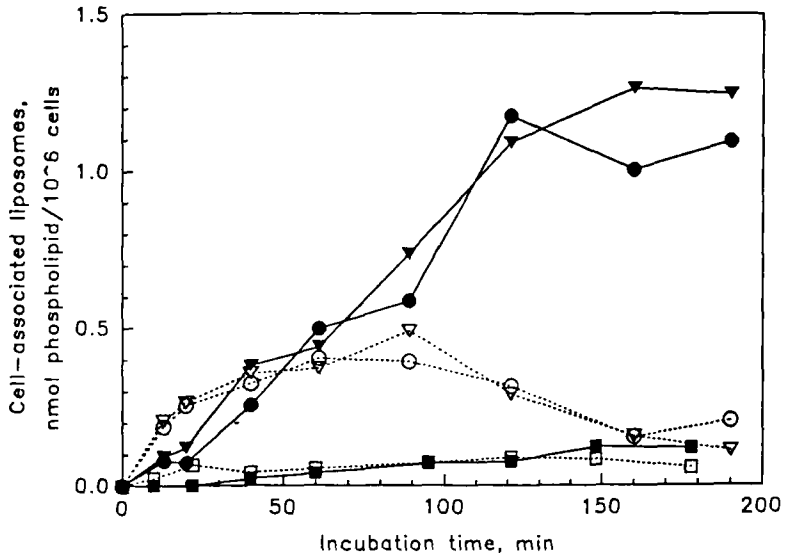

FIG. 2. Effect of PEG-DSPE (PEG M_r=1,900) content on the kinetics of anti-HER2 liposome association with SKBR-3 cells. Liposomes (POPC/cholesterol 3:2, with or without 5.7 mol.% PEG-DSPE) containing entrapped 35 mM HPTS adjusted to pH 7.2 (NaOH) and osmolarity of 290 mOsmol/kg (NaCl) were incubated with the cells in serum-supplemented cell growth medium at concentration of 25 μM liposomal phospholipid and 37°C. Following incubation, the cells were repeatedly rinsed with buffered saline, harvested with 5 mM EDTA in buffered saline, pH 7.2, and cell fluorescence (excitation: 413 nm, 454 nm; emission: 512 nm) was examined using SPEX Fluorolog 2 photon-counting spectrofluorometer. Amounts of cell surface-bound and endocytosed liposomes were calculated as described earlier (18,61). (○) Anti-HER2 Fab' attached via short linker MMC-DSPE; no PEG-DSPE; (□) Anti-HER2 Fab' attached via short linker, 5.7 mol.% PEG-DSPE; (▽) Anti-HER2 Fab' attached to the distal end of PEG chain (linker MP-PEG-DSPE); 5.7 mol.% PEG-DSPE. Surface-bound liposomes: dashed line, hollow symbols. Endocytosed liposomes: solid line, filled symbols.

and low temperature (4 °C) that completely inhibits endocytosis. For anti-HER2 liposomes without PEG coating, K_d normalized to the amount of liposome-conjugated Fab' was 12.0±1.7 nM (mean±SE), close to the reported values for the whole murine prototype anti-HER2 MAb 4D5 (6.0 nM) and its free Fab' fragment (19.0 nM) (41). As one may expect from the size of Fab' fragment, 6 nm in the longer aspect (63), and the thickness of PEG layer on the liposome surface, 5-7 nm for PEG with M_r=2,000 in the "brush" regime (55,64), when Fab' fragments were conjugated through the "short" linker MMC-PE, the increase of PEG (Mr=2,000)-DSPE amount to 1.2 mol.% reduced the affinity approximately three-fold, and at 3.5-5.7 mol.% of PEG-DSPE the binding affinity was 20 to 75 times lower (K_d=320-900 nM). On the contrary, conjugation of Fab' to the termini of PEG chains did not affect liposome binding to the target cells at increasing PEG-DSPE content (K_d 13-15 nM for 1.2-5.7 mol.% PEG-DSPE) (17). First-order rate constants of liposome endocytosis (k_e) were determined from the kinetic curves of cell surface-bound and endocytosed liposomes obtained by HPTS method (Fig. 2), as the ratio of liposome internalization rate to the steady-state surface concentration of liposomes (65). Compared to K_d, k_e of anti-HER2 SSL prepared with the "short" linker MMC-PE was somewhat reduced at increased PEG-DSPE content (k_e decreased 2 times at 5.7 mol.% PEG-DSPE vs. 0%), and was not affected at all when MP-PEG-DSPE was used as a linker. Thus, PEG interfered with the ability of liposome-conjugated Fab' to bind to the cell surface antigen, and to the less extent, with its ability to induce endocytosis or the liposome; however, this interference was completely avoided by conjugation of anti-HER2 Fab' at the distal termini of liposome-grafted PEG chains.

The number of liposome-conjugated anti-HER2 Fab' and fthe cellular level of HER2 protein expression required to achieve specificity and effectiveness of liposome uptake were studied using anti-HER2 liposomes without PEG coating (17,18). Binding of liposomes with the target cells increased in a linear manner as a function of Fab' density, and reached saturation (plateau) at ≈40 Fab'/vesicle; endocytosis of cell-bound liposomes occurred with 60% efficiency already at ≈10 Fab'/vesicle. That is, relatively few conjugated Fab' were needed for specific uptake. Similarly, the uptake of anti-HER2 liposomes by the cells with minimally elevated cellular levels of HER2 to allow classification as "HER2-positive" (MDA-MB-453, approx. 10^5 molecules/cell) was comparable to that by the cells with extremely high levels of HER2 expression (SK-BR-3, BT-474; approx. 10^6 molecules/cell). There was, however, pronounced difference in the uptake of anti-

HER2 liposomes between the cells with elevated levels of HER2 and those with only basal HER2 expression (MCF-7; approx. 10^4 molecules/cell) (Fig. 3).

Liposomes with conjugated via MMC-PE linker to rhuMAbHER2 Fab' were loaded with doxorubicin using ammonium sulfate gradient method. In contrast to the liposomes conjugated via aromatic maleimide derivative, MPB-PE (16,54), the drug loading was practically quantitative even though the linker constituted 1.2 mol.% of total liposome lipid. Doxorubicin-loaded anti-HER2 liposomes showed efficient and specific *in vitro* cytotoxicity against HER2-overexpressing cancer cells. After 1 hour incubation, doxorubicin delivered by anti-HER2 liposomes to SKBR-3 cells was as cytotoxic as free doxorubicin (IC_{50}=0.3 μg/ml), while cytotoxicity of doxorubicin in the liposomes with conjugated irrelevant Fab', or in anti-HER2 liposomes incubated with lung fibroblast cells (WI-38) expressing only minimal levels of HER2 was 20-30 times less than that of free drug (18). Goren and co-workers (66) reported doxorubicin-loaded anti-HER2 SSL bearing whole antibodies attached to PEG terminal groups via hydrazone formation with periodate-oxidized carbohydrate moieties. These liposomes bound quite well to HER2-overexpressing gastric cancer cells (N-87), but their cytotoxicity was equal to that of non-targeted doxorubicin-loaded liposomes, and much less than that of the free drug, presumably because of the inability of these liposomes to be internalized by the target cells (66). In contrast, doxorubicin-loaded immunoliposomes bearing conjugated anti-HER2 MAb SER4 were endocytosed by HER2 overexpressing cells SKBR-3 and MKN-7, and were 25 times more cytotoxic to these cells than the matching liposomes conjugated to an antibody against non-internalizable surface protein gp125 (67). Moreover, compared with anti-gp125 immunoliposomes, SER4-conjugated immuno-liposomes required 4.3-4.5 times less doxorubicin association with HER2 overexpressing cells for equal cytotoxicity (67). Evidently, endocytosis of doxorubicin-loaded anti-HER2 liposomes is important for increased cytotoxicity of the liposomal drug, presumably by creating an acidic environment and transmembrane pH gradients which favor escape of the drug from the liposome and its further distribution throughout the cell.

IN VIVO STUDIES WITH ANTI-HER2 SSL

Pharmacokinetics of doxorubicin in anti-HER2 SSL was studied in normal Lewis rats with indwelling jugular vein catheters. The animals received 5 μmol of

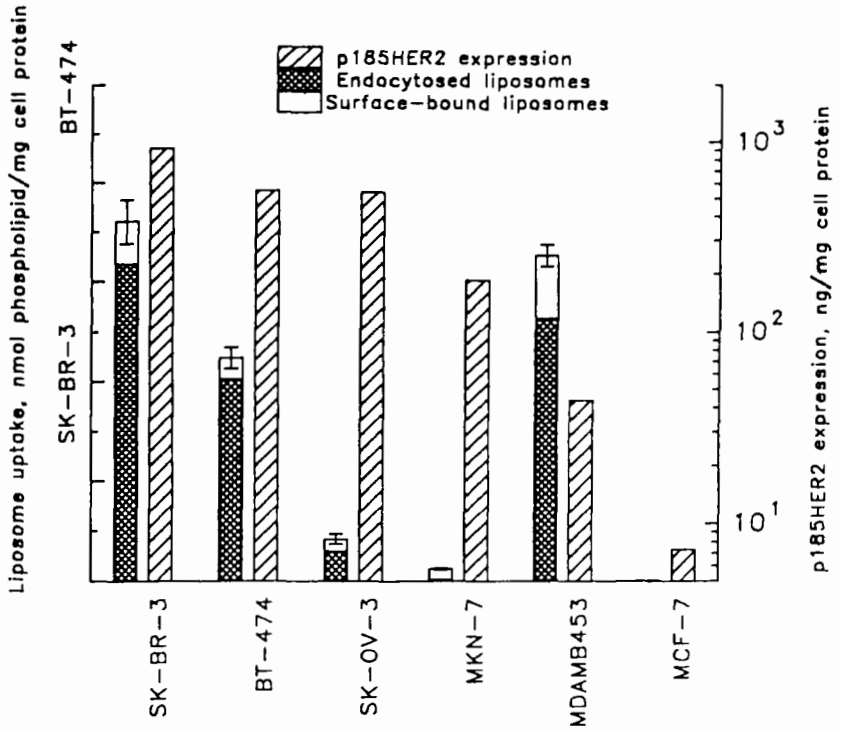

FIG. 3. *In vitro* uptake of anti-HER2 liposomes by the cells with various levels of HER2 expression. For methodology see caption to Fig. 2; incubation time 4 hours. Values in brackets indicate total cellular HER2 expression (ng/mg cell protein) (from Ref. 37). Error bars, SEM (n=3).

liposomal phospholipid (0.8-1 mg of doxorubicin) intravenously at time 0; the blood was repeatedly sampled within 48 hours post injection and assayed for doxorubicin by spectrofluorometry after separation of blood cells and extraction witn acidified alcohol. The drug showed biphasic elimination profile with $t_{1/2\alpha}$= 6.1 min., $t_{1/2\beta}$=976 min., AUC=93,100 min%, and blood MRT=1460 min. (19,21). This pharmacokinetic behavior is characteristic of long-circulating liposomes and similar to that of non-targeted doxorubicin-loaded SSL, in contrast to free doxorubicin which had plasma half-life of about 5 min. (19). The use of Fab' instead of the whole antibody was of importance, since the analogous constructs bearing conjugated whole antibodies show lower circulation half-lives than corresponding non-conjugated SSL (16,66). To analyze possible drug

leakage or dissociation of Fab' from the liposomes in circulation, plasma pharmacokinetics of the anti-HER2 SSL-entrapped drug was compared to that of liposome-conjugated Fab' fragments. In this study, plasma concentration of anti-HER2 Fab' was determined by ELISA using extracellular domain of HER2 for capture, and HPR-linked goat anti-human IgG for detection. Drug and anti-HER2 Fab' showed identical pharmacokinetic profiles indicating the lack of drug or Fab' dissociation in from the liposomes in circulation (19,20).

The effect of tumor cell targeting on the therapeutic efficacy of sterically stabilized liposomes containing doxorubicin was studied in nude mice with subcutaneous xenografts of HER2-overexpressing human breast adenocarcinomas (BT-474 and MDA-MB-453) Treatment of animals with established (approximately 200 mm^3) xenografts of HER2-overexpressing human breast carcinomas (BT-474, MDA-MB-453) by three weekly injections of free or SSL-encapsulated doxorubicin revealed superior activity of doxorubicin in anti-HER2 SSL (20,21). The average ratio of the volume of BT-474 tumors at the end of the experiment (48-60 days post tumor inoculation) to that at the beginning of the experiment (12-14 days post inoculation) was 19.13±1.14 in the group treated with free doxorubicin at a total maximum tolerated dose (MTD) of 7.5 mg/kg, 2.59±0.28 in the group receiving non-targeted doxorubicin-loaded SSL (MTD, 15 mg/kg), and 0.63±0.12 in the group injected with doxorubicin-loaded anti-HER2 SSL (15 mg/kg); in the case of MDA-MB-453 tumors (with lower expression of HER2), these values were 3.54±0.53, 2.15±0.29, and 1.17±0.16, respectively. The difference between growth rates of tumors in the groups receiving doxorubicin in HER2-targeted vs. non-targeted SSL was statistically significant at p=0.001 (BT-474) and p=0.004 (MDA-MB-453) according to a modified Norton-Simon model of tumor growth (69). Administration of "empty" anti-HER2 SSL at equal dose/schedule did not produce antitumor effect in these models, ruling out the inhibitory effect of immunoliposome itself. Therefore the increased antitumor activity must be attributed to the targeting.

Enhanced antitumor activity of immunotargeted drugs is often attributed to their enhanced accumulation in tumor tissue. To find out if this was the case for anti-HER2 SSL as well, we prepared non-targeted or HER2-targeted sterically stabilized liposomes with encapsulated chelating agent (DTPA) and remote-loaded them with a radiotracer ^{67}Ga *via* oxine complex (68). Nude mice bearing BT-474 or MCF-7 xenografts were injected via tail vein with ^{67}Ga-labeled anti-

HER2 SSL or non-targeted SSL (in the latter, the Fab'-conjugation step was omitted during the liposome preparation) in the dose of 1 μmol of liposome phospholipid per animal, which is equivalent to the dose of doxorubicin liposomes administered in the treatment efficacy studies. The animals were sacrificed 24 hours later, and the tissue concentrations of liposomes (% of injected dose/g tissue) were determined by radioactivity measurements (Fig. 4). Both HER2-targeted and non-targeted SSL were found in tumor tissue in high concentration, 4-8 % of injected dose (i.d.)/g, which, assuming 10^9 cells per 1 g of tumor tissue and 80,000 phospholipid molecules per liposome, was consistent with an average of 300-600 vesicles per tumor cell. Bioistribution of ^{67}Ga-labeled anti-HER2 SSL in non-tumor tissues was characteristic of PEG-coated liposomes and was not significantly different from that of the similar non-targeted SSL. Interestingly, no statistically significant difference was found between the accumulation of HER2-targeted SSL (8.34±1.54 % i.d./g) or non-targeted SSL (7.32±1.05 % i.d./g) in HER2-overexpressing BT-474 tumors. In similarly established xenografts of MCF-7 tumors which express low levels of HER2, the difference of tumor uptake between targeted and non-targeted SSL was also insignificant, and both liposomes accumulated in MCF-7 tumors in similar amounts (7.2-8.6 % i.d./g) regardless of the presence of anti-HER2 Fab' (Fig. 4).

Since the biodistribution study ruled out the increased tumor uptake of targeted liposomes as an explanation of higher antitumor efficacy, the mechanisms of this phenomenon had to be sought elsewhere. In fact, the lack of increase in the tumor concentration of tumor-cell targeted SSL over non-targeted ones is in accord with the view that the limiting step in the tumor accumulation of the blood-borne liposomes is extravasation (70) upon which this type of targeting evidently would have no effect. One cannot exclude that specific interaction with HER2-overexpressing tumor cells resulted in the extended residence time of anti-HER2 SSL in the tumor tissue, which calls for further pharmacokinetic studies of tumor cell-targeted vs. non-targeted SSL in tumor tissue at longer post injection times. However, studies of liposome disposition within the tumor tissue revealed another mechanism that may explain higher antitumor efficacy of tumor cell targeted cytotoxic SSL.

To visualize the location and distribution of anti-HER2 SSL in the tumor tissue, we used anti-HER2 SSL, as well as non-targeted SSL labeled with entrapped colloidal gold (71). Liposomes of egg PC, cholesterol,

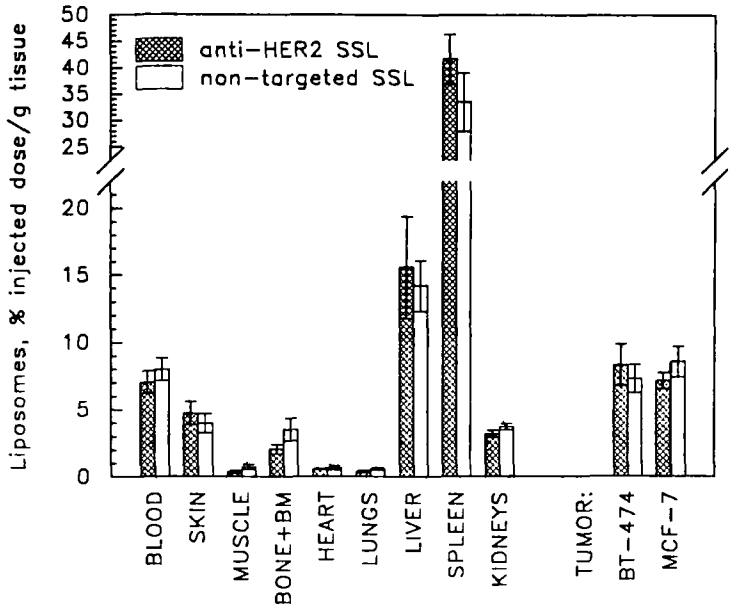

FIG. 4. Biodistribution of anti-HER2 SSL and non-targeted SSL in nude mice with breast cancer xenorgafts. 4-6 week old NCR nu/nu mice with subcutaneously implanted slow-release estrogen pellets (in the neck area) were injected subcutaneously in the flank with $2x10^7$ cells of human breast adenocarcinoma: HER2-overexpressing (BT-474), or low HER2-expressing (MCF-7). 12-14 days later (tumor size 200-300 mm^3) the animals were injected via tail vein with 1 μmol (liposomal phospholipid) of SSL labeled by remote loading with ^{67}Ga-DTPA complex (68). The animals were sacrificed, and liposome concentration in the tissues was determined by radioactivity measurements 24 hours post injection. Error bars, SEM (n=5).

PEG(M_r=1,900)-DSPE, and maleimidopropionyl-amido-PEG-DSPE in molar ratio 3:2:0.24:0.06 were prepared in aqueous solution of $HAuCl_4$ by reverse phase evaporation, followed by sizing through 0.1 μm and 0.05 μm polycarbonate membranes. Colloidal gold particles were precipitated within liposomes by pH shift using citrate-carbonate buffer; unentrapped gold particles were removed by passing of the liposomes through Sephacryl S-400 in 144 mM NaCl, 20 mM HEPES-Na, pH 7.2. Nude mice bearing subcutaneous xenografts of the above tumors (200-600 mm^3 in size) were injected with gold-labeled liposomes or immunoliposomes via tail vein at the dose of 5 μmol phospholipid/animal, and

sacrificed 48 hours later. Tumors were collected, fixed with 4% formaldehyde in phosphate-buffered saline, embedded in glycol metacrylate, and cut in 3 μm sections. Entrapment of gold had no effect on liposome stability or anti-HER2 Fab' conjugation. Tumor sections were enhanced with silver to visualize the location of gold particles, and microscopically examined at lower magnification (x400) using dark field illumination, and at higher magnification (x1,250) using regular bright field illumination (Fig. 5). In HER2-positive tumor (BT-474) anti-HER2 SSL were observed abundantly throughout the tumor tissue. In the absense of targeting, either through the lack of liposome-conjugated anti-HER2 Fab' fragments (non-targeted SSL), or through insufficient expression of HER2 protein (MCF-7 tumors), the label was concentrated mostly within tumor-resident macrophages and in perivascular areas, in agreement with previous observations on non-targeted SSL (72). At higher magnification, anti-HER2 SSL were frequently revealed within the cytoplasm and in the perinuclear spaces of HER2-positive cancer cells within the tumor tissue. In the absence of targeting, there was no clear deposition of silver granules within cancer cells: the label was detected in tumor stroma and especially within tumor-resident macrophages. Thus, anti-HER2 SSL not only crossed the vascular barrier into the solid tumor, but, in the case of HER2-overexpression, frequently became endocytosed by the cancer cells as they would be *in vitro*. Increased deposition in the intercellular spaces within the tumor tissue outside tumor-resident macrophages, as well as the intracellular delivery of the encapsulated drug *in vivo* may have contributed to superior antitumor efficacy of doxorubicin-loaded anti-HER2 SSL.

CONCLUSION

Liposomal pharmacology has been greatly advanced over the last decade, by introduction of long-circulating liposomes (6-10), refinement of liposome preparation techniques (50,52) and "remote loading" methods for drug loading into liposomes (60,73). This is clearly evidenced by the recent approval of liposome-encapsulated drugs (Doxil, DaunoXome) for cancer treatment (74). It has also brought new enthusiasm to the area of liposome targeting. In this communication we presented a case study that illustrates, in our view, a "rational design" approach to antibody-targeted liposomes. Each element of this design addresses certain issues relating to the ultimate medical use of the targeted

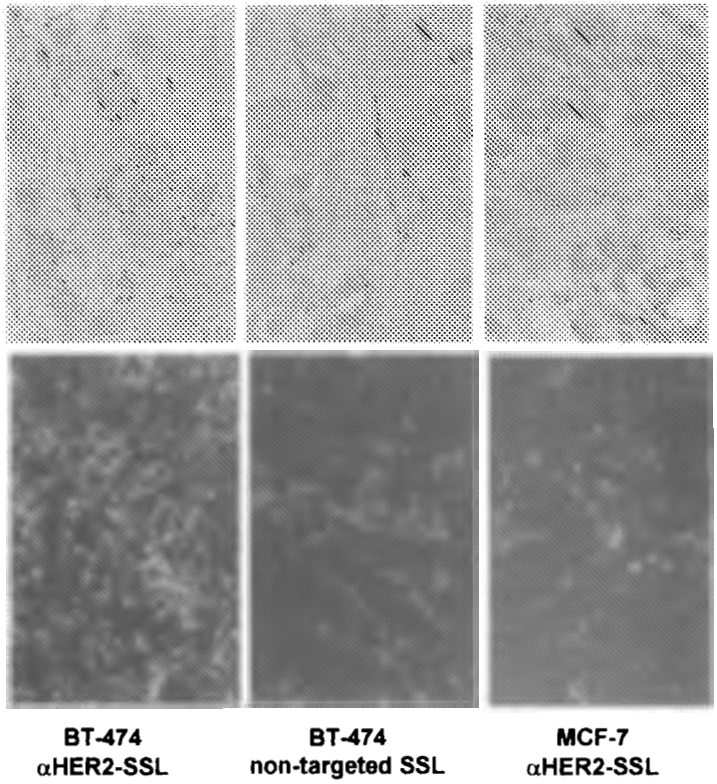

FIG. 5. Localization of anti-HER2 SSL and matched non-targeted SSL in HER2-overexpressing (BT-474) and low-HER2 expressing (MCF-7) human breast carcinoma xenografts in nude mice. Liposomes were visualized on tumor sections by silver enhancement of liposome-entrapped colloidal gold, and appear as black grains on bright field images (top panels; x1,250), or as bright dots on dark field images (bottom panels, x400).

liposomal drug carrier. This design may be readily applied to other targeted drug delivery systems utilizing different drugs and/or different target-specific molecules, such as, for example, single chain anti-HER2 antibodies produced by phage display libraries (44,45). Doxorubicin-loaded HER2-targeted SSL constructed according to such design had superior antitumor activity compared to matched non-targeted liposomes in established solid tumor xenografts overexpressing HER2 oncoprotein. Interestingly, this phenomenon was not associated with the increased accumulation of targeted liposomes in HER2-

overexpressing tumors, but apparently resulted from a different pattern of liposome disposition within the tumor tissue. This unexpected but important finding includes improved tumor tissue penetration and internalization of liposomes into HER2-overexpressing cancer cells. Such "rational design" of cancer cell-targeted sterically stabilized liposomes leads to a re-evaluation of the potential for tumor targeting and opens new avenues for better treatment of cancer.

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REFERENCES

1. Papahadjopoulos, D. 1995. 30-Year progress for liposomes: from serendipity to molecular design. J. Liposome Res. 5:ix-xiv.

2. Lasic, D.D. and Papahadjopoulos, D. 1995. Liposomes revisited. Science 267:1275-1276.

3. Leserman, L. and Machy, P. 1987. Ligand targeting of liposomes. In "Liposomes. From Biophysics to Therapeutics". M.J. Ostro (Ed.). Marcell Dekker, New York, p.157-194.

4. Ranade, V.V. 1989. Drug delivery systems. 1. Site-specific drug delivery using liposomes as carriers. J. Clin. Pharmacol. 29:685-694.

5. Straubinger, R.M., Lopez, N.G., Debs, R.J, Hong, K. and Papahadjopoulos, D. 1988. Liposome-based therapy of human ovarian cancer: parameters determining potency of negatively charged and antibody-targeted liposomes. Cancer Res. 48:5237-5245.

6. Allen, T.M. and Chonn, A. 1987. Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS Lett. 223:42-46.

7. Gabizon, A. and Papahadjopoulos, D. 1988. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc. Natl. Acad. Sci. USA 85: 6949-6953.

8. Blume, G., and Cevc, G. 1990. Liposomes for the sustained drug release in vivo. Biochim. Biophys. Acta 1029:91-97.

9. Klibanov, A.L., Maruyama, K., Torchilin, V.P. and Huang, L. 1990. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett. 268:235-237.

10. Papahadjopoulos, D., Allen, T., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.-D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. 1991. Sterically stabilized liposomes: Improvements in pharmacokinetics and anti-tumor therapeutic efficacy. Proc. Natl. Acad. Sci. USA 88:11460-11464.

11. Torchilin, V.P., Klibanov, A.L., Huang, L., O'Donnell, S., Nossiff, N.D. and Khaw, B.A. 1992. Targeted accumulation of polyethylene glycol-coated immunoliposomes in infarcted rabbit myocardium. FASEB J. 6:2716-2719.

12. Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P. and Huang, L. 1991. Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation lifetime of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. Biochim. Biophys. Acta 1062:142-148.

13. Torchilin, V.P., Papisov, M.I., Bogdanov, A.A., Trubetskoy, V.S. and Omelyanenko, V.G. 1995. Molecular mechanism of liposome and immunoliposome steric protection with poly(ethylene glycol). In "Stealth Liposomes". D. Lasic, F. Martin (Eds.). CRC Press, Boca Raton, FL, p.51-62.

14. Allen, T.M., Brandeis, E., Hansen, C.B., Kao, G.Y. and Zalipsky, S. 1995. A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. Biochim. Biophys. Acta 1237:99-108.

15. Hansen, C.B., Kao, G.Y., Moase, E.H., Zalipsky, S. and Allen, T.M. 1995. Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. Biochim. Biophys. Acta 1239:133-144.

16. Zalipsky, S., Hansen, C.B., Lopes de Menezes, D., E and Allen, T.M. 1996. Long-circulating, polyethylene glycol-grafted immunoliposomes. J. Controlled Release 39:153-161.

17. Kirpotin, D., Park, J.W., Hong, K., Zalipsky, S., Li, W.-L., Carter, P., Benz, C.C. and Papahadjopoulos, D. 1997. Sterically stabilized anti-HER2 immunoliposomes: desin and targeting to human breast cancer cells *in vitro*. Biochemistry 36:66-75.

18. Park, J.W., Hong, K., Carter, P., Asgari, H., Guo, L.Y., Wirth, C., Shalaby, R., Kotts, C., Keller, G.A., Wood, W.I., Papahadjopoulos, D and Benz, C.C. 1995. Development of anti-p185HER2 immunoliposomes for cancer therapy. Proc. Natl. Acad. Sci. USA 92:1327-1331.

19. Park, J.W., Hong, K., Kirpotin, D.B., Papahadjopoulos, D. and Benz C.C. 1997. Immunoliposomes for cancer treatment. Adv. Pharmacol. 40:399-436.

20. Park, J.W., Hong, K., Kirpotin, D.B., Meyer, O., Papahadjopoulos, D. and Benz C.C. 1997. Anti-HER2 immunoliposomes for targeted therapy of human tumors. Cancer Lett. 118:153-160.

21. Park, J.W., Colbern, G., Hong, K., Kirpotin, D., Shao, Y., Baselga, J., Moore, D., Papahadjopoulos, D. and Benz, C.C. 1997. Targeted intracellular drug delivery via anti-HER2 immunoliposomes yields superior antitumor efficacy. Proc. ASCO, 16:430a.

22. Meyer, O., Kirpotin, D., Hong, K., Sternberg, B., Park, J.W., Woodle, M.C. and Papahadjopoulos, D. 1997. Poly(ethylene glycol)-coated cationic liposomes as carriers for cellular delivery of antisense oligonucleotides. J. Biol. Chem. (submitted)

23. Hynes, N.E. and Stern, D.F. 1994. The biology of erbB-2/neu/HER2 and its role in cancer. Biochim. Biophys. Acta 1198:165-184.

24. Carraway, K.L. and Cantley, L.C. 1994. A neu acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerization in growth signaling. Cell 78:5-8.

25. Slamon, D.J., Clark, G.Mm, Wong, S.G., Levin, W.J., Ullrich, A. and McGuire, W.L. 1987. Human breast cancer: correlation of relapse and survival with amplification of HER2/neu oncogene. Science 235:177-182.

26. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. and Press, M. 1989. Studies of the HER2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707-712.

27. Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J.T., Dodge, R., Clarke-Pearson, D.L., Marks, P., McKenzie, S., Yin, S. and Bast, R.C. 1990. Overexpression of HER2/neu is associated with poor susvival in advanced epithelial ovarian cancer. Cancer Res. 50:4087-4091.

28. Berchuck, A., Rodriguez, G., Kinney, R.B., Soper, J.T., Dodge, R.K., Clarke-Pearson, D.L. and Bast, R.C. 1991. Overexpression of HER2/neu in endometrial cancer is associated with advanced stage disease. Am. J. Obstetrics Gynecol. 164:15-21

29. Kern, J.A., Schwartz, D.A., Nordberg, J.E., Weiner, D.B., Greene, M.I., Torney, L. and Robinson, R.A. 1990. p185neu expression in human lung adenocarcinomas predict shortened survival. Cancer Res. 50:5184-5191.

30. Yokota, J., Yamamoto, T., Miyajima, N., Toyoshima, K., Nomura, N., Sakamoto, H., Yoshida, T., Terada, M. and Sugimura, T. 1988. Genetic alterations of the c-erbB-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of v-erbA homologue. Oncogene 2:283-287.

31. Park, J.B., Rhim, J.S., Park, S.C., Kimm, S.W. and Kraus, M.H. 1989. Amplification, overexpression and rearrangement of the erbB-2 proto-oncogene in primary human stomach carcinomas. Cancer Res. 49:6605-6609.

32. Lei, A., Appert, H.E., Nakata, B., Domenico, D.R., Kim, K. and Howard, J.M. 1995. Overexpression of HER2/neu oncogene in pancreatic cancer correlates with shortened survival. Int. J. Pancreatology 17:15-21.

33. Zhau, H.E,, Zhang, X., von Eschenbach, A.C., Scorsone, K., Babaian, R.J., Ro, J.Y. and Hung, M.C. 1990. Amplification and expression of the c-erbB-2/neu proto-oncogene in human bladder cancer. Molec. Carcinogenesis 3:254-257.

34. Gu, K., Mes-Masson, A.-M., Gauthier, J. and Saad, F. 1996. Overexpression of her-2/*neu* in human prostate cancer and benign hyperplasia. Cancer Lett. 99:185-189.

35. VandeVijver, M.J., Peterse , M.L., Mooi, W.J., Wisman, O., Lomans, J., Dalesio, O. and Nusse, R. 1988. Neu-protein expression in breast cancer. New Engl. J. Med. 319:1239-1245.

36. Liu, E., Thor, A., He, M., Barcos, M., Ljung, B.M. and Benz, C.C. 1992. The HER2 (c-erbB-2) oncogene is frequently amplified in *in situ* carcinomas of the breast. Oncogene 7:1027-1032.

37. Lewis, G.D., Figari, I., Fendly, B.M., Wong, W.-L., Carter, P., Gorman, C. and Shepard, H.M. 1993. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol. Immunother. 37:255-263.

38. Press, M.F., Cordon-Cardo, C. and Slamon, D.J. 1990. Expression of the HER-2/neu proto-oncogene in normal humad adult and fetal tissues. Oncogene 5: 953-962.

39. Niehans, G.A., Singleton, T. P., Dykoski, D. and Kiang, D.T. 1993. Stability of HER-2/neu expression over time and at multiple metastatic sites. J. Natl. Cancer Inst. 85:1230-1235.

40. Tagliabue, E., Centis, P., Campiglio, M., Mastroiannini, A., Martignone, S, Pellegrini, R., Casalani, P., Lanzi, C., Menard, S. and Colnaghi, M.I. 1991. Selecion of monoclonal antibodies which induce internalization and phosphorylation of p185HER2 and growth inhibition of cells with HER2/neu gene amlpification. Int. J. Cancer 47: 933-937.

41. Sarup, J.C., Johnson, R.M., King, K.L., Fendly, B.M., Lipari, M.T., Napier, M.A., Ullrich, A. and Shepard, H.M. 1991. Characterization of an anti-p185HER2 monoclonal antibody that stimulated receptor function and inhibits tumor cell growth. Growth Regulation 37: 72-82.

42. Hurwitz, E., Stancovski, I., Sela, M. and, Yarden, Y. 1995. Suppression and promotion of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake. Proc. Natl. Acad. Sci. USA 92:3553-3557.

43. Fendly, B.M., Winget, M., Hudziak, R.M., Lipari, M.T., Napier, M.A. and Ullrich, A. 1990. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res 50:1550-1558.

44. Schier, R., Marks, J.D., Wolf, E.J., Apell, G., Wong, C., McCartney, J.E., Bookman, M.A., Huston, J.A., Houston, L.L., Weiner, L.M., Adams, G.P. 1995. *In vitro* and *in vivo* characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. Immunotechnology 1:73-81.

45. Schier, R., McCal, A., Adams, G.P., Marshall, K.W., Merritt, H., Yim, M., Craford, R.S., Weiner, L.M., Marks, C., Marks, J.D. 1996. Isolation of picomolar affinity anti-c-erbB-2 single chain Fv by molecular evolution of the complementary determining regions in the center of the antibody binding site. J. Mol. Biol. 263:551-567.

46. Park, J.W., Stagg, R., Lewis, G.D., Carter, P., Maneval, D., Slamon, D.J., Jaffe, H. and Shepard, H.M., 1992. Anti-p185HER2 monoclonal antibodies: biological properties and potential for immunotherapy. In "Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer". R.B. Dickson and M.E. Lippman (Eds). Kluwer, Boston, MA, p.193-211.

47. Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B.B., Henner, D., Wong, W.L.T., Rowland, A.M., Kotts, C., Carver, M.E. and Shepard, H.M. 1992. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc. Natl. Acad. Sci. USA 89:4285-4289.

48. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C.C., Dantis, L., Sklarin, N.T., Seidman, A.D., Hudis, C.A., Moore, J., Rosen, P.P., Twaddell, T., Henderson, I.C. and Norton, L. 1996. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin. Oncol. 14:737-744.

49. Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. 1973. Phase transitions in phospholipid vesicles, fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochim. Biophys. Acta 311:330-348.

50. Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. 1979. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim. Biophys. Acta 557: 9-23.

51. Martin, F. and Papahadjopoulos, D. 1982. Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting. J. Biol. Chem. 257:286-288.

52. Shahinian, S. and Silvius, J.R. 1995. A novel strategy affords high-yield coupling of antibody Fab' fragments to liposomes. Biochim. Biophys. Acta 1239: 157-167.

53. Loughrey, H.C., Choi, L.S., Cullis, P.R. and Bally, M.B. 1990. Optimized procedures for the coupling of proteins to liposomes. J. Immunol. Methods 132:25-35.

54. Bredehorst, R., Ligler, F.S., Kusterbeck, A.W., Chang, E.L., Gaber, B.P. and Vogel, C.-W. 1986. Effect of covalent attachment of immunoglobulin fragments on liposomal integrity. Biochemistry 25:5693-5698.

55. Hristova, K. and Needham, D. 1995. Physical properties of polymer-grafted bilayers. In "Stealth Liposomes". D. Lasic, F. Martin (Eds). CRC Press, Boca Raton, FL, p. 35-49.

56. Mori, A., Klibanov, A.L., Torchilin, V.P. and Huang, L. 1991. Influence of the steric barrier activity of amphipathic poly(ethylene glycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes *in vivo*. FEBS Lett. 284:263-266.

57. Huang, S.K., Mayhew, E., Gilani, S., Lasic, D.D., Martin, F.J. and Papahadjopoulos, D. 1992. Pharmacokinetics and therapeutics of sterically stabilized liposomes in mice bearing C-26 colon carcinoma. Cancer Res. 52:6774-6781.

58. Working, P.W., Newman, M., Huang, S.K., Vaage, J., Mayhew, E. and Lasic, D.D. 1994. Pharmacokinetics, biodistribution and therapeutic efficacy of doxorubicin encapsulated in Stealth liposomes. J. Liposome Res. 4:667-687.

59. Bogner, J.R. and Goebel, F.-D. 1995. Efficacy of Dox-SL (Stealth® liposomal doxorubicin) in the treatment of advanced AIDS-related Kaposi's sarcoma. In "Stealth Liposomes". D. Lasic, F. Martin (Eds). CRC Press, Boca Raton, FL, p.267-278.

60. Haran, G., Cohen, R., Bar, L.K. and Barenholz, Y. 1993. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim. Biophys. Acta 1149:180-184.

61. Daleke, D.L., Hong, K. and Papahadjopoulos, D. 1990. Endocytosis of liposomes by macrophages: binding, acidification, and leakage of liposomes monitored by a new fluorescence assay. Biochim. Biophys. Acta 1024:352-366.

62. Maruyama, K., Takizawa, T., Yuda, T., Kennel, S.J., Huang, L. and Iwatsuru, M. 1995. Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies. Biochim. Biophys. Acta 1234:74-80.

63. Nezlin, R.C. 1977. Structure and biosynthesis of antibodies. Consultants Bureau, New York, NY, p.174.

64. Kenworthy, A.K., Simon, S.A. and McIntosh, T.J. 1995. Structure and phase behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol). Biophys. J. 68:1903-1920.

65. Lee, K.-D., Nir, S. and Papahadjopoulos, D. 1993. Quantitative analysis of liposome-cell interactions *in vitro*: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes. Biochemistry 32: 889-899.

66. Goren, D., Horowitz, A.T., Zalipsky, S., Woodle, M.C., Yarden, Y. and Gabizon, A. 1996. Targeting of stealth liposomes to erbB-2 (Her/2) receptor: *in vitro* and *in vivo* studies. Br. J. Cancer 74:1749-1756.

67. Suzuki, S., Uno, S., Fukuda, Y., Aoki, Y., Masuko, T. and Hashimoto, Y. 1995. Cytotoxicity of anti-c-erbB-2 immunoliposomes containing doxorubicin on human cancer cells. Br. J. Cancer 72:663-668.

68. Gabizon, A., Huberty, J., Straubinger, R., Price, D.C. and Papahadjopoulos, D. 1988. An improved method for *in vivo* tracing and imaging of liposomes using Gallium-67- deferoxamine complex. J. Liposome Res. 1:123-135.

69. Heitjan, D.F. 1991. Generalized Norton-Simon models of tumour growth. Statistics in Medicine 10:1075-1088.

70. Matzku, S., Krempel, H., Weckenmann, H.-P., Schirrmacher, V., Sinn, H. and Stricker, H. 1990. Tumour targeting with antibody-coupled liposomes: failure to achieve accumulation in xenografts and spontaneous liver metastases. Cancer Immunol. Immunother. 31:285-291.

71. Hong, K., Friend, D.S., Glabe C.G. and Papahadjopoulos, D. 1983. Liposomes containing colloidal gold are a useful probe of liposome-cell interactions. Biochim. Biophys. Acta 732:320-323.

72. Huang, S.K., Lee, K.-D., Hong, K., Friend, D.S. and Papahadjopoulos, D. 1992. Microscopic localization of sterically stabilized liposomes in colon-carcinoma bearing mice. Cancer Res. 52:5135-5143.

73. Madden, T.D., Harrigan, P.R., Tai, L.C., Bally, M.B., Mayer, L.D., Redelmeier, T.E., Loughrey, H.C., Tilcock, C.P., Reinish, L.W. and Cullis, P.R. 1990. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. Chem. Phys. Lipids 53:37-46.

74. Janknegt, R. 1996. Liposomal formulations of cytotoxic drugs. Supportive Care Cancer, 4:298–304.

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CHAPTER 4.7

Targeting of sterically stabilized liposomes to cancers overexpressing HER2/neu proto-oncogene

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Overview

I. Introduction

The potential of liposomes as vehicles for targeted delivery of drugs to diseased tissues has been long recognized.[1] There is a copious and convincing experimental evidence for specific association of the cells exposing a characteristic molecular "tag" with liposomes bearing "tag" recognition molecules, such as antibodies or antigen-binding antibody fragments.[2–5] Specific association with the target should lead to better therapeutic efficacy and less systemic side effects of liposome-encapsulated pharmaceuticals[2,3] which is especially important in cytotoxic chemotherapy of cancer where therapeutic indices are narrow and systemic toxicities are high. Introduction of liposome designs allowing avoidance of early clearance by the cells of mononuclear phagocytic system (MPS), longer circulation times, and increased likelihood for a liposome drug to reach its intended destination in the body[6–9] brought about a new interest in the liposome targeting. Grafting of

hydrophilic, flexible chains of poly(ethylene glycol) with M_r of 2,000–5,000 (PEG) on the liposome surface (sterically stabilized, of Stealth® liposomes) proved to be among the most successful methods for introduction of long-circulating properties.[10] Targeting of PEG-stabilized liposomes was at first achieved by attachment of antibodies, via a hydrophobic anchor, onto the surface of liposomes containing also phospholipids with poly-(ethylene glycol)-modified head groups (PEG-DSPE).[11] Such liposomes had long-circulating properties, but their interaction with the target was reduced presumably by the same "steric repulsion" phenomenon which contributed to the mechanism of MPS avoidance.[12,13] Later, target-specific molecules (antibodies, sugars, or peptides) were attached to the distal termini of liposome-grafted poly(ethylene glycol) chains, leading to targeted sterically stabilized liposomes with fully preserved target-binding capability.[14–16]

This fruitful approach will be illustrated here by the studies of sterically stabilized liposomes targeted to cancer cells overexpressing the HER2/neu protooncogene. In contrast to a number of previous liposomal targeting systems for anticancer agents, sterically stabilized anti-HER2 immunoliposomes (anti-HER2 SIL) were not only capable of target-specific binding and internalization by cancer cells in culture,[17,18] but were also able to cross the vascular barrier and to be internalized by the target cancer cells in solid tumor xenografts following intravenous injection.[19] Anti-HER2 SSL carried the anticancer drug doxorubicin to HER2 overexpressing cancer xenografts more efficiently than their non-targeted counterparts.[20,21] Inclusion of a cationic lipid in the composition of anti-HER2 SIL resulted in the specific delivery of nucleic acids into HER2-overexpressing cancer cells.[22]

II. HER2 oncoprotein as a target recognition molecule in cancer

Malignant phenotype is often associated with the expression of protooncogenes. The HER2 (c-erbB-2, neu) protooncogene encodes a 185 KDa (1255 amino acids) receptor tyrosine kinase (p185HER2, ErbB-2, or HER2) which belongs to the family of receptor tyrosine kinases including also the products of epidermal growth factor (EGFR), HER3 (erbB-3), and HER4 (erbB-4) genes.[23,24] Overexpression of HER2 was first observed in 20–30% of breast carcinomas and was associated with aggressive tumor growth, high recurrence rate, and poor prognosis for the patients.[25,26] Further studies showed ubiquitous overexpression of HER2 in a variety of malignancies, including cancers of the ovary,[27] endometrium,[28] lung (non-small cell),[29] stomach,[30,31] pancreas,[32] bladder,[33] and prostate.[34] Especially high incidence of HER2 overexpression (up to 50%) was found in the breast ductal carcinoma in situ (DCIS), particularly in the lesions having high risk of recurrence.[35,36] The accumulated evidence suggests that HER2 is an important mediator of tumor growth which directly contributes to tumor onset and progression, and confers especially aggressive malignant phenotype.[23]

HER2 offers a number of advantages as a recognition marker for targeted delivery of anticancer agents. HER2 is a readily accessible cell surface protein

with substantial levels of overexpression (10^5–10^6 copies/cell) in various malignancies.[37] In normal adult tissues HER2 occurs only in certain epithelial types, and at very low levels.[38] HER2-overexpression is relatively homogenous within primary tumors, and is maintained at metastatic sites, suggesting continuous requirement for high levels of HER2 throughout the malignant process,[39] while many other tumor-associated antigens show variable expression patterns within the tumor tissue and/or in the course of tumor progression. Last, but not least, is the fact that HER2 activation is accompanied by its internalization into the cell, which may occur upon interaction with an agonistic antibody.[40–42] Therefore, ligands targeted to HER2 by means of such antibodies would have better chance to enter the cell rather than stay attached to the cell surface.

The role of HER2 in the malignant progression brought about considerable effort to achieve antitumor effect by blocking the function of this receptor protein. A variety of monoclonal antibodies[40–43] as well as phage-display library generated single-chain Fv's[44,45] reactive with the extracellular portion of HER2 have been reported, offering a vast palette of target-recognition molecules suitable for liposome attachment. The variants with the highest antiproliferative effect also demonstrated the highest rate of cell internalization.[41] One such antibody, muMAb4D5, is highly reactive toward HER2[43] and inhibitory for the growth of HER2 overexpressing tumor cells in vitro[37] and in animal models.[46] This antibody was engineered into a fully humanized version, rhuMAbHER2, to reduce the potential for immunogenicity,[47] and entered clinical trials showing objective, but infrequent (12%) antitumor responses in patients with metastatic HER2-overexpressing breast cancer.[48]

III. Design of sterically stabilized anti-HER2 immunoliposomes

Successful design of antibody-targeted pharmaceutical liposomes for the treatment of solid tumors should satisfy a number of requirements aimed at maximum targeting effect of immunoliposomes administered systemically in the bloodstream. Antigen binding sites of the liposome-conjugated antibody must be accessible for unperturbed interaction with antigens on the surface of target cells. To ensure that immunoliposomes will reach their target cells, the rate of MPS clearance or other "non-productive" elimination of blood-borne immunoliposomes must be minimized in comparison with the rate of extravasation in the tumor. Since the liposome-conjugated antibody is a foreign protein likely to elicit host immune response, their immunogenicity must be minimized. Immunoliposomes must allow efficient loading and retention of a selected anticancer drug. And finally, the drug and antibody incorporation must be stable enough to permit liposomal entry into the tumor tissue without the loss of either of these agents.

The elements of immunoliposome design were chosen to maximally satisfy these requirements. Lipid composition of anti-HER2 immunoliposomes was based on hydrogenated soy phosphatidylcholine (T_m = 54°C) so that at the body temperature the liposome bilayer maintained the "solid" (gel) state. Cholesterol (40 mol.%) was included to increase the bilayer stability in the presence of

plasma.[49] To reduce MPS clearance rate, the liposomes contained up to 5.7 mol.% of DSPE modified with methoxypoly (ethylene glycol) with molecular mass 1900.[7] To assist extravasation, the liposomes were of uniform, small size (90–110 nm) achieved by extrusion of hydrated lipid suspension (multilamellar vesicles) through track-etched polycarbonate membranes (pore sizes 100 and 50 nm) after several cycles of freezing and thawing.[50,52]

To avoid immunogenic effects of Fc portion and increased MPS clearance through specific recognition of immunoliposomes by the phagocytic cells carrying Fc receptor, we used Fab′ fragments instead of the whole anti-HER2 MAb. Fab′ fragments also allowed better way of conjugation to the liposome through unique thiol groups in the hinge region[51,52] resulting in the definite, correct (outward) orientation of the antigen-binding sites. The binding affinity of liposome-conjugated Fab′ was not compromised in comparison with the binding of the whole antibody (see below) probably because the presence of multiple Fab′ fragments on the liposome restored the multicenter interaction characteristic for the whole antibody which carries two antigen-binding sites. The Fab′ fragments were a recombinant protein, a portion of the fully humanized anti-HER2 MAb (rhuMAbHER2) developed by Genentech, Inc. (South San Francisco, CA, USA) for the therapy of cancers overexpressing HER2.[47,48] This antibody was derived from a murine prototype muMAb4D5 which induces endocytosis of HER2 receptor upon binding to its extracellular domain.[43] The use of Fab′ antibody fragments with humanized sequence further reduced the risk of immune reaction in human patients; recombinant technology provided stable, reproducible and more economical source of this protein, and the ability to induce internalization upon binding to the target antigen was favorable for intracellular delivery of liposome-associated pharmaceuticals.

Two types of conjugation linkers were used for attachment of anti-HER2 Fab′ to the sterically stabilized liposomes. Both linkers contained a hydrophobic anchor (DSPE or DPPE), and a thiol-reactive maleimide function. The use of aromatic maleimides, such as maleimidophenylbutyric acid derivatives (MPB-PE)[51,53] was avoided because of their reported ability to cause leakage of encapsulated drugs from liposomes.[54] Linkers of the first type consisted of DPPE or DSPE with their amino groups acylated by hydroxysuccinimide esters of (N-maleimido)methylcyclohexylcarboxylic acid (MMC-PE) or β-(N-maleimido)propionic acid (MP-PE). These linkers positioned the conjugated Fab′ fragment "in parallel" with the surface-attached PEG chains. The second type of linkers additionally included PEG chain (M_r = 2,000) between DSPE and maleimido group (MMC or MP) (Figure 1).[18,52] Linkers of this type positioned Fab′ fragments "in series" with surface-attached PEG, i.e., outside of the PEG "cloud" surrounding the liposome. The second type of conjugation was essential for preservation of the target-binding and, to the less extent, internalization of the anti-HER2 liposomes containing more than 1.2 mol.% of PEG-DSPE, as illustrated below.

Liposome-Fab′ conjugations were performed by incubation of SSL containing 1.2 mol.% of appropriate linker with anti-HER2 Fab′ (approx. 30 μmol of liposomal phospholipid) for 2 hours to overnight at 4°C and pH 7.2–7.4 in neutal

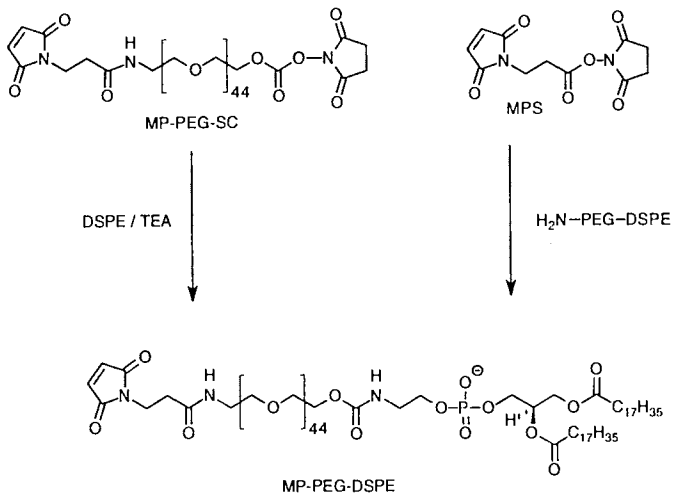

Fig. 1. Synthesis of maleimido-terminated PEG-DSPE derivatives.

atmosphere. Under these conditions, major portion of the protein became covalently attached to the liposomes and co-eluted with the liposomes in the void volume fraction during gel-exclusion chromatography on Sepharose 4B. More than 90% of Fab′ bearing free thiol groups were coupled to the liposomes containing MP-PEG-DSPE as a conjugation linker, resulting in 50–60 Fab′/liposome, while the Fab′ coupling to MMCC-PE-containing liposomes somewhat decreased at PEG-DSPE content more than 2 mol.%, perhaps due to the increasing expulsion of the protein from the liposome surface by overlapping PEG chains. However, even at PEG densities characteristic for the emerging "brush" regime (at PEG(M_r = 2,000)-DSPE)5 mol.% of the liposome phospholipid, or, in our case, >3.3 mol.% or total lipid[55]), conjugation of Fab′ fragments (Mol. weight 46 kD) was efficient enough (40%), i.e., Fab′ molecules still were able to reach the liposome bilayer. Therefore, permeability of the PEG "cloud" for protein molecules may be higher than previously predicted.[12,56]

The anticancer drug of choice for this study was doxorubicin which is well characterized in a similar, non-targeted sterically stabilized liposome system (Doxil[57–59]). Practically quantitative drug loading (0.09–0.15 mg of doxorubicin/μmol of liposomal phospholipid) was performed prior to the conjugation of anti-HER2 Fab′ by ammonium sulfate gradient method[60] above the liposome T_m, using 250 mM ammonium sulfate in the inner space of the liposomes, and isoosmotic NaCl-MES-buffer, pH 5.5, as the outer buffer.

IV. Interaction of anti-HER2 SIL with cancer cells in vitro

Targeting properties of SSL with conjugated Fab′ fragments of rhuMAbHER2 were studied in the cultures of human breast carcinoma cells with high (SK-

BR-3, 10^6 HER2/cell) or low (MCF-7, 10^4 HER2/cell) expression of HER2.[37] Liposomes of HSPC and cholesterol, containing 2.6 mol.% of methoxyPEG-DSPE, 1.2 mol.% of MMC-PEG-DSPE, and 0.2 mol.% of a fluorescent lipid marker N-lissamine-rhodamine B-dihexadecanoyl phosphatidylethanolamine (Rh-PE) were prepared and conjugated to anti-HER2 Fab′ as described above. The liposomes were incubated with the cells at 37°C in the presence of fluorescein-labeled (FITC) transferrin which served as a marker for endosomes due to its ability to undergo endocytosis via transferrin receptors present at the surface of the cells. The liposomes were at first deposited on the surface of SKBR-3 cells, and further entered the cells and became co-localized with FITC-transferrin, as evidenced by yellow color produced by overlapping green (FITC) and red (Rh-PE) fluorescent colors (Figure 2A,B). Under the same experimental conditions, "target-negative" MCF-7 cells displayed only green punctate fluorescence of endocytosed FITC-transferrin, but not of the lipid label (Figure 2C); similar results were obtained with SKBR-3 cells incubated with Rho-PE labeled SSL lacking conjugated anti-HER2 Fab′.[18] These results confirmed the ability of anti-HER2 SIL to specifically bind to target cells and undergo endocytosis. Endocytosis of anti-HER2 liposomes without PEG-DSPE by SKBR-3 cells was also shown by electron microscopy using liposomes labeled with colloidal gold.[17]

Quantitative assessment of cell binding and endocytosis was performed with liposomes containing pH-sensitive probe 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS, pyranine). Fluorescence excitation spectrum of liposome-entrapped HPTS undergoes rapid changes in response to the liposome entry into acidic environment of endosomes and lysosomes, but it also has a pH-independent isosbestic point for accurate quantitation of the probe.[61] To ensure fast equilibration of proton concentrations across the liposome bilayer, in the studies using HPTS method we replaced HSPC with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) which forms bilayers maintaining more proton-permeable liquid crystalline structure rather than gel state at ambient conditions. Upon incubation with HER2-overexpressing SKBR-3 cells, HPTS-loaded anti-HER2 SSL became rapidly associated with the cells in neutral compartment (cell surface), followed by acidification of the liposome environment indicating endocytosis. The amount of cell-associated anti-HER2 SSL reached plateau after 3–4 hours of incubation, with more than 80% of liposomes endocytosed. At saturating concentrations, the uptake of anti-HER2 SIL by SKBR-3 cells, estimated from the fluorescence at HPTS isosbestic point, was in the range of 8,000–25,000 vesicles/cell, while the uptake of liposomes by "target-negative" MCF-7 cells was less than 100 vesicles/cell.[18]

Attachment of Fab′ to the liposomes via hinge thiol group and maleimide-activated hydrophobic linker[51] resulted in high conjugation yield without the loss of antigen-binding activity; however, the relative position of liposome-conjugated Fab′ and PEG was crucial for maintaining high uptake of anti-HER2 SIL by target cells. During incubation of SKBR-3 cells at the constant concentration of liposomes in the cell growth medium, the increasing PEG-DSPE content inhibited the uptake of anti-HER2 SSL prepared with MMC-DSPE linker, while no such inhibition occurred when anti-HER2 Fab′ were attached via MP-PEG-DSPE

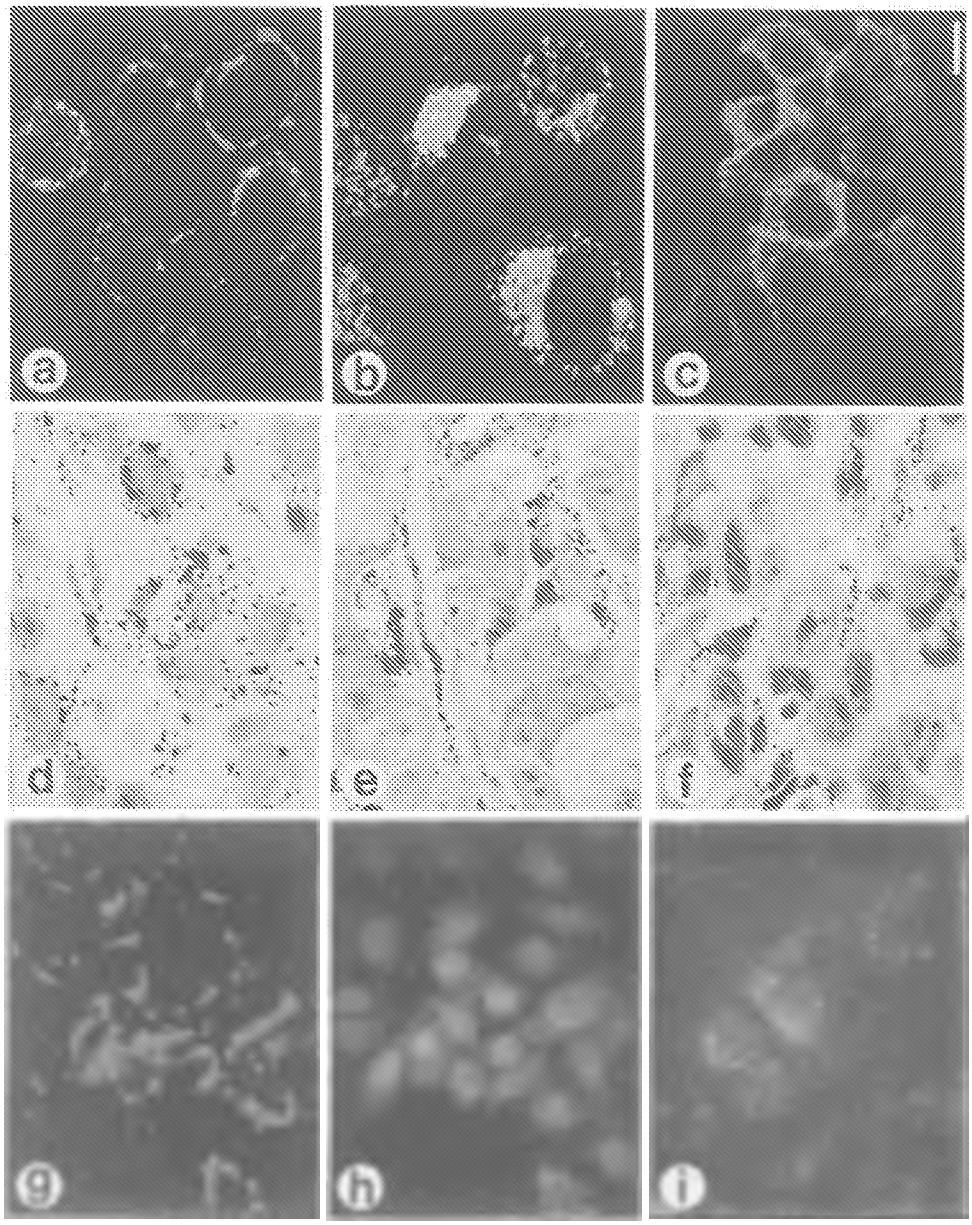

Fig. 2. *Panels A–C*: Confocal fluorescent microscopy of Rh-PE-labeled anti-HER2 SIL (red) and FITC-transferrin (green) co-incubated with breast cancer cells in cell culture at 37°C. SKBR-3 cells: 10 min. incubation (a); 30 min. incubation (b). MCF-7 cells: 30 min. incubation (c). *Panels d–f*: Localization of anti-HER2 SIL (d,f) and matched non-targeted SSL (e) in HER2-overexpressing (SK-BR-3, d,e) and low-expressing (MCF-7, f) breast cancer xenografts in nude mice 48 hours after intravenous administration. Liposomes are visualized as black grains by silver enhancement of liposome-entrapped colloidal gold. Staining with hematoxylin-eosin. *Panels g–i*: Uptake of FITC-ODN (green) and liposome lipid (Rh-PE-labeled, red) after incubation of SKBR-3 cells with PEG-coated anti-HER2 cationic liposome-ODN complex ([ODN]/[Lipid] = 0.007/22. (g) no free Fab′, lipid fluorescence; (h) no free Fab′, ODN fluorescence; (i) HER2 blocked by preincubation with excess free antiHER2 Fab′, ODN fluorescence.

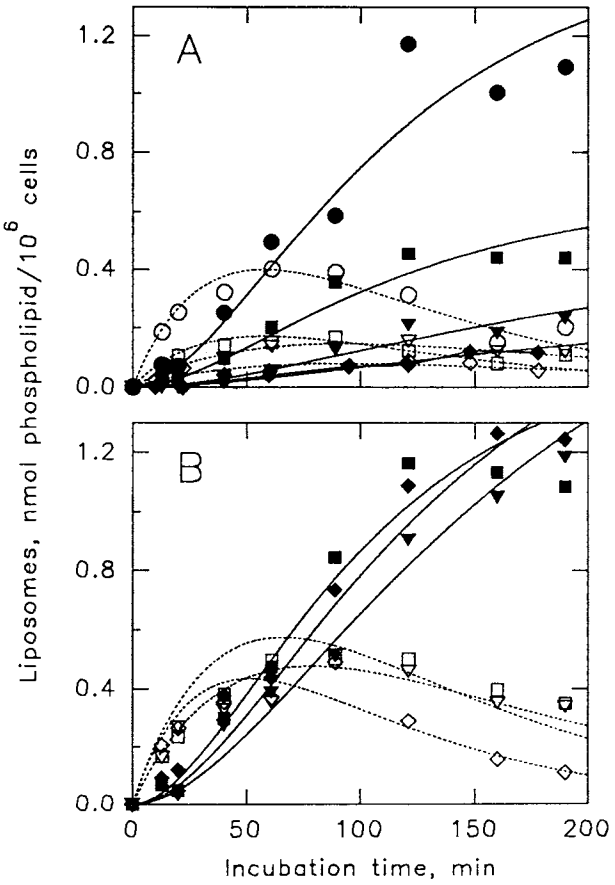

Fig. 3. Effect of PEG-DSPE content on the kinetics of anti-HER2 liposome association with SKBR-3 cells. (A) Anti-HER2 Fab′ attached via short linker MMC-DSPE (A) or to the distal end of PEG chain (linker MP-PEG-DSPE). Surface-bound liposomes: dashed line, hollow symbols. Endocytosed liposomes: solid line, filled symbols. PEG-DSPE (mol.%): none (○), 1.2 (□), 3.5 (▽), 5.7 (◇).

linker (Figure 3). Similar effect of the placement of a targeting antibody on the uptake of SIL by target cells/tissues was reported earlier[16,62] in the studies that used whole antibodies rather than Fab′ fragments. Since the cellular uptake of anti-HER2 liposomes includes steps of binding and subsequent endocytosis, we have studied the effect of PEG-DSPE and Fab′ placement on each of these steps separately. Liposome-cell binding was characterized by dissociation constants (K_d) estimated from the amounts of cell-associated anti-HER2 liposomes after incubation of SKBR-3 cells with various liposome concentrations at low temperature (4°C) that completely inhibits endocytosis. For anti-HER2 liposomes without PEG coating, K_d normalized to the amount of liposome-conjugated Fab′ was 12.0 ± 1.7 nM (mean ± SE), close to the reported values for the whole murine prototype anti-HER2 MAb 4D5 (6.0 nM) and its free Fab′ fragment (19.0 nM).[41]

When Fab′ fragments were conjugated through the "short" linker MMC-PE, 1.2 mol.% of PEG(M_r = 2,000)-DSPE decreased the binding affinity approximately three-fold, and at 3.5–5.7 mol.% of PEG-DSPE the binding affinity was 20 to 75 times lower (K_d = 320–900 nM). This is to be expected from the size of Fab′ fragment (6 nm in length[63]) and the thickness of PEG layer on the liposome surface (5–7 nm for PEG with M_r = 2,000 in "brush" regime[55,64]). On the contrary, conjugation of Fab′ to the termini of PEG chains did not affect liposome binding to the target cells at increasing PEG-DSPE content (K_d 13–15 nM for 1.2–5.7 mol.% PEG-DSPE).[18] First-order rate constants of liposome endocytosis (k_e) were determined from the kinetic curves of cell surface-bound and endocytosed liposomes obtained by HPTS method (Figure 3), as the ratio of liposome internalization rate to the steady-state surface concentration of liposomes.[65] Compared to K_d, k_e of anti-HER2 SIL prepared with the "short" linker MMC-PE was somewhat affected by increasing PEG-DSPE content (k_e decreased 2 times at 5.7 mol.% PEG-DSPE vs. 0%), and was not affected at all when MP-PEG-DSPE was used as a linker.[18] Thus, PEG interfered with the ability of liposome-conjugated Fab′ to bind to the cell surface antigen, and to the less extent, with its ability to induce endocytosis of the liposomes; however, this interference was completely abolished by conjugation of anti-HER2 Fab′ at the distal termini of liposome-grafted PEG chains.

Minimum requirements for the number of liposome-conjugated anti-HER2 Fab′ and for the cellular level of HER2 protein to achieve specificity and effectiveness of liposome uptake were established using anti-HER2 liposomes without PEG coating.[17,18] Binding of liposomes with the target cells increased in a linear manner as a function of Fab′ density, and reached saturation (plateau) at ~40 Fab′/vesicle; endocytosis of cell-bound liposomes occurred with 60% efficiency already at ~10 Fab′/vesicle.[18] Therefore, relatively few conjugated Fab′ were needed for the targeting. Similarly, the uptake of anti-HER2 liposomes by the cells with minimally elevated cellular levels of HER2 to allow classification as "HER2-positive" (MDA-MB-453: 44 ng HER2/mg cell protein, 6.52 ± 0.22 nmol of liposome phospholipid/mg cell protein)) was comparable to that by the cells with extremely high levels of HER2 expression (SKBR-3: 920 ng HER2/mg cell protein, 7.21 ± 0.45 nmol of liposome phospholipid/mg cell protein; BT-474: 550 ng HER2/mg cell protein, 4.47 ± 0.21 nmol of liposome phospholipid/mg cell protein). There was, however, pronounced difference in the uptake of anti-HER2 liposomes between the cells with elevated levels of HER2 and those with only basal HER2 expression (MCF-7: 7.3 ng HER2/mg cell protein, <0.01 nmol of liposome phospholipid/mg cell protein) (Figure 4).

Liposomes with Fab′ fragments of rhuMAbHER2 conjugated via MMC-PE linker were loaded with doxorubicin using ammonium sulfate gradient method. In contrast to the liposomes with aromatic maleimide derivative, MPB-PE,[16,54] the drug loading was practically quantitative even though the linker constituted 1.2 mol.% of total liposome lipid. Doxorubicin-loaded anti-HER2 liposomes showed efficient and specific in vitro cytotoxicity against HER2-overexpressing cancer cells. After 1 hour incubation, doxorubicin delivered by anti-HER2 lipo-

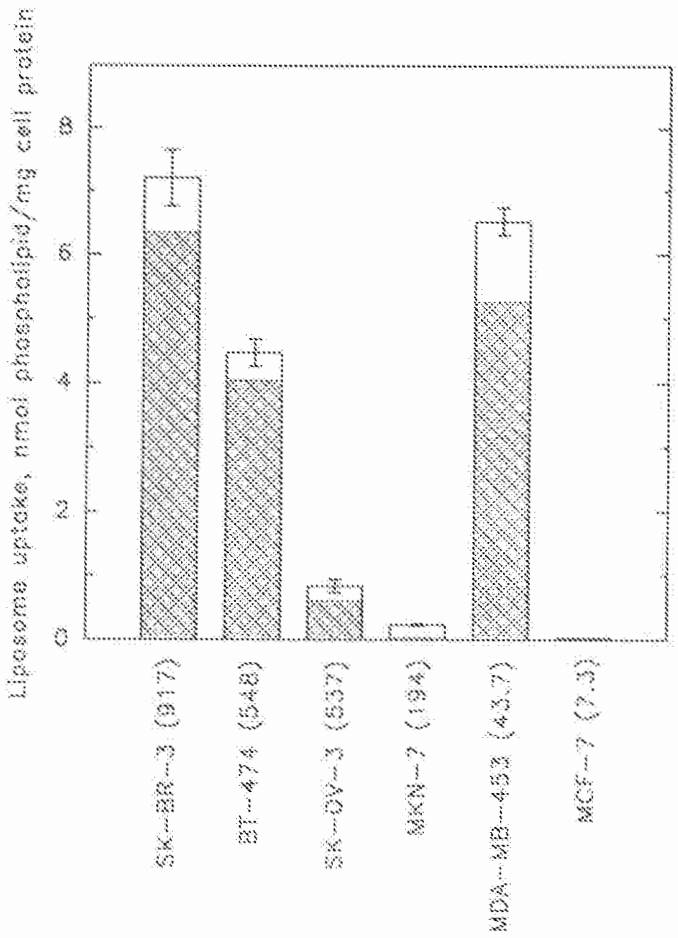

Fig. 4. Uptake of anti-HER2 liposomes by the cells with various levels of HER2 expression. Values in brackets indicate total cellular HER2 expression (ng/mg cell protein). Cross-hatched areas on the bars correspond to endocytosed liposomes; hollow areas, to surface-bound liposomes. Error bars, SEM ($n = 3$).

somes to SKBR-3 cells was as cytotoxic as free doxorubicin ($IC_{50} = 0.3$ μg/ml), while cytotoxicity of doxorubicin in the liposomes with conjugated irrelevant Fab′, or in anti-HER2 liposomes incubated with lung fibroblast cells (WI-38) expressing only minimal levels of HER2 was 20–30 times less than that of free drug.[17] Goren and co-workers[66] reported doxorubicin-loaded anti-HER2 SIL bearing whole antibodies attached to PEG terminal groups via hydrazone formation with periodate-oxidized carbohydrate moieties. These liposomes bound quite well to HER2-overexpressing gastric cancer cells (N-87), but their cytotoxicity was equal to that of non-targeted doxorubicin-loaded liposomes, and much less than that of the free drug, presumably because of the inability of these liposomes to be internalized

by the target cells.[66] In contrast, doxorubicin-loaded immunoliposomes bearing conjugated anti-HER2 MAb SER4 were endocytosed by HER2 overexpressing cells SKBR-3 and MKN-7, and were 25 times more cytotoxic to these cells than the matching liposomes conjugated to an antibody against non-internalizable surface protein gp125.[67] Moreover, compared with anti-gp125 immunoliposomes, SER4-conjugated immunoliposomes required 4.3–4.5 times less doxorubicin association with HER2 overexpressing cells for equal cytotoxicity.[67] Evidently, endocytosis of doxorubicin-loaded anti-HER2 liposomes is important for increased cytotoxicity of the liposomal drug, presumably by creating acidic environment and transmembrane pH gradients which favor escape of the drug from the liposome and its further distribution throughout the cell.

V. Properties of anti-HER2 SIL in vivo

Plasma pharmacokinetics of doxorubicin loaded in anti-HER2 SIL was studied in healthy Lewis rats following intravenous injection at the dose of 5 μmol of liposomal phospholipid (0.8–1 mg of doxorubicin) per animal. The liposomes showed biphasic elimination profile with $t_{1/2}\alpha = 6.1$ min., $t_{1/2}\beta = 976$ min., AUC = 93,100 min%, and blood MRT = 1460 min.[21,67] This pharmacokinetic behavior was characteristic for long-circulating liposomes and similar to that of similarly designed non-targeted doxorubicin-loaded SSL, in contrast to free doxorubicin which had plasma half-life of about 5 min.[68] The use of Fab′ instead of the whole antibody was of importance, since the analogous constructs bearing conjugated whole antibodies show lower circulation half-lives than corresponding non-conjugated SSL.[16,66] To analyze possible drug leakage or dissociation of Fab′ from the liposomes in circulation, plasma pharmacokinetics of the anti-HER2 SIL-entrapped drug was compared to that of liposome-conjugated Fab′ fragments (Plasma concentration of anti-HER2 Fab′ was assayed by ELISA using microtiter plates coated with extracellular domain of HER2 for capture, and horseradish peroxidase-linked goat anti-human IgG for detection). These two markers showed identical pharmacokinetic profiles indicating excellent stability of the drug-loaded anti-HER2 SIL in circulation.[19,20]

Nude mice with established subcutaneous xenografts of HER2-overexpressing human breast carcinoma (BT-474) were used to study biodistribution and tumor localization of anti-HER2 SIL. For quantitation in tissues, the liposomes were prepared in the presence of a chelator (DTPA) and loaded with radiotracer ^{67}Ga using remote loading via oxine complex.[69] Biodistribution of anti-HER2 SIL in non-tumor tissues was characteristic for PEG-coated liposomes and was not significantly different from that of the similar non-targeted SSL (Table 1). There was also no statistically significant difference between the accumulation of HER2-targeted SIL or matching non-targeted SIL in HER2-overexpressing BT-474 tumors, or between the accumulation of anti-HER2 SIL in similarly established xenografts of MCF-7 tumors which express low levels of HER2 (Table 1). Nonetheless, treatment of animals with established (approximately 200 mm^3) xenografts of HER2-overexpressing human breast carcinomas(BT-474, MDA-MB-453) by

Table 1

Biodistribution of ^{67}Ga-labeled anti-HER2 SIL and matching non-targeted SSL in nude mice with breast cancer xenografts. Liposomes (1 μmol of phospholipid) were injected via tail vein 24 hours prior to sacrifice. Data: mean ± SE of 6 animals/group

Tissue	Anti-HER2 SSL	Non-targeted SSL % injected dose per g tissue
Blood	7.04 ± 0.82	7.98 ± 0.85
Skin	4.71 ± 0.86	3.97 ± 0.72
Muscle	0.37 ± 0.12	0.74 ± 0.20
Bone	2.06 ± 0.35	3.50 ± 0.82
Heart	0.58 ± 0.05	0.68 ± 0.16
Lungs	0.42 ± 0.10	0.61 ± 0.10
Liver	15.6 ± 3.8	14.2 ± 1.9
Spleen	41.6 ± 4.7	33.5 ± 5.5
Kidneys	3.21 ± 0.24	3.72 ± 0.24
Tumor: HER2-positive (BT-474)	8.34 ± 1.54	7.32 ± 1.05
Tumor: HER2-negative (MCF-7)	7.18 ± 0.60	8.59 ± 1.16

three weekly injections of free or SIL-encapsulated doxorubicin revealed superior activity of doxorubicin in anti-HER2 SIL.[20,21] The average ratio of the volume of BT-474 tumors at the end of experiment (48–60 days post tumor inoculation) to that at the beginning of experiment (12–14 days post inoculation) was 19.13 ± 1.14 in the group treated with free doxorubicin at a total maximum tolerated dose (MTD) of 7.5 mg/kg, 2.59 ± 0.28 in the group receiving non-targeted doxorubicin-loaded SSL (MTD, 15 mg/kg), and 0.63 ± 0.12 in the group injected with doxorubicin-loaded anti-HER2 SIL (15 mg/kg); in the case of MDA-MB-453 tumor (with lower expression of HER2), these values were 3.54 ± 0.53, 2.15 ± 0.29, and 1.17 ± 0.16, respectively.[19,21] The difference between growth rates of tumors in the groups receiving doxorubicin in HER2-targeted vs. non-targeted SIL was statistically significant at $p = 0.001$ (BT-474) and $p = 0.004$ (MDA-MB-453) according to a modified Norton-Simon model of tumor growth.[70] Administration of "empty" anti-HER2 SIL at equal dose/schedule did not produce antitumor effect in these models, ruling out the inhibitory effect of immunoliposome itself. Therefore the increased antitumor activity had to be attributed to the targeting.

The mechanisms by which the targeting of doxorubicin-loaded SIL to a surface antigen on cancer cells may increase therapeutic efficacy of the drug are at present not fully understood. The biodistribution data ruled out the increased uptake of targeted SIL over non-targeted ones in the tumor overexpressing target antigen; this observation is in accord with the view that the major bottleneck in the tumor accumulation of the circulating liposomes is crossing of the vascular wall,[71] a process on which this type of targeting evidently has no effect. One can not exclude that specific interaction with HER2-overexpressing tumor cells resulted in the extended residence time of anti-HER2 SIL in the tumor tissue. Another mechanism by which anti-HER2 SIL may be more efficient as carriers of cytotoxic drugs into HER2-positive tumors was revealed by the studies of liposome disposition in the tumor. To visualize the location and distribution of anti-HER2 SIL in the

tumor tissue, the liposomes were labeled with entrapped colloidal gold as described.[72] Entrapment of gold has no effect on the liposome stability or anti-HER2 Fab′ conjugation. Two days after intravenous administration of colloidal gold-labeled anti-HER2 SIL (5 μmol of phospholipid/animal), tumors were excised, fixed, embedded in glycol metacrylate, and gold-labeled liposomes were visualized on tumor sections by silver enhancement method. In HER2-positive tumor (BT-474) anti-HER2 SIL were abundantly deposited in the intercellular spaces throughout the tumor tissue, while in MCF-7 tumors with low HER2 expression, or if non-targeted gold-labeled SIL were given, the label was concentrated mostly within tumor-resident macrophages and in perivascular areas, in agreement with previous observations on non-targeted SIL.[73] At higher magnification, anti-HER2 SIL were frequently revealed within the cytoplasm and in the perinuclear spaces of HER2-positive cancer cells within the tumor tissue (Figure 2d), while non-targeted SIL (Figure 2e), or anti-HER2 SIL in the HER2-negative tumor (MCF-7) (Figure 2f) showed no clear deposition of silver granules within the cancer cells, but prominent intercellular deposition and localization of the silver grains in macrophages was apparent. Thus, anti-HER2 SIL not only crossed the vascular barrier into the solid tumor, but, in the case of HER2-overexpression, frequently became endocytosed by the cancer cells as they would in vitro. Increased deposition in the intercellular spaces within the tumor tissue outside tumor-resident macrophages, as well as the intracellular delivery of the encapsulated drug in vivo may contribute to superior antitumor efficacy of doxorubicin-loaded anti-HER2 SIL.

VI. Targeted delivery of nucleic acids by cationic anti-HER2 SIL

Liposomes that incorporate cationic lipids and therefore bear overall positive charge (cationic liposomes) have been established as non-viral vectors for introducing functional DNA, RNA, and oligonucleotides into cells.[74–76] The use of cationic liposomes as delivery vehicles in gene therapy and antisense oligonucleotide therapy is attractive because of the high loading capacity driven by electrostatic interactions of DNA or RNA with the liposome surface and ability to deliver at least part of its DNA/RNA load into cytoplasmic/nuclear compartments necessary for gene expression or other appropriate function. This use is now limited, however, by the instability of cationic liposomes against aggregation and dissociation in the physiological media, and by unfavorable pharmacokinetic properties. Other limiting aspects of cationic liposomes as in vivo delivery carriers for nucleic acids are non-specific reactivity and lack of targeting. As mentioned above, stability and pharmacokinetics of "neutral" liposomes used to carry encapsulated drugs is improved by "steric stabilization" with liposome-grafted PEG.[10] The similar approach to cationic liposomes would face a principal difficulty because steric hindrance created by PEG-coating may interfere with the liposome ability to form complexes with nucleic acids and, most importantly, to interact with cell membrane and deliver them into appropriate intracellular compartments, either by fusion with plasma membrane,[74] endocytosis,[77,78] or formation of pores.[79] Combination

of steric stabilization by amphiphilic PEG derivatives and HER2-targeting by an internalizable antibody construct may help to overcome these limitations and utilize the potential of steric stabilization for creation of cationic liposome-based systems for in vivo systemic delivery of therapeutic genes and/or oligonucleotides into HER2-overexpressing cancer cells.

The design of HER2-targeted SIL described in the preceding sections was used to demonstrate the feasibility of this approach. Cationic liposomes composed of equimolar amounts of dioctadecyldimethylammonium bromide (DDAB) and dioleoyl-phosphatidylethanolamine (DOPE) formed complexes with expression plasmid DNA carrying luciferase reporter gene (Lux) under the control of early CMV promoter. SKBR-3 cells incubated with such complexes for 4 hours at a dose of 1 μg DNA (16 nmol lipid)/10^6 cells showed high levels of Lux expression 48 hours later. Addition of 5 mol.% of methoxyPEG(M_r = 2,000)-DSPE substantially increased the stability of complexes against aggregation and destruction in the serum,[80] but the expression was down approximately 20-fold. When the same complex was prepared with cationic liposomes containing 4 mol.% of methoxy-PEG-DSPE and 1 mol.% of MP-PEG-DSPE, and conjugated to anti-HER2 Fab′ as described above, the level of Lux expression increased to the value observed without PEG-modification (Figure 5A) while the stability of such construct was the same as without conjugated Fab′ (K. Hong & W.-W. Zheng, unpublished data). In MCF-7 cells (low expression of HER2 receptor) modification of cationic liposomes with PEG led to an equal reduction of transfection by HER2-targeted or nontargeted complexes (Figure 5B). These results suggest that, first, PEG-

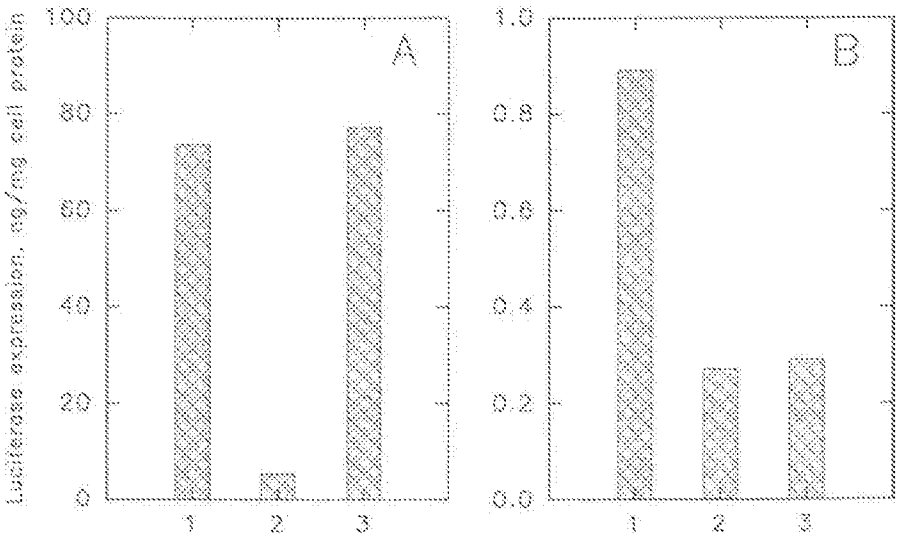

Fig. 5. Expression of luciferase reporter gene in SKBR-3 (A) and MCF-7 (B) cells transfected with DDAB/DOPE-plasmid DNA complexes: (1) no PEG-DSPE; (2) 5 mol.% of methoxyPEG (M_r = 1,900)-DSPE; (3) methoxyPEG-DSPE (5 mol.%) + anti-HER2 Fab′ conjugate of MP-PEG-DSPE.

modification at the above level did not preclude interaction between plasmid DNA and cationic liposomes, and, second, that internalization of PEG-modified anti-HER2 cationic liposome-DNA-complex through HER2-mediated endocytosis can substitute for internalization pathway of the complex between DNA and "conventional" cationic liposome (without PEG coating), leading to functional intracellular delivery of DNA.

Similar construct was developed for the delivery of oligonucleotides to HER2-overexpressing cancer cells, revealing more subtle targeting effects.[22] Cationic liposomes of 1,2-di(oleoyloxy)-3-trimethylammoniopropane (DOTAP), DOPE, and 6 mol.% of methoxyPEG-DSPE were capable of binding substantial amount of 18-mer antisense phosphorothiate oligodeoxynucleotide (ODN); unlike their prototypes without PEG-coating, these complexes were stable against aggregation and upon incubation in 50% human plasma. Interaction of such complexes with SKBR-3 cells resulted in the appearance of both lipid (followed by rhodamine-labeled lipid) and oligonucleotide (followed by FITC-labeled ODN) in the punctate cytoplasmic patterns and on the cell surface, but not in the cell nuclei. Conjugation of anti-HER2 Fab′ via MP-PEG-DSPE linker at such complexes resulted in the nuclear delivery of ODN into every cell (Figure 2h), while the lipid components remained associated with cytoplasmic, but not nuclear, compartments (Figure 2g). Nuclear delivery of ODN was specific to the uptake of the complexes mediated by specific interaction of the conjugated Fab′ with extracellular domains of HER2, since preincubation of the cells with excess of free anti-HER2 Fab′ completely abolished nuclear localization (Figure 2i). In "target-negative" MCF-7 cells neither HER2-targeted, nor non-targeted PEG-coated cationic liposome-ODN complexes could deliver ODN into the cell nuclei.[22] Nuclear localization of ODN is considered a prerequisite for its functional activity and can be normally achieved by incubation of the cells with ODN and cationic liposomes without PEG-coating.[76,81,82] Therefore, in the above two examples, the likely role of HER2-targeting in the delivery of DNA by sterically stabilized cationic liposomes was to substitute HER2-mediated endocytosis for the cell internalization otherwise mediated by the cationic lipid, while preserving the "correct" intracellular address for the delivered DNA.

VII. Conclusion

Over the last decade, introduction of "long-circulating" liposomes,[6–10] refinement of liposome preparation techniques[50,52] and "remote loading" methods for drug loading into liposomes[60,83] greatly advanced liposomal pharmacology. This advance is clearly evidenced by the recent appearance of anticancer liposomal drugs (see Section VIII) in the pharmaceutical market.[84] It also brought new enthusiasm to the area of liposome targeting.[1,2] Here we presented a case study that illustrates, in our view, a "rational design" approach to antibody-targeted pharmaceutical liposomes (Table 2). Each element of this design answers certain demand related to the ultimate medical use of the targeted liposomal drug carrier. This design may be readily applied to other targeted drug delivery systems utilizing different

Table 2

Components of the immunoliposome design

Component	Considerations for optimal design
Target Antigen	*Expression*: Highly and homogeneously overexpressed in target tissue. *Function*: Vital to tumor progression, so that down-modulation does not occur or is associated with therapeutic benefit. *Shedding of antigen*: Limited, to avoid binding to soluble antigen and accelerated clearance.
Antibody	*Affinity*: High enough to ensure binding at low liposome concentrations. *Immunogenicity*: Humanized MAb, to remove murine sequences. Use fragments without Fc portion (Fab', scFv) *Internalization*: Efficiently endocytosed by target cells. *Biological activity*: Intrinsic antitumor activity may enhance antitumor effect. *Scale-up*: Easy and economical scale-up, e.g., by efficient bacterial expression system Stability during storage.
Linkage	*Stability*: Covalent attachment to hydrophobic anchor, stable in blood. *Attachment site*: Away from the binding site, to ensure correct orientation of antibody molecule. Well defined, to ensure reproducibility and uniformity of coupling. Avoids steric hindrance (e.g., from PEG) of MAb binding and internalization. *Chemical nature of the linker*: Non-toxic. Non-immunogenic.Avoids opsonization. Does not affect drug loading and membrane stability. Excess linker may be quenched to avoid non-specific coupling to biomolecules. Availability, economical manufacturing process.
Liposome	*Stability*: Stable as intact construct in vivo. *Pharmacokinetics*: Long circulating. *Tumor penetration*: Capable of extravasation in tumors. Small diameter improves tumor penetration.
Drug	*Encapsulation*: Efficient, high capacity (e.g., by remote loading). Encapsulated drug storage-stable and resists leakage. *Bystander Toxicity*: Drug affects tumor cells not directly targeted (bystander cells) *Interaction with target cells*: Effective against target cell population. Cytotoxicity enhanced by binding of MAb.

drugs and/or different target-specific molecules, such as, for example, single chain anti-HER2 antibody fragments produced by phage display libraries.[44,45] Doxorubicin-loaded HER2-targeted SIL constructed according to such design had superior antitumor activity compared to matched non-targeted liposomes in established solid tumor xenografts overexpressing HER2 oncoprotein. Unexpectedly, this phenomenon was not associated with an increased accumulation of targeted liposomes in HER2-overexpressing tumors, but rather resulted from a different pattern of liposome disposition (improved penetration and internalization into HER2-overexpressing cancer cells) within the tumor tissue. Finally, the same design showed promise in the development of HER2-targeted sterically stabilized cationic liposomes for the delivery of therapeutic genes and oligonucleotides. The "rational design" of cancer cell-targeted sterically stabilized liposomes leads to a re-evaluation of tumor targeting paradigms and opens new avenues for better treatment of cancer.

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References

1. Papahadjopoulos D. 30-Year progress for liposomes: from serendipity to molecular design. J Liposome Res 1995;5:ix–xiv.
2. Lasic DD, Papahadjopoulos D. Liposomes revisited. Science 1995;267:1275–1276.
3. Leserman L, Machy P. Ligand targeting of liposomes. In: Ostro MJ, ed. Liposomes. From Biophysics to Therapeutics. New York: Marcel Dekker, 1987;157–194.
4. Ranade VV. Drug delivery systems. 1. Site-specific drug delivery using liposomes as carriers. J Clin Pharmacol 1989;29:685–694.
5. Straubinger RM, Lopez NG, Debs RJ, Hong K, Papahadjopoulos D. Liposome-based therapy of human ovaruan cancer: parameters determining potency of negatively charged and antibody-targeted liposomes. Cancer Res 1988;48:5237–5245.
6. Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 1988;85:6949–6953.
7. Papahadjopoulos D, Allen T, Gabizon A, Mayhew E, Matthay, Huang, SK, Lee, K-D, Woodle MC, Lasic DD, Redemann C, Martin FJ. Sterically stabilized liposomes: Improvements in pharmacokinetics, and anti-tumor therapeutic efficacy. Proc Natl Acad Sci USA 1991;88:11460–11464.
8. Klibanov AL, Huang L. Long-circulating liposomes: development and perspectives. J. Liposome Res 1992;2:321–332.
9. Allen TM, Chohn A. Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS Lett 1987;223:42.
10. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta 1992;1113:171–199.
11. Torchilin VP, Klibanov AL, Huang L, O'Donnell S, Nossiff ND, Khaw BA. Targeted accumulation of polyethylene glycol-coated immunoliposomes in infarcted rabbit myocardium. FASEB J 1992;6:2716.
12. Klibanov AL, Maruyama K, Beckerleg AM, Torchilin VP, Huang, L. Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation lifetime of liposomes depends on the liposome

size and is unfavorable for immunoliposome binding to target. Biochim Biophys Acta 1991;1062:142–148.
13. Torchilin VP, Papisov MI, Bogdanov AA, Trubetskoy VS, Omelyanenko VG. Molecular mechanism of liposome and immunoliposome steric protection with poly(ethylene glycol). In: Lasic D, Martin F, eds. Stealth Liposomes. Boca Raton, FL: CRC Press, 1995;51–62.
14. Allen TM, Brandeis E, Hansen CB, Kao GY, Zalipsky S. A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. Biochim Biophys Acta 1995;1237:99–108.
15. Zalipsky S, Hansen CB, Lopes de Menezes DE, Allen TM. Long-circulating, polyethylene glycol-grafted immunoliposomes. J Controlled Release 1996;39:153–161.
16. Hansen CB, Kao GY, Moase EH, Zalipsky S, Allen TM. Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. Biochim Biophys Acta 1995;1239:133–144.
17. Park JW, Hong K, Carter P, Asgari H, Guo LY, Wirth C, Shalaby R, Kotts C, Keller GA, Wood WI, Papahadjopoulos D, Benz CC. Development of anti-p185^{HER2} immunoliposomes for cancer therapy. Proc Natl Acad Sci USA 1995;92:1327–1331.
18. Kirpotin D, Park JW, Hong K, Zalipsky S, Li W-L, Carter P, Benz CC, Papahadjopoulos D. Sterically stabilized anti-HER2 immunoliposomes: desin and targeting to human breast cancer cells in vitro. Biochemistry 1997;36:66–75,
19. Park JW, Hong K, Kirpotin DB, Papahadjopoulos D, Benz C. Immunoliposomes for cancer treatment. Adv Pharmacol 1997;40:399–436.
20. Park JW, Hong K, Kirpotin DB, Meyer O, Papahadjopoulos D, Benz C. Anti-HER2 immunoliposomes for targeted therapy of human tumors. Cancer Lett, 1997;118:153–160.
21. Park JW, Colbern G, Hong K, Kirpotin D, Shao Y, Baselga J, Moore D, Papahadjopoulos D, Benz CC. Targeted intracellular drug delivery via anti-HER2 immunoliposomes yields superior antitumor efficacy. Proc ASCO 1997;16.
22. Meyer O, Kirpotin D, Hong K, Sternberg B, Park JW, Woodle MC, Papahadjopoulos D. Poly(ethylene glycol)-modified cationic liposomes as carriers for cellular delivery of antisense oligonucleotides. J Biol Chem 1998; in press.
23. Hynes NE, Stern DF. The biology of erbB-2/neu/HER2 and its role in cancer. Biochim Biophys Acta 1994;1198:165–184.
24. Carraway KL, Cantley LC. A neu acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerization in growth signaling. Cell 1994;78:5–8.
25. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of HER2/neu oncogene. Science 1987;235:177–182.
26. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press M. Studies of the HER2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–712.
27. Berchuck A, Kamel A, Whitaker R, Kerns B, Olt G, Kinney R, Soper JT, Dodge R, Clarke-Pearson DL, Marks P, McKenzie S, Yin S, Bast RC. Overexpression of HER2/neu is associated with poor susvival in advanced epithelial ovarian cancer. Cancer Res 1990;50:4087–4091.
28. Berchuck A, Rodriguez G, Kinney RB, Soper JT, Dodge RK, Clarke-Pearson DL, Bast RC. Overexpression of HER2/neu in endometrial cancer is associated with advanced stage disease. Am J Obstetrics Gynecol 1991;164:15–21.
29. Kern JA, Schwartz DA, Nordberg JE, Weiner DB, Greene MI, Torney L, Robinson RA. p185neu expression in human lung adenocarcinomas predict shortened survival. Cancer Res 1990;50:5184–5191.
30. Yokota J, Yamamoto T, Miyajima N, Toyoshima K, Nomura N, Sakamoto H, Yoshida T, Terada M, Sugimura T. Genetic alterations of the c-erbB-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of v-erbA homologue. Oncogene 1988;2:283–287.
31. Park JB, Rhim JS, Park SC, Kimm SW, Kraus MH. Amplification, overexpression, and rearrangement of the erbB-2 proto-oncogene in primary human stomach carcinomas. Cancer Res 1989;49:6605–6609.
32. Lei A, Appert HE, Nakata B, Domenico DR, Kim K, Howard JM. Overexpression of HER2/neu oncogene in pancreatic cancer correlates with shortened survival. Int J Pancreatology 1995;17:15–21.
33. Zhau HE, Zhang X, von Eschenbach AC, Scorsone K, Babaian RJ, Ro JY, Hung MC. Amplifi-

cation and expression of the c-erbB-2/neu proto-oncogene in human bladder cancer. Molec Carcinogen 1990;3:254–257.
34. Zhau HE, Wan DS, Zhou J, Miller GJ, von Eschenbach AC. Expression of c-erbB-2/neu proto-oncogene in human prostatic cancer tisues and cell lines. Molec Carcinogen 1990;3:254–257.
35. VandeVijver MJ, Peterse ML, Mooi WJ, Wisman O, Lomans J, Dalesio O, Nusse R. Neu-protein expression in breast cancer. New Engl J Med 1988;319:1239–1245.
36. Liu E, Thor A, He M, Barcos M, Ljung BM, Benz C. The HER2 (c-erbB-2) oncogene is frequently amplified in in situ carcinomas of the breast. Oncogene 1992;7:1027–1032.
37. Lewis GD, Figari I, Fendly BM, Wong W-L, Carter P, Gorman C, Shepard HM. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol Immunother 1993;37:255–263.
38. Press MF, Cordon-Cardo C, Slamon DJ. Expression of the HER-2/neu proto-oncogene in normal humad adult and fetal tissues. Oncogene 1990;5:953–962.
39. Niehans GA, Singleton TP, Dykoski D, Kiang DT. Stability of HER-2/neu expression over time and at multiple metastatic sites. J Natl Cancer Inst 1993;85:1230–1235.
40. Tagliabue E, Centis P, Campiglio M, Mastroiannini A, Martignone S, Pellegrini R, Casalani P, Lanzi C, Menard S, Colnaghi MI. Selecion of monoclonal antibodies which induce internalization and phosphorylation of p185HER2 and growth inhibition of cells with HER2/neu gene amplification. Int. J. Cancer 1991;47:933–937.
41. Sarup JC, Johnson RM, King KL, Fendly BM, Lipari MT, Napier MA, Ullrich A, Shepard HM. Characterization of an anti-p185HER2 monoclonal antibody that stimulated receptor function and inhibits tumor cell growth. Growth Regulation 1991;37:72–82.
42. Hurwitz E, Stancovski I, Sela M, Yarden Y. Suppression and promotion of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake. Proc Natl Acad Sci USA 1995;92:3553–3557.
43. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res 1990;50:1550–1558.
44. Schier R, Marks JD, Wolf EJ, Apell G, Wong C, McCartney JE, Bookman MA, Huston JA, Houston LL, Weiner LM, Adams GP. In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. Immunotechnology 1995;1:73–81.
45. Schier R, McCall A, Adams GP, Marshall KW, Merritt H, Yim M, Craford RS, Weiner LM, Marks C, Marks JD. Isolation of picomolar affinity anti-c-erbB-2 single chain Fv by molecular evolution of the complementary determining regions in the center of the antibody binding site. J Mol Biol 1996;263:551–567.
46. Park JW, Stagg R, Lewis GD, Carter P, Maneval D, Slamon DJ, Jaffe H, Shepard HM. Anti-p185HER2 monoclonal antibodies: biological properties and potential for immunotherapy. In: Dickson RB, Lippman ME, eds. Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer. Boston, MA: Kluwer Academic Publishers, 1992;193–211.
47. Carter P, Presta L, Gorman CM, Ridgway JBB, Henner D, Wong WLT, Rowland AM, Kotts C, Carver ME, Shepard HM. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA 1992;89:4285–4289.
48. Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin Oncol 1996;14:737–744.
49. Papahadjopoulos D, Jacobson K, Nir S, Isac T. Phase transitions in phospholipid vesicles, fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochim Biophys Acta 1973;311:330–348.
50. Olson F, Hunt CA, Szoka FC, Vail WJ, Papahadjopoulos D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim Biophys Acta 1979;557:9–23.
51. Martin F, Papahadjopoulos D. Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting. J Biol Chem 1982;257:286–288.
52. Shahinian S, Silvius JR. A novel strategy affords high-yield coupling of antibody Fab′ fragments to liposomes. Biochim Biophys Acta 1995;1239:157–167.
53. Loughrey HC, Choi LS, Cullis PR, Bally MB. Optimized procedures for the coupling of proteins to liposomes. J Immunol Methods 1990;132:25–35.

54. Bredehorst R, Ligler FS, Kusterbeck AW, Chang EL, Gaber BP, Vogel C-W. Effect of covalent attachment of immunoglobulin fragments on liposomal integrity. Biochemistry 1986;25:5693–5698.
55. Hristova K, Needham D. Physical properties of polymer-grafted bilayers. In: Lasic D, Martin F, eds. Stealth Liposomes. Boca Raton. FL: CRC Press, 1995;35–49.
56. Mori A, Klibanov AL, Torchilin VP, Huang L. Influence of the steric barrier activity of amphipathic poly(ethylene glycol) and ganglioside GM_1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo. FEBS Lett 1991;284:263–266.
57. Huang SK, Mayhew E, Gilani S, Lasic DD, Martin FJ, Papahadjopoulos D. Pharmacominetics and therapeutics of sterically stabilized liposomes in mice bearing C-26 colon carcinoma. Cancer Res 1992;52:6774–6781.
58. Working PW, Newman M, Huang SK, Vaage J, Mayhew E, Lasic DD. Pharmacokinetics, biodistribution and therapeutic efficacy of doxorubicin encapsulated in Stealth liposomes. J Liposome Res. 1994;4:667–687.
59. Bogner JR, Goebel F-D. Efficacy of Dox-SL (Stealth® liposomal doxorubicin) in the treatment of advanced AIDS-related Kaposi's sarcoma. In: Lasic D, Martin F, eds. Stealth Liposomes. Boca Raton. FL: CRC Press, 1995;267–278.
60. Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta 1993;1149:180–184.
61. Daleke DL, Hong K, Papahadjopoulos D. Endocytosis of liposomes by macrophages: binding, acidification, and leakage of liposomes monitored by a new fluorescence assay. Biochim Biophys Acta 1990;1024:352–366.
62. Maruyama K, Takizawa T, Yuda T, Kennel SJ, Huang L, Iwatsuru M. Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies. Biochim Biophys Acta 1995;1234:74–80.
63. Nezlin RC. Structure and Biosynthesis of Antibodies. New York: Consultants Bureau, 1977;174.
64. Kenworthy AK, Simon SA, McIntosh TJ. Structure and phase behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol). Biophys J 1995;68:1903–1920.
65. Lee K-D, Nir S, Papahadjopoulos D. Quantitative analysis of liposome-cell interactions in vitro: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes. Biochemistry 1993;32:889–899.
66. Goren D, Horowitz AT, Zalipsky S, Woodle MC, Yarden Y, Gabizon A. Targeting of stealth liposomes to erbB-2 (Her/2) receptor: in vitro and in vivo studies. Br J Cancer 1996;74:1749–1756.
67. Suzuki S, Uno S, Fukuda Y, Aoki Y, Masuko T, Hashimoto Y. Cytotoxicity of anti-c-erbB-2 immunoliposomes containing doxorubicin on human cancer cells. Br J Cancer 1995;72:663–668.
68. Park JW, Colbern G, Baselga J, Hong K, Shao Y, Kirpotin D, Nuijens A, Wood W, Papahadjopoulos D, Benz C. Antitumor efficacy of anti-p185HER2 liposomes: enhanced therapeutic index due to targeted delivery. Proc ASCO 1996;15:501.
69. Gabizon A, Huberty J, Straubinger R, Price DC, Papahadjopoulos D. An improved method for in vivo tracing and imaging of liposomes using Gallium-67-deferoxamine complex. J Liposome Res 1988;1:123–135.
70. Heitjan DF. Generalized Norton-Simon models of tumour growth. Statistics in Medicine 1991;10:1075–1088.
71. Matzku S, Krempel H, Weckenmann H-P, Schirrmacher V, Sinn H, Stricker H. Tumour targeting with antibody-coupled liposomes: failure to achieve accumulation in xenografts and spontaneous liver metastases. Cancer Immunol Immunother 1990;31:285–291.
72. Hong K, Friend DS, Glabe CG, Papahadjopoulos D. Liposomes containing colloidal gold are a useful probe of liposome-cell interactions. Biochim Biophys Acta 1983;732:320–323.
73. Huang SK, Lee K-D, Hong K, Friend DS, Papahadjopoulos D. Microscopic localization of sterically stabilized liposomes in colon-carcinoma bearing mice. Cancer Res. 1992;52:5135–5143.
74. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA 1987;84:7413–7417.
75. Malone RW., Felgner PL, Verma IM. Cationic liposome-mediated RNA transfection. Proc Natl Acad Sci USA 1989;86:6077–6081.
76. Zelphati O, Szoka FC Jr. Cationic liposomes as an oligonucleotide carrier: mechanism of action. J Liposome Res 1997;7:31–49.

77. Friend DS, Papahadjopoulos D, Debs RJ. Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. Biochim Biophys Acta 1996;1278:41–50.
78. Zelphati O, Szoka FC Jr. Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. Pharm Res 1996;13:1367–1372.
79. Boulikas T. Cancer gene therapy and immunotherapy (review). Int J Oncology 1996;9:941–954.
80. Hong K, Zheng, W-W, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery.FEBS Letters 1997;400:233–237.
81. Lewis JG, Lin K-Y, Kothavale A, Flanagan WM, Mateucci MD, DePrince RB, Mook Jr RA, Hendren RW, Wagner RW. A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. Proc Natl Acad Sci USA 1966;93:3176–3181.
82. Bennett CF, Chiang M-Y, Chan H, Shoemaker JEE, Mirabelli CK. Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. Molec Pharmacol 1992;41:1023–1033.
83. Madden TD, Harrigan PR, Tai LC, Bally MB, Mayer LD, Redelmeier TE, Loughrey HC, Tilcock CP, Reinish LW, Cullis PR. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. Chem Phys Lipids 1990;53:37–46.
84. Janknegt R. Liposomal formulations of cytotoxic drugs. Supportive Care Cancer 1996;4:298–304.

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Preliminary observations indicate variable patterns of plasma 5-fluorouracil (5-FU) levels during dose optimization of infusional 5-FU in colorectal cancer patients

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Efforts to improve efficacy and minimize toxicity have led to pharmacokinetic monitoring of plasma 5-fluorouracil (5-FU) levels in colorectal cancer patients undergoing chemotherapy. We observed variation in basal 5-FU levels in 21 patients and significant variation during subsequent dose optimization. Tumor KRAS, BRAF mutations and TS mRNA levels were determined. Regimens included FOLFOX6+Avastin (n = 8), FOLFOX6 (n = 11), FOLFIRI (n = 1) and FOLFOX4 (n = 1). Mutations identified in tumors included G12V KRAS (n = 2), G12A KRAS (n = 1), and V600E BRAF (n = 3). Six-of-11 patients with normalized tumor TS mRNA levels < 4.0 had a 5-FU AUC of 20 mg.h/L or greater, and 80% of patients (four of five) with TS levels > 4.0 had a plasma 5-FU AUC of less than or equal to 20 mg.h/L. Approximately 2/3 of patients achieved therapeutic 5-FU AUC levels with 0–2 dose adjustments while a sub-group of ~1/3 of patients slowly achieved therapeutic levels (> 3–4 dose increases leading to supra-therapeutic 5-FU and subsequent reductions to lesser than original doses). Liver metastases and tumor TS levels did not fully account for variable 5-FU AUC optimization patterns. The 5-FU level during continuous infusion was half-therapeutic in one patient who received FOLFOX4. The observed heterogeneous patterns at baseline and during dose optimization of 5-FU levels suggest variations in 5-FU metabolism among treated patients. Physiological and/or genetic differences underlying heterogeneity in 5-FU levels during dose optimization require further study of patient demographics, single nucleotide polymorphisms in Dihydropyrimidine Dehydrogenase (DPD), TS, or other genes that impact 5-FU metabolism and gene expression changes in liver after 5-FU therapy.

Introduction

5-Fluorouracil (5-FU) has been the cornerstone of colorectal cancer chemotherapy regimens for over five decades. However more than 80% of patients undergoing treatment will experience adverse side-effects from the chemotherapy.[1] The efficacy and side effects of chemotherapy are influenced by the dose calculated for each patient. The dose of 5-FU is conventionally calculated using the body surface area (BSA) method.

It has been shown that the efficacy of 5-FU is not optimized by BSA-based dosing. On the other hand, the use of AUC (defined as the area-under-the-curve when plasma 5-FU levels are plotted against a defined period of time) has been demonstrated to improve outcomes.[2-7] Although the data vary, most studies indicate that 5-FU may lack its desired therapeutic effect below an AUC of 20 mg.h/L, whereas it may cause excessive toxicity above an AUC of 24 mg.h/L.[3,4] Therefore, the optimum range for 5-FU AUC is considered to lie between an AUC of 20 and 24 mg.h/L to derive the greatest therapeutic benefit with minimal toxicity. Studies indicate that when using the conventional BSA-based method of 5-FU dose calculation, about 70–80% of patients do not achieve the desired 5-FU AUC range of 20–24 mg.h/L.[5]

The impact of keeping 5-FU plasma levels at therapeutic concentrations can be gleaned from observations that the overall survival with a 5-FU/leucovorin (FU/LV) regimen with pharmacokinetically-guided monitoring of 5-FU levels, is nearly equivalent to that observed with combination regimens involving FU/LV and oxaliplatin, and FU/LV and the topoisomerase inhibitor irinotecan. Thus, 5-FU (in the presence of leucovorin) may be as effective as these more toxic combinations, as long as it is present at the optimum levels in the blood.[2] There is also limited evidence suggesting that 5-FU pharmacokinetic monitoring may further improve the efficacy of a modified FOLFOX regimen.[5,6]

Given previous results that therapeutic benefit can be gained by achieving optimum plasma 5-FU levels, we have adopted

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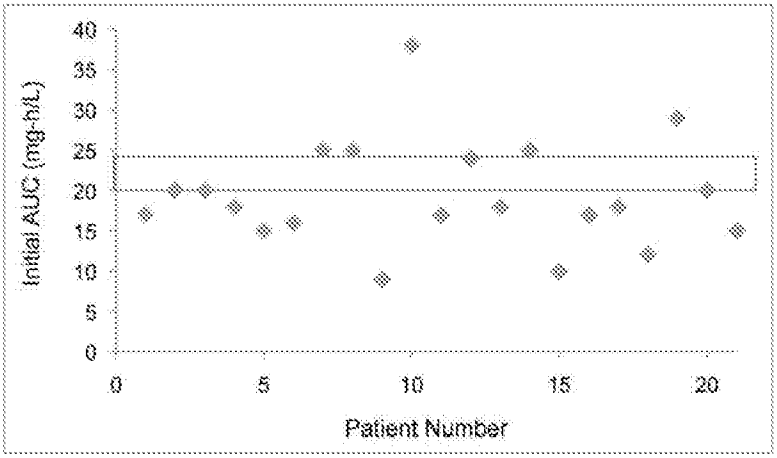


Figure 1. A scatter plot of initial AUC of individual patients observed in the study. The box outlines data points that are within the therapeutic range of 5-FU (20–24 mg.h/L). Four data points lie within this area.

routine pharmacokinetic monitoring of 5-FU in colorectal cancer patients receiving 5-FU-based chemotherapy regimens in our clinical practice. Our study has identified novel and heterogeneous patterns in 5-FU levels, at baseline and during dose optimization, suggesting variations in 5-FU metabolism among patients.

Results

Patient demographics and baseline 5-FU AUC following initial body surface area (BSA) dosing. Patient characteristics are summarized in Table 1. The median age of the patients was 59 y. Both genders were equally represented in the sample (11 male and ten female). Stages III and IV were also equally represented (12 Stage III and 9 Stage IV). The patients have been assigned to groups based on the pattern of the variability of their 5-FU AUC levels. These patterns are further described below. Of 21 patients, only four patients achieved an AUC within the target range without any dose titration (Fig. 1). These patients are referred to as Group A. In another eight patients (Group B), the target 5-FU AUC was achieved after adjusting the administered dose once or twice (Fig. 2).

Identification of a sub-group of colorectal cancer patients requiring multiple dose adjustments to achieve a 5-FU AUC in the therapeutic range. In a subset of our patients (n = 6) the observed changes in actual AUC did not correlate well with the adjustments made on the administered dose. In two patients (assigned to Group C), sub-therapeutic 5-FU plasma levels did not increase up to the target range despite an incremental (recommended) dose increase in the first two consecutive treatments but were seen to exceed the upper limit of the target range after the third dose adjustment. In one patient, an increase of 120 mg/m^2 in the first two consecutive doses of 5-FU failed to increase the AUC levels but caused an increase after the third adjustment from an AUC level of 15–28 mg.h/L (Fig. 3A). A similar observation can be made from the results of another patient (Fig. 3B).

Our observations demonstrate the importance of monitoring plasma 5-FU levels even after the therapeutic range has been achieved. In two patients (Group D), 5-FU AUC levels showed an upward trend exceeding past the target range of 20–24 mg.h/L after a continued use of the fixed therapeutic dose in the absence of any dose adjustments. This phenomenon was also observed when a therapeutic dose was reduced leading to an increase in the AUC levels beyond the upper limit of the target range. In the first patient, the therapeutic range was reached after one adjustment. Yet, even after using the same dose in the next cycle, the AUC levels exceeded the therapeutic range. (Fig. 4A). In the second patient, the therapeutic range was also reached after one adjustment. Yet, even when the administered dose was decreased, the AUC levels increased (Fig. 4B).

Lower plasma 5-FU AUC level in a patient treated with FOLFOX4 as compared with patients treated with FOLFOX6. FOLFOX 6 was administered to the majority of our patients in this study. However, we report the case of a patient who received FOLFOX4 (Fig. 5). The plasma levels in this patient were found to be half the lower limit of the therapeutic range. This patient was assigned to Group E.

Relationships between tumor thymidylate synthase (TS) expression and baseline 5-FU AUC levels. To begin to understand the possible mechanisms that contribute to the variability of 5-FU levels observed in the study, the thymidylate synthase (TS) expression levels of the tumors of the patients were plotted against the initial patient AUC's (Fig. 6A). A TS level below 4 is considered low, according to the manufacturer that routinely analyzes this parameter. Patients that had high TS levels (i.e., above 4) tended to have AUC's lower than the minimum therapeutic range of 20 mg.h/L. However, the difference in the distribution of the AUC levels between the low and high TS level patient groups was not statistically significant by the Wilcoxon rank sum test ($p > 0.05$). The initial AUC levels of the patients in different groups were plotted (Fig. 6B). Although the number of patients in Groups C and D were small, preliminary observations indicate that patients with low AUC's needed more than 3–4 dose adjustments before therapeutic levels of 5-FU can be achieved.

Discussion

Although the advantages of administering 5-FU doses based on actual 5-FU plasma levels of patients have been reported in the literature,[5,6] its use in the clinic has been slow. We have presented in our preliminary case series evidence to show that actual 5-FU plasma levels of patients cannot be taken for granted for its therapeutic efficacy using BSA methodology alone. There is a marked individual variation in the bioavailability of plasma 5-FU levels that has been well documented in the literature and reaffirmed in our series of 21 patients. In addition, we have reported data that indicate that 5-FU metabolism in patients changes as they undergo cycles of chemotherapy. We, thus, propose that the practice of treating patients with doses that are based on their height and weight (BSA methodology), should be

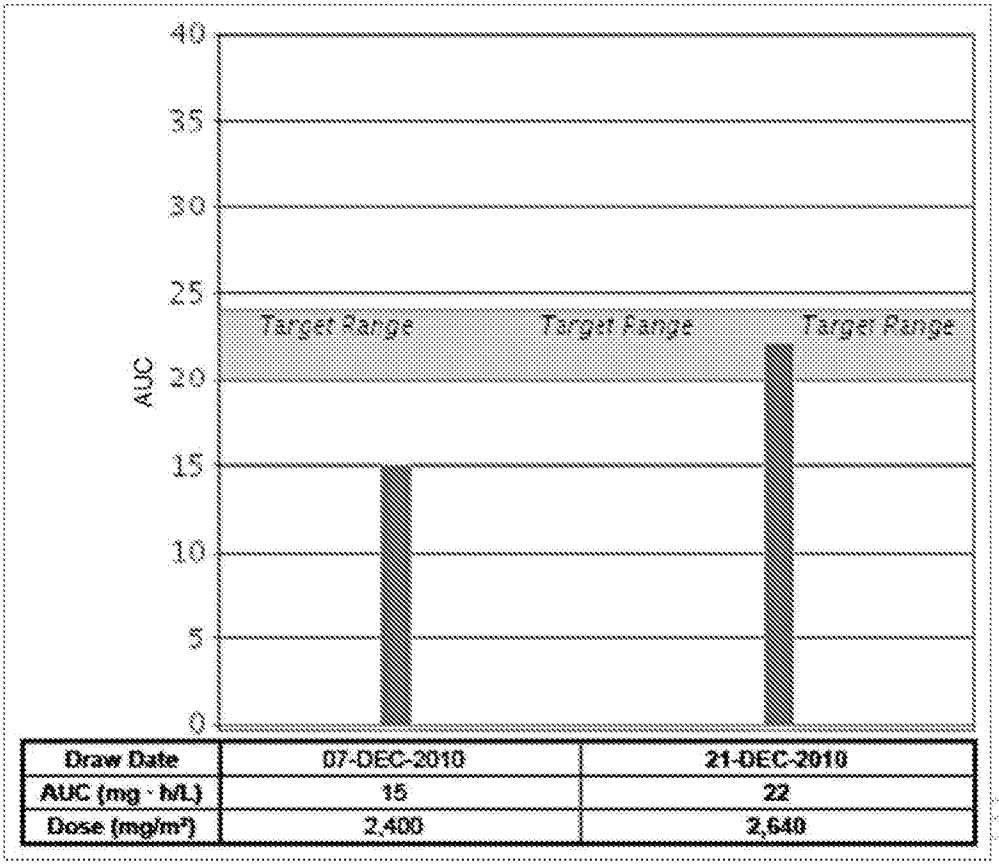
Table 1. Patient Characteristics.

Patient	Age	Sex	Diagnosis	KRAS/ BRAF	TS	Stage	Regimen	Initial AUC	Group	Liver mets	5-FU dose reduction
1	75	M	Colon cancer	WT/WT	1.87	IV	FOLFOX6 + Avastin	17	B/C	Yes	Yes based on AUC of 32
2	67	M	Colon cancer	G12A/WT	1.34	III	FOLFOX6	20	A	No	No (yes for oxaliplatin)
3	50	M	Colon cancer	WT/WT	6.41	III	FOLFOX6	20	A	No	No (yes for oxaliplatin)
4	53	F	Rectal cancer	NA	NA	III	FOLFIRI	18	B	No	No
5	40	M	Rectal cancer	WT/WT	6.99	IV	FOLFOX6 + Avastin	15	B	Yes	No (yes for oxaliplatin)
6	51	F	Colon cancer	WT/WT	NA	III	FOLFOX6	16	C/D	No	No (yes for oxaliplatin)
7	60	F	Colon cancer	WT/V600E	1.75	IV	FOLFOX6 + Avastin	25	D	Yes	Yes based on AUC of 25
8	76	F	Colon cancer	G12V/WT	2.71	III	FOLFOX6	25	B	No	Yes based on AUC of 25
9	34	M	Colon cancer	NA	NA	III	FOLFOX4	9	E	No	No (yes for oxaliplatin)
10	62	F	Colon cancer	WT/WT	5.72	III	FOLFOX6	38	B	No	Yes based on AUC of 38
11	55	M	Colon cancer	WT/WT	5.56	III	FOLFOX6	17	C*	No	No (yes for oxaliplatin)
12	43	F	Colon cancer	WT/WT	3.5	IV	FOLFOX6 + Avastin	24	A	No	Yes based on later toxicity (yes for oxaliplatin)
13	47	F	Colon cancer	G12V/WT	1.64	IV NED	FOLFOX6	18	B	No	No
14	61	F	Rectal cancer	NA	NA	III	FOLFOX6	25	C	No	Yes based on AUC of 25 (yes for oxaliplatin)
15	57	M	Colon cancer	WT/WT	NA	III	FOLFOX6	10		No	No due to diarrhea
16	61	M	Colon cancer	WT/WT	1.99	III	FOLFOX6	17	B	No	Yes based on later AUC of 25 (yes for oxaliplatin)
17	63	M	Colon cancer	WT/WT	1.03	IV	FOLFOX6 + Avastin	18	C	No	Yes based on AUC of 27, 29
18	43	M	Colon cancer	WT/V600E	1.9	IV	FOLFOX6 + Avastin	12	C**	Yes	Yes based on later AUC of 30 (yes for oxaliplatin)
19	63	M	Rectal cancer	WT/WT	3.58	III	FOLFOX6	29	B	No	Yes based on later AUC of 30
20	68	F	Rectal cancer	WT/WT	3.68	IV	FOLFOX6 + Avastin	20	A	No	No (therapy in progress)
21	59	F	Colon cancer	WT/V600E	6.76	IV	FOLFOX6 + Avastin	15		No	No (therapy in progress)

The patients were assigned to an AUC optimization group based on the pattern of variability of their 5-FU levels. The groups were designated as follows: A) patients that achieved an AUC within the target range without any dose titration; B) patients where the target 5-FU was achieved by adjusting the administered dose once or twice; C) patients that did not respond to two dose adjustments but at the third adjustment, experienced a spike in 5-FU level, exceeding the target range; D) patients that demonstrated increases in 5-FU levels (past the target range) despite receiving previously established therapeutic dose or even less than the therapeutic dose and E) patient that received FOLFOX4.

re-evaluated especially in the era of readily available commercial testing for 5-FU.

In a subset of our patients, multiple dose adjustments were required to achieve the desired therapeutic range as determined by Gamelin et al.[4] Moreover, we observed that some patients despite repeated dose adjustments over 3–4 two-week cycles did not reach target AUC levels initially but suddenly exceeded the upper limit of the target range with the following dose adjustments.



Draw Date	07-DEC-2010	21-DEC-2010
AUC (mg · h/L)	15	22
Dose (mg/m²)	2,400	2,640

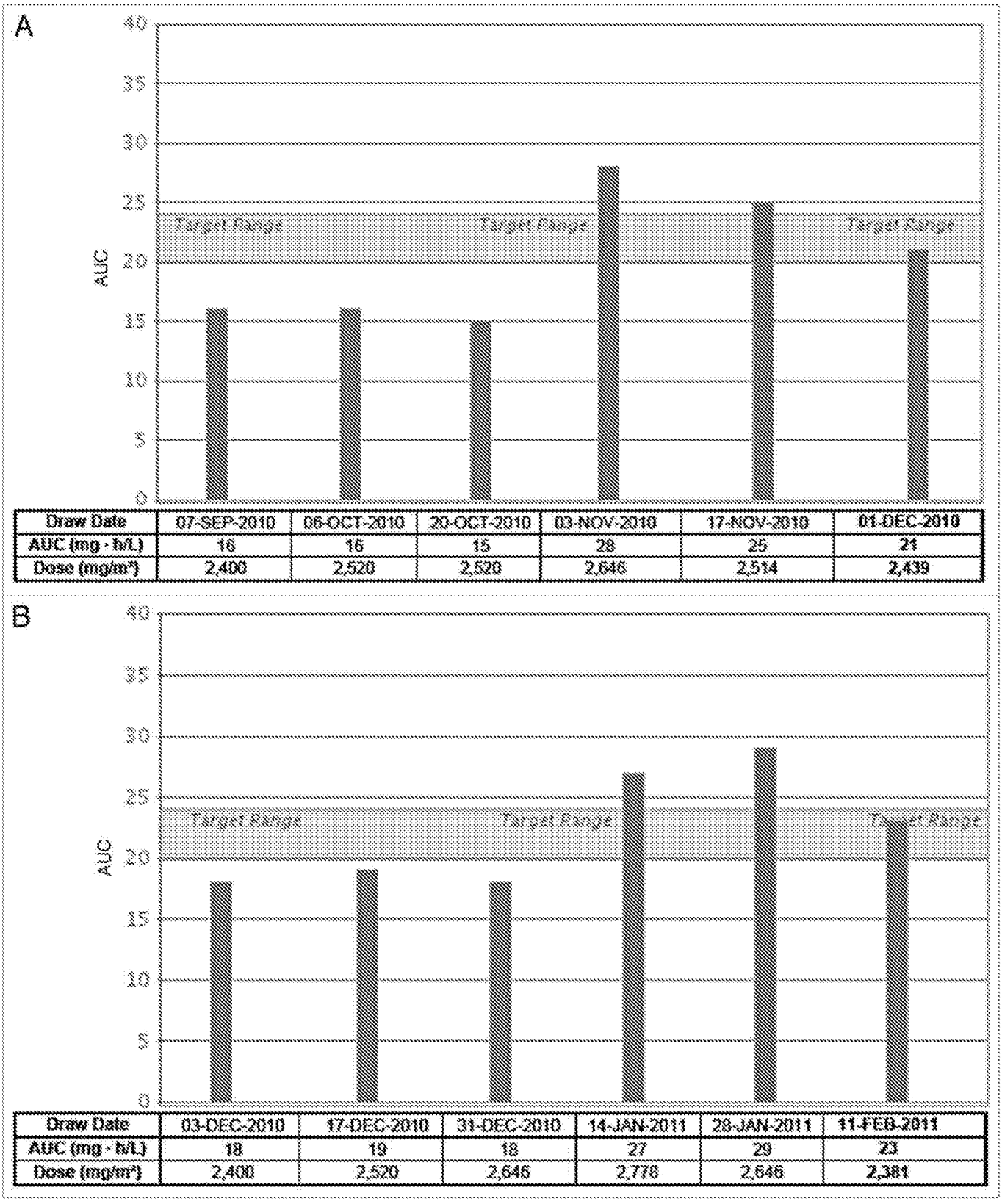
Figure 2. A representative Ondose test result on a patient that had therapeutic 5-FU level after one or two dose adjustments.

This necessitated a dose reduction to an amount less than their first dose that was initially deemed sub-therapeutic. The increase in 5-FU plasma levels seen after a small dose increase can be an indication of a reduction in 5-FU clearance. A similar conclusion can perhaps be made from the observations of increasing 5-FU plasma levels seen despite the continued use of therapeutic 5-FU dose or even dose reductions.

The elimination kinetics of 5-FU has been shown to be non-linear. When administered doses are increased, the half-life and bioavailability of 5-FU also increase,[9] and systemic clearance decreases. By contrast, the amount of 5-FU in the liver decreases.[10] The reduction in clearance with increased 5-FU doses is also seen when 5-FU is taken orally.[11] The mechanism behind the decrease in 5-FU clearance in response to 5-FU-based chemotherapy has not been elucidated. Although it has been proposed by Gamelin et al.[4] that this is due to a saturable metabolic process, the molecular basis for this saturation is not completely understood. In our study and in Gamelin's study, the change in clearance is observable after three cycles of chemotherapy. The time lag between the third and the fourth cycle is different between the patients in our study, and the population in Gamelin's study. Yet, the observations are similar. This raises the question on the impetus behind this change in 5-FU metabolism.

One possible mechanism behind the change in 5-FU clearance may involve the 5-FU catabolic enzyme dihydropyrimidine dehydrogenase (DPD). 5-FU is degraded via a 3-step catabolic pathway: a) conversion to 5-fluoro-5,6-dihydrouracil via dihydropyrimidine dehydrogenase; b) formation of α-fluoro-β-ureidopropionic acid via dihydropyrimidinase and c) conversion to α-fluoro-β-alanine via β-alanine synthase. These reactions occur primarily in the liver cytosol[12] (Fig. 7). The enzyme that catalyzes the rate-limiting step in this pathway is DPD.[13] The critical role that DPD plays in 5-FU metabolism is underscored by the deleterious effects in patients that have complete or near-complete deficiency of the enzyme.[14] Colorectal cancer patients exposed to a single bolus of 5-FU before surgery had significantly lower DPD mRNA levels in their primary tumors.[15] DPD activity in human PBMC's was decreased in colorectal cancer patients with intravenous treatment. This decrease was also seen in livers of rats that received a bolus injection of 5-FU.[16] An in vitro study on cervical carcinoma cells showed that extended exposure to 5-FU has a different effect on DPD expression than that of short-term exposure. With short-term 5-FU treatment, DPD mRNA is inhibited only by high 5-FU concentrations. On the other hand, treating for an extended period of time with lower 5-FU concentrations was sufficient to inhibit DPD mRNA expression.[17] In the case of nude mice with gastric cancer xenografts, a short-term exposure to 5-FU also resulted in a decrease in DPD activity.[18] Despite this mounting evidence that 5-FU treatment affects DPD, the impact on long-term 5-FU based chemotherapy on DPD has not been investigated.

Aside from potential differences in the rate-limiting enzyme of 5-FU catabolism, DPD, there are other plausible sources of variability that can explain the heterogeneity in 5-FU levels reported in this study. In a continuous infusion, the 5-FU levels of a patient depend on the time of day the blood sample was drawn. This is at least, in part due to a circadian variability in DPD activity.[19] However, it has been shown that the circadian variability of 5-FU levels did not account for most of the intra-patient variability observed in a study of 61 patients receiving 24 h continuous infusion.[20] Gender has been shown to influence 5-FU clearance, with women having a lower ability to clear 5-FU.[21-23] This could, at least in part, explain why 5-FU-related toxicities are more prevalent in women.[24] The influence of age on 5-FU clearance has been more equivocal. In one study, it was concluded that age did not affect clearance, as long as corrections



Draw Date	07-SEP-2010	06-OCT-2010	20-OCT-2010	03-NOV-2010	17-NOV-2010	01-DEC-2010
AUC (mg · h/L)	16	16	15	28	25	21
Dose (mg/m²)	2,400	2,520	2,520	2,646	2,514	2,439

Draw Date	03-DEC-2010	17-DEC-2010	31-DEC-2010	14-JAN-2011	28-JAN-2011	11-FEB-2011
AUC (mg · h/L)	18	19	18	27	29	23
Dose (mg/m²)	2,400	2,520	2,646	2,778	2,646	2,381

Figure 3. Ondose test results of two patients (A) and (B) whose 5-FU AUC did not respond with increases in administered doses in the first three cycles. After two adjustments, however, a small increase in the administered dose resulted in a marked increase in 5-FU AUC, exceeding the target range of 5-FU plasma level.

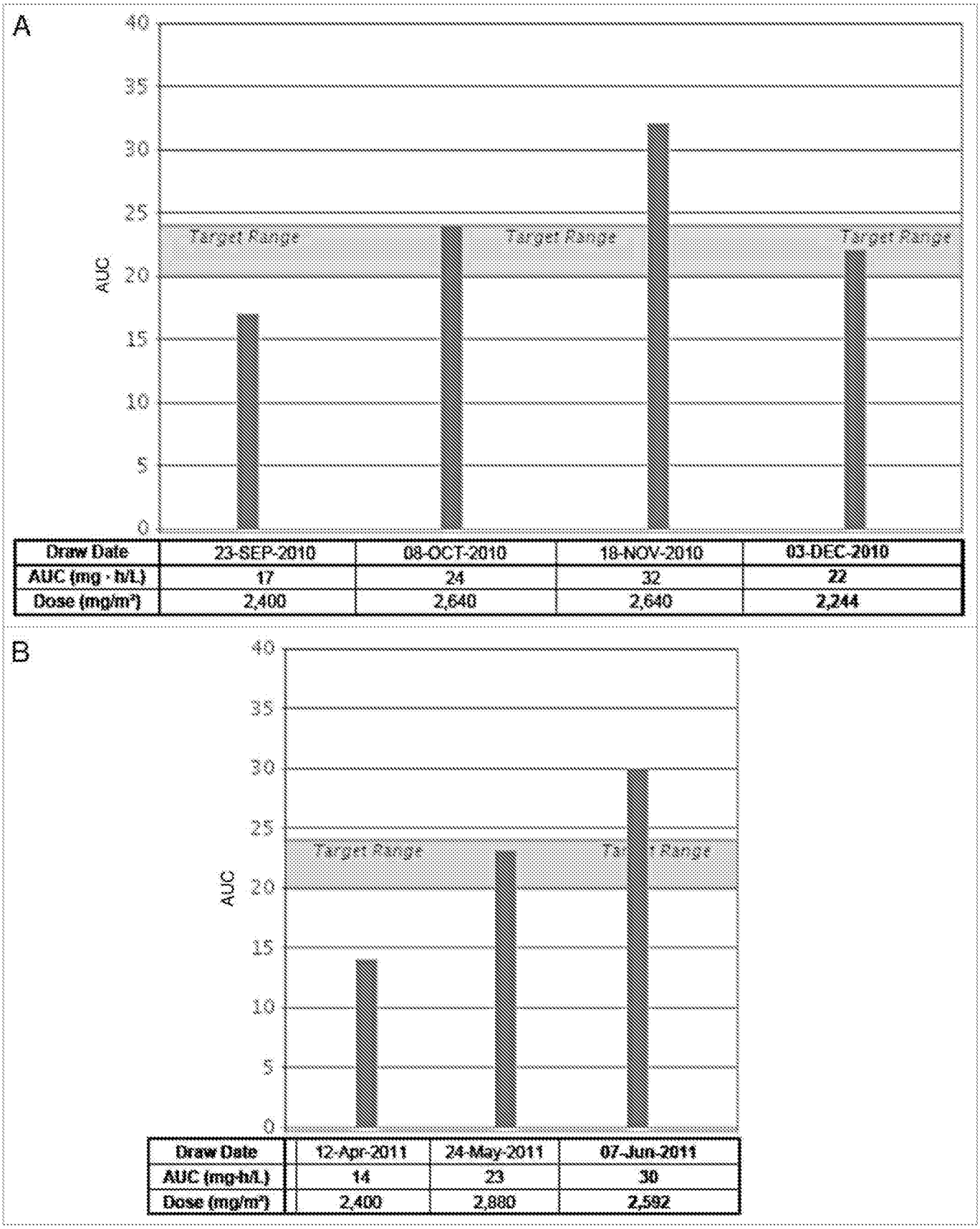
for gender and dose were made.[19] Yet, there are also indications that increasing age may reduce clearance.[23,25]

Treating patients with the regimen of 5-FU, leucovorin and oxaliplatin (FOLFOX) has proven to be more effective than FU/LV alone, in terms of a higher objective response rate, longer time to tumor progression, and a higher rate of relief from tumor-related symptoms.[26,27] Different concentrations of oxaliplatin have been combined with different modes of administration of 5-FU and leucovorin, leading to the development of different regimens.[28] Continuous infusion (CI) of 5-FU has been shown to have advantages possibly due to the short half-life of 5-FU and its cell cycle-dependent effect on thymidylate synthase.[29]

Currently, the combination of short-term infusional 5-FU/LV and oxaliplatin (FOLFOX) is considered a standard first-line therapy for mCRC. At least seven modifications of this combination exist based on a difference in the dose intensity of 5-FU/LV and oxaliplatin. In the North America, FOLFOX6 is more commonly employed over FOLFOX4 largely due a convenience in the administration of chemotherapy in the community oncology practice. Both regimens use the same amount of oxaliplatin but with a significant difference in the dose intensity of short-term infusional 5-FU/LV (bolus 5-FU 400 mg/m^2 followed by 600 mg/m^2 of 22 h CI 5-FU on day 1 and day 2 vs. bolus 5-FU 400 mg/m^2 followed by 2400 mg/m^2 of 46 h CI 5-FU on day 1 only). Whether this difference in dose intensity translates into a meaningful clinical benefit is not clear as there are no prospectively designed clinical trials comparing the two regimens in a head to head fashion. In one small retrospective study of Japanese patients with refractory or advanced colorectal cancer treated with FOLFOX4 or mFOLFOX6 regimen, mFOLFOX6 produced a higher observed partial response rate (35.5 vs. 25%) without any significant differences in toxicity. However, this study was not primarily designed to compare the two regimens and only evaluated the efficacy, feasibility and tolerability of the two regimens in a Japanese cohort.[30] In the randomized OPTIMOX1 trial, FOLFOX4 was compared with FOLFOX7 in a stop and go fashion in mCRC patients as a novel strategy to mitigate the cumulative side effects of neurotoxicity resulting from oxaliplatin. The FOLFOX7 regimen contained the same amount of CI 5-FU/LV as FOLFOX6 without the 5-FU bolus but a higher oxaliplatin dose. Previously untreated patients were randomly assigned to either FOLFOX4 administered every 2 weeks until progression (arm A) or FOLFOX7 for six cycles, maintenance bolus and CI 5-FU/LV without oxaliplatin for 12 cycles, and reintroduction of FOLFOX7 upon progression. The two arms were equal in their duration of disease control (DDC), progression-free survival (PFS), overall survival (OS), objective tumor response and the toxicity profile. These two studies, although not primarily designed to compare the efficacy and toxicity of the two FOLFOX regimens, did not show a clinically meaningful difference in the outcomes such as overall survival, PFS and response rate based on a difference in 5-FU/LV intensity alone.[31]

While the outcomes data in the combination regimens of 5FU/LV with oxaliplatin is unequivocal in terms of difference in dose intensity, there is evidence that 5-FU/LV as a single agent does translate into clinical benefit if delivered at an optimum dose based on pharmacokinetic adjustments vs. conventional body-surface-area calculations. Gamelin et al.[2] compared in a multicenter Phase III randomized study conventional dosing of fluorouracil (FU) plus folinic acid with pharmacokinetically guided 5-FU dose adjustment in terms of response, tolerability and survival. The 5-FU doses were adjusted weekly until the patients reached the therapeutic plasma range (AUC 20–24 mg.h/L). In the intent-to-treat analysis of the 208 patients, objective response rate was 18.3% in arm A, in which the 5-FU dose was calculated based on body-surface area, and 33.7% in arm B ($p = 0.004$) in which the 5-FU dose was individually determined using pharmacokinetically guided adjustments. Median overall survival was 16 mo in arm A and 22 mo in arm B ($p = 0.08$). The higher median OS of 22 mo reached in the pharmacokinetically adjusted arm was comparable to the other commonly used 5-FU/LV combination regimens, with a low toxicity and financial costs. Whether this clinical benefit resulting from the optimization of a weekly single agent 8 h infusion of 5-FU (1500 mg/m^2) dose can be reproduced in FOLFOX4 or FOLFOX6 regimen with 5-FU dose optimization to an AUC of 20–24 is currently unknown. Furthermore, there is limited evidence assessing the optimum AUC needed for oxaliplatin and irinotecan based regimens. These studies are critical given the observation that oxaliplatin can actually reduce the clearance of 5-FU.[32] There is limited evidence that suggests with a modified FOLFOX4 regimen (resembling FOLFOX4 with oxaliplatin 85 mg/m and leucovorin 200 mg/m^2 day 1, however 5-FU comparable to FOLFOX6 with fluorouracil 400 mg/m^2 bolus day 1 and 2500 mg/m^2 over 44 h) that over 80% of patients required a 5-FU infusion increase targeting a concentration of 0.6 mg/mL to achieve an AUC of 28.8 mg.h/L. Despite this titration there was a smaller number of patients reported to have grade ¾ toxicity (diarrhea/mucositis) and suggestive increase in median overall survival (22 vs. 28 mo).[6] A single-arm trial involving 90 patients treated with FOLFIRI with dose optimization of 5-FU CI starting at 2500 mg/m^2 over 46 h and modified to target an AUC of 25–30 mg.h/L (Css 0.55–0.65 mg/mL) reported a median overall survival of 28 mo with minimal grade ¾ toxicity.[33]

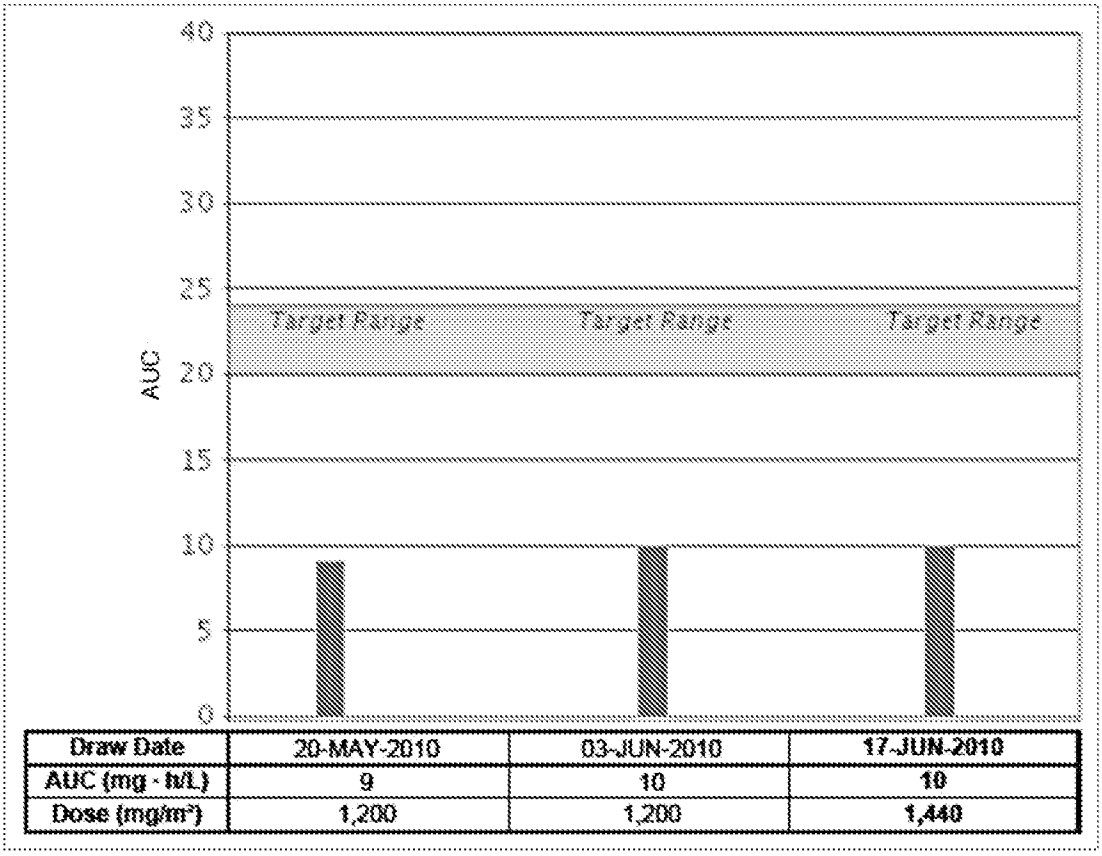
We noted a suboptimal 5-FU AUC of 10 in one patient who received FOLFOX4 regimen. This observation is not surprising given that CI 5-FU is half of what is used in FOLFOX6. Similar data has been observed in other reports of patients undergoing the FOLFOX4 regimen with an average AUC of 12.4 compared with an average AUC of 20.4 for FOLFOX6 regimen.[34] Given the prevalence of this observation across the patient population receiving FOLOFX4 regimen, it cannot be entirely attributed to individual variability in 5-FU metabolism. At the same time it does pose an important question regarding achieving an optimal dose with FOLFOX4 regimen given the correlation of higher 5-FU AUC of 20–24 levels with therapeutic efficacy. Our observations in a patient treated with FOLFOX4 showing a level of 5-FU that is half of the minimum value for the therapeutic range brought to our attention an important issue as various 5-FU containing regimens are tested and combined with novel targeted agents in patients with colorectal cancer. It would seem to be



Draw Date	23-SEP-2010	08-OCT-2010	18-NOV-2010	03-DEC-2010
AUC (mg · h/L)	17	24	32	22
Dose (mg/m²)	2,400	2,640	2,640	2,244

Draw Date	12-Apr-2011	24-May-2011	07-Jun-2011
AUC (mg·h/L)	14	23	30
Dose (mg/m²)	2,400	2,880	2,592

Figure 4. Ondose test results of two patients (A) and (B) that demonstrate that 5-FU levels can be supertherapeutic in subsequent cycles even if a previously established effective dose is administered.



Draw Date	20-MAY-2010	03-JUN-2010	17-JUN-2010
AUC (mg · h/L)	9	10	10
Dose (mg/m²)	1,200	1,200	1,440

Figure 5. Ondose test results of a patient that received the FOLFOX4 regimen.

important to ensure that 5-FU levels are optimized in such trials in order to not overestimate the benefit from targeted therapy. For example, if 5-FU levels are sub-optimal with regard to therapeutic range in a regimen such as FOLFOX4 one might expect a lesser effect in terms of response rate or patient survival for cohorts treated with FOLFOX4 and such result might contribute to a greater apparent benefit from regimens where 5-FU is more optimally dosed or from those regimens that involve the addition of a combined targeted agent.

Aside from analyzing 5-FU levels of the patients as they undergo repeated cycles of chemotherapy, we also took note of the ratios of administered-dose: AUC in the patients. In ten out of 15 patients (67%), the respective ratio of administered-dose: AUC had a decreasing trend as a patient underwent chemotherapy. This indicates that as a patient goes through multiple cycles of chemotherapy, they may need to receive less 5-FU during treatment to continue to have therapeutic plasma 5-FU levels. The decrease in 5-FU metabolism that we have observed with chemotherapy is supported by a recent study by Ibrahim et al.[36] They reported that 5-FU clearance is decreased even in the course of one cycle of chemotherapy. They compared 5-FU clearance on day 1 to that on day 5 of the first chemotherapy cycle of 81 patients. In another study, an increase in AUC was observed in patients given weekly doses of 5-FU.[37] The mechanism behind the decrease in 5-FU clearance in response to 5-FU based chemotherapy needs to be further elucidated. Low 5-FU clearance has been shown to be a predictor of severe toxicity.[36] This research problem is significant given the current practice that doses given to patients are only reduced when toxic side-effects have been experienced. These side-effects can be avoided with the understanding that 5-FU-based chemotherapy may decrease the ability of patients to metabolize/clear 5-FU.

In this study, the possibility that tumor TS expression levels might correlate with the variability of 5-FU plasma levels was explored. Patients that had higher tumor TS levels tended to have lower AUC's, although the difference between the distribution of the AUC levels of the high and low TS patient groups was not statistically significant. This lack of statistical significance may be due to the limited sample size. On the other hand, it may also be an indication that TS level is not a major determinant of 5-FU AUC's. In cells, including in tumor cells, 5-FU is metabolized in a two-step reaction to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP).[27] FdUMP forms a complex with the cofactor 5,10-methylenetetrahydrofolate and TS.[38-40] This makes TS less available to catalyze the formation of thymidylate, a critical step in DNA synthesis. The inhibition of thymidylate synthase is one of the primary modes of action of 5-FU. Thus, studies have been done to correlate TS expression with survival[41-43] and response.[45] These studies show that low TS expression is predictive of better survival and response to 5-FU-based chemotherapy. Polymorphisms of the TS gene, have also been analyzed and correlated with response to 5-FU. The TS gene has

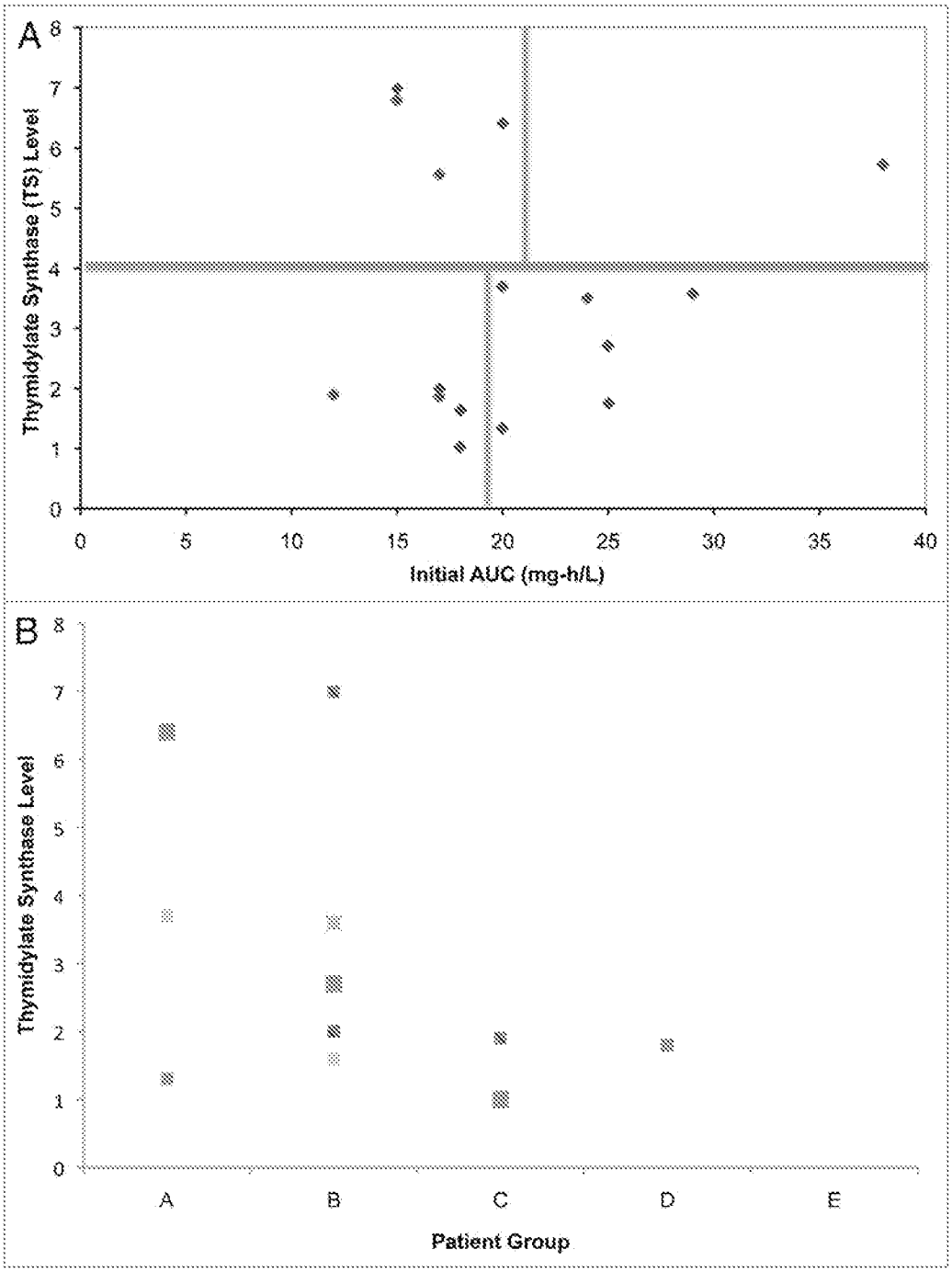


Figure 6. Thymidylate synthase (TS) levels of patients in this study. (A) Thymidylate synthase mRNA expression levels of the tumors of the patients were plotted against the initial 5-FU AUC values of the patients. The horizontal line indicates the normalized mRNA cut-off value of 4, with TS values higher than 4 indicative of high TS. Vertical lines arbitrarily separate AUC levels based on TS expression into potentially meaningful groups as described in the text. (B) TS levels of the patients in different groups based on the pattern of variability of their 5-FU levels, as described in Figure 1 and in the Results section.

a tandem repeat in its 5'-untranslated region. The number of repeats is polymorphic.[46] This polymorphism has been correlated with response and toxicity of 5-FU chemotherapy, with patients having the triple repeat experiencing fewer side effects. These same patients, however, also had a low response rate. Although 5-FU AUC's were not reported in the study, it is possible that these patients had low 5-FU AUC's. Thus, patient AUC's may be influenced more by TS gene polymorphisms than TS expression levels. This, however, needs further elucidation.

In the future, in addition to greater attention to 5-FU levels, it will be important to gain a better understanding of what physiological and genetic differences might underlie the observed

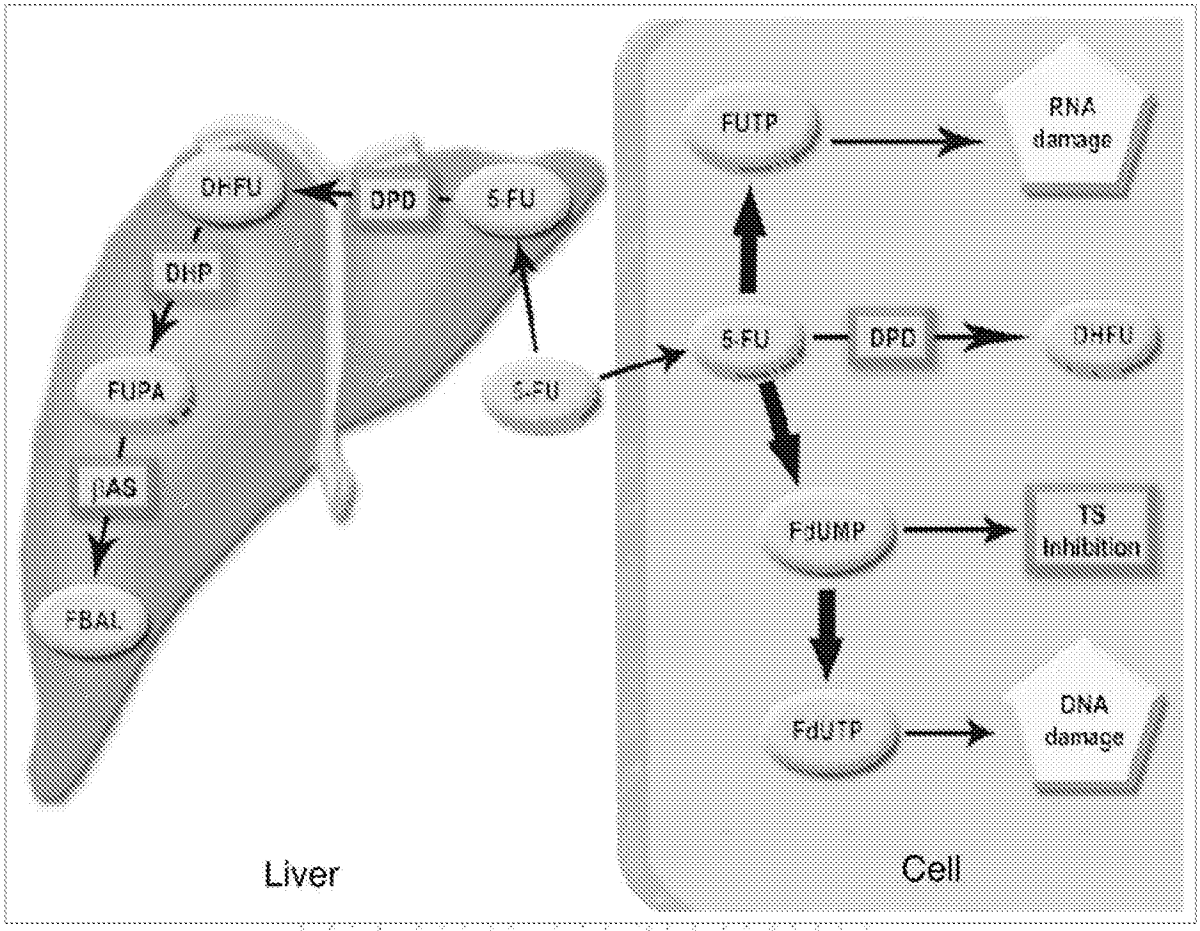


Figure 7. 5-FU is largely metabolized in the liver. The rate-limiting enzyme for 5-FU, DPD, is shown. In the liver and in cells, 5-FU is converted by DPD to dihydrofluorouracil (DHFU). In the cell, 5-FU undergoes catabolic reactions and conversion reactions, resulting in thymidylate synthase inhibition (TS), DNA and RNA damage. Only a subset of the reactants and enzymes involved in the 5-FU metabolic pathways are shown. For an excellent review of these processes, the readers are referred to Longley et al.[9] Ovals denote the reactants/products of the reactions and rectangles enclose the enzymes. Abbreviations: 5FU, 5-fluorouracil; DPD, Dihydropyrimidine dehydrogenase; DHFU, dihydrofluorouracil; DHFU, 5-fluoro-5,6-dihydrouracil; DHP, dihydropyrimidinase; FUPA, α-fluoro-β-ureidopropionic acid; βAS, β-alanine synthase; FBAL, α-fluoro-β-alanine; FUMP, fluorouridine monophosphate; FdUMP, fluorodeoxyuridine monophosphate; FUTP, fluorouridine triphosphate; FdUTP, fluorodeoxyuridine triphosphate. Thick arrows denote multiple reactions are necessary to convert the reactant to the product shown. Walt Kline II prepared the artwork for this Figure.

heterogeneity among patients during optimization of infusional 5-FU dose. Larger studies will need to examine patient demographics including gender, age, ethnicity and disease state and it would be of interest to examine single nucleotide polymorphisms in DPD, TS, as well as other genes. It will also be of interest to more closely examine in vivo gene expression changes in liver to unravel other potential pathways that might impact on 5-FU levels over time after exposure to 5-FU therapy. The liver studies are particularly important given that 5-FU catabolism occurs mainly in the liver.[12]

Materials and Methods

Patient characteristics. The observations described here involved the study of existing data, including patient medical records, blood test results, pathological specimens and diagnostic images in the course of routine clinical care in a colorectal cancer clinic. The information in this manuscript was recorded in such a manner that subjects could not be identified. Patient confidentiality was maintained and the work was performed in compliance with institutional and federal guidelines, and with approval from our Institutional Review Board.

A total of 21 patients with colorectal cancer as part of routine clinical care had 5-FU pharmacokinetic testing using the commercially available OnDose test (Myriad Genetic Laboratories Inc., Salt Lake City, UT) to target a plasma AUC level of 20–24 mg.h/L. The 21 subjects ranged in age from 34–76 y old, included 11 men and ten women, and included 16 patients with colon cancer and five with rectal cancer. The patients included nine with Stage IV disease, including one with Stage IV and no evidence of disease (NED) following resection, and 12 patients with Stage III disease.

Chemotherapy regimens. Regimens used included FOLFOX6 + Avastin for the eight patients with measurable Stage IV disease, and FOLFOX6 for 11 patients with Stage III or Stage IV NED disease. One patient with Stage III disease

received the FOLFIRI regimen and one patient received the FOLFOX4 regimen.

Blood collection for 5-FU AUC determination. Routine blood collection for 5-FU levels was drawn from an upper extremity peripheral vein on the contra-lateral side where the patients had a port for infusional 5-FU. Blood was collected at the 26th hour of the continuous 5-FU infusion of FOLFOX6. In the case of the patient receiving FOLFOX4, blood was drawn at 23 ± 3 h of infusion.

KRAS, BRAF mutation status and thymidylate synthase expression level. The analyses of the K-Ras and B-Raf mutation status, and the thymidylate synthase expression level in patient tumor tissue were performed by Response Genetics Inc. (Los Angeles, CA).

Disclosure of Potential Conflicts of Interest statement

No potential conflicts of interest were disclosed.

References

1. Schnell B, Gruenberger T, Kornek GV, Dworan N, Depisch D, Lang F, et al. Side effects during chemotherapy predict tumour response in advanced colorectal cancer. Br J Cancer 2005; 93:744-8; PMID:16160693; http://dx.doi.org/10.1038/sj.bjc.6602783.
2. Gamelin E, Delva R, Jacob J, Merrouche Y, Raoul JL, Pezet D, et al. Individual fluorouracil dose adjustment based on pharmacokinetic follow-up compared with conventional dosage: results of a multicenter randomized trial of patients with metastatic colorectal cancer. J Clin Oncol 2008; 26:2099-105; PMID:18445839; http://dx.doi.org/10.1200/JCO.2007.13.3934.
3. Gamelin E, Boisdron-Celle M, Delva R, Regimbeau C, Cailleux PE, Alkaaume C, et al. Long-term weekly treatment of colorectal metastatic cancer with fluorouracil and leucovorin: results of a multicentric prospective trial of fluorouracil dosage optimization by pharmacokinetic monitoring in 152 patients. J Clin Oncol 1998; 16:1470-8; PMID:9552054.
4. Gamelin EC, Danquechin-Dorval EM, Dumesnil YF, Maillart PJ, Goudier MJ, Burtin PC, et al. Relationship between 5-fluorouracil (5-FU) dose intensity and therapeutic response in patients with advanced colorectal cancer receiving infusional therapy containing 5-FU. Cancer 1996; 77:441-51; PMID:8630950; http://dx.doi.org/10.1002/(SICI)1097-0142(19960201)77:3<441::AID-CNCR4>3.0.CO;2-N
5. Wenstrup R, Saam J, Kaldate R, Rhode M, Hamilton S, Eisenbraun A, et al. Excessive 5-FU inter-patient pharmacokinetic variability may result in suboptimal 5-FU dosing of metastatic and adjuvant colon cancer patients on FOLFOX regimens. ASCO-GI, 2010.
6. Gamelin E, Capitain O, Morel A, et al. Comparison of two patient cohorts treated in parallel for advanced colorectal cancer with a simplified FOLFOX 4 regimen with or without 5-FU therapeutic dose management. ASCO 2009 Gastrointestinal Tumors Symposium, 2009.
7. Ychou M, Duffour J, Kramar A, Debrigode C, Gourgou S, Bressolle F, et al. Individual 5-FU dose adaptation in metastatic colorectal cancer: results of a phase II study using a bimonthly pharmacokinetically intensified LV5FU2 regimen. Cancer Chemother Pharmacol 2003; 52:282-90; PMID:12827293; http://dx.doi.org/10.1007/s00280-003-0658-0.
8. Saif MW, Choma A, Salamone SJ, Chu E. Pharmacokinetically guided dose adjustment of 5-fluorouracil: a rational approach to improving therapeutic outcomes. J Natl Cancer Inst 2009; 101:1543-52; PMID:19843331; http://dx.doi.org/10.1093/jnci/djp328.
9. Ensminger WD, Rosowsky A, Raso V, Levin DC, Glode M, Come S, et al. A clinical-pharmacological evaluation of hepatic arterial infusions of 5-fluoro-2'-deoxyuridine and 5-fluorouracil. Cancer Res 1978; 38:3784-92; PMID:151583.
10. Wagner JG, Gyves JW, Stetson PL, Walker-Andrews SC, Wellner IS, Cochran MK, et al. Steady-state nonlinear pharmacokinetics of 5-fluorouracil during hepatic arterial and intravenous infusions in cancer patients. Cancer Res 1986; 46:1499-506; PMID:3943107.
11. Obrecht JP, Weber W, Cano JP, Crevoisier C, Alberto P, Forgo I, et al. 5-Fluorouracil: a comparative pharmacokinetic study and preliminary results of a clinical phase I study. Recent Results Cancer Res 1981; 79:101-7; PMID:7302355.
12. Naguib FN, el Kouni MH, Cha S. Enzymes of uracil catabolism in normal and neoplastic human tissues. Cancer Res 1985; 45:5405-12; PMID:3931905.
13. Zhang X, Diasio RB. Regulation of human dihydropyrimidine dehydrogenase: implications in the pharmacogenetics of 5-FU-based chemotherapy. Pharmacogenomics 2007; 8:257-65; PMID:17324113; http://dx.doi.org/10.2217/14622416.8.3.257.
14. Van Kuilenburg AB, van Lenthe H, Blom MJ, Mul EP, Van Gennip AH. Profound variation in dihydropyrimidine dehydrogenase activity in human blood cells: major implications for the detection of partly deficient patients. Br J Cancer 1999; 79:620-6; PMID:10027339; http://dx.doi.org/10.1038/sj.bjc.6690097.
15. Maurizi R, van Groeningen CJ, Smid K, Jansen G, Pinedo HM, Peters GJ. Thymidylate synthase and dihydropyrimidine dehydrogenase mRNA expression after administration of 5-fluorouracil to patients with colorectal cancer. Int J Cancer 2007; 120:2609-12; PMID:17330233; http://dx.doi.org/10.1002/ijc.22626.
16. McLeod HL, Sludden J, Hardy SC, Lock RE, Hawkeworth GM, Cassidy J. Autoregulation of 5-fluorouracil metabolism. Eur J Cancer 1998; 34:1623-7; PMID:9893640; http://dx.doi.org/10.1016/S0959-8049(98)00175-0.
17. Kashima N, Ueda M, Kanazawa J. Effect of 5-fluorouracil and epidermal growth factor on cell growth and dihydropyrimidine dehydrogenase regulation in human uterine cervical carcinoma SKG-IIIb cells. Cancer Sci 2003; 94:821-5; PMID:12967482; http://dx.doi.org/10.1111/j.1349-7006.2003.tb01525.x.
18. Sakurai Y, Uraguchi T, Imazu H, Hasegawa S, Matsubara T, Ochiai M, et al. Changes in thymidylate synthase and its inhibition rate and changes in dihydropyrimidine dehydrogenase after the administration of 5-fluorouracil with cisplatin to nude mice with gastric cancer xenograft SC-1-NU. Gastric Cancer 2004; 7:110-6; PMID:15224198; http://dx.doi.org/10.1007/s10120-004-0280-9.
19. Harris BE, Song R, Soong SJ, Diasio RB. Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. Cancer Res 1990; 50:197-201; PMID:2293556.
20. Friberg G, Schumm P, Ratain MJ, Schilsky RL, Fleming GF. Circadian variations in plasma 5-fluorouracil (5-FU) levels during 24-hour infusions. Journal of Clinical Oncology, 2004 ASCO Annual Meeting Proceedings (Post-Meeting Edition), 2004:2055.
21. Zhu AX, Puchalski TA, Stanton VP Jr., Ryan DP, Clark JW, Nesbitt S, et al. Dihydropyrimidine dehydrogenase and thymidylate synthase polymorphisms and their association with 5-fluorouracil/leucovorin chemotherapy in colorectal cancer. Clin Colorectal Cancer 2004; 3:225-34; PMID:15025795; http://dx.doi.org/10.3816/CCC.2004.n.003.
22. Milano G, Etienne MC, Cassuto-Viguier E, Thyss A, Santini J, Frenay M, et al. Influence of sex and age on fluorouracil clearance. J Clin Oncol 1992; 10:1171-5; PMID:1607921.
23. Port RE, Daniel B, Ding RW, Herrmann R. Relative importance of dose, body surface area, sex, and age for 5-fluorouracil clearance. Oncology 1991; 48:277-81; PMID:1891168; http://dx.doi.org/10.1159/000226942.
24. Stein BN, Petrelli NJ, Douglass HO, Driscoll DL, Arcangeli G, Meropol NJ. Age and sex are independent predictors of 5-fluorouracil toxicity. Analysis of a large scale phase III trial. Cancer 1995; 75:11-7; PMID:7804963; http://dx.doi.org/10.1002/1097-0142(19950101)75:1<11::AID-CNCR2820750104>3.0.CO;2-N.
25. Bertino J, Gamelin E, Milano G. 5-Fluorouracil drug management: Pharmacokinetics and Pharmacogenomics workshop meeting summary 5-fluorouracil drug management Pharmacokinetics and Pharmacogenomics workshop meeting summary Orlando, Florida: Clinical Colorectal Cancer, 2007.
26. Rothenberg ML, Oza AM, Bigelow RH, Berlin JD, Marshall JL, Ramanathan RK, et al. Superiority of oxaliplatin and fluorouracil-leucovorin compared with either therapy alone in patients with progressive colorectal cancer after irinotecan and fluorouracil-leucovorin: interim results of a phase III trial. J Clin Oncol 2003; 21:2059-69; PMID:12775730; http://dx.doi.org/10.1200/JCO.2003.11.126.
27. de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, Cassidy J, et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 2000; 18:2938-47; PMID:10944126.
28. Maindrault-Goebel F, Louvet C, Andre T, Carola E, Lotz JP, Molitor JL, et al. Oxaliplatin added to the simplified bimonthly leucovorin and 5-fluorouracil regimen as second-line therapy for metastatic colorectal cancer (FOLFOX6). GERCOR. Eur J Cancer 1999; 35:1338-42; PMID:10658524; http://dx.doi.org/10.1016/S0959-8049(99)00149-5.
29. Ainalm L. Individualization of 5-Fluorouracil in the Treatment of Colorectal Cancer. SRX Pharmacology 2010; 2010.
30. Shimizu T, Satoh T, Tamura K, Ozaki T, Okamoto I, Fukuoka M, et al. Oxaliplatin/fluorouracil/leucovorin (FOLFOX4 and modified FOLFOX6) in patients with refractory or advanced colorectal cancer: post-approval Japanese population experience. Int J Clin Oncol 2007; 12:218-23; PMID:17566846; http://dx.doi.org/10.1007/s10147-007-0658-x.

31. Tournigand C, Cervantes A, Figer A, Lledo G, Flesch M, Buyse M, et al. OPTIMOX1: a randomized study of FOLFOX4 or FOLFOX7 with oxaliplatin in a stop-and-Go fashion in advanced colorectal cancer--a GERCOR study. J Clin Oncol 2006; 24:394-400; PMID:16421419; http://dx.doi.org/10.1200/JCO.2005.03.0106.
32. Boisdron-Celle M, Craipeau C, Brienza S, Delva R, Guerin-Meyer V, Cvitkovic E, et al. Influence of oxaliplatin on 5-fluorouracil plasma clearance and clinical consequences. Cancer Chemother Pharmacol 2002; 49:235-43; PMID:11935216; http://dx.doi.org/10.1007/s00280-001-0406-2.
33. Capitain O, Asevoaia A, Boisdron-Celle M, Poirier AL, Maillart E, Morel A, et al. Influence of pharmacogenetic polymorphisms on 5-fluorouracil and irinotecan efficacy and tolerance in patients treated for advanced colorectal cancer. ASCO 2008 Gastrointestinal Cancers Symposium, 2008.
34. Saam J, Critchfield GC, Hamilton SA, Rae BB, Wenstrup RJ, Kaldare RR. Body surface area-based dosing of 5-fluorouracil results in extensive interindividual variability in 5-fluorouracil exposure in colorectal cancer patients on FOLFOX regimens. Clinical Colorectal Cancer 2011.
35. Ibrahim T, Di Paolo A, Amatori F, Mercatali L, Ravaioli E, Flamini E, et al. Time-dependent pharmacokinetics of 5-fluorouracil and association with treatment tolerability in the adjuvant setting of colorectal cancer. J Clin Pharmacol 2011; PMID:21383342.
36. Guglia M, Frigo AC, Bolzonella C, Marinelli F, Barile C, Bononi A, et al. Predictors of survival and toxicity in patients on adjuvant therapy with 5-fluorouracil for colorectal cancer. Br J Cancer 2009; 100:1549-57; PMID:19384296; http://dx.doi.org/10.1038/sj.bjc.6605052.
37. Maier-Martino M, Martino R. Clinical studies of three oral prodrugs of 5-fluorouracil (capecitabine, UFT, S-1): a review. Oncologist 2002; 7:288-323; PMID:12185293; http://dx.doi.org/10.1634/theoncologist.7-4-288.
38. Blakley RL, Ramasastry BV, McDougall BM. The Biosynthesis of Thymidylic Acid. V. Hydrogen Isotope Studies with Dihydrofolic Reductase and Thymidylate Synthetase. J Biol Chem 1963; 238:3075-9; PMID:14081927.
39. Langenbach RJ, Danenberg PV, Heidelberger C. Thymidylate synthetase: mechanism of inhibition by 5-fluoro-2'-deoxyuridylate. Biochem Biophys Res Commun 1972; 48:1565-71; PMID:4263280; http://dx.doi.org/10.1016/0006-291X(72)90892-3.
40. Santi DV, Pena VA, Lam SS. On the structure of the cofactor in the complex formed with thymidylate synthetase, 5,10-methylenetetrahydrofolate and 5-fluoro-2'-deoxyuridylate. Biochim Biophys Acta 1976; 438:324-31; PMID:938684.
41. Leichman CG, Lenz HJ, Leichman L, Danenberg K, Baranda J, Groshen S, et al. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. J Clin Oncol 1997; 15:3223-9; PMID:9336359.
42. Edler D, Glimelius B, Hallstrom M, Jakobsen A, Johnston PG, Magnusson I, et al. Thymidylate synthase expression in colorectal cancer: a prognostic and predictive marker of benefit from adjuvant fluorouracil-based chemotherapy. J Clin Oncol 2002; 20:1721-8; PMID:11919227; http://dx.doi.org/10.1200/JCO.2002.07.039.
43. Shirota Y, Stoehlmacher J, Brabender J, Xiong YP, Uetake H, Danenberg KD, et al. ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. J Clin Oncol 2001; 19:4298-304; PMID:11731512.
44. Aschele C, Debernardis D, Casazza S, Antonelli G, Tunesi G, Baldo C, et al. Immunohistochemical quantitation of thymidylate synthase expression in colorectal cancer metastases predicts for clinical outcome to fluorouracil-based chemotherapy. J Clin Oncol 1999; 17:1760-70; PMID:10561213.
45. Cascinu S, Aschele C, Barni S, Debernardis D, Baldo C, Tunesi G, et al. Thymidylate synthase protein expression in advanced colon cancer: correlation with the site of metastasis and the clinical response to leucovorin-modulated bolus 5-fluorouracil. Clin Cancer Res 1999; 5:1996-9; PMID:10473077.
46. Horie N, Aiba H, Oguro K, Hojo H, Takeishi K. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. Cell Struct Funct 1995; 20:191-7; PMID:7586009; http://dx.doi.org/10.1247/csf.20.191.
47. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 2003; 3:330-8; PMID:12724731; http://dx.doi.org/10.1038/nrc1074.

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Emerging Antibody-Based HER2 (ErbB-2/*neu*) Therapeutics

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ABSTRACT: Targeting HER2(ErbB-2/*neu*) overexpressing tumor cells to selectively deliver anticancer agents and thereby reduce host toxicity represents a rational and emerging strategy for the treatment of breast and other epithelial cancers. The extracellular domain of the HER2 receptor tyrosine kinase is readily accessible to systemically administered antibody-based therapeutics, including growth-inhibiting monclonals such as rhuMAbHER2 (trastuzumab/Herceptin©) as well as anti-HER2 immunotoxins, antibody-dependent enzyme prodrug therapy (ADEPT), and immune cell recruiting bispecific antibodies. In addition to summarizing recent advances in these antibody-based strategies, this review focuses on preclinical advances in the development of anti-HER2 immunoliposomes (ILs) as a platform technology for targeted drug delivery. Extensive *in vitro* and *in vivo* testing including efficacy and tumor uptake studies in multiple human tumor xenograft models now provide conclusive evidence for the superior therapeutic efficacy of anti-HER2 ILs-doxorubicin (dox) over free dox or liposomal (Ls)-dox, and even over combinations of dox and Ls-dox with rhuMAbHER2. As anti-HER2 ILs-dox approaches clinical testing in patients with advanced HER2 overexpressing breast cancer, future applications of this novel targeting strategy will also broaden to include intracellular delivery of other anticancer agents as well as therapeutic nucleic acids (oligonucleotides, genes).

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ACTIVATED HER2(ErbB-2/*NEU*) RECEPTOR AS A TARGET FOR NEW THERAPEUTICS

Role of Amplified/Overexpressed HER2 in Breast and other Epithelial Malignancies

The HER2/ErbB-2/*neu* proto-oncogene encodes p185HER2, a 1255 amino acid and 185 kDa transmembrane receptor that is a member of a large family of receptor tyrosine kinases [1–3]. Other members of this subfamily include the epidermal growth factor receptor (EGFR/HER1), HER3 (ErbB-3), and HER4 (ErbB-4). Unlike the other subfamily members, HER2 is an orphan growth factor receptor in that it has no known high-affinity growth factor ligand, and it is thought to be activated in two general ways. Activation occurs independent of ligand when mutated or overexpressed and via spontaneous homodimerization of the surface receptor; as well, HER2 undergoes ligand-dependent hetero-dimerization with a related ErbB subfamily member that is bound to either an EGF-like or heregulin-like growth factor ligand.

Clinical studies have revealed that overexpression of HER2 occurs in a variety of human malignancies, including cancer of the breast, ovary, endometrium, lung (non-small cell), stomach, pancreas, bladder and prostate [4–15]. An especially high frequency of HER2 amplification and overexpression (40–80%) has been found in breast ductal carcinoma in situ (DCIS), particularly those preinvasive lesions with the highest risk of recurrence (comedo-type DCIS). Up to 30% of invasive ductal breast cancers exhibit HER2 overexpression, and this malignant phenotype is associated with poor patient prognosis and increased risk of death from metastatic disease.

These clinical observations and an increasing appreciation of the tumorigenic role played by activated HER2 over the past decade fostered a concerted effort to develop novel strategies for therapeutically targeting epithelial tumors that overexpress HER2. Given its surface accessibility at levels up to 10^6 receptors/cell in various malignancies bearing the genomically amplified oncogene, and the additional observation that breast tumors continuously overexpress HER2 throughout their clinical progression while normal adult tissues express less than 10^4 receptors/cell [16,17], the development of growth-inhibiting anti-HER2 monoclonals ushered in an exciting era of clinical trials that culminated in recent FDA approval of the first anti-HER2 antibody therapy (trastuzumab/Herceptin©) for epithelial cancer [3,18]. In addition to several other antibody-based anti-HER2 therapeutics currently in clinical trials, small molecule inhibitors of the p185HER2 receptor tyrosine kinase and additional compounds capable of inhibiting its intracellular signaling are now under active preclinical and clinical development [1].

Endocytosis and other Receptor-Induced Early Responses to Anti-HER2 Antibodies

As for other receptor tyrosine kinases, ligands and antibodies that bind to EGFR subfamily members are capable of inducing a variety of early (< 15 min.) intracellular responses (e.g. agonist-like induction of receptor phosphorylation, second messenger release, actin cytoskeletal changes, and immediate-early gene transcription), few of which are predictive of the net cellular effect produced upon more sustained (≥ 8 h.) exposure to a given receptor-binding agent [3]. Classical ligand-induced endocytosis, as with EGF binding to EGFR, is associated with rapid cellular internalization by clathrin-coated pits; in contrast, following antibody binding to the orphan receptor HER2 internalization has been shown to occur by both clathrin-coated and non-coated pathways into internal endosomal structures and vesicles [19]. It appears that the process of endocytosis, however mediated, may be generally associated with some agonist-like early cellular responses [20,21]. Nonetheless, different receptors even within the same EGFR subfamily have different propensities and triggering mechanisms for endocytosis as well cell signaling induction and membrane recycling vs. cytosolic degradation fates following antibody binding and endocytosis [22–24]. Unlike other early agonist-like intracellular responses to receptor binding, endocytosis with internalization of receptor-bound antibody complexes offers an important functional mechanism that can be exploited by future antibody-based anti-HER2 therapeutics (e.g. anti-HER immunoliposomes) as well as in the design of potentially predictive assays of HER2 receptor function and anti-HER2 antibody responsiveness [25].

DEVELOPMENT OF ANTIBODY-BASED ANTI-HER2 THERAPEUTICS

Growth-Inhibiting Anti-HER2 Monoclonals as Breast Cancer Therapeutics

After two decades of research, progress in monoclonal-based cancer therapeutics has finally been clinically validated [26]. Two years after HER2 amplification and overexpression were first observed in a collection of primary human breast tumors and found to correlate with early disease relapse and poor patient survival [6], the first murine monoclonal antibodies with binding specificity against human HER2 were described [18,27]. One of these murine monoclonals, muMAb4D5, was fully characterized and shown to cytostatically inhibit growth of HER2 overexpressing human breast tumor cells as well as sensitize HER2 transformed cells to macrophage and cytokine (e.g. tumor necrosis factor alpha, TNFα) mediated cell lysis [27]. Studies using another similarly acting anti-HER2 monoclonal, Tab250, first demonstrated the potential of combining a cytostatic anti-HER2 antibody with a chemotherapeutic agent to achieve a synergistic tumoricidal response against HER2 overexpressing tumors [28]. Humanization of muAb4D5 to rhuMAbHER2 (ultimately renamed Herceptin©) by Genentech investigators in

preparation for clinical trials intentionally incorporated the human Fc antibody element to elicit a host (NK cell)-mediated antitumor immune response known as ADCC, antibody-directed cell-mediated cytotoxicity [29]. With preclinical reports of synergy in combination with chemotherapy and clinical reports of durable antitumor responses and acceptable patient toxicity in Phase II and III multi-institutional trials [30–33], the FDA approved rhuMAbHER2 therapy for metastatic breast cancer in late 1998 [34].

Immunotoxins and Antibody-Dependent Enzyme Prodrug Therapy (ADEPT)

In contrast to growth-inhibiting anti-HER2 monoclonal antibodies, recombinantly produced fusions of monoclonal fragments with other toxic agents are yielding novel multifunctional antibody therapeutics that can target HER2 overexpressing human tumors. Anti-HER2 immunotoxins derive their cytotoxic potential from the recombinant fusion of one of several potent cell toxins to the targeting (epitope binding) moiety of an anti-HER2 immunoglobulin derivative, typically a single-chain variable region-containing fragment (scFv). A recent review highlights the general advances made in designing ligand/toxin conjugates for therapy against human cancers [35]; and while immunotoxins targeting different EGFR family members have been designed and are currently under evaluation, several different anti-HER2 immunotoxins have now advanced rapidly through preclinical development and into clinical trials [36–42]. Fused to the targeting antibody sequence may be either a truncated bacterial toxin (e.g. *Pseudomonas* exotoxin A, ETA) or a recombinant plant toxin (e.g. gelonin). Alone, neither the truncated bacterial toxin nor the full-length plant toxin are able to enter mammalian cells, and both rely on the fused antibody fragment for receptor-mediated internalization into HER2 positive cells. The targeting specificity and reputed ability of a single internalized toxin molecule to induce cell kill may account for the preclinical success of anti-HER2 immunotoxins to eradicate both primary and metastatic lesions in animal xenograft models of HER2 overexpressing human tumors. Since these immunotoxins show specific binding to human HER2 receptor their potential for host toxicity can only be assessed in clinical trials, thus it remains to be seen if the $\leq 10^4$ HER2 receptors/cell expressed by those normal human tissues that passively accumulate these potent immunotoxins (e.g. liver) are sufficient to result in receptor-mediated internalization and development of organ toxicity following human administration of anti-HER2 immunotoxins.

Using a similarly constructed targeting agent in a two-step therapeutic approach, the ADEPT strategy first employs treatment with an antibody fragment linked to a prodrug activating enzyme followed by systemic administration of an otherwise innocuous prodrug. The interval following initial treatment with the antibody-enzyme fusion protein must be sufficient to enable tumor localization and serum clearance of the unbound activating enzyme, such that subsequently administered prodrug is only activated at the tumor site. To target HER2 overexpressing tumors, a disulfide-linked anti-HER2 Fv (dsFv) β-lactamase fusion protein was recently constructed for extracellular activation of cytotoxic doxorubicin from a cephalosporin-based prodrug conjugate [43]. Compared to the use of a cytostatic anti-HER2 antibody in a xenografted HER2 overexpressing tumor model, this anti-HER2 ADEPT strategy was shown to selectively amplify and render cytotoxic the *in vivo* antitumor activity of the antibody-doxorubicin combination, while sparing normal host tissues from doxorubicin toxicity.

Bispecific Anti-HER2 Antibodies

Another multifunctional antibody approach used against HER2 overexpressing human tumors is the bispecific antibody, designed to both bind the tumor target antigen and recruit host immune effector cells to enhance cell-mediated tumor lysis [44]. With these novel constructs, one of the Fv-containing bivalent antibody arms targets the HER2 receptor while the other Fv-containing arm targets a selected lymphocyte or monocyte/macrophage receptor (e.g. CD16 or CD64). In theory, this approach stimulates a cell-mediated

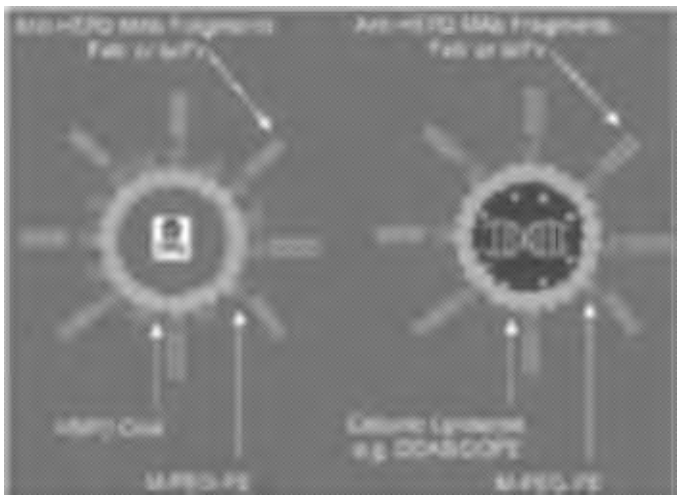


Fig. 1. Schematic illustration of anti-HER2 immunoliposomes (ILs) currently under preclinical evaluation and designed for intracellular tumor delivery of either drug (left panel) or DNA (right panel). In both these ILs designs the HER2 receptor-targeting antibody fragment is rhuMAbHER2 Fab' or scFv covalently linked to the termini of derivatized polyethylene glycol (maleimide-terminated PEG phosphatidylethanolamine, M-PEG-PE), which also provides steric stabilization contributing to their long circulation *in vivo*. Linkage typically yields 40–100 antibody fragments per liposome particle. A neutral lipid (HSPC:Chol) formulation is used for encapsulation of drugs such as doxorubicin, while a cationic lipid (e.g. DDAB/DOPE) formulation is used for nucleic acid complexes of anti-HER2 ILs.

host immune response to the tumor without introduction of foreign agents or toxins. Several different groups of academic and industrial investigators have pursued development of anti-HER2 bispecific antibodies and at least one of these agents has entered clinical trials [45–48]. However, more clinical analysis is required to determine the extent to which these bispecifics augment tumor infiltration by appropriate immune cells, and the resulting degree of tumor lysis that can be induced by these recruited host immune cells.

Anti-HER2 Immunoliposomes (ILs)

Encapsulated anticancer drug delivery, through the use of liposomes, has been used for more than two decades to reduce bystander toxicity to normal host tissues [49–53]. As well, advances in both liposomal technology and antibody engineering have produced more specific and efficient means for *in vivo* tumor delivery of liposomally encapsulated antitumor agents, with the resulting emergence of immunoliposome therapy [53]. Such immunoliposomes consist of monoclonal antibody fragments (Fab' or scFv) externally and covalently linked to the lipidic surface of long-circulating small diameter particles containing either antitumor drug (e.g. doxorubicin) or nucleic acid (e.g. DNA oligonucleotide or expression plasmid), whose therapeutic efficacy is enhanced by or dependent upon intracellular uptake. Figure 1 schematically illustrates anti-HER2 immunoliposomes currently under clinical development and designed for intracellular tumor delivery of either drug or DNA [53–60].

ANTI-HER2 ILS: ENHANCED ANTITUMOR ACTIVITY BY TARGETED INTRACELLULAR DELIVERY

Construction of Anti-HER2 ILs for Doxorubicin Delivery

Anti-HER2 ILs incorporate multiple design elements to optimize intracellular delivery of encapsulated agent to tumor cells; these elements are summarized in Table 1 and are reviewed in full detail elsewhere [53,56,60]. Antibody fragments (Fab' or scFv) are preferred to avoid accelerated clearance and immunogenicity; as well, the antibody of choice should demonstrate rapid internali-

zation when bound to the targeted tumor cell receptor. Of note in this regard, anti-HER2 antibodies with comparable receptor binding affinity do not all internalize with equal efficiency. Steric stabilization of the liposome surface enhances ILs circulation time as well as its selective tumor extravasation; this can be achieved by using small (~100 nm diameter), unilamellar liposomes consisting of hydrogenated soy phosphatidylcholine/cholesterol (HSPC/Chol, 3:2 molar ratio) and polyethylene glycol (PEG_{2000})-derivatized distearoylphosphatidylethanolamine (PEG-PE) at varying concentrations (0–12 mol%). After exploring several methods of securing antibody fragments to the surface of sterically stabilized liposomes (converting Ls to ILs), we have observed optimum *in vitro* and *in vivo* anti-HER2 ILs performance when $\geq$ 40 antibody fragments per lipid particle are covalently linked to maleimide-terminated PEG-PE, as schematically illustrated in Figure 1. Lastly, not all drugs are suitable for liposome encapsulation; doxorubicin (dox) is efficiently and actively encapsulated within either Ls or ILs, stably maintaining for days (*in vitro* or *in vivo*) ~ 10^4 molecules of drug per lipid particle. Given this formulation advantage and the resulting dramatic improvement it confers in both dox pharmacokinetics and total tumor dox uptake, the FDA has recently approved several versions of liposomal dox for treatment of human malignancies. While liposomal versions of other water soluble anticancer drugs are also under development, liposomal dox is the only FDA-approved liposomal antineoplastic agent currently available; thus, it was reasonable to establish proof-of-concept for anti-HER2 ILs therapy by encapsulating dox and comparing the properties of ILs-dox with those of the free drug and its liposomal version, Ls-dox.

In vitro Binding and Internalization of Anti-HER2 ILs

Anti-HER2 ILs binding and internalization were studied on cells in culture using a pH-sensitive fluorescent probe (1-hydroxypyrene-

Table 1
Component considerations for design of anti-HER2 ILs

Component	Considerations for optimal design
Target Antigen (p185HER2)	*Expression:* excess homogeneous antigen expression in target tissue.
	Function: internalizable receptor functionally linked to tumor growth.
	Proteolytic shedding: limited to minimize antigen binding in circulation.
Antibody (anti-HER2)	*Affinity:* high enough to assure binding at low ILs concentrations.
	Immunogenicity: avoid by humanization and use of Fab' or scFv fragments (no Fc).
	Internalization: monovalent or multivalent binding that triggers receptor endocytosis.
	Other biological activity: optional.
Linkage	*Stability:* covalently attach antibody to lipid anchor for *in vivo* stability.
	Attachment site: avoid steric hindrance that compromises antibody binding and/or internalization.
	Linker: nontoxic, non-immunogenic and does not affect drug loading or ILs stability.
Liposome	*Stability:* stable *in vivo* without leaking.
	Pharmacokinetics: long circulating.
	Tumor penetration: small ILs diameter to enhance extravasation and tumor penetration.
Encapsulated Agent	*Loading:* efficient for high drug capacity.
	Bystander toxicity: if desired, nontargeted cellular effects when released extracellularly.
	Interaction with target cell: most effective when delivered intracellularly.

3,6,8-trisulfonic acid; HPTS/pyranine), and results showed rapid ILs uptake into HER2 overexpressing SKBR3 cells via a receptor-mediated endocytotic pathway [55]. Total uptake of ILs into these cells reached 23,000 ILs/cell, while total uptake of non-targeted control liposomes was essentially undetectable. In contrast, total uptake of ILs into the low HER2 expressing MCF-7 cells was 700-fold lower than that in SKBR3 cells. Cell internalization was further studied by electron microscopy using anti-HER2 ILs containing colloidal gold particles [54]. Treated SKBR3 cells showed gold-loaded ILs at the cell surface and intracellularly in coated pits, coated vesicles, endosomes, multivesicular bodies and lysosomes, consistent with anti-HER2 ILs internalization occuring via a coated pit pathway.

In Vivo Pharmacokinetics and Tumor Cell Uptake by Anti-HER2 ILs

Studies in non-tumor bearing rats have demonstrated that following a single intravenous injection, dox-loaded anti-HER2 ILs exhibit long circulation *in vivo*, comparable to dox-containing sterically stabilized liposomes (Ls-dox) and unlike the < 5 min. plasma half-life of free dox. Measuring plasma levels of both the dox and Fab' components of the anti-HER2 ILs-dox, the terminal half-lives of both components were $\geq$ 10 h, demonstrating the prolonged *in vivo* stability of this ILs complex. As well, both Ls-dox and ILs-dox showed identical pharmacokinetics indicating lack of any significant effect on liposomal clearance due to the covalently linked anti-HER2 Fab'. Comparisons of tumor uptake by anti-HER2 ILs and Ls have been performed by two different techniques: quantitative radionuclide tumor uptake using ^{67}Ga-encapsulated liposomes, and microscopic tumor evaluation following injection with gold encapsulated liposomes. HER2 overexpressing human tumor xenografts in nude mice (either BT-474 or MCF7/HER2) were compared with low HER2 expressing tumor xenografts (MCF-7); and both overexpressing and low expressing tumor models were treated with either anti-HER2 ILs or non-targeted Ls containing label (^{67}Ga or gold). Surprisingly, anti-HER2 ILs showed identical quantitative uptake into the xenografted tumors as the non-targeted Ls (~ 8.5% of injected dose/g tissue at 48 h), verifying the efficient extravasation and passive tumor delivery potential of sterically stabilized liposomes. However, significant qualitative differences in tumor uptake between anti-HER2 ILs and Ls were apparent upon microscopic evaluation of tumors treated with the gold labeled liposomes. Both sets of controls (low HER2 expressing MCF-7 xenografts treated with anti-HER2 ILs and BT-474 xenografts treated with gold-labeled Ls) showed gold label concentrated either in extracellular stromal gaps and tumor interstitium or within tumor-resident macrophages. In contrast and as illustrated in Figure 2, anti-HER2 ILs treatment of BT-474 xenografts produced not only occasional interstitial tumor localization of the label but a marked predominance of intracellular and perinuclear particle localization within the HER2 overexpressing tumor cells in a labeling pattern that was well dispersed across the entire tumor mass. This important qualitative difference is thought to account for the improved therapeutic efficacy of anti-HER2 ILs-dox over Ls-dox, as observed in these same animal models.

Comparative *In Vivo* Efficacy of Anti-HER2 ILs-Dox

Four different HER2 overexpressing human tumor xenograft models (ranging from the MDA-MB-453 model expressing ~10^5 HER2 receptors/tumor cell, to the BT-474 and MCF7/HER2 models expressing ~10^6 receptors/tumor cell) and nearly three hundred tumor-bearing mice have been tested to compare the antitumor efficacy of anti-HER2 ILs-dox over free dox and Ls-dox, with each agent given at its maximum tolerated dose (MTD). By every *in vivo* parameter measured (tumor growth ratio, tumor growth delay, cures) anti-HER2 ILs-dox proved significantly superior to Ls-dox (p values ranging from < 0.0001 to 0.04); and, as expected, Ls-dox proved significantly superior over free dox. Moreover, only the anti-HER2 ILs-dox treatment resulted in histologically confirmed tumor cures

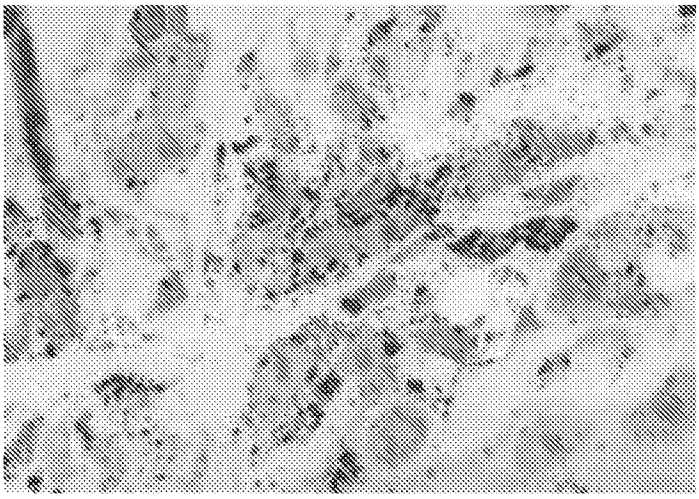


Fig. 2. Intracellular localization of colloidal gold-loaded anti-HER2 ILs within a HER2 overexpressing BT-474 tumor xenograft 48 h after intravenous injection. Sterically stabilized anti-HER2 ILs were prepared and labeled with entrapped colloidal gold as previously described (53), and injected (8 μmol total lipid per animal) into nude mice bearing established BT-474 tumor xenografts. Mice were sacrificed and tumors excised 48 h after injection, tissues were fixed, embedded and sectioned for silver-enhanced visualization of the gold-labeled particles. Control experiments (not shown) employed both low HER2 expressing MCF-7 xenografts treated identically and BT-474 xenografts treated with gold-labeled Ls (without anti-HER2 antibody fragment); both sets of controls showed gold label concentrated either in extracellular stromal gaps and tumor interstitium or within tumor-resident macrophages. In contrast and as shown above, anti-HER2 ILs produced not only occasional interstitial tumor localization of the label but a marked predominance of intracellular and perinuclear particle localization within the HER2 overexpressing tumor cells and in a labeling pattern dispersed across the entire tumor mass.

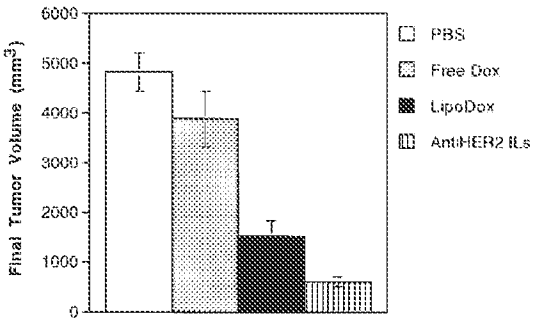
in these animal models. Figure 3A shows a representative experiment, with ILs-dox vs. Ls-dox vs. free dox treatment results compared in terms of final tumor volumes. As well, additional experiments using different preparations of anti-HER2 ILs-dox containing either rhuMAbHER2 Fab' or either of two different anti-HER2 scFv's (C6.5, F5) all showed comparable superiority over Ls-dox. Lastly, more recent studies have demonstrated that anti-HER2 ILs-dox is also superior to combination therapies consisting of free dox plus free rhuMAbHER2 (Herceptin©) or Ls-dox plus free rhuMAbHER2, as illustrated in Figure 3B. Collectively, these efficacy results in conjunction with the *in vivo* tumor uptake studies constitute proof-of-concept that anti-HER2 ILs can produce enhanced therapeutic efficacy *in vivo* as a result of reduced host toxicity and targeted intracellular tumor delivery. In collaboration with the NCI, who have recently accepted sponsorship for further clinical development of anti-HER2 ILs-dox (NSC-701315), this novel antibody-based therapeutic is currently undergoing scale-up GMP production with formal toxicology and initiation of Phase I clinical testing anticipated in the near future.

FUTURE DIRECTIONS FOR ANTI-HER2 ILs THERAPEUTICS

Targeted Delivery of other Anticancer Compounds

Although dox and other anthracyclines have been the most widely used cancer drugs in liposomes, a number of other cytotoxic drugs have been or are being encapsulated as part of preclinical and clinical development efforts (e.g. vinorelbine and other vinca alkaloids, cisplatin analogs, camptothecins, taxanes). Those that appear most promising and with intracellular mechanisms of action that may be further enhanced by intracellular delivery into HER2 over-

A



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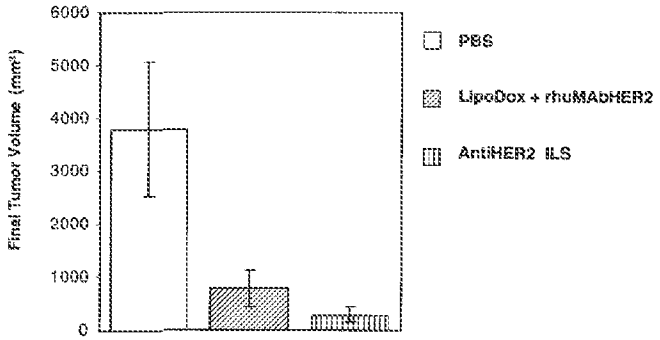

Fig. 3. Superior *in vivo* antitumor activity of doxorubicin (dox)-loaded anti-HER2 ILs against the HER2 overexpressing human breast tumor xenograft model, BT-474. Panel A. Superior efficacy of anti-HER2 ILs over free dox and sterically stabilized liposomal dox (LipoDox) when each agent was given intravenously once weekly x 3 at its maximum tolerated dose (MTD free dox total dose = 7.5 mg/kg; MTD LipoDox or anti-HER2 ILs total dox dose = 15 mg/kg), beginning 14 days after tumor innoculation. Control animals were treated with saline (PBS), final tumor volumes were measured 44 days after tumor innoculation, and data represent mean (8 mice per group) tumor volumes ± SEM. Panel B. Superior efficacy of anti-HER2 ILs over LipoDox given in combination with a tumoristatic dose of rhuMAbHER2. Experimental conditions were as described in Panel A except that LipoDox treated mice also received six, twice weekly intraperitoneal injections of rhuMAbHER2 (Herceptin©), at a dose (7.5 μg/injection) shown to significantly retard growth of this tumor model and with rhuMAbHER2 injections concurrent with the LipoDox treatments. In this experiment all treatments were initiated 16 days after tumor innoculation, and final tumor volumes (10 mice per group, mean values ± SEM) were measured 43 days after tumor innoculation.

expressing tumor cells are being evaluated for use in anti-HER2 ILs.

Delivery of Nucleic Acids by Cationic Anti-HER2 ILs

While cationic lipids have long been used to deliver RNA, DNA and oligonucleotides (ODN) into cells in culture [61–63], such cationic liposomes exhibit poor pharmacokinetic properties *in vivo* and lack potential for tumor-specific targeting. We have recently demonstrated that with some modifications to our current anti-HER2 ILs, targeted delivery of ODN and genes can be achieved both *in vitro* and *in vivo* [56–59]. For antisense ODN delivery, we have constructed anti-HER2 ILs in which cationic lipids have been included for efficient complexation with the nucleic acid [58]. The nonspecific reactivity of these lipids was reduced by addition of a PEG coat analogous to that used in sterically stabilized neutral Ls and ILs. These cationic anti-HER2 ILs-ODN complexes internalized into cultured SKBR3 cells, resulting in intracellular delivery

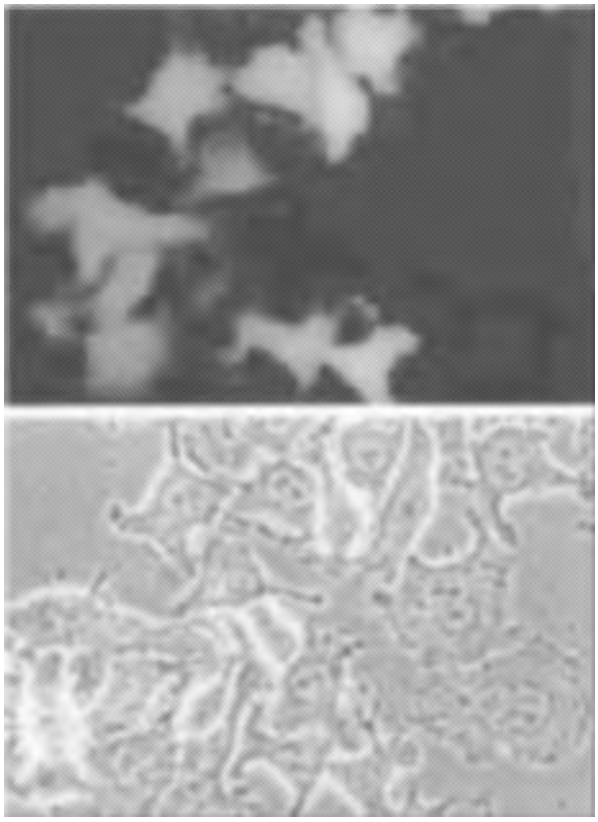

Fig. 4. Gene expression following transfection of HER2 overexpressing breast cancer cells with cationic anti-HER2 ILs bearing a GFP expression plasmid. Cultured SKBR3 cells were treated with anti-HER2 ILs complexed with condensed plasmid DNA encoding the reporter protein, green fluorescence protein (GFP). The expressed level of intracellular GFP is visualized by fluorescence microscopy (upper panel); and the relatively high level of cellular transfection and expression efficiency is apparent by comparison with the phase contrast image of the transfected monolayer cells (lower panel).

and nuclear accumulation of the labeled ODN. Delivery of antisense ODN directed against bcl-2 via cationic anti-HER2 ILs produced a significant reduction in bcl-2 expression *in vitro*, greater than that associated with non-targeted cationic liposomes or free ODN. More recently we have constructed similar cationic anti-HER2 ILs for delivery of ribozymes and genes that have produced similar intracellular responses specific to HER2 overexpressing tumor cells [59,64,65]. *In vitro*, these constructs efficiently transfect HER2 overexpressing cells and show minimal transfection into low HER2 expressing cells. Figure 4 illustrates cellular expression following transfection of HER2 overexpressing breast tumor cells with cationic anti-HER2 ILs bearing a green fluorescence protein (GFP) expressing reporter gene.

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REFERENCES

[1] Noonberg SB, Benz CC. Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents. Drugs 2000; in press.

[2] Hynes NE, Stern DF. The biology of erbB-2/neu/HER-2 and its role in cancer. Biochim Biophys Acta 1994; 1198:165–184.

[3] Benz CC, Tripathy T. ErbB2 overexpression in breast cancer: biology and clinical translation. Women and Cancer 2000; (in press).

[4] Tripathy D, Benz CC. Activated oncogenes and putative tumor suppressor genes involved in human breast cancers. In: Benz CC, Liu E, eds. *Oncogenes and Tumor Suppressor Genes in Human Malignancies*. Boston, Kluwer Academic Publishers 1993; 15–60.

[5] Tripathy D, Benz C. Growth factors and their receptors. In: Shapiro CL, Henderson IC, eds. *New Directions in Breast Cancer Research and Therapeutics*. Hematology/Oncology Clin N Am 1994; vol 8:29–50.

[6] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of HER2/neu oncogene. Science 1987; 235:177–182.

[7] Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER2/neu proto-oncogene in human breast and ovarian cancer. Science 1989; 244:707–712.

[8] Berchuck A, Kamel A, Whitaker R, et al. Overexpression of HER2/neu is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res 1990; 50:4087–4091.

[9] Berchuch A, Rodriguez G, Kinney RB, et al. Overexpression of HER2/neu in endometrial cancer is associated with advanced stage disease. Am J Obstet Gynecol 1991; 164:5–21.

[10] Kern JA, Schwartz DA, Nordberg JE, et al. P185 neu expression in human lung adenocarcinomas predict shortened survival. Cancer Res 1990; 50:5184–5191.

[11] Yokota J, Yamamoto T, Miyajima N, et al. Genetic alterations of the c-erbB-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of v-erbA homologue. Oncogene 1988; 2:283–287.

[12] Park JB, Rhim JS, Park SC, Kimm SW, Kraus MH. Amplification, overexpression, and rearrangement of the erbB-2 proto-oncogene in primary human stomach carcinomas. Cancer Res 1989; 49:6605–6609.

[13] Lei A, Appert HE, Nakata B, Domenico DR, Kim K, Howard JM. Overexpression of HER2/neu oncogene in pancreatic cancer correlates with shortened survival. Int J Pancreatol 1995; 17:15–21.

[14] Zhau HE, Zhang X, Von Eschenbach AC, et al. Amplification and expression of the c-erbB-2/neu proto-oncogene in human bladder cancer. Mol Carcinog 1990; 3:254–257.

[15] Zhau HE, Wan DS, Zhou J, Miller GJ, von Eschenbach AC. Expression of c-erbB-2/neu proto-oncogene in human prostatic cancer tissues and cell lines. Mol Carcinog 1990; 3:254–257.

[16] Press MF, Cordon-Cardo C, Slamon DJ. Expression of the HER2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene 1990; 5:953–962.

[17] Niehans GA, Singleton TP, Dylkoski D, Kiang DT. Stability of HER-2/neu expression over time and at multiple metastatic sites. J Natl Can Inst 1993; 85:1230–1235.

[18] Lewis GD, Figari I, Fendly B, et al. Differential responses of human tumor cell lines to anti-p185 HER2 monoclonal antibodies. Cancer Immunol Immunother 1993; 37:255–263.

[19] Lotti LV, Dilazzaro C, Zompetta C, Frati L, Torrisi MR. Surface distribution and internalization of erbB-2 proteins. Exp Cell Res 1992; 202:274–280.

[20] Riezman H, Woodman PG, van Meer G, Marsh M. Molecular mechanism of endocytosis. Cell 1997; 731–738.

[21] Trowbridge IS, Collawn JF, Hopkins CR. Signal-dependent membrane protein trafficking in the endocytic pathway. Annu Rev Cell Biol 1993; 9:129–161.

[22] Sunada H, Magun BE, Mendelsohn J, MacLeod CL. Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. Proc Natl Acad Sci 1986; 83:3825–3834.

[23] Sarup JC, Johnson RM, King KL, et al. Characterization of an anti-p185 HER2 monoclonal antibody that stimulated receptor function and inhibits tumor cell growth. Growth Regul 1991; 37:72–82.

[24] Scott GK, Dodson JM, Montgomery P, et al. $p185^{HER2}$ signal transduction in breast cancer cells. J Biol Chem 1991; 266:14300–14305.

[25] Hurwitz E, Stancovski I, Sela M, Yarden Y. Suppression and promotion of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake. Proc Natl Acad Sci 1995; 92:3353–3357.

[26] Sznol M, Holmlund J. Antigen-specific agents in development. Semin Oncol 1997; 24:173–186.

[27] Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. $p185^{HER2}$ monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol 1989; 9:1165–1172.

[28] Hancock MC, Langton BC, Chan T, et al. A monoclonal antibody against the c-*erb*B2 protein enhances the cytotoxicity of cis-diamminedichloroplatinum against human breast and ovarian tumor cell lines. Cancer Res 1991; 51:4575–4580.

[29] Carter P, Presta L, Gorman CM, et al. Humanization of an anti-$p185^{HER2}$ antibody for human cancer therapy. Proc Natl Acad Sci USA 1992; 89:4285–4289.

[30] Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/*neu*-overexpressing metastatic breast cancer. J Clin Oncol 1996; 14:737–744.

[31] Baselga J, Norton L, Albanell J, Kim Y-M, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. Cancer Res 1998; 58:2825–2831.

[32] Cobleigh MA, Vogel CL, Tripathy D, et al. Efficacy and safety of Herceptin (humanized anti-HER2 antibody) as a single agent in 222 women with HER2 overexpression who relapsed following chemotherapy for metastatic breast cancer. Proc Amer Soc Clin Oncol 1998; 17:376.

[33] Slamon D, Leyland-Jones B, Shak S, et al. Addition of Herceptin (humanized anti-HER2 antibody) to first line chemotherapy for HER2 overexpressing metastatic breast cancer (HER2+/MBC) markedly increases anticancer activity: a randomized multinational controlled phase III trial. Proc Amer Soc Clin Oncol 1998; 17:377.

[34] Trastuzumab/Herceptin©, package insert (FDA-approved Sept. 25, 1998), USPI.

[35] Fitzgerald D. Why toxins! Seminars in cancer biology 1996; 7:87–95.

[36] Wels W, Harwerth IM, Mueller M, Groner B, Hynes NE. Selective inhibition of tumor cell growth by a recombinant single-chain antibody-toxin specific for the erbB2 receptor. Cancer Res 1992; 52:6310–6317.

[37] Batra JK, Kasprzyk PG, Bird RE, Pastan I, King CR. Recombinant anti-erbB2 immunotoxins containing *Pseudomonas* exotoxin. Proc Natl Acad Sci USA 1992; 89:5867–5871.

[38] Fischer PH, Bird RE, Kasprzyk PG, et al. *In vitro* and *in vivo* activity of a recombinant toxin OLX-209, which targets the erbB2 oncoprotein. Adv Enzymol 1994; 34:119–128.

[39] Kasprzyk PG, Sullivan TL, Hunt JD, et al. Activity of anti-erbB2 recombinant toxin OLX-209 on lung cancer cell lines in the absence of erbB2 gene amplification. Clin Cancer Res 1996; 2(1):75–80.

[40] King CR, Kasprzyk PG, Fischer PH, et al. Preclinical testing of an anti-erbB2 recombinant toxin. Breast Cancer Res Treat 1996; 38(1):19–25.

[41] Maurer-Gebhard M, Schmidt M, Azemar M, et al. Systemic treatment with a recombinant erbB2 receptor-specific tumor toxin efficiently reduces pulmonary metastases in mice injected with genetically modified carcinoma cells. Cancer Res 1998; 58(12):2661–2666.

[42] Rosenblum MB, Shawver LK, Marks JW, et al. Recombinant immunotoxins directed against the c-erbB2/HER2/neu oncogene product: *in vivo* cytotoxicity, pharmacokinetics, and *in vivo* efficacy studies in xenograft models. Clin Cancer Res 1999; 5:865–874.

[43] Rodrigues ML, Presta LG, Kotts CE, et al. Development of a humanized disulfide stabilized anti-p185 HER2 Fv-β-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug. Cancer Res 1995; 55:63–70.

[44] Curnow RT. Clinical experience with CD64-directed immunotherapy: an overview. Cancer Immunol 1997; 45:210–215.

[45] Shalaby, MR, Shepard HM., Presta L, et al. Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. J Exp Med 1992; 175:217–225.

[46] Weiner LM, Holmes M, Adams GP, LaCreta F, Watts P, Garcia de Palazzo, I. A human tumor xenograft model of therapy with a bispecific monoclonal antibody targeting c-*erb*B-2 and CD16. Cancer Res 1993; 53:94–100.

[47] Valone FH, Kaufman PA, Guyre PM, et al. Phase II/Ib trial of bispecific antibody MDX-210 in patients with advanced breast or ovarian cancer that overexpress the proto-oncogene HER2/neu. J Clin Invest 1995; 13:2281–2292.

[48] Keler T, Graziano RF, Mandal A, et al. Bispecific antibody-dependent cellular cytotoxicity of HER2/neu overexpressing tumor cells by Fc gamma receptor type 1 expressing effector cells. Cancer Res 1997; 57(18):4008–4014.

[49] Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 1988; 85:6949–6953.

[50] Papahadjopoulos D, Allen T, Gabizon A, et al. Sterically stabilized liposomes: improvements in pharmacokinetics and anti-tumor therapeutic efficacy. Proc Natl Acad Sci USA 1991; 88:11460–11464.

[51] Klibanov AL, Huan L. Long circulating liposomes: development and perspectives. J Liposome Res 1992; 2:321–332.

[52] Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta 1992; 1113: 171–199.

[53] Park JW, Hong K, Kirpotin DB, Papahadjopoulos D, Benz CC. Immunoliposomes for cancer treatment. Adv Pharmacol 1997; 40:399–435.

[54] Park JW, Hong K, Carter P, et al. Development of anti-p185 HER2 immunoliposomes for cancer therapy. Proc Natl Acad Sci USA 1995; 92:1327–1331.

[55] Kirpotin D, Park JW, Hong K, et al. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro*. Biochemistry 1997; 36:66–75.

[56] Park JW, Hong K, Kirpotin DB, Meyer O, Papahadjopoulos D, Benz CC. Anti-HER2 immunoliposomes for targeted therapy of human tumors. Cancer Lett 1997; 118:153–160.

[57] Hong K, Zheng WW, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient *in vivo* gene delivery. FEBS Lett 1997; 400:233–237.

[58] Meyer O, Kirpotin D, Hong K, et al. Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides. J Biol Chem 1998, 273:15621–15627.

[59] Kirpotin DB, Park JW, Hong K, et al. Targeting of sterically stabilized liposomes to cancers overexpressing HER2/neu proto-oncogene. In: Lasic DD, Papahadjopoulos D, eds. *Medical Applications of Lipo-*

somes. Amsterdam, Elsevier Science B.V. 1998; 325–345.

[60] Park JW, Kirpotin D, Hong K, et al. Anti-HER2 immunoliposomes for targeted drug delivery. Med Chem Res 1998; 8:383–391.

[61] Fiegner PL, Gadek TR, Holm M, et al. Lipofection: a highly efficient, lipid mediated DNA transfection procedure. Proc Natl Acad Sci USA 1987; 84:7413–7417

[62] Malone RW, Felgner PL, Verma IM. Cationic liposome-mediated RNA transfection. Proc Natl Acad Sci USA 1989; 86:6077–6081.

[63] Zelphati O, Szoka FC. Cationic liposomes as an oligonucleotide carrier: mechanism of action. J Liposome Res 1997; 7:31–49.

[64] Park JW, Hong K, Zheng W, Benz CC, Papahadjopoulos D. Development of liposome- and anti-HER2 immunoliposome-plasmid complexes for gene delivery. Proc Amer Assoc Cancer Res 1997; 38:342.

[65] Ren S, Scott G, Zheng W, et al. Anti-HER2 immunoliposomesribozyme constructs mediate targeted gene transfer and HER2 downmodulation. Proc Amer Assoc Cancer Res 1999; 40:418.

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Confirmation Number:	5137
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
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First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Gollamudi S KISHORE
Attorney Docket Number	01208-0007-01US

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	Filing Date	2017-11-10
	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Gollamudi S KISHORE
	Attorney Docket Number	01208-0007-01US

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	1	BOUCHÉ O, et al. "Randomized Multicenter Phase II Trial of a Biweekly Regimen of Fluorouracil and Leucovorin (LV5FU2), LV5FU2 Plus Cisplatin, or LV5FU2 Plus Irinotecan in Patients With Previously Untreated Metastatic Gastric Cancer: A Fédération Francophone De Cancérologie Digestive Group Study—FFCD 9803," J Clin Oncol. 22 (21):4319-28 (2004).	
	2	CHIANG, N-J, et al., "Development of Nanoliposomal Irinotecan (nal-IRI, MM-398, PEP02) in the Management of Metastatic Pancreatic Cancer," Expert Opin Pharmacother. 17(10):1413-20 (2016).	
	3	EP3337478: EPO Notice of Sandoz AG Opposition dated May 6, 2021, 5 pages.	
	4	EP3337478: Sandoz AG Opposition dated May 6, 2021, 22 pages.	
	5	EP3337478: Sandoz AG Opposition dated May 6, 2021, D1 (History of Changes for Study NCT02551991, retrieved from ClinicalTrials.gov archive on May 3, 2021, 4 pages).	
	6	EP3337478: Sandoz AG Opposition dated May 6, 2021, D2 (Abstract O-0003. VON HOFF D, et al., "NAPOLI 1: Randomized Phase 3 Study of MM-398 (nal-IRI), With or Without 5-Fluorouracil and Leucovorin, Versus 5-Fluorouracil and Leucovorin, in Metastatic Pancreatic Cancer Progressed on or Following Gemcitabine-Based Therapy." Ann Oncol. 25(Suppl 2):ii105 (2014)).	
	7	EP3337478: Sandoz AG Opposition dated May 6, 2021, D3 (MARSH R, et al., "Pancreatic Cancer and FOLFIRINOX: A New Era and New Questions," Cancer Med. 4(6):853-63 (2015)).	
	8	EP3337478: Sandoz AG Opposition dated May 6, 2021, D4 (ONIVYDE [MM-398] package insert, revision October 22, 2015, 18 pages).	
	9	EP3337478: Sandoz AG Opposition dated May 6, 2021, D5 (CARNEVALE J and KO A, "MM-398 (Nanoliposomal Irinotecan): Emergence of a Novel Therapy for the Treatment of Advanced Pancreatic Cancer," Future Oncol. 12 (4):453-64 (2016). Epub 2015).	
	10	EP3337478: Sandoz AG Opposition dated May 6, 2021, D6 (DEAN A, et al., Abstract TPS482. "A Randomized, Open-Label Phase II Study of Nanoliposomal Irinotecan (nal-IRI)-Containing Regimens Versus nab-Paclitaxel Plus Gemcitabine in Patients With Previously Untreated Metastatic Pancreatic Adenocarcinoma (mPAC)," J Clin Oncol. 34 (4_Suppl):tps482 (2016), DOI: 10.1200/jco.2016.34.4_suppl.tps482, 5 printed pages).	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15809815
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	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Gollamudi S KISHORE
	Attorney Docket Number	01208-0007-01US

	11	EP3337478: Sandoz AG Opposition dated May 6, 2021, D7 (ZHANG H, "Onivyde for the Therapy of Multiple Solid Tumors," Onco Targets Ther. 9:3001-3007 (2016)).
	12	EP3337478: Sandoz AG Opposition dated May 6, 2021, D8 (GADDY D, et al., "Abstract 4830: Preclinical Anti-tumor Activity of Nanoliposomal Irinotecan (Nal-IRI, MM-398) + 5-FU + Oxaliplatin in Pancreatic Cancer." In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Cancer Res. 76(14 Suppl):Abstract nr 4830 (2016), 4 printed pages).
	13	EP3337478: Sandoz AG Opposition dated May 6, 2021, D9 (PRIYAMBADA P, et al., "Nanotechnology-Based Combinational Drug Delivery: An Emerging Approach for Cancer Therapy," Drug Discov Today. 17(17-18):1044-52 (2012)).
	14	EP3337478: EPO Notice of Generics [UK] Limited Opposition dated May 12, 2021, 5 pages.
	15	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, 9 pages.
	16	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, D10 (CONROY T, et al., "FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer," N Engl J Med. 364(19):1817-25 (2011)).
	17	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, D11 (GOURGOU-BOURGADE S, et al., "Impact of FOLFIRINOX Compared With Gemcitabine on Quality of Life With Metastatic Pancreatic Cancer: Results From the PRODIGE 4/ACCORD 11 Randomized Trial," J Clin Oncol. 31(1):23-9 (2013). Epub 2012.).
	18	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, D12 (KO A, et al., "A Multinational Phase 2 Study of Nanoliposomal Irinotecan Sucrosofate (PEP02, MM-398) for Patients with Gemcitabine-Refractory Metastatic Pancreatic Cancer," Br J Cancer. 109(4):920-5 (2013)).
	19	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, D13 (HANN B, et. al., Abstract 5648. "Lipidic Nanoparticle CPT-11 in a Bioluminescent Orthotopic Pancreas Cancer Model," Cancer Res. 67(9 Suppl):5648 (2007), 4 printed pages).
	20	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, D14 (CHANG T, et al., "Phase I Study of Nanoliposomal Irinotecan (PEP02) in Advanced Solid Tumor Patients," Cancer Chemother Pharmacol. 75(3):579-86 (2015)).
	21	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, D15 (CHEN L, et al., "Phase I Study of Liposome Irinotecan (PEP02) in Combination with Weekly Infusion of 5-FU/LV in Advanced Solid Tumors," J Clin Oncol., 2010 ASCO Annual Meeting Abstracts, 28(15_suppl) (May 20 Suppl):e13024 (2010), 4 printed pages).

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	22	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, D16 (Pubmed abstract retrieved on May 6, 2021 for MAHASETH H, et al., "Modified FOLFIRINOX Regimen With Improved Safety and Maintained Efficacy in Pancreatic Adenocarcinoma," Pancreas. 42(8):1311-5 (2013), 2 printed pages).
	23	EP3337478: Generics [UK] Limited Opposition dated May 12, 2021, D17 (DRUMMOND D, et al., "Development of a Highly Active Nanoliposomal Irinotecan Using a Novel Intraliposomal Stabilization Strategy," Cancer Res. 66 (6):3271-77 (2006)).
	24	KOIZUMI W, at al., "Phase I/II Study of Bi-weekly Irinotecan plus Cisplatin in the Treatment of Advanced Gastric Cancer," Anticancer Res. 25(2B):1257-62 (2005).
	25	LORUSSO P, et al., "Phase I Study of the Safety, Pharmacokinetics (PK), and Pharmacodynamics (PD) of the Poly (ADP-ribose) Polymerase (PARP) Inhibitor Veliparib (ABT-888; V) in Combination with Irinotecan (CPT-11; Ir) in Patients (pts) with Advanced Solid Tumors," Journal of Clinical Oncology 29.15_suppl: Abstract 3000 (2011), 3 printed pages.
	26	MORISE M, et al., "Low-dose Irinotecan as a Second-line Chemotherapy for Recurrent Small Cell Lung Cancer," Jpn J Clin Oncol. 44(9):846-51 (2014).
	27	WAINBERG Z, et al., "First-line Liposomal Irinotecan With Oxaliplatin, 5-Fluorouracil and Leucovorin (NALIRIFOX) in Pancreatic Ductal Adenocarcinoma: A Phase I/II Study," Eur J Cancer. 151:14-24 (2021).

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ORIGINAL REPORT

Randomized Multicenter Phase II Trial of a Biweekly Regimen of Fluorouracil and Leucovorin (LV5FU2), LV5FU2 Plus Cisplatin, or LV5FU2 Plus Irinotecan in Patients With Previously Untreated Metastatic Gastric Cancer: A Fédération Francophone de Cancérologie Digestive Group Study—FFCD 9803

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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
To determine the efficacy and safety of a biweekly regimen of leucovorin (LV) plus fluorouracil (FU) alone or in combination with cisplatin or irinotecan in patients with previously untreated metastatic gastric adenocarcinoma and to select the best arm for a phase III study.

Patients and Methods
One hundred thirty-six patients (two were ineligible) were enrolled onto the randomized multicenter phase II trial. Patients received LV 200 mg/m^2 (2-hour infusion) followed by FU 400 mg/m^2 (bolus) and FU 600 mg/m^2 (22-hour continuous infusion) on days 1 and 2 every 14 days (LV5FU2; arm A), LV5FU2 plus cisplatin 50 mg/m^2 (1-hour infusion) on day 1 or 2 (arm B), or LV5FU2 plus irinotecan 180 mg/m^2 (2-hour infusion) on day 1 (arm C).

Results
The overall response rates, which were confirmed by an independent expert panel, were 13% (95% CI, 3.4% to 23.3%), 27% (95% CI, 14.1% to 40.4%), and 40% (95% CI, 25.7% to 54.3%) for arms A, B, and C, respectively. Median progression-free survival and overall survival times were 3.2 months (95% CI, 1.8 to 4.6 months) and 6.8 months (95% CI, 2.6 to 11.1 months) with LV5FU2, respectively; 4.9 months (95% CI, 3.5 to 6.3 months) and 9.5 months (95% CI, 6.9 to 12.2 months) with LV5FU2-cisplatin, respectively; and 6.9 months (95% CI, 5.5 to 8.3 months) and 11.3 months (95% CI, 9.3 to 13.3 months) with LV5FU2-irinotecan, respectively.

Conclusion
Of the three regimens tested, the combination of LV5FU2-irinotecan is the most promising and will be assessed in a phase III trial.

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INTRODUCTION

Although the incidence of gastric cancer has decreased in most western countries, it remains a significant problem in global health terms and is the second most common cause of cancer mortality worldwide.[1] Surgery is the only potentially curative treatment for localized gastric cancer, but most cases present at an advanced stage. The prognosis for the disease is extremely poor, with overall 5-year survival rates ranging from 10% to 15% in the United States and most developed countries.[2]

The efficacy of chemotherapy with palliative intent compared with supportive care alone is now widely accepted.[3] Studies showed the benefit of combination regimens, such as fluorouracil (FU), doxorubicin, and methotrexate (FAMTX)[4,5] or etoposide, leucovorin (LV), and FU (ELF),[6] over best supportive care.[7-9] Other combination regimens investigated include epirubicin, cisplatin, and infusional FU (ECF) and 5-day infusional FU plus cisplatin (FUP).[4,6] The survival advantage is small, however, and no internationally accepted standard regimen has emerged.[10]

ECF is one standard regimen and is associated with median survival times of around 9 months.[11,12] Infusional FU plus cisplatin is another standard treatment that is active, but it failed to demonstrate its superiority over FU monotherapy or other combination regimens in three randomized studies.[6,13-15] Although the current regimens yield overall response rates (ORR) of up to 51%,[15] the median survival time in patients with advanced disease remains consistently below 10 months.[6,11,12]

An important issue in patients with gastric cancer is toxicity. The elderly patient population cannot tolerate aggressive combination chemotherapy. The anthracycline-containing regimens can be particularly toxic.[16]

FU is one of the most effective and widely used drugs in the treatment of advanced gastric cancer, and it forms part of all the current reference regimens. FU monotherapy, a standard treatment in Japan, is associated with a response rate of approximately 20%, manageable toxicity,[17] and overall survival (OS) times of between 5 and approximately 7 months in phase III randomized studies.[14,15,18] The modulation of FU by LV has generally enhanced antitumor efficacy (ORR, 22% to 48%) and produced some complete responses (5% to 9%).[19-22] The biweekly FU and LV regimen (LV5FU2), which is popular in Europe,[20,23] combined with low-dose cisplatin was less toxic than FUP in a retrospective study,[24] and therefore, LV5FU2 was chosen as the reference FU regimen in this study.

There is a clear need for more convenient and active new agents and regimens. Irinotecan is a new cytotoxic agent with promising activity in combination with FU in gastrointestinal cancers.[25-27] Irinotecan monotherapy is active in patients with gastric cancer, with response rates in phase II trials of 14% to 23%.[28-31] The drug is also active when administered with FU-LV, a combination that yields response rates of 21% to 29% and OS times of 6.4 to 7.6 months.[27,32,33] Irinotecan plus cisplatin is another active combination with response rates of 27% to 58% and an OS time of 9.0 months.[34-36]

Therefore, a multicenter randomized phase II study was conducted to compare LV5FU2 administered alone or in combination with cisplatin or irinotecan in patients with previously untreated metastatic gastric or cardial adenocarcinoma. The aim of the study was to select the best regimen for comparison with a reference treatment in a future phase III trial. The end points were ORR, progression-free survival (PFS), OS, safety, duration of hospital stay, and quality of life (QOL).

PATIENTS AND METHODS

Patients

The study conformed to the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. The protocol was reviewed and approved by the Ethics Review Committee of Champagne Ardennes (Reims, France). All patients provided written informed consent before inclusion in the trial. Eligible patients had histologically proven metastatic gastric or cardial adenocarcinoma without linitis, at least one measurable metastatic lesion located outside a previously irradiated area and measuring more than 15 mm in diameter, no symptomatic brain metastases, an age between 18 and 75 years, and a WHO performance status ≤ 2 with a life expectancy of more than 2 months. Adjuvant chemotherapy without cisplatin or irinotecan was allowed if completed at least 6 months before randomization. Prior radiotherapy was allowed if completed more than 4 weeks before randomization. All patients had adequate hematologic (neutrophils $\geq 1.5 \times 10^9$/L and platelets $\geq 100 \times 10^9$/L), hepatic (bilirubin ≤ 25 μmol/L and AST and ALT $\leq 5 \times$ the upper normal limit), renal (creatinine ≤ 135 μmol/L and no contraindication to hyperhydration), and cardiac function. The main exclusion criteria were chronic diarrhea, prior enteropathy, or extensive intestinal resection.

Study Design and Randomization

The study was an open-label, multicenter, phase II, randomized trial with three treatment arms. After obtaining informed consent, eligible patients were registered at the Fédération Francophone de Cancérologie Digestive center and randomized with stratification by institution, tumor site (cardia *v* other localization), prior adjuvant chemotherapy (yes *v* no), and WHO performance status (0-1 *v* 2).

Chemotherapy Administration and Dose Adjustments

Patients assigned to the LV5FU2 arm (arm A) received LV 200 mg/m^2 intravenous (IV) over 2 hours followed by FU 400 mg/m^2 IV bolus then FU 600 mg/m^2 continuous infusion over 22 hours on days 1 and 2, repeated every 14 days (one cycle = 15 days). No systematic prophylactic premedication was administered. Patients assigned to the LV5FU2-cisplatin arm (arm B) received cisplatin 50 mg/m^2 IV over 1 hour on day 1 or 2 with LV5FU2 (one cycle = 15 days). Prophylactic medication consisted of IV antiemetics (setrons) and methylprednisolone 120 mg 10 minutes before cisplatin administration, hydration (1 L over 3 hours before and after cisplatin), oral antiemetics, and corticosteroids from days 2 to 5. Patients assigned to the LV5FU2-irinotecan arm (arm C) received irinotecan 180 mg/m^2 IV over 90 minutes on day 1 with LV5FU2 and no systematic prophylactic premedication (one cycle = 15 days).

Treatment was continued for at least four cycles or until disease progression, unacceptable toxicity, patient refusal, or physician decision. In the event of toxicity (WHO), the following dose reductions and treatment delays were planned. In cases of insufficient hematologic function (neutrophil count $< 1.5 \times 10^9$/L and platelet count $< 100 \times 10^9$/L) on day 14 of any cycle, treatment was delayed for up to 14 days. If recovery did not occur at this point, the treatment was discontinued. Any FU dose

reductions were only applied to the continuous infusion. For grade 3 to 4 gastrointestinal toxicities, thrombocytopenia, and neutropenia, there were 25% FU, cisplatin, and irinotecan dose reductions. For grade 2 or greater cardiotoxicity, FU treatment was discontinued. Cisplatin administration was delayed if creatinine levels were more than 135 μmol/L, and irinotecan administration was delayed if bilirubin levels were more than 25 μmol/L. Patients showing a complete response received treatment for up to 1 year.

Study Evaluations

In the 4 weeks preceding treatment, patients underwent a chest x-ray and a computed tomography scan of the abdomen and of all measurable and assessable sites. In the week preceding treatment, patients underwent a complete medical history evaluation, a physical examination, a QOL evaluation, and an ECG. Baseline biologic analyses (blood cell count, serum creatinine, bilirubin, AST, ALT, lactate dehydrogenase, and alkaline phosphatase) were measured at baseline and before each cycle of chemotherapy. QOL evaluations were carried out every 2 months.

All adverse events were graded using the WHO toxicity criteria.[37] Planned tumor evaluations were carried out every four cycles during therapy with the appropriate clinical and radiologic examinations and confirmation of responses by further radiologic examinations within 4 weeks. All objective tumor responses and cases of disease stabilization were reviewed retrospectively by an external expert committee. PFS was calculated from the date of randomization to either the date of first progression, the date of the last assessment in the absence of progression if the patient was alive, the date of death from any cause, or the date of last contact. In patients with subsequent complete surgical resection, PFS was measured from the time of randomization to the date of documentation of progression after surgery. OS was measured from the date of randomization until death from any cause.

QOL

Patients were requested to complete the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire C30 (QLQ-C30) before randomization and every 2 months thereafter.[38] Completed questionnaires were scored according to guidelines provided by the European Organization for Research and Treatment of Cancer.[39] The questionnaire comprises a global QOL scale, five functional scales (physical, role, cognitive, emotional, and social), and nine symptom scales (fatigue, pain, nausea and vomiting, constipation, diarrhea, sleep, dyspnea, appetite, and financial). The functional and global scores range from 0 (worst) to 100 (best), and the symptom scores range from 0 (best) to 100 (worst). The reliability and validity of this measure has been reported elsewhere.[40,41]

Statistical Analysis

The primary end point was ORR. Secondary end points were PFS, OS, safety, duration of hospitalization, and QOL. The expected number of patients for this study was calculated according to an Ensign-Minimax optimal three-stage design.[42] The ORR according to WHO criteria was used with the following hypotheses and estimations for the stopping rules in each arm: stop if ORR is less than 20% or more than 40% with alpha and beta levels of 0.05 and 0.10, respectively. An interim analysis was carried out after the first nine assessable patients had been recruited in each arm. If at least one objective response was observed, 16 additional patients were included in the second stage (total = 25 patients). For the second interim analysis, if more than five objective responses and less than 14 objective responses were observed, 20 additional patients were included in the third and last stage (total = 45 patients). If at least 14 responses were observed in a treatment arm, a phase III study was to be considered against the reference treatment (ECF or simplified ECF regimen yet to be determined). A sample of 135 patients was necessary with 45 patients per arm. PFS and OS were updated until October 1, 2002. Statistical comparisons were not planned in this selection study, with small numbers of patients in each arm. The criterion for choosing the best arm for a phase III study was at least 14 objective responses according to the external expert committee.

The QLQ-C30 scores were described as a mean, standard deviation, median, and range at the start of the study and at each 2-month follow-up visit; the mean of available global health scores was graphically reported at each follow-up. The missing data were described as a percentage of the calculated score among patients with follow-up. Prestudy scores were compared between treatment arms using analysis of variance and a Bonferroni test to adjust for multiple comparisons. During the first three follow-ups, the longitudinal change of QLQ-C30 scores was analyzed using a mixed model analysis of variance for repeated measurements[43] to study a global time effect whatever the treatment and to calculate differences in mean QOL scores between treatment arms whatever the follow-up (contrast analysis).

RESULTS

Patient Characteristics

One hundred thirty-six patients were enrolled between January 1999 and October 2001 in 41 centers in France. Two patients were considered ineligible; one had a lymphoma and the other had no metastatic disease. No arm was closed after the two interim analyses. Thus, the analyses were carried out on an intent-to-treat (ITT) basis with the remaining 134 enrolled patients. All eligible patients received treatment allocated by randomization. The patient characteristics, which are listed in Table 1, were similar between the three arms except for the number of patients with weight loss more than 10%; this was higher in arm C than in the other two treatment arms ($P = .05$). The median age of all patients was 65 years (range, 37 to 76 years). The most frequent metastatic site was the liver (79% of all patients), and half of the patients had undergone prior curative or palliative surgery for their primary tumor.

Response Rate

All of the 134 eligible patients were assessed for response. The response rate data per independent review on ITT basis are listed in Table 2. Ten patients (7%) were not evaluated for response review by the external expert committee because of the loss of computed tomography images. Three additional patients (2%) received insufficient treatment (fewer than four cycles) because of early toxicity (n = 2) or toxic death (n = 1). Early deaths (6%) related to disease progression occurred in three, two, and three patients in arms A, B, and C, respectively. The expert-assessed per protocol ORRs (eligible patients receiving at least four

Table 1. Patient Characteristics

Characteristic	% of Patients		
	LV5FU2 (n = 45)	LV5FU2-Cisplatin (n = 44)	LV5FU2-Irinotecan (n = 45)
Sex			
Male	82	80	84
Female	18	20	16
Age, years			
Median	64	64	65
Range	45-75	43-76	37-76
WHO performance status			
0 or 1	73	75	78
2	27	25	22
Primary tumor location			
Cardia	29	30	33
Gastric	71	70	67
Prior surgery	51	50	51
Curative	25	34	27
Palliative	25	16	22
Unknown	2	0	2
Prior radiotherapy	0	0	2
Prior chemotherapy	0	2	0
Histology of adenocarcinoma			
Well differentiated	69	61	69
Poorly differentiated	22	32	27
Signet-ring cell	2	2	0
Unknown	7	5	4
Metastatic sites			
Liver	78	84	76
Lymph nodes	58	52	62
Peritoneum	22	14	20
Lung	18	11	16
Bone	4	5	5
Others	9	14	9
No. of organs			
1	33	46	36
2	47	39	47
> 2	20	16	18
Symptom			
Weight loss			
No	20	27	29
≤ 10%	49	34	18
> 10%	27	32	51*
Anorexia			
No	53	50	67
Yes	47	50	33
Dysphagia			
No	76	82	84
Yes	24	18	16
Pain			
No	49	57	62
Yes	51	43	38

Abbreviation: LV5FU2, biweekly regimen of leucovorin plus fluorouracil.
*The number of patients with weight loss greater than 10% was higher in the LV5FU2-irinotecan arm compared with the other two arms ($P = .05$).

cycles of chemotherapy) were 14% (95% CI, 3.6% to 24.3%; six of 43 patients), 30% (95% CI, 15.8% to 44.2%; 12 of 40 patients), and 47% (95% CI, 31.5% to 63.2%; 18 of 38 patients) for arms A, B, and C, respectively. The investigator-assessed ITT ORRs were 24% (95% CI, 11.9% to 37.0%), 30% (95% CI, 16.1% to 43.0%), and 40% (95% CI, 25.7% to 54.3%), of which 0%, 2%, and 4% were complete responses, for arms A, B, and C, respectively. The rate of agreement between investigator and expert evaluation was 83%. Three patients in arm C underwent subsequent complementary locoregional treatment (one had a resection of liver metastases, one had a resection of pulmonary metastases, and one had radiofrequency ablation of liver metastases). The primary tumor was also resected in one patient.

Survival

The median follow-up time was 26 months (95% CI, 20 to 33 months). One hundred sixteen patients (87%) were dead at the cutoff date of October 1, 2002. The numbers of patients still alive were four, eight, and six for arms A, B, and C, respectively. Table 3 lists the survival data, and Figures 1 and 2 show the OS and PFS of the patients in the study. The median PFS times were 3.2 months (95% CI, 1.8 to 4.6 months), 4.9 months (95% CI, 3.5 to 6.3 months), and 6.9 months (95% CI, 5.5 to 8.3 months) for arms A, B, and C, respectively. The median OS times were 6.8 months (95% CI, 2.6 to 11.1 months), 9.5 months (95% CI, 6.9 to 12.2 months), and 11.3 months (95% CI, 9.3 to 13.3 months) for arms A, B, and C, respectively. Patients receiving LV5FU2-irinotecan seemed to have a longer PFS and OS.

Safety

The median number of cycles administered per patient, the number of cycles delayed, and the median relative dose-intensities for the three treatment arms are listed in Table 4. Patients received a median of seven cycles (range, one to 20 cycles), seven cycles (range, one to 18 cycles), and 10 cycles (range, one to 25 cycles) of treatment in arms A, B, and C, respectively. The main reason for stopping treatment in all arms was disease progression (37 patients, 82%; 24 patients, 55%; and 27 patients, 60% in arms A, B, and C, respectively). Treatment was discontinued as a result of toxicity in 4%, 16%, and 11% of patients in arms A, B, and C, respectively (Table 5).

The grade 3 and 4 toxicities experienced during treatment are listed in Table 5. The main toxicity was hematologic (neutropenia, febrile neutropenia, and anemia), which was highest in the arm B and lowest in arm A. Gastrointestinal toxicity was also common, with nausea and vomiting experienced by more patients in arm B and diarrhea experienced by more patients in arm C. Stomatitis was uncommon with any treatment. Two deaths occurred that were considered likely to be related to treatment (one each in arms A and B, neutropenic infections). The overall mean duration of hospitalization for toxicity was 1.2 days (range, 0 to 14 days).

Table 2. Response Rates for ITT Population Per Independent Review and Further Therapy

Response	LV5FU2 (n = 45) No. of Patients	LV5FU2 (n = 45) %	LV5FU2-Cisplatin (n = 44) No. of Patients	LV5FU2-Cisplatin (n = 44) %	LV5FU2-Irinotecan (n = 45) No. of Patients	LV5FU2-Irinotecan (n = 45) %
Complete response	0	0	0	0	0	0
Partial response	6	13	12	27	18	40
Overall response	6	13	12	27	18	40
95% CI, %	3.4 to 23.3		14.1 to 40.4		25.7 to 54.3	
Stable disease	16	36	17	39	9	20
PD	21	47	11	25	11	24
Early death caused by PD	3	7	2	5	3	7
Not evaluated	2	4	4	9	7	6
Images not available	2	4	2	5	6	13
Early toxicity	0	0	1	2	1	2
Toxic death	0	0	1	2	0	0
Further therapy	32	71	33	75	32	71
Surgical resection	0	0	0	0	3	7
Second-line chemotherapy	24	53	23	52	23	51
Third-line chemotherapy	8	18	10	23	6	13

Abbreviations: ITT, intent to treat; LV5FU2, biweekly regimen of leucovorin plus fluorouracil; PD, progressive disease.

Hospital Stay

The median duration of hospital stay was 53 days (range, 7 to 300 days), 59 days (range, 3 to 124 days), and 56 days (range, 13 to 139 days) for arms A, B, and C, respectively. When converted to days per month of life, the median duration was 8.2 days per months (range, 2.1 to 30.5 days), 6.3 days per month (range, 0.7 to 19.9 days), and 5.7 days per month (range, 1.1 to 30.5 days) for arms A, B, and C, respectively. The two main reasons for hospitalization were chemotherapy administration, with a median of 30 days (range, 3 to 148 days) or 4 days per month of life (range, 1 to 15 days), and palliative care, with a median of 12 days (range, 0 to 103 days) or 1.1 days per month of life (range, 0 to 30.4 days).

QOL

Global QOL data were available for 82%, 75%, and 84% of patients at the time of inclusion compared with 41% (n = 22 patients with follow-up), 38% (n = 21), and 48% (n = 29) of patients at the third evaluation in arms A, B, and C, respectively. Thereafter, the number of patients with follow-up was small (fewer than 10 patients in each arm), whereas the rate of missing scores was maintained. A similar pattern was observed for the other 14 QOL scales. There was no difference in pretreatment global QOL scores between the study arms. However, patients in arms B and C had less constipation than patients in arm A ($P < .01$), and patients in arm C slept better than patients in arm A ($P < .05$). The trend in global health score was graphically equivalent between arms (Fig 3); compared with pretreatment scores, there was an increase in the global health score at all three evaluations, although the third evaluation revealed a slightly lower value than the second evaluation. However,

Table 3. Survival Data

Survival	LV5FU2 (n = 45)	LV5FU2-Cisplatin (n = 44)	LV5FU2-Irinotecan (n = 45)
OS, months			
Median	6.8	9.5	11.3
95% CI	2.6 to 11.1	6.9 to 12.2	9.3 to 13.3
1-Year OS, %	31	43	43
PFS, months			
Median	3.2	4.9	6.9
95% CI	1.8 to 4.6	3.5 to 6.3	5.5 to 8.3
9-Month PFS, %	7	18	24

Abbreviations: LV5FU2, biweekly regimen of leucovorin plus fluorouracil; OS, overall survival; PFS, progression-free survival.

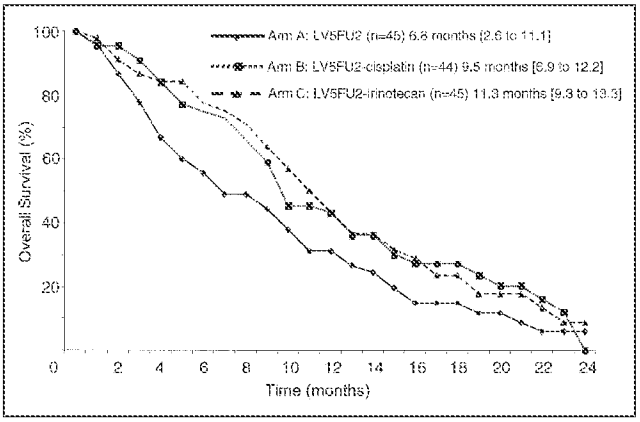

Fig 1. Overall survival according to treatment arm. The median survival times with 95% CIs are shown. LV5FU2, biweekly regimen of leucovorin plus fluorouracil.

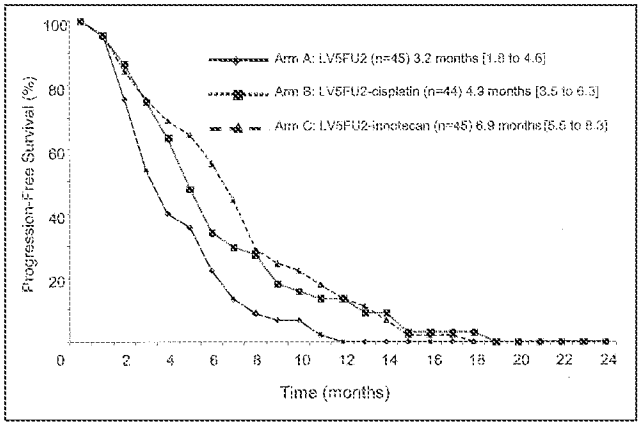

Fig 2. Progression-free survival according to treatment arm. The median progression-free survival times with 95% CIs are shown. LV5FU2, biweekly regimen of leucovorin plus fluorouracil.

longitudinal analysis showed that 14 mean scores were respectively higher in arm C than in arms A and B, regardless of the first three follow-ups (Table 6). The patients in all three arms had a significant improvement in QOL scores compared with pretreatment values (global QOL, $P < .0001$; role, $P < .01$; emotional, $P < .0001$; social, $P < .01$; pain, $P < .0001$; sleep, $P < .0001$; and appetite loss, $P < .01$; Table 6).

Comparison between arms during the third QOL assessment showed that six functional scores were higher in arm C compared with arm A (mean difference in scores: global, 2.2; physical, 2.4; role, 4.6; emotional, 4.1; cognitive, 8.3; and social, 4.7). In addition, with the exception of a worse financial score (2.1), all the symptom scores were improved (range, −1.1 for pain to −11.9 for constipation). Comparison of arms B and C showed that the irinotecan-based therapy was associated with higher global QOL (mean difference in score, 0.8) and functional scores (mean difference in scores ranging from 2.5 for social to 6.7 for emotional) and lower symptom scores (mean difference in scores ranging from −0.3 for constipation to −8.2 for sleep). The only exception was an improvement in dyspnea

Table 4. Treatment Delivery

Treatment	LV5FU2 (n = 45)	LV5FU2-Cisplatin (n = 44)	LV5FU2-Irinotecan (n = 45)
No. of cycles			
Median	7	7	10
Range	1-20	1-18	1-25
Cycles delayed for toxicity			
No.	12	45	28
%	3	13	6
RDI			
FU bolus			
Median	0.99	0.96	0.98
Range	0.82-1.07	0.24-1.18	0.63-1.17
FU CI			
Median	0.99	0.97	0.98
Range	0.84-2.00	0.72-1.92	0.73-1.18
Cisplatin			
Median	—	0.97	—
Range	—	0.73-1.04	—
Irinotecan			
Median	—	—	0.97
Range	—	—	0.69-1.01

Abbreviations: LV5FU2, biweekly regimen of leucovorin plus fluorouracil; RDI, relative dose-intensity; CI, continuous infusion; FU, fluorouracil.

Table 5. Grade 3/4 Toxicity by Patient According to WHO Grade

Toxicity	% of Patients: LV5FU2 (n = 45)	LV5FU2-Cisplatin (n = 44)	LV5FU2-Irinotecan (n = 45)
Hematologic toxicity	22	71	44
Neutropenia	11	61	40
Febrile neutropenia ± infection	9	18	11
Anemia	16	30	16
Thrombocytopenia	2	2	0
Gastrointestinal toxicity	18	25	33
Nausea and vomiting	11	23	9
Diarrhea	2	2	22
Stomatitis	4	0	7
Other toxicity			
Alopecia	0	0	13
Cutaneous	2	5	0
Neurosensory	0	5	0
Cardiac	0	0	2
Toxic deaths			
No.	1	1	0
%	2*	2*	0
Treatment stopped for toxicity	4	16	11

Abbreviation: LV5FU2, biweekly regimen of leucovorin plus fluorouracil.
*Neutropenic infection.

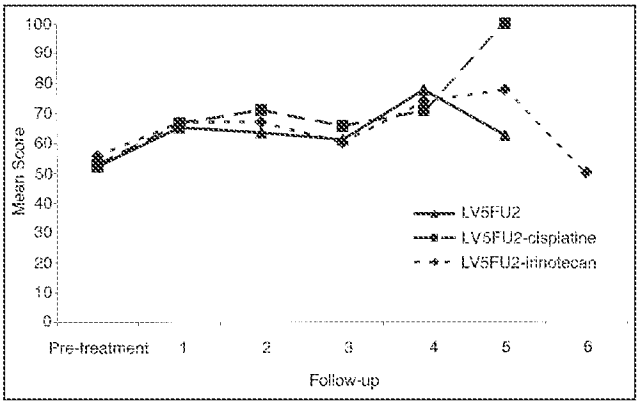

Fig 3. Quality of life global health score according to treatment arm. LV5FU2, biweekly regimen of leucovorin plus fluorouracil.

Table 6. Main Results of the Logitudinal QLQ-C30 Analysis Using a Mixed Model Analysis of Variance for Repeated Measurement: Baseline and the First Three Follow-Ups

QLQ-C30 Scores	Time Effect,* P	Treatment Effect, P	Arm C *v* Arm A, Mean Difference in Scores	Arm C *v* Arm B, Mean Difference in Scores
Global health	< .0001	.89	+ 2.2	+ 0.8
Functional scales				
Physical	.45	.41	+ 2.4	+ 4.9
Role	< .01	.68	+ 4.6	+ 3.7
Emotional	< .0001	.29	+ 4.1	+ 6.7
Cognitive	.79	.15	+ 8.3	+ 2.6
Social	< .01	.71	+ 4.7	+ 2.5
Symptom scales				
Fatigue	.16	.12	− 10.2	− 4.4
Nausea	.99	.55	− 2.6	− 4.7
Pain	< .0001	.72	− 1.1	− 3.9
Dyspnea	.36	.17	− 3.5	+ 5.2
Insomnia	< .0001	.13	− 10.1	− 8.2
Appetite loss	< .01	.31	− 8.8	− 8.1
Constipation	.41	< .05	− 11.9	− 0.3
Diarrhea	.97	.27	− 4.7	− 5.9
Financial	.36	.72	+ 2.1	− 0.5

NOTE. $P < .05$ is significant.
Abbreviation: QLQ-C30, Quality of Life Questionnaire C30.
*Global change during the first three follow-ups. Scores vary from 0 (worst) to 100 (best) for functional and global health scales and from 0 (best) to 100 (worst) for symptom scales.

in patients receiving cisplatin-based therapy (mean difference in score for arm C *v* arm B, 5.2).

DISCUSSION

The externally reviewed ORRs reported here for the various regimens studied fall within the range (6% to 56%)[6,12-14,44-46] reported in phase II and III studies using FU, FU plus cisplatin, FAMTX, ELF, EAP (epirubicin, doxorubicin, cisplatin), and ECF and more recently in studies using taxane- or oxaliplatin-based regimens. For example, in the recent interim analysis of a randomized phase III trial, a response rate of 39% was reported for a docetaxel, cisplatin, and FU combination.[47] The 13% ORR (14% per protocol) obtained with the LV5FU2 regimen is similar to the 6% to 15% ORR found in two phase II studies using another infusional FU regimen.[13,48] The 27% ORR (30% as per protocol) for the LV5FU2-cisplatin regimen is similar to the response rates found in studies with other FU-cisplatin combination regimens (20%,[6] 23%,[47] 34%,[14] and 37%[13]). It is possible that the two patients considered inassessable may have achieved partial responses, and if this were the case, the number of responses would have been 14, and the ORR would have been 32%. The externally reviewed ORR of 40% (47% per protocol) for the LV5FU2-irinotecan regimen is similar to the 42% ORR reported in abstract form only for another randomized phase II study.[44] In that study, irinotecan 80 mg/m^2, LV 500 mg/m^2, and FU 2 g/m^2 over 22 hours were administered weekly for 6 weeks followed by a 1-week rest.[44] In both cases, the ORRs for irinotecan combined with infusional FU-LV were higher than the 22% ORR reported for irinotecan combined with bolus FU-LV.[27]

The median PFS and OS (6.9 and 11.3 months, respectively) for the LV5FU2-irinotecan combination were promising when compared with the PFS and OS reported in previously published randomized studies, and this suggests that this combination is one of the most active to date. The results are even more noteworthy in view of greater pretreatment weight loss in this group, indicating a potentially worse prognostic group, and the fact that all the patients had metastatic disease, in contrast with other studies that included patients with locally advanced gastric cancer. OS times of 8.7 and 6.1 months have been reported for ECF and FAMTX, respectively,[4,12] and OS times of 6.7, 7.2, and 7.2 months have been reported for FAMTX, ELF, and FUP, respectively.[6] The results have recently been published from a phase II study of oxaliplatin 100 mg/m^2, LV 400 mg/m^2 (2-hour infusion), and FU 400 mg/m^2 (bolus) followed by 3 g/m^2 (46-hour continuous infusion) every 14 days. Although a higher dose of FU was used, the combination resulted in a median time to progression and OS of only 6.2 months and 8.6 months, respectively.[49] Also, the interim analysis of a randomized phase III trial involving docetaxel 75 mg/m^2 and cisplatin 75 mg/m^2 on day 1, then FU 750

mg/m^2/d repeated every 3 weeks showed a time to progression of 5.2 months and OS of 10.2 months.[47]

The LV5FU2-irinotecan regimen was not only active but also well tolerated. Indeed, treatment compliance for all the regimens studied was very good, with the median relative dose-intensity profile favoring the LV5FU2-irinotecan combination and with no study deaths in this arm. As expected, the LV5FU2 regimen was less toxic when delivered alone than when combined with cisplatin or irinotecan. The LV5FU2-cisplatin regimen was associated with the highest rate of nausea and vomiting and hematologic toxicity. As known from studies in patients with colorectal cancer, the LV5FU2-irinotecan regimen was associated with diarrhea.[26] Stomatitis was uncommon with any treatment, and the duration of hospital stay was similar for the three regimens. The irinotecan-containing regimen seemed to be less toxic than the regimens used in other studies involving patients with gastric carcinoma. In particular, severe nausea and vomiting occurred less frequently compared with cisplatin-based regimens.

Compared with pretreatment scores, chemotherapy seemed to improve social, emotional, and global QOL in the early first three follow-ups. The global QOL increased after the treatment induction and was maintained for 6 months. This finding is consistent with the QOL benefits reported with the ELF regimen.[7] The finding also suggests an advantage of the LV5FU2-irinotecan combination over the ECF regimen, which was associated with a maintained but not improved global QOL.[11,12] The reduction in the availability of QOL data during follow-up, together with the small number of patients, prevented an analysis of QOL after the third follow-up. This lack of data could have biased the longitudinal QOL analyses; patients with a shorter survival time and/or progression had a poor compliance in completing the QLQ-C30 assessment and probably a poor QOL. Therefore, it is likely that there is an overestimation in the mean scores, especially in the later follow-ups.

To further increase survival of patients with gastric cancer, future studies should investigate new strategies with novel drugs in different settings, including neoadjuvant, adjuvant, first line, and second line. Recent preliminary data have shown that neoadjuvant ECF significantly increases the curative resection rate from 69% to 79%.[50] Our observation that three patients in the LV5FU2-irinotecan arm were able to undergo surgery or radiofrequency ablation after their chemotherapy supports the evaluation of this regimen in the neoadjuvant setting. Although the role of chemotherapy in the adjuvant treatment of gastric cancer remains controversial, recent literature-based meta-analyses have suggested a small but statistically significant benefit.[51-55] Postoperative bolus FU-LV chemoradiotherapy is emerging as an internationally accepted standard,[56] but it is recognized that there is a need for large well-designed randomized trials in this area. The efficacy and tolerability of the LV5FU2-irinotecan regimen reported here support the evaluation of this combination in the adjuvant setting.

On the basis of ORR alone, the LV5FU2-cisplatin regimen might have warranted consideration for the phase III study. However, the decision regarding the phase III trial was based on the benefit to risk ratio and the high activity and better safety profile of the LV5FU2-irinotecan regimen makes it a more attractive treatment option. We are awaiting the results of a randomized phase III trial comparing irinotecan plus infusional FU-LV with FUP. A planned randomized French intergroup phase III study aims to compare first-line simplified LV5FU2 plus irinotecan (FOLFIRI) followed by second-line epirubicin, cisplatin, and capecitabine (ECC)[46] with ECC followed by FOLFIRI.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

REFERENCES

1. Parkin DM, Whelan SL, Ferlay J, et al: Cancer Incidence in Five Continents (volume VIII). Lyon, France, International Agency for Research on Cancer, 2002

2. Karpeh MS, Kelsen DP, Tepper JE: Cancer of the stomach, in DeVita VT, Hellman S, Rosenberg SA (eds): Cancer: Principles and Practice on Oncology (ed 6). Philadelphia, PA, Lippincott, Williams & Wilkins, 2001, pp 1092-1126

3. Kohne CH, Wils JA, Wilke HJ: Developments in the treatment of gastric cancer in Europe. Oncology (Huntingt) 14:22-25, 2000

4. Waters JS, Norman A, Cunningham D, et al: Long-term survival after epirubicin, cisplatin and fluorouracil for gastric cancer: Results of a randomized trial. Br J Cancer 80:269-272, 1999

5. Wils JA, Klein HO, Wagener DJ, et al: Sequential high-dose methotrexate and fluorouracil combined with doxorubicin: A step ahead in the treatment of advanced gastric cancer—A trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cooperative Group. J Clin Oncol 9:827-831, 1991

6. Vanhoefer U, Rougier P, Wilke H, et al: Final results of a randomized phase III trial of sequential high-dose methotrexate, fluorouracil, and doxorubicin versus etoposide, leucovorin, and fluorouracil versus infusional fluorouracil and cisplatin in advanced gastric cancer: A trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group. J Clin Oncol 18:2648-2657, 2000

7. Glimelius B, Ekstrom K, Hoffman K, et al: Randomized comparison between chemotherapy plus best supportive care with best supportive care in advanced gastric cancer. Ann Oncol 8:163-168, 1997

8. Pyrhonen S, Kuitunen T, Nyandoto P, et al: Randomised comparison of fluorouracil, epidoxorubicin and methotrexate (FEMTX) plus supportive care with supportive care alone in patients with non-resectable gastric cancer. Br J Cancer 71:587-591, 1995

9. Murad AM, Santiago FF, Petroianu A, et al: Modified therapy with 5-fluorouracil, doxorubicin, and methotrexate in advanced gastric cancer. Cancer 72:37-41, 1993

10. Ajani JA: Standard chemotherapy for gastric carcinoma: Is it a myth? J Clin Oncol 18: 4001-4003, 2000

11. Ross P, Nicolson M, Cunningham D, et al: Prospective randomized trial comparing mitomycin, cisplatin, and protracted venous-infusion fluorouracil (PVI 5-FU) with epirubicin, cisplatin, and PVI 5-FU in advanced esophagogastric cancer. J Clin Oncol 20:1996-2004, 2002

12. Webb A, Cunningham D, Scarffe JH, et al: Randomized trial comparing epirubicin, cisplatin, and fluorouracil versus fluorouracil, doxorubicin, and methotrexate in advanced esophagogastric cancer. J Clin Oncol 15:261-267, 1997

13. Vanhoefer U, Wagner T, Lutz M, et al: Randomized phase II study of weekly 24h infusion of high dose 5-FU±folinic acid (HD-FU±FA) versus HD-FU/FA/biweekly cisplatin in advanced gastric cancer. EORTC trial 40953. Eur J Cancer 37:S27, 2001 (suppl 6, abstr 88)

14. Ohtsu A, Shimada Y, Shirao K, et al: Randomized phase III trial of fluorouracil alone versus fluorouracil plus cisplatin versus uracil and tegafur plus mitomycin in patients with unresectable, advanced gastric cancer: The Japan Clinical Oncology Group Study (JCOG9205). J Clin Oncol 21:54-59, 2003

15. Kim NK, Park YS, Heo DS, et al: A phase III randomized study of 5-fluorouracil and cisplatin versus 5-fluorouracil, doxorubicin, and mitomycin C versus 5-fluorouracil alone in the treatment of advanced gastric cancer. Cancer 71:3813-3818, 1993

16. Kelsen D, Atiq OT, Saltz L, et al: FAMTX versus etoposide, doxorubicin, and cisplatin: A random assignment trial in gastric cancer. J Clin Oncol 10:541-548, 1992

17. Findlay M, Cunningham D: Chemotherapy of carcinoma of the stomach. Cancer Treat Rev 19:29-44, 1993

18. Coombes RC, Chilvers CE, Amadori D, et al: Randomised trial of epirubicin versus fluorouracil in advanced gastric cancer. An International Collaborative Cancer Group (ICCG) study. Ann Oncol 5:33-36, 1994

19. Johnson PW, Thompson PI, Seymour MT, et al: A less toxic regimen of 5-fluorouracil and high-dose folinic acid for advanced gastrointestinal adenocarcinomas. Br J Cancer 64:603-605, 1991

20. Louvet C, de Gramont A, Dermuynck B, et al: High-dose folinic acid, 5-fluorouracil bolus and continuous infusion in poor-prognosis patients with advanced measurable gastric cancer. Ann Oncol 2:229-230, 1991

21. Rubin J, Gallagher JG, Schroeder G, et al: Phase II trials of 5-fluorouracil and leucovorin in patients with metastatic gastric or pancreatic carcinoma. Cancer 78:1888-1891, 1996

22. Machover D, Goldschmidt E, Chollet P, et al: Treatment of advanced colorectal and gastric adenocarcinomas with 5-fluorouracil and high-dose folinic acid. J Clin Oncol 4:685-696, 1986

23. de Gramont A, Bosset JF, Milan C, et al: Randomized trial comparing monthly low-dose leucovorin and fluorouracil bolus with bimonthly high-dose leucovorin and fluorouracil bolus plus continuous infusion for advanced colorectal cancer: A French intergroup study. J Clin Oncol 15:808-815, 1997

24. Lecomte TL, Mitry E, Taïeb J, et al: Combination of 5-FU and cisplatin for metastatic pancreatic (P) and gastric (G) cancers: A nonrandomized comparison of two regimens (LV5FU2-P vs FUP). Gut 49, 2001 (suppl III, abstr 2777)

25. Saltz LB, Cox JV, Blanke C, et al: Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer: Irinotecan Study Group. N Engl J Med 343:905-914, 2000

26. Douillard JY, Cunningham D, Roth AD, et al: Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: A multicentre randomised trial. Lancet 355:1041-1047, 2000

27. Blanke CD, Haller DG, Benson AB, et al: A phase II study of irinotecan with 5-fluorouracil and leucovorin in patients with previously untreated gastric adenocarcinoma. Ann Oncol 12. 1575-1580, 2001

28. Kohne CH, Catane R, Klein B, et al: Irinotecan is active in chemonaive patients with metastatic gastric cancer: A phase II multicentric trial. Br J Cancer 89:997-1001, 2003

29. Lin-Shin L, Hecht J: A phase II trial of irinotecan in patients with advanced adenocarcinoma of the gastroesophageal (GE) junction. Proc Am Soc Clin Oncol 19:289a, 2000 (abstr 1130)

30. Hecht J, Parson M, Lee R: A phase II trial of irinotecan (CPT-11) in patients with adenocarcinoma of the esophagus and gastric cardia. Proc Am Soc Clin Oncol 18:287a, 1999 (abstr 1100)

31. Futatsuki K, Wakui A, Nakao I, et al: Late phase II study of irinotecan hydrochloride (CPT-11) in advanced gastric cancer: CPT-11 Gastrointestinal Cancer Study Group. Gan To Kagaku Ryoho 21:1033-1038, 1994

32. Assersohn L, Brown G, Cunningham D, et al: Phase II study of irinotecan and 5-fluorouracil/leucovorin in patients with primary refractory or relapsed advanced oesophageal and gastric carcinoma. Ann Oncol 15:64-69, 2004

33. Findlay MPN, Ackland S, Gebski V, et al: Phase II study of irinotecan, leucovorin and 5FU (ILF) in advanced gastric cancer. Proc Am Soc Clin Oncol 20:165a, 2001 (abstr 655)

34. Ajani JA, Baker J, Pisters PW, et al: CPT-11 plus cisplatin in patients with advanced, untreated gastric or gastroesophageal junction carcinoma: Results of a phase II study. Cancer 94:641-646, 2002

35. Er O, Coskun HS, Solak Y, et al: Irinotecan plus cisplatin combination against metastatic gastric adenocarcinoma: Preliminary results of phase II study. Proc Am Soc Clin Oncol 21:2310, 2002 (abstr 2310)

36. Takiuchi H, Kurihara M, Koizumi W, et al: Phase I/II study of CPT-11 plus CDDP in patients with advanced gastric carcinoma. Proc Am Soc Clin Oncol 19:275a, 2000 (abstr 1074)

37. Miller AB, Hoogstraten B, Staquet M, et al: Reporting results of cancer treatment. Cancer 47:207-214, 1981

38. Aaronson NK, Ahmedzai S, Bergman B, et al: The European Organization for Research and Treatment of Cancer QLQ-C30: A quality-of-life instrument for use in international clinical trials in oncology. J Natl Cancer Inst 85:365-376, 1993

39. Fayers PM, Aaronson NK, Bjordal K, et al: The EORTC QLC-C30 Scoring Manual (ed 3). Brussels, Belgium, European Organization for Research and Treatment of Cancer, 2001

40. Hjermstad MJ, Fossa SD, Bjordal K, et al: Test/retest study of the European Organization for Research and Treatment of Cancer Core Quality-of-Life Questionnaire. J Clin Oncol 13: 1249-1254, 1995

41. Ringdal GI, Ringdal K: Testing the EORTC Quality of Life Questionnaire on cancer patients with heterogeneous diagnoses. Qual Life Res 2:129-140, 1993

42. Ensign LG, Gehan EA, Kamen DS, et al: An optimal three-stage design for phase II clinical trials. Stat Med 13:1727-1736, 1994

43. Cnaan A, Laird NM, Slasor P: Using the general linear mixed model to analyse unbalanced repeated measures and longitudinal data. Stat Med 16:2349-2380, 1997

44. Pozzo C, Bugat R, Peschel C, et al: Irinotecan in combination with CDDP or 5-FU and

folinic acid is active in patients with advanced gastric or gastro-oesophageal junction adenocarcinoma: Final results of a randomised phase II study. Proc Am Soc Clin Oncol 20:134a, 2001 (abstr 531)

45. Van Cutsem E, Ajani JA, Tjulandin S, et al: Docetaxel (T) in combination with cisplatinum (C) with or without 5-fluorouracil (F) in patients (pts) with advanced gastric or GE junction adenocarcinoma (AGC): Preliminary results. Ann Oncol 11:2860a, 2000 (suppl 4, abstr 2860)

46. Sumpter KA, Harper-Wynne C, Cunningham D, et al: Randomised, multicenter phase III study comparing capecitabine with fluorouracil and oxaliplatin with cisplatin in patients with advanced oesophagogastric cancer: Confirmation and dose escalation. Proc Am Soc Clin Oncol 22:257a, 2003 (abstr 1031)

47. Ajani JA, Van Cutsem E, Moiseyenko V, et al: Docetaxel (D), cisplatin, 5-fluorouracil compare to cisplatin (C) and 5-fluorouracil (F) for chemotherapy-naive patients with metastatic or locally recurrent, unresectable gastric carcinoma (MGC): Interim results of a randomized phase III trial (V325). Proc Am Soc Clin Oncol 22:249, 2003 (abstr 999)

48. Berenberg JL, Tangen C, Macdonald JS, et al: Phase II study of 5-fluorouracil and folinic acid in the treatment of patients with advanced gastric cancer: A Southwest Oncology Group study. Cancer 76:715-719, 1995

49. Louvet C, Andre T, Tigaud JM, et al: Phase II study of oxaliplatin, fluorouracil, and folinic acid in locally advanced or metastatic gastric cancer patients. J Clin Oncol 20:4543-4548, 2002

50. Allum W, Cunningham D, Weeden S, et al: Perioperative chemotherapy in operable gastric and lower oesophageal cancer: A randomised, controlled trial (the MAGIC trial, ISRCTN 93793971). Proc Am Soc Clin Oncol 22:249, 2003 (abstr 998)

51. Hu JK, Chen ZX, Zhou ZG, et al: Intravenous chemotherapy for resected gastric cancer: Meta-analysis of randomized controlled trials. World J Gastroenterol 8:1023-1028, 2002

52. Hermans J, Bonenkamp JJ, Boon MC, et al: Adjuvant therapy after curative resection for gastric cancer: Meta-analysis of randomized trials. J Clin Oncol 11:1441-1447, 1993

53. Earle CC, Maroun JA: Adjuvant chemotherapy after curative resection for gastric cancer in non-Asian patients: Revisiting a meta-analysis of randomised trials. Eur J Cancer 35:1059-1064, 1999

54. Mari E, Floriani I, Tinazzi A, et al: Efficacy of adjuvant chemotherapy after curative resection for gastric cancer: A meta-analysis of published randomised trials—A study of the GISCAD (Gruppo Italiano per lo Studio dei Carcinomi dell'Apparato Digerente). Ann Oncol 11:837-843, 2000

55. Panzini I, Gianni L, Fattori PP, et al: Adjuvant chemotherapy in gastric cancer: A meta-analysis of randomized trials and a comparison with previous meta-analyses. Tumori 88:21-27, 2002

56. Macdonald JS, Smalley SR, Benedetti J, et al: Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. N Engl J Med 345:725-730, 2001

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DRUG EVALUATION

Development of nanoliposomal irinotecan (nal-IRI, MM-398, PEP02) in the management of metastatic pancreatic cancer

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ABSTRACT

Introduction: Systemic chemotherapy remains the standard of care for patients with advanced pancreatic ductal adenocarcinoma (PDAC). The introductions of FOLFIRINOX and nab-paclitaxel/gemcitabine combinations have improved the first-line treatment outcomes of patients with metastatic PDAC; while second-line therapy options are limited. Based on the results of pivotal NAPOLI-1 study, nanoliposomal irinotecan (nal-IRI) plus 5-fluorouracil and leucovorin (5-FU/LV) became the first US Food and Drug Administration (FDA) approved regimen for patients with metastatic PDAC with previous gemcitabine-based chemotherapy in November 2015.

Areas covered: We reviewed and summarized the rationale, pharmacokinetics, therapeutic efficacy and adverse events of nal-IRI alone or combined with 5-FU/LV for metastatic PDAC with previous gemcitabine-based chemotherapy.

Expert opinion: In the NAPOLI study, nal-IRI plus 5-FU/LV significantly improved the overall survival, progression-free survival and objective response rate compared to 5-FU/LV alone. The nal-IRI plus 5-FU/LV treatment was associated with a manageable toxicity profile and comparable outcomes in patients with negative demographic characteristics. The relatively sparse of neurotoxicity makes nal-IRI plus 5-FU/LV as a more favorable option than oxaliplatin-containing regimens and the current recommended standard treatment for patients with metastatic PDAC after frontline nab-paclitaxel/gemcitabine treatment. The front-line therapeutic role of nal-IRI is currently under investigation.

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common histological type and accounts for over 90% of malignant pancreatic neoplasms. With the increase in its incidence and persistent high mortality rates, and the concurrently rapid decline of colorectal, breast and prostate cancer-related death rates by 50%, 36% and 50%, respectively, from their peak rates, PDCA was the third most common cause of cancer-related death in the US in 2015, and is expecting to be the second before 2020.[1,2] Early detection is uncommon with 80–85% of the patients being diagnosed at an advanced stage. Systemic chemotherapy remains the standard of care for patients with advanced or metastatic PDAC. As the emergence of gemcitabine as the front-line chemotherapy for advanced PDAC in the mid-1990s, the median survival of patients who received first-line gemcitabine monotherapy in the majority of the randomized phase III trials has consistently ranged between 5.5 and 6.5 months in the past two decades.[3] The recent introductions of FOLFIRINOX (oxaliplatin, irinotecan plus infusional 5-fluorouracil and leucovorin [5-FU/LV]) and nab-paclitaxel/gemcitabine, and the Asian countries-restricted oral S-1 have improved the median overall survival (OS) of patients with metastatic PDAC from 8.5 to 11.1 months.[4–6] Compared to the increasing treatment options for front-line chemotherapy, second-line treatment option is limited.

Previously, a series of prospective single-arm or small randomized phase II studies that evaluated cytotoxic or targeted agents, or both in patients with gemcitabine-refractory diseases provided unsatisfactory outcomes.[7] Although an investigator-initiated randomized phase III trial (CONKO-003) demonstrated that oxaliplatin plus fluorouracil and folinic acid (OFF) significantly prolonged the survival of patients with gemcitabine-refractory advanced PDAC compared to fluorouracil and folinic acid,[8] the regimen has not been approved by the U.S. Food and Drug Administration (FDA) for such an indication. In addition, the overlapping peripheral neuropathy between OFF and the current standard, first-line nab-paclitaxel/gemcitabine treatment further limits the clinical usefulness of OFF. In this article, we review the development of the first-FDA approved regimen, nanoliposomal irinotecan (nal-IRI) plus 5-FU/LV for use in patients with metastatic PDAC after previous gemcitabine-based therapy.

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Box 1. Drug summary

Drug name (generic)	Nanoliposomal irinotecan (nal-IRI, MM-398, PEP02)
Phase (for indication under discussion)	Phase III
Indication (specific to discussion)	Metastatic adenocarcinoma of the pancreas failure to gemcitabine-based therapy
Pharmacology description/ mechanism of action	MM-398 (irinotecan liposome injection), also known as 'nal-IRI,' is a novel encapsulation of irinotecan in a long-circulating liposomal formulation. The activated form of irinotecan is SN-38, which functions by inhibiting topoisomerase I (an essential enzyme involved in DNA transcription and replication) and promoting cell death
Route of administration	Intravenous injection
Chemical structure	Novel nanoparticle formulation of irinotecan encapsulated with polyethylene glycolated liposome with 80–120 nm of particle size
Pivotal trial(s)	An international phase III study (NAPOLI-1) conducted in patients with metastatic pancreatic cancer who previously received gemcitabine-based therapy. MM-398 in combination with 5-fluorouracil (5-FU) and leucovorin achieved its primary and secondary end points by demonstrating a statistically significant improvement in overall survival, progression free survival and overall response rate compared to the control group of patients who received a combination of 5-FU and leucovorin[30]

2. Market overview

Globally, the estimated numbers of newly diagnosed cases of pancreatic cancer and related deaths were 337,872 and 330,372 respectively, in the year 2012.[9] Furthermore, according to the estimations in the StatFact Sheet of the Surveillance, Epidemiology and Results (SEER) Program, National Cancer Institute (NCI), the numbers in the US will be 53,070 and 41,780, respectively, in the year 2016.[10]

However, perhaps due to the modest activity of 5-FU and gemcitabine monotherapy, and the advanced age and poor general condition of patients, earlier data from SEER registries showed that only 50% or less of patients with advanced PDAC received chemotherapy treatment in the US before 2010.[11,12] There is little, if any, population data on the administration of second-line chemotherapy in patients with advanced PDAC after the failure of front-line chemotherapy. In the three pivotal, front-line chemotherapy trials, only 40–65% of patients received second-line therapy.[3–5] However, we can expect the requirement for second-line treatment to further increase after the emergence of more effective front-line treatment and the approval of second-line treatment.

Currently, existing treatment options are limited with nal-IRI plus 5-FU/LV as the first and the only FDA-approved therapy for patients with metastatic PDAC after front-line gemcitabine-based chemotherapy. Recently, a randomized, phase III study of ruxolitinib or placebo in combination with capecitabine for the second-line treatment of patients with advanced or metastatic PDAC (JANUS 1 study, ClinicalTrials.gov Identifier: NCT02117479) has been discontinued early because of unsatisfactory efficacy after an interim analysis in February, 2016. The other phase III study of ^{90}Y-clivatuzumab tetraxetan or the best supportive care in combination with low-dose gemcitabine in patients with metastatic PDAC who have received at least two prior therapies (ClinicalTrials.gov Identifier: NCT01956812) is still ongoing. Therefore, there is no immediate competitor for nal-IRI plus 5-FU/LV as a second-line therapy for metastatic PDAC.

3. Introduction of Nal-IRI (MM-398, PEP02)

Irinotecan, a topoisomerase 1 inhibitor, has been investigated in numerous studies as a monotherapy or combination therapy in several tumor types, including colorectal, gastric, hepatobilary and pancreatic cancers. Despite earlier phase III trials showed that the combination of irinotecan and gemcitabine failed to improve the clinical outcomes of patients with chemo-naïve advanced PDAC compared to gemcitabine monotherapy.[13,14] However, several phase II or III studies have provided preliminary evidence for the activity of irinotecan, especially in combination with 5-FU/LV, as first- or second-line treatments for metastatic PDAC.[4,15,16]

Nal-IRI has been studied in patients with various solid tumors, including breast, cervical, gastric, pancreatic, lung and colorectal cancers as well as brain tumors in phase I and II trials to define the dose-limiting toxicities (DLT), maximum tolerated dose (MTD) and characterize the safety profile in biweekly (Q2W) and triweekly (Q3W) schedules (Box 1). Before the pivotal NAPOLI-1 trial, there were four phase I trials that evaluated the MTD of intravenous nal-IRI alone or in combination with infusional 5-FU/LV. One of these trials was conducted in the US for recurrent high-grade gliomas (ClinicalTrials.gov Identifier: NCT00734682), and the patients were stratified, based on their UDP-glucuronosyl transferase 1A1*28 (*UGT1A1*28)* genotype to receive different initial dose of nal-IRI (starting with 120 and 60 mg/m^2 in patients with wild/heterozygous and homozygous mutant genotype, respectively). The other three were conducted in Asian populations without *UGT1A1* pre-testing. The clinical trials involved in the development of nal-IRI for metastatic PDAC are summarized in Figure 1.

3.1. Chemistry

Nal-IRI is a novel nanoparticle formulation of irinotecan encapsulated with polyethylene glycolated liposomes with an 80–120-nm particle size. The intra-liposomal trapping agent allows not only high drug loading (800 g irinotecan per mole of phospholipid) but also preserves the active lactone form of the drug within the liposome interior to protect it from hydrolysis.[17]

3.2. Pharmacokinetics

Treatment with nal-IRI is theoretically to increase the levels of both irinotecan and its active metabolite (SN-38) and, thereby, prolonging their actions within the tumor tissue compared to the free irinotecan.[17,18] SN-38 can be inactivated by glucuronidation by the UGT1A1 enzyme to SN-38G.[19] The favorable pharmacokinetics (PK) of lower maximum plasma concentration (C_{max}) of SN-38, as well as longer half-life ($t_{1/2}$) and higher area under curve (AUC) of DTI-31 and

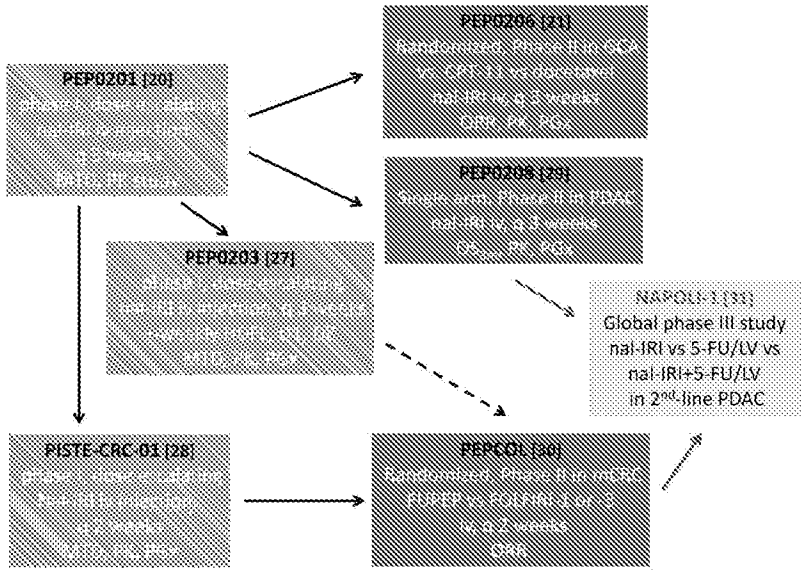

Figure 1. The development of nanoliposomal irinotecan (nal-IRI, MM-398, PEP02) in the management of metastatic pancreatic cancer. From 2005 to 2013, there were three phase I, three phase II and one phase III studies focusing on nanoliposomal irinotecan in metastatic solid tumors. MTD, maximum tolerable dosage; PK, pharmacokinetics; GCA, gastric adenocarcinoma; PGx, pharmacogenetics; HDFL, high-dose fluorouracil and leucovorin; mCRC, metastatic colorectal cancer; PDAC, pancreatic ductal adenocarcinoma; $OS_{3\ m}$, 3-month overall survival; FUPEP. Fluorouracil and nanoliposomal irinotecan; FOLFIRI, irinotecan plus fluorouracil and leucovorin; ORR, objective response rate.

SN-38 after the administration of nal-IRI than free-form irinotecan were demonstrated in the first-in-human phase I study (PEP0201 trial) of refractory solid tumors. At the MTD of nal-IRI at 120 mg/m^2 Q3W, the C_{max}, $t_{1/2}$, and $AUC_{0\text{-}169.5}$ for SN-38 were 9.20 ± 3.5 ng/ml, 75.4 ± 43.8 h and 710 ± 395 ng·h/ml, respectively, compared to 26.30 ± 11.9 ng/ml, 10.4 ± 3.1 h and 229 ± 108 ng·h/ml, respectively, with 125 mg/m^2 of the free-form irinotecan previously reported in the literature.[20] The PK advantage of nal-IRI over free-form irinotecan was further confirmed by a direct head-to-head comparison of 120 mg/m^2 nal-IRI Q3W versus 300 mg/m^2 irinotecan in the PEP0206 study of patients with fluoropyrimidine/platinum-resistant advanced gastric cancer.[21] In that study, the dose-normalized C_{max} value of SN-38 following nal-IRI infusion was approximately 50% of that after free-form irinotecan. Furthermore, the dose-normalized AUC0–∞ value of SN-38 was five times higher after nal-IRI than it was after free-form irinotecan. The concentration-time curves of CPT-11, SN-38 and SN-38G in the PEP0206 study are shown in Figure 2.

3.3. Pharmacodynamics

A prolonged retention of circulating irinotecan along with the characteristic passive diffusion of nanoliposomal particles through leaky tumor vasculature can preferentially localize nal-IRI in tumor tissue and, thereby, enhance its permeability and retention. Based on the known phenomenon of the uptake of intra-tumor deposited liposome by tumor-associated macrophage (TAM), Drummond et al. demonstrated the activity of macrophage to metabolically convert nal-IRI into SN-38 with an *ex vivo* assay by incubating nal-IRI with macrophages isolated from the peritoneum of nude mice.[17] In human studies, nal-IRI also promoted local activation and release of SN-38 leading to extended tumor exposure of SN-38, which in turn drives response.[22]

Current evidence suggests that drug resistance mechanisms against PDAC chemotherapies are driven largely by inadequate drug penetration into the poorly vascularized, desmoplastic and hypoxia tumor tissues. In animal models of colorectal and pancreatic cancers, tumors treated with nal-IRI

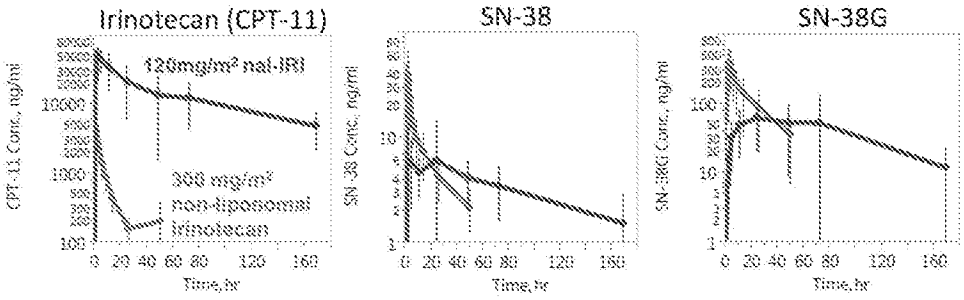

Figure 2. Pharmcokinetics of irinotecan, SN-38 and SN-38G after either 120 mg/m^2 of nanoliposomal irinotecan (nal-IRI, blue lines) or 300 mg/m^2 of non-liposomal, conventional irinotecan (red lines) in PEP0206 study (modified from Roy AC, et al. Ann Oncol 2013;24:1567–73, Supplementary data).

showed a greater and lower degree of CD31 (microvessel density) and CAIX (hypoxia marker) staining, respectively. This observation indicates that nal-IRI may change the vascular tumor microenvironment by reducing hypoxia, which possibly could partially reverse drug resistance in PDAC.[23,24] Recently, Kalra et al. extended the findings with xenograft animal models and *ex vivo* assay on human tumors, and concluded that liposome permeability and tumor carboxylesterase (CES, a critical enzyme in the conversion of irinotecan to SN-38) activity were the critical factors on determining the tumor SN-38 exposure duration, which were experimentally shown to vary across and within tumor types.[25] In the other study, a mice model was used to demonstrate that the duration of intratumoral residence of SN-38 is significantly affected by CES in the tumor, rather than CES in the serum.[26] The local conversion of nal-IRI to SN-38 by tumor CES is the main factor drives SN-38 tumor residence, which in turn drives its response.

3.4. Clinical development

3.4.1. Phase I trials

The first-in-human phase I trial of nal-IRI in refractory solid tumors, the PEP0201 study, was conducted in 2005.[20] Nal-IRI was administered as a 90-min intravenous infusion Q3W. A prophylactic muscarinic acetylcholine receptor inhibitor to prevent free-form irinotecan-associated acute cholinergic reaction was not routinely given before the administration of nal-IRI. This phase I study used an accelerated titration design, and 11 patients were enrolled at three dose levels: 60, 120 and 180 mg/m^2 (one, six and four patients, respectively). Two of the patients at the 180 mg/m^2 dose level experienced DLT, which manifested as prolonged grade 4 neutropenia in one and grade 4 hematological toxicities and diarrhea in the other. Finally, the MTD of the nal-IRI monotherapy administered Q3W was determined as 120 mg/m^2. None of the 11 patients experienced any acute cholinergic reaction.

In clinical practice, irinotecan is commonly used in combination with 5-FU/LV for treating gastrointestinal malignancies. We subsequently explored the MTD of nal-IRI in combination with weekly 24-h infusional 5-FU/LV in patients with refractory, advanced solid tumors in the PEP0203 study between March 2006 and August 2008.[27] In this multicenter, phase I trial, nal-IRI was administered at doses of 60–120 mg/m^2 at day 1 followed by 24-h infusion of 5-FU/LV (2000 and 200 mg/m^2, respectively) on day 1 and 8, Q3W. A total of 16 patients were accrued at four dose levels, 60, 80, 100 and 120 mg/m^2 (three, six, five, and two patients, respectively). DLT was observed in four patients, consisting of two each at doses of 100 and 120 mg/m^2. The MTD of nal-IRI in this combination therapy was determined as 80 mg/m^2.

In addition, to comply with the modern Q2W schedule of the FOLFIRI regimen (irinotecan and infusional 5-FU/LV), which is a standard of care in the management of metastatic colorectal cancer, an investigator-initiated phase I trial (the PISTE-CRC-01 study, ClinicalTrials.gov Identifier: NCT00940758) to determine the MTD of nal-IRI monotherapy administered Q2W was conducted between March 2009 and September 2011.[28] Patients with metastatic colorectal cancer, whose diseases had progressed after first-line oxaliplatin-based chemotherapy and without prior exposure to irinotecan, were eligible for the study. Patients were included in cohorts of three to receive nal-IRI at doses of 80, 90, and 100 mg/m^2. A total of 18 patients were enrolled, with six at each dose level. The DLT, which manifested as grade 3 diarrhea was observed in one patient per dose level. The target dose of 100 mg/m^2 was determined as the MTD of nal-IRI monotherapy, administered Q2W.

3.4.2. Phase II trials

Of the subsequent three phase II trials, the PEP0206 and PEP0208 studies (ClinicalTrials.gov Identifier: NCT00813072 and NCT00813163, respectively) evaluated nal-IRI monotherapy, 120 mg/m^2 Q3W, as a second-line therapy in fluoropyrimidine/platinum-failed advanced gastric cancer and gemcitabine-based therapy-refractory metastatic PDAC, respectively.[21,29] The GERCOR conducted the PEPCOL randomized phase II study (ClinicalTrials.gov Identifier: NCT01375816) between May 2011 and August 2013, to evaluate the feasibility and efficacy of Q2W nal-IRI in combination with infusional 5-FU/LV (the FUPEP regimen) as a second-line therapy in patients with metastatic colorectal cancer (Figure 1). The FUPEP regimen consisted of an intravenous infusion of nal-IRI and LV 80 and 400 mg/m^2 over 90 min and 2 h, respectively, followed by a 46-h infusion of 5-FU 2400 mg/m^2, Q2W. Of the 28 patients who received FUPEP, grade 3–4 diarrhea and neutropenia occurred in 21.4% and 10.7%, respectively. This outcome indicated this regimen had a comparable safety profiles to that of the FOLFIRI arm, and confirmed the feasibility of the Q2W nal-IRI and 5-FU/LV combination chemotherapy.[30]

3.4.3. Activity of nal-IRI on gemcitabine-refractory advanced PDAC in phase I/II studies

In the first-in-human PEP0201 trial, one of the two patients with gemcitabine-based chemotherapy-refractory metastatic PDAC achieved a partial response after nal-IRI at the dose of 180 mg/m^2.[20] Based on the finding, five more patients with gemcitabine-based therapy-refractory metastatic PDAC were included into the second phase I trial (PEP0203 study). Among the five patients, four had durable stable diseases.[27]

Therefore, based on such encouraging results of 71% of disease control rate (DCR: one partial response and four stable diseases) in heavily pre-treated gemcitabine-based therapy-refractory metastatic PDAC, an international phase II study which evaluated nal-IRI monotherapy as a second-line treatment for metastatic PDAC (PEP0208) was launched in 2009. Of the 40 patients with metastatic PDAC after treated with gemcitabine-based therapy, 120 mg/m^2 of nal-IRI monotherapy Q3W resulted in 7.5% of objective response rate (ORR), 3-month and 12-month survival rates of 75% and 25%, respectively, 5.2 months of median OS and a manageable toxicity profile.[29] These promising results in a highly unmet need patient population led the U.S. FDA to grant an orphan status for the treatment of metastatic PDAC in July 2011 and the launch of a randomized, pivotal NAPOLI-1 trial in January 2012.

3.4.4. Phase III trial (NAPOLI-1 study)

This international, multicenter, randomized, phase III NAPOLI-1 study evaluated nal-IRI alone or in combination with 5-FU/LV (nal-IRI+5-FU/LV) versus a common control (5-FU/LV) in patients with metastatic PDAC previously treated with gemcitabine-based therapies.[31] Adult patients (age ≥18 years) with documented disease progression after gemcitabine or gemcitabine-containing therapy were enrolled. Other inclusion criteria included a Karnofsky Performance Status (KPS) ≥70 (from 100 to 0 with standard intervals of 10, where 100 is 'perfect' health and 0 is death) and adequate hematologic, hepatic, and renal function. Randomization was pre-stratified based on patient baseline albumin levels, KPS, and ethnicity. Patients were initially randomized at a 1:1 ratio to receive nal-IRI 120 mg/m^2 Q3W or 5-FU/LV 2000 and 200 mg/m^2 (over 24 h and 30 min, respectively) weekly for 4 weeks in a 6-week cycle. After the first 63 patients were enrolled under the original protocol, the safety data of a Q2W nal-IRI+5-FU/LV (80 and 400 mg/m^2 over 90 and 30 min, respectively), followed by 5-FU 2400 mg/m^2 over 46 h, the FUPEP regimen from a concurrent GERCOR phase II PEPCOL trial in metastatic colorectal cancer became available.[30] Therefore, the protocol was amended to include a third arm for the nal-IRI+5-FU/LV treatment.[31] *UGT1A1*28* genotyping was required for all patients, and those with the homozygous *UGT1A1*28(TA)$_7$* allele in either nal-IRI-containing arms, had their the initial nal-IRI dose reduced by 20 mg/m^2. However, an allowance was made for dose escalation to standard dose in the absence of drug-related toxicities during the first cycle of treatment. The primary end point was OS while secondary end points included progression-free survival (PFS), ORR, change in CA19-9, and safety. For the efficacy end points, the nal-IRI+5-FU/LV and 5-FU/LV control arms were compared, in an analysis comprising only of patients randomized under the amended protocol. Furthermore, the nal-IRI monotherapy and 5-FU/LV control arms comprised of all patients randomized under both versions of the protocol, were compared and the results are shown in Figure 3. The radiographic tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 every 6 weeks.

The study completed 417 patients accrual between January 2012 and September 2013. The primary results of the NAPOLI-1 study were first disclosed at the European Society for Medical Oncology (ESMO) World Congress on Gastrointestinal Cancer 2014. The results showed that nal-IRI+5-FU/LV significantly improved the OS of the intent-to-treat (ITT) population compared to 5-FU/LV alone, with the media OS of 6.1 months (95% CI, 4.8–8.9) and 4.2 months (95% CI, 3.6–4.9), respectively. However, nal-IRI alone (OS, 4.9 months, 95% CI, 4.2–5.6) failed to demonstrate its superiority over the 5-FU/LV arm.[31] The hazard ratio of the OS of the combination vs. control arm was 0.67 (95% CI: 0.49–0.92, p = 0.012, unstratified log-rank). Nal-IRI+5-FU/LV also achieved significantly better ORR (16% vs. 1%) and median progression-free survival (3.1 months vs. 1.5 months, HR = 0.56, 95% CI: 0.41–0.75, $p < 0.001$), and higher proportion of patients with abnormal baseline CA 19.9 had ≥50% reduction in CA 19.9 (29% vs. 9%, $p < 0.001$). The nal-IRI+5-FU/LV treatment was associated with 10–30% of manageable grade ≥3 neutropenia, fatigue, diarrhea and vomiting. The pre-planned subgroup analyses showed that nal-IRI+5-FU/LV treatment achieved comparable treatment outcome in patients with negative demographic characteristics, such as worse KPS (<90), lower baseline serum albumin level (<4.0 g/l), abnormal baseline CA 19.9 level (≥40 U/ml) and the presence of liver metastases.[31] In the pre-planned analysis for the per protocol population (PPP, defined as eligible patients who received ≥80% dose intensity of the protocol assigned treatment during the first 6 weeks of treatment), the nal-IRI+5-FU/LV combination achieved a median OS of 8.9 months (vs. 5.1 months in the control arm, stratified HR = 0.47, p = 0.002). Surprisingly, the combination treatment also significantly improved the OS (median 4.4 vs. 2.8 months in the control arm, stratified HR: 0.56, p = 0.037) in the non-PPP.[32] The drug was granted the Fast Track designation by the U.S. FDA in November 2014 and was approved for the treatment of patients with metastatic PC after previous gemcitabine-based therapy by both the U.S. and Taiwan FDAs on 22 October 2015.

3.5. Safety and tolerability

The incidence of grade 3–4 adverse events that were reported in previous phase II or III studies is summarized in Table 1, which shows a certain degree of variation in the incidence of major adverse events among the studies. For example, in the NAPOLI-1 study, despite similar dose density of administered nal-IRI in both the nal-IRI-containing arms, patients in the nal-

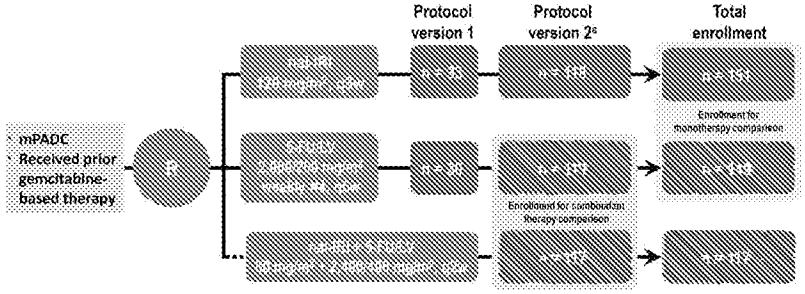

Figure 3. The schema of NAPOLI-1 study design, denote a: NAPOLI-1 was amended to add the nal-IRI + 5-FU/LV arm once safety data on the combination became available. Only those patients enrolled in the 5-FU/LV arm after the amendment (n = 119) were used as the control for the combination arm.

Table 1. The incidence of major significant adverse events of nal-IRI either alone or in combination with 5-FU/LV in previous studies.

	Nal-IRI alone nal-IRI 120 mg/m^2, q 3 weeks			Nal-IRI + 5-FU/LV nal-IRI 80 mg/m^2, q 2 weeks	
Grade 3–4 AEs,	PEP0206 n = 44	PEP0208 n = 40	NAPOLI-1 n = 147	PEPCOL n = 28	NAPOLI-1 n = 117
Diarrhea	27%	15%	21%	21%	13%
Vomiting	5%	8%	14%	4%	11%
Nausea	11%	10%	5%	4%	8%
Anorexia	7%	10%	19%	NA	4%
Fatigue	5%	20%[1]	6%	NA	14%
Neutropenia	18%[2]	30%	15%[2]	11%	27%[2]
Anemia	5%	15%	11%	0%	9%

[1]Including asthenia.
[2]Including febrile neutropenia.
AEs: adverse events; Nal-IRI: nanoliposomal irinotecan; NA: not available; 5-FU/LV: fluorouracil and leucovorin.

IRI+5-FU/LV arm had less grade 3–4 diarrhea (13% vs. 21%) but more grade 3–4 neutropenia (27% vs. 15%) compared to those in the nal-IRI monotherapy arm. The former adverse event was considered to be attributable to the higher C_{max} and perhaps $AUC_{0\text{-}48\ h}$ of SN-38 following the administration of 120 mg/m^2 of nal-IRI in the monotherapy arm compared to the 80 mg/m^2 dose in the combination arm.[31] On the other hand, co-administered infusional 5-FU/LV is likely the main etiology for the higher incidence of grade 3–4 neutropenia in the nal-IRI+5-FU/LV arm. These trends were not as obvious in those small phase II trials with patient numbers ranging from 28 to 44.[21,29,30] In general, as an effective second-line chemotherapy for metastatic PDAC, the 13% incidence of grade 3–4 diarrhea and 27% grade 3–4 neutropenia are manageable. Continuous monitoring and collection of the adverse event reports in phase IV trials after the launch of nal-IRI for daily clinical practice use is warranted.

3.6. Factors and biomarkers related to its efficacy and toxicity

Ferumoxytol (FMX) is a 30 nm iron-oxide, super-paramagnetic nanoparticle, known to be taken up by macrophages, and exhibits magnetic resonance imaging (MRI) properties. In a patient-derived PDAC tumor xenograft model, a baseline MRI scan was performed with intravenous injection of FMX followed by fluorescently labeled nal-IRI. The IHC analysis showed that both liposomes and FMX were co-localized with TAM. A significant correlation between the tumor FMX-based MRI (Fe-MRI) signal and intratumoral levels of irinotecan was also noted.[33] In a small series study, the Fe-MRI was performed in patients undergoing nal-IRI treatment to evaluate the feasibility of correlating Fe-MRI acquisition with tissue drug-metabolite levels. The relationship between the FMX levels in tumor lesions and nal-IRI activity suggested that lesion permeability to FMX may be a useful biomarker for tumor response to nal-IRI in patients with solid tumors.[22] However, the clinical relevance of such a molecular imaging diagnostic procedure in nanoliposomal drug-based therapy requires further validation in large-scale prospective studies.

UGT1A1 variants have been shown to correlate with irinotecan treatment-related toxicities.[34] In the PEP0201 study, one patient who died of grade 4 diarrhea, neutropenia and infection after first dose of 180 mg/m^2 nal-IRI had a combination heterozygosity of *UGT1A1*6* and **28* in *post hoc* analysis. The SN-38 AUC of this particular patient was almost threefold higher than those of the other three patients without the *UGT1A1* genetic alternations, at the same nal-IRI dose level.[20] In addition, of the, 36 patients who had PK study after nal-IRI in the PEP0206 study,[21] the incidence of grade 3–4 neutropaenia was higher in patients with *UGT1A1*6* heterozygotes [40% (2 of 5) vs. 3% (11 of 30) in those with the wild-type genotype, $p = 0.022$]. No correlation between *UGT1A* gene polymorphism and PEP02/irinotecan PK was demonstrated. The clinical relevance of *UGT1A1* polymorphisms, especially *UGT1A1*28* vs. *UGT1A1*6*, on the therapeutic efficacies and adverse events of nal-IRI-containing regimens requires further investigation.

4. Conclusion

Nal-IRI, a new nano-liposome encapsulated irinotecan formulation, which exhibited favorable pharmacokinetic characteristics in both preclinical models and human studies, has become the first and the only FDA-approved agent for the treatment of metastatic PDAC after previous gemcitabine-based therapy, based on the results of the NAPOLI-1 study. Following the approval of their use as the first- and second-line treatments, nab-paclitaxel/gemcitabine followed by nal-IRI plus 5-FU/LV will be the standard of care for patients with metastatic PDAC. However, a recent French multi-center, single arm study suggested that nab-paclitaxel/gemcitabine could be a highly effective regimen, with a 17.5% of response rate, 5.1 months of median PFS, and 8.8 months OS, after FOLFIRINOX in metastatic PDAC.[35] This observation suggests that the reverse sequence of the nal-IRI-based regimen followed by nab-paclitaxel/gemcitabine could be an attractive alternative approach and deserves further investigation. Currently, a randomized, open-label phase II study of nal-IRI-containing regimens vs. nab-paclitaxel/gemcitabine in previously untreated, metastatic PDAC (ClinicalTrials.gov Identifier: NCT02551991) is ongoing. It seeks to determine a suitable nal-IRI-containing front-runner regimen to compare with the nal-paclitaxel/gemcitabine as a first-line therapy for metastatic PDAC.

Table 2. The comparison of the patients' characteristics and therapeutic efficacy parameters between NAPOLI-1 and CONKO-003 trials.

	NAPOLI-1 (n = 117)	CONKO-003 (n = 76)
Study period	2012–2013	2004–2007
Backbone of regimen	nal-IRI plus 5-FU/LV	oxaliplatin plus 5-FU/LV
Sites	14 countries[1]	Germany
Median age (years-old)	63	62
KPS, ≤70–80/90–100 (%)	41/59	46/54
Disease status, LA/Meta (%)	0/100	12/88
Previous treatment allowed	Gem-based therapy with 34% had ≥2 line of metastatic therapy	Only Gem monotherapy
Response rate (%)	16%	NA
Median PFS (months)	3.1	2.9
Median OS (months)	6.1	5.9

[1]Argentina, Australia, Brazil, Canada, Czech Republic, France, Germany, Hungary, Italy, South Korea, Spain, Taiwan, UK and USA.
Nal-IRI: nanoliposomal irinotecan; 5-FU/LV: fluorouracil and leucovorin; LA: locally advanced; Meta: metastasis; Gem: gemcitabine; PD: disease progression after previous treatment; PFS: progression-free survival; OS: overall survival.

5. Expert opinion

To date, two positive phase III trial demonstrated the survival benefit of second-line therapy with either oxaliplatin plus 5-FU/LV (OFF regimen, CONKO-003 study) or nal-IRI plus 5-FU/LV (NAPOLI-1 study) in patients with PDAC previously treated with a gemcitabine-based therapy.[8,31] The median PFS and OS after the second-line OFF (2.9 and 5.9 months, respectively) and nal-IRI plus 5-FU/LV (3.1 and 6.1 months, respectively) were quite similar. However, of patients in the nal-IRI plus 5-FU/LV arm of the NALPOLI-1 study, 45% had previous gemcitabine monotherapy only, and 34% had two or more lines of therapy. In contrast, the CONKO-003 study enrolled only patients who failed to previous gemcitabine monotherapy. The comparison of the patients' characteristics and therapeutic efficacy parameters between the NAPOLI-1 and CONKO-003 trials is shown in Table 2. Both regimens also had different safety profiles with more grade 3–4 neutropenia and diarrhea for nal-IRI plus 5-FU/LV, and grade 2–3 sensory neuropathy for OFF. Currently, nab-paclitaxel/gemcitabine is a standard regimen for the first-line therapy of metastatic PDAC that is associated with substantial neurotoxicity. Therefore, the relatively sparse of neurotoxicity of the nal-IRI plus 5-FU/LV regimen appears to be one of its major advantages over the oxaliplatin-containing OFF or FOLFOX, to serve as the second-line therapy after nab-paclitaxel/gemcitabine, as suggested by Oettle and Lehmann.[36] Therefore, nab-paclitaxel/gemcitabine followed by nal-IRI plus 5-FU/LV is the recommended standard of care for patients with metastatic PDAC.

However, we can expect that investigators and pharmaceutical companies will be interested to advance nal-IRI as the front-line treatment for PDAC, and search for opportunities in other gastrointestinal malignancies such as gastric cancer, CRC and biliary tract cancer over the next few years. Furthermore, the combinations of nal-IRI with either targeted agents or immunotherapy or both are other strategies for further drug development. Currently, a phase I study of nal-IRI and veliparib (ClinicalTrials.gov Identifier: NCT02631733) in solid tumors is ready to commence.

Declaration of interests

Y-S. Shen received honorariums from TTY Biopharm. L-T. Chen received honorariums from Merck Serono, TTY Biopharm, Sanofi, and PharmaEngine. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1. Rahib L, Smith BD, Aizenberg R, et al. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res. 2014;74:2913–2921.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016;66:7–30.
3. Vaccaro V, Sperduti I, Vari S, et al. Metastatic pancreatic cancer: Is there a light at the end of the tunnel? World J Gastroenterol. 2015;21:4788–801.
•• **FOLFIRINOX is superior to gemcitabine alone for metastatic PDAC.**
4. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med. 2011;364:1817–1825. doi:10.056/NEJMoa1011923.
•• **Oral S-1 is non-inferior to gemcitabine alone for advanced PDAC.**
5. Ueno H, Ioka T, Ikeda M, et al. Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. J Clin Oncol. 2013;31:1640–1648.
•• **Abraxane and gemcitabine treatment superior to gemcitabine alone for metastatic PDAC.**
6. Von Hoff DD, Ervin T, Arena FP, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med. 2013;369:1691–1703.
7. Nagrial AM, Chin VT, Sjoquist KM, et al. Second-line treatment in inoperable pancreatic adenocarcinoma: A systematic review and synthesis of all clinical trials. Crit Rev Oncol Hematol. 2015;26:007.
8. Oettle H, Riess H, Stieler JM, et al. Second-line oxaliplatin, folinic acid, and fluorouracil versus folinic acid and fluorouracil alone for gemcitabine-refractory pancreatic cancer: outcomes from the CONKO-003 trial. J Clin Oncol. 2014;32:2423–2429.
• **Oxaliplatin plus 5-FU/LV is superior to 5-FU/LV as second-line treatment for gemcitabine-failure metastatic PDAC.**
9. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136:E359–86.
10. SEER Stat Fact Sheets: Pancreas Cancer. Bethesda (MD): National Cancer Institute; 2016. http://seer.cancer.gov/statfacts/html/pancreas.html
11. Boyd CA, Branch DW, Sheffield KM, et al. Hospital and medical care days in pancreatic cancer. Ann Surg Oncol. 2012;19:2435–2442.
12. Enewold L, Harlan LC, Tucker T, et al. Pancreatic cancer in the USA: persistence of undertreatment and poor outcome. J Gastrointest Cancer. 2015;46:9–20.
13. Rocha Lima CM, Green MR, Rotche R, et al. Irinotecan plus gemcitabine results in no survival advantage compared with gemcitabine monotherapy in patients with locally advanced or metastatic pancreatic cancer despite increased tumor response rate. J Clin Oncol. 2004;22:3776–3783.

14. Stathopoulos GP, Syrigos K, Aravantinos G, et al. A multicenter phase III trial comparing irinotecan-gemcitabine (IG) with gemcitabine (G) monotherapy as first-line treatment in patients with locally advanced or metastatic pancreatic cancer. Br J Cancer. 2006;95:587–592.
15. Taieb J, Lecomte T, Aparicio T, et al. FOLFIRI.3, a new regimen combining 5-fluorouracil, folinic acid and irinotecan, for advanced pancreatic cancer: results of an Association des Gastro-Enterologues Oncologues (Gastroenterologist Oncologist Association) multicenter phase II study. Ann Oncol. 2007;18:498–503.
16. Yoo C, Hwang JY, Kim JE, et al. A randomised phase II study of modified FOLFIRI.3 vs modified FOLFOX as second-line therapy in patients with gemcitabine-refractory advanced pancreatic cancer. Br J Cancer. 2009;101:1658–1663.
17. Drummond DC, Noble CO, Guo Z, et al. Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. Cancer Res. 2006;66:3271–3277.
18. Tsai C-S, Park JW, Chen L-T. Nanovector-based therapies in advanced pancreatic cancer. J Gastrointest Oncol. 2011;2:185–194.
19. Chen S, Yueh MF, Bigo C, et al. Intestinal glucuronidation protects against chemotherapy-induced toxicity by irinotecan (CPT-11). Proc Natl Acad Sci U S A. 2013;110: 19143–19148. doi:10.1073/pnas.1319123110Epub 2013 Nov 4
20. Chang TC, Shiah HS, Yang CH, et al. Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients. Cancer Chemother Pharmacol. 2015;75:579–586.
•• **PEP0201 study: the first-in-human study of nal-IRI.**
21. Roy AC, Park SR, Cunningham D, et al. 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. Ann Oncol. 2013;24:1567–1573.
•• **PEP0206 study: head-to-head comparison of PK and AE of nal-IRI and irinotecan in advanced gastric cancer patients.**
22. Ramanathan RK, Korn RL, Sachdev JC, et al. Abstract CT224: Pilot study in patients with advanced solid tumors to evaluate feasibility of ferumoxytol (FMX) as tumor imaging agent prior to MM-398, a nanoliposomal irinotecan (nal-IRI). Cancer Research. 2014;74:CT224.
23. Klinz SG, Paz N, Kalra A, et al. Abstract C207: Identifying differential mechanisms of action for MM-398/PEP02, a novel nanotherapeutic encapsulation of irinotecan. Mol Cancer Ther. 2011;10:C207.
24. Paz N, Laivins P, Niyikiza C, et al. Abstract A63: MM-398/PEP02, a novel liposomal formulation of irinotecan, demonstrates stromal-modifying anticancer properties. Cancer Research. 2012;72:A63.
25. Kalra AV, Kim J, Klinz SG, et al. Preclinical activity of nanoliposomal irinotecan is governed by tumor deposition and intratumor prodrug conversion. Cancer Res. 2014;74:7003–7013.
26. Kim J, Bayever E, Laivins P, et al. Abstract A6: Sustained intratumoral activation of MM-398 results in superior activity over irinotecan demonstrated by using a systems pharmacology approach. Cancer Research. 2012;72:A6.
27. Chen L, Shiah H, Chao T, et al. Phase I study of liposome irinotecan (PEP02) in combination with weekly infusion of 5-FU/LV in advanced solid tumors. ASCO Meet Abstr. 2010;28:e13024.
•• **PEP0203 study: to determine the MTD and feasibility of nal-IRI plus weekly infusion 5-FU/LV.**
28. Chen L-T, Shiah H-S, Lin P-C, et al. Phase I study of biweekly liposome irinotecan (PEP02, MM-398) in metastatic colorectal cancer failed on first-line oxaliplatin-based chemotherapy. ASCO Meet Abstr. 2012;30:613.
•• **PISTE-CRC-01 study: to define the MTD of biweekly nal-IRI monotherapy.**
29. Ko AH, Tempero MA, Shan YS, et al. A multinational phase 2 study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer. Br J Cancer. 2013;109:920–925.
•• **PEP0208 study: the first phase II study to show the efficacy of second-line nal-IRI in metastatic PDAC.**
30. Chibaudel B, Maindrault-Goebel F, Bachet JB, 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 Med. 2016; doi:10.1002/cam4.635.
•• **PEPCOL study: to demonstrate the feasibility and safety of biweekly nal-IRI plus 46-h infusion of 5-FU/LV.**
31. Wang-Gillam A, Li CP, Bodoky G, et al. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global, randomised, open-label, phase 3 trial. Lancet. 2015;387:545–557.
• **NAPOLI study, the pivotal trial to demonstrate the superiority of nal-IRI plus 5-FU/LV over 5-FU/LV in metastatic PDAC after gemcitabine treatment.**
32. Chen L-T, Von Hoff DD, Li C-P, et al. Expanded analyses of napoli-1: phase 3 study of MM-398 (nal-IRI), with or without 5-fluorouracil and leucovorin, versus 5-fluorouracil and leucovorin, in metastatic pancreatic cancer (mPAC) previously treated with gemcitabine-based therapy. ASCO Meet Abstr. 2015;33:234.
33. Kalra AV, Spernyak J, Kim J, et al. Abstract 2065: Magnetic resonance imaging with an iron oxide nanoparticle demonstrates the preclinical feasibility of predicting intratumoral uptake and activity of MM-398, a nanoliposomal irinotecan (nal-IRI). Cancer Research. 2014;74:2065.
34. Hoskins JM, McLeod HL. UGT1A and irinotecan toxicity: keeping it in the family. J Clin Oncol. 2009;27:2419–2421.
35. Portal A, Pernot S, Tougeron D, et al. Nab-paclitaxel plus gemcitabine for metastatic pancreatic adenocarcinoma after Folfirinox failure: an AGEO prospective multicentre cohort. Br J Cancer. 2015;113:989–995.
36. Oettle H, Lehmann T. Gemcitabine-resistant pancreatic cancer: a second-line option. Lancet. 2016;387:507–508.

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Notice of opposition to a European patent

I. Patent opposed

Patent No.	EP3337478
Application No.	EP16758337.6
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	12 August 2020
Title of the invention	Drug combination comprising liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin for treating metastatic pancreatic cancer

II. Proprietor of the patent

first named in the patent specification	Ipsen Biopharm Ltd.
Opponent's or representative's reference	ESP00506SAN

III. Opponent

Name	Sandoz AG
Address:	Lichtstrasse 35 4056 Basel Switzerland
State of residence or of principal place of business	Switzerland
Multiple opponents (see additional sheet)	☐

IV. Authorisation

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Additional representative(s) on additional sheet/see authorisation ☐

Authorisation(s)

is/are enclosed ☐

has/have been registered under No. ☐

V. **Opposition is filed against**

the patent as a whole ☒

claim(s) No(s).

VI. **Grounds for opposition:**

Opposition is based on the following grounds:

(a) the subject-matter of the European patent opposed is not patentable (Art. 100(a) EPC) because:

• it is not new (Art. 52(1); Art. 54 EPC) ☐

• it does not involve an inventive step (Art. 52(1); Art. 56 EPC) ☒

• patentability is excluded on other grounds, namely articles ☐

(b) the patent opposed does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art (Art. 100(b) EPC; see Art. 83 EPC). ☐

(c) the subject-matter of the patent opposed extends beyond the content of the application/of the earlier application as filed (Art. 100(c) EPC, see Art. 123(2) EPC). ☐

VII. **Facts (Rule 76(2)(c) EPC)**

presented in support of the opposition are submitted herewith on an attached document ☒

VIII. **Other requests:**

• Oral proceedings are hereby requested auxiliarily.

IX. Evidence presented

D1	Other evidence	Protocol of the clinical trial NCT02551991 original file name: D1.pdf attached as: Other-evidence-1.pdf
D2	Other evidence	D. Von Hoff et al., Ann. Oncol. 2014, 25(2), ii105–ii117 original file name: D2.pdf attached as: Other-evidence-2.pdf
D3	Other evidence	R.D.W. Marsh et al., Cancer Med 2015, 4(6), 853–863 original file name: D3.pdf attached as: Other-evidence-3.pdf
D4	Other evidence	Onivyde prescribing information, October 2015 original file name: D4.pdf attached as: Other-evidence-4.pdf
D5	Other evidence	J. Carnevale et al., Fut. Oncol. 2016, 12(4), 453-464 original file name: D5.pdf attached as: Other-evidence-5.pdf
D6	Other evidence	A. Dean et al., J. Clin. Oncol. 2016, 34, 4(Supp 1), Abstract TPS482 original file name: D6.pdf attached as: Other-evidence-6.pdf
D7	Other evidence	H. Zhang et al., Onco Targets Ther. 2016, 9, 3001-3007 original file name: D7.pdf attached as: Other-evidence-7.pdf
D8	Other evidence	D.F. Gaddy et al., Cancer Res. 2016, 76, 14(Supp), Abstract 4830, presented at the AACR 107th Annual Meeting in New Orleans, LA original file name: D8.pdf attached as: Other-evidence-8.pdf
D9	Other evidence	P. Parhi et al., Drug Discov. Today 2012, 17(17/18), 1044 original file name: D9.pdf attached as: Other-evidence-9.pdf

X. **Payment**

Method of payment	Debit from deposit account

The European Patent Office is hereby authorised, to debit from the deposit account with the EPO any fees and costs indicated in the fees section below.

Currency:	EUR
Deposit account number:	28000381
Account holder:	Lederer & Keller

Refunds

Any refunds should be made to EPO deposit account:	28000381
Account holder:	Lederer & Keller

Fees	Factor applied	Fee schedule	Amount to be paid
010 Opposition fee	1	815.00	815.00
Total:		**EUR**	**815.00**

	A **Forms**	Details:	System file name:
A-1	Form for notice of opposition		ep-oppo.pdf

	B **Attached document files**	Original file name:	System file name:
B-1	1. Facts and arguments	opposition.pdf	OPPO.pdf

	C **Attached evidence files**	Original file name:	System file name:
C-1	1. Other evidence	D1.pdf	Other-evidence-1.pdf
C-2	2. Other evidence	D2.pdf	Other-evidence-2.pdf
C-3	3. Other evidence	D3.pdf	Other-evidence-3.pdf
C-4	4. Other evidence	D4.pdf	Other-evidence-4.pdf
C-5	5. Other evidence	D5.pdf	Other-evidence-5.pdf
C-6	6. Other evidence	D6.pdf	Other-evidence-6.pdf
C-7	7. Other evidence	D7.pdf	Other-evidence-7.pdf

C-8	8. Other evidence	D8.pdf	Other-evidence-8.pdf
C-9	9. Other evidence	D9.pdf	Other-evidence-9.pdf

Signature of opponent or representative

Place	**München**
Date:	**06 May 2021**
Signed by:	**Marco Fachini 46014**
Association:	**Lederer & Keller Patentanwälte Partnerschaft mbB**
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Your reference	Our reference	06 May 2021
EP 3 337 478	ESP00506SAN	MF/SB

Re.: European Patent No. 3 337 478
European Patent Application No. 16758337.6
Patentee: Ipsen Biopharm Ltd.
Opponent: Sandoz AG

On behalf of

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OPPOSITION

is lodged according to Article 99 EPC against the above-referenced patent of Ipsen Biopharm Ltd. entitled

"Drug combination comprising liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin for treating metastatic pancreatic cancer".

The opposition fee amounting to EUR 815.-- is paid via online fee payment.

The opponent has appointed us as their representatives, and it is respectfully requested to effect all notifications to our address.

The European patent EP 3 337 478 (hereinafter referred to as the "contested patent") is opposed in its full extent (claims 1 to 14).

The opposition is based on the grounds of Article 100(a) EPC. In particular, it is submitted that the subject matter of the contested patent does not involve an inventive step.

It is requested to revoke the European patent in its entirety. Oral proceedings in accordance with Article 116 EPC are requested in the event that the Opposition Division does not reach the decision to revoke the patent on the basis of the written submissions of the opponent.

Detailed statement of the grounds for opposition

1. Bibliographic information

The contested patent is based on European application no. 16758337.6, which is the European phase of international application PCT/US2016/047727, published as WO 2017/034957 A1. Said application was filed on August 19, 2016, claiming the 7 following priorities:

1. August 21, 2015,
2. September 10, 2015,
3. December 30, 2015,
4. January 21, 2016,
5. March 2, 2016,
6. April 15, 2016 and
7. May 31, 2016.

2. Cited documents

2.1 Reference will be made to the following documents:

D1: Protocol of the clinical trial NCT02551991 entitled *"Study of Nanoliposomal Irinotecan (Nal-IRI)-Containing Regimens Versus Nab-paclitaxel Plus Gemcitabine in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma"*, version 1 of September 15, 2015;

D2: D. Von Hoff *et al.*, *Ann. Oncol.* **2014**, 25(2), ii105–ii117;

D3: R.D.W. Marsh *et al.*, *Cancer Med* **2015**, 4(6), 853–863;

D4: Onivyde prescribing information, October **2015**;

D5: J. Carnevale *et al.*, *Fut. Oncol.* **2016**, 12(4), 453-464;

D6: A. Dean *et al.*, *J. Clin. Oncol.* **2016**, 34, 4(Supp 1), Abstract TPS482, presented at the 2016 ASCO Gastrointestinal Cancers Symposium in San Francisco, CA, on January 22, 2016;

D7: H. Zhang *et al.*, *Onco Targets Ther.* **2016**, 9, 3001-3007;

D8: D.F. Gaddy *et al.*, *Cancer Res.* **2016**, 76, 14(Supp), Abstract 4830, presented at the AACR 107th Annual Meeting in New Orleans, LA, on April 16-20, 2016

D9: P. Parhi *et al.*, *Drug Discov. Today* **2012**, 17(17/18), 1044.

2.2 Documents D2, D3 and D9 were published prior to the earliest priority date of the contested patent and are thus full prior art for all claims.

2.3 Documents D1, D4 and D5 were published between the 2nd and the 3rd priority dates, document D6 between the 4th and the 5th priority dates, and documents D7 and D8 between the 6th and the 7th priority dates of the contested patent.

As we will show in the following, none of the claims of the contested patent are entitled to any of the claimed priorities. Hence, documents D1 and D4 to D8 are prior art according to Article 54(2) EPC for all claims.

2.4 Document D4 is the prescribing information of an authorised drug. Documents D3, D5, D7 and D9 are review articles. These documents are thus, by definition, an account of the common general knowledge and the state of the art prior to their own publication date (see e.g. T 777/08, r. 5.2, or T 1641/11).

2.5 Irrespective of the finding of the Opposition Division in relation to the priority entitlement, documents D5 and D7 are cited as evidence of the common general knowledge prior to the effective date of the contested patent.

3. The subject matter of the contested patent

3.1 Technical field and background

3.1.1 The contested patent relates to the first-line treatment of pancreatic cancer using liposomal irinotecan in combination with 5-fluorouracil and oxaliplatin (contested patent, paragraph [0001]).

3.1.2 The contested patent explains that pancreatic cancer is chemotherapy-resistant, with an extremely poor prognosis and median survival rates of less than one year for patients with metastatic disease (contested patent, paragraph [0002]).

3.1.3 One combination chemotherapy regimen that has emerged as standard of care for first-line treatment of metastatic pancreatic cancer is the combination therapy of 5-fluorouricil (5-FU)/leucovorin (LV) + irinotecan + oxaliplatin (FOLFIRINOX). However, FOLFIRINOX is known to have significant toxicity and its use is limited to patients with better performance status (contested patent, paragraph [0003]).

3.1.4 The contested patent further explains in the background art section that MM-398 is a liposomal irinotecan formulation marketed in the U.S. as the FDA-approved product ONIVYDE® in combination with 5-fluorouracil and leucovorin for the treatment of patients with metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy (contested patent, paragraph [0003]).

3.1.5 The object of the contested patent appears to be the provision of new treatment options for metastatic pancreatic cancer (contested patent, paragraph [0002]).

3.2 The common general knowledge of the skilled person

3.2.1 Review article D3 explains that the FOLFIRINOX regimen was introduced to clinical practice for the treatment of metastatic pancreatic cancer in 2010. Despite initial concerns over toxicity, there has been rapid uptake of this regimen, both revolutionizing management and opening the door to innovative research. As experience with FOLFIRINOX has accrued, many questions have arisen including the impact of frequent modifications to this regimen (D3, abstract).

3.2.2 The FOLFIRINOX regimen consists of oxaliplatin 85 mg/m^2, leucovorin 400 mg/m^2, irinotecan 180 mg/m^2, 400 mg/m^2 bolus 5-fluorouracil (5FU) and infusional 5FU 2400 mg/m^2 over 46 h, every 14 days. This regimen was found to be superior to gemcitabine in patients with metastatic pancreatic cancer (D3, paragraph bridging pages 853-854). While toxicity was not inconsequential, oncologists rapidly adopted the FOLFIRINOX regimen following the 2010 ASCO meeting (D3, page 854, left-hand column, second paragraph).

3.2.3 Many modifications to the FOLFIRINOX regimen have been made. Initially, physicians removed the bolus of 5FU, which is notably myelosuppressive: commonly referred to as "mFOLFIRINOX," this was the way it was often used at the priority date of the contested patent (D3, page 855, second paragraph). Commenting on a table with selected studies using FOLFIRINOX (table 3), the authors of D3 highlight again the frequent modification to the FOLFIRINOX regimen:

> *"It may once again be noted that FFX is very frequently modified"*
> (D3, page 859, right-hand column, second paragraph).

3.2.4 Document D4 is the US prescribing information of Onivyde (irinotecan liposome injection for intravenous use) and thus represents the general knowledge of the skilled person (see T 734/12, item 21). Onivyde is indicated, in combination with fluorouracil and leucovorin, for the treatment of patients with metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy (D4, page 1, left-hand column, *"Indications and usage"*). The recommended dose of ONIVYDE is 70 mg/m^2 intravenous infusion over 90 minutes every two weeks (D4, page 1, left-hand column, *"Dosage and administration"*).

3.2.5 Document D5 is a review article on Onivyde (also called MM-398, see D5, page 462, left-hand column, second paragraph) and its use for the treatment of advanced (i.e., metastatic) pancreatic cancer (D5, title). D5 highlights in its abstract the favourable safety and tolerability profile of liposomal irinotecan compared to standard irinotecan (i.e., not encapsulated in liposomes). D5 further teaches that liposomal irinotecan, due to its optimized pharmacokinetic and safety profile, may be an ideal substitute of standard irinotecan in the first-line FOLFIRINOX regimen (D5, paragraph bridging pages 462 and 463).

3.2.6 Review article D7 explains that Onivyde has been designed and developed as a nanoliposomal formulation of irinotecan, which improves the pharmacokinetics of the drug by increasing drug encapsulation and loading efficiency, protecting the drug in the active lactone configuration, prolonging circulation time, providing sustained release, rerouting the drug from

sites of toxicity such as the gastrointestinal tract, increasing tumour accumulation via the EPR effect, and reducing host toxicity (D7, page 3002, left-hand column, second paragraph). This novel nanoliposomal formulation of irinotecan could present a safe therapeutic option in the FOLFIRINOX regimen, i.e. as an alternative to standard irinotecan (D7, page 3005, left-hand column, first paragraph).

3.6.7 D7 thus notes that a randomized, open-label, phase II study of Onivyde-containing regimens vs nab-Paclitaxel + gemcitabine in patients with previously untreated, metastatic pancreatic adenocarcinoma is actively recruiting (D7, sentence bridging pages 3005 and 3006). The protocol of this study (NCT02551991) is document D1.

3.2.8 In conclusion, the person skilled in the art knew at the relevant date that the FOLFIRINOX regimen (irinotecan, oxaliplatin, leucovorin, 5-fluorouracil every 14 days), notwithstanding its toxicity, was a standard first-line treatment for metastatic adenocarcinoma of the pancreas and that said regimen was frequently modified. He or she also knew that the new liposomal formulation of irinotecan was safer and better tolerated than the standard one. For this reason, a clinical study of the FOLFIRINOX regimen using liposomal instead of standard irinotecan in the first-line treatment of metastatic pancreatic cancer was ongoing.

3.3 The claims of the contested patent

3.3.1 The contested patent comprises 14 claims. Claim 1 represents an independent claim. Claims 2, 6, 9 and 11, although formally drafted as independent claims, contain all technical features of claim 1 and are thus *de facto* dependent claims. Dependent claims 3, 4, 5, 7, 8, 10, 12, 13 and 14 refer directly or indirectly back to said claims.

3.3.2 Independent claim 1 relates to

1. *Liposomal irinotecan*
   for use in a method of treating metastatic adenocarcinoma of the pancreas
   in a human patient who has not previously received chemotherapy to treat the metastatic adenocarcinoma of the pancreas, wherein
   the liposomal irinotecan is administered in combination with oxaliplatin, leucovorin, and 5-fluorouracil,
   the method comprising administering an antineoplastic therapy to the patient a total of once every two weeks,
   the antineoplastic therapy consisting of:

a. 60 mg/m^2 of liposomal irinotecan,

b. 60 mg/m^2 oxaliplatin,

c. 200 mg/m^2 of the (l)-form of leucovorin or 400 mg/m^2 of the (l+d) racemic form of leucovorin, and

d. 2,400 mg/m^2 5-fluorouracil.

3.3.3 Claim 2 specifies that the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposome vesicles consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethyleneglycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).

Claim 3 specifies that the 5-fluorouracil is administered as an infusion over 46 hours.

Claim 4 specifies that the leucovorin is administered immediately prior to the 5-fluorouracil.

Claim 5 specifies that the liposomal irinotecan, oxaliplatin and leucovorin are administered on days 1 and 15 of a 28-day treatment cycle. Claim 6 is identical to claim 5 when depending from claim 1 and is thus redundant.

Claim 7 specifies that the liposomal irinotecan is administered as an infusion over a total of about 90 minutes.

Claim 8 specifies that the liposomal irinotecan is administered, followed by administering the oxaliplatin, followed by administering the leucovorin, followed by administering the 5-fluorouracil. Claim 9 is identical to claim 8 when depending from claim 1 and is thus redundant.

Claim 10 specifies that the administration of the oxaliplatin begins 2 hours after completing each administration of the liposomal irinotecan. Claim 11 is identical to claim 10 when depending from claims 1 and 5 and is thus redundant.

Claim 12 specifies that the administration of the 5-fluorouracil is initiated on days 1 and 15 of a 28-day treatment cycle.

Claim 13 specifies that the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposomes.

Claim 14 specifies that the liposomal irinotecan comprises irinotecan encapsulated in liposome vesicles consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethyleneglycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).

3.4 The examples of the contested patent

The contested patent contains 5 examples.

3.4.1 Example 1 (described in paragraph [0048] of the contested patent) discloses an *in vitro* simulation of exposure of tumour cells to liposomal irinotecan by means of prolonged exposure to SN-38 (an active metabolite of irinotecan). These results were disclosed in document D8.

3.4.2 Example 2 (described in paragraphs [0049-56] of the contested patent) discloses an evaluation of *in vivo* tolerability and efficacy of combination therapies in mice models using liposomal irinotecan, irinotecan, oxaliplatin and 5-fluorouracil.

3.4.3 Example 3 (described in paragraphs [0057-137] of the contested patent) discloses the protocol of a phase II comparative study to assess the safety, tolerability, and efficacy of MM-398 in combination with other anticancer therapies, compared to nab-paclitaxel + gemcitabine, in patients with metastatic pancreatic adenocarcinoma who have not received prior chemotherapy. The study will assess the following regimens: (1) MM-398 + 5-FU/LV + oxaliplatin (arm 1), (2) MM-398 + 5-FU/LV (arm 2) and (3) nab-paclitaxel + gemcitabine (arm 3). In the study, MM-398 will be administered instead of conventional irinotecan to improve the safety, tolerability, and ultimately efficacy of a FOLFIRINOX regimen. The protocol of this study was disclosed in prior art document D1.

The study will be conducted in two parts. Part 1 consists of an open-label safety run-in of the combination regimen in arm 1 (MM-398 + 5-FU/LV + oxaliplatin). Part 2 consists of an open-label, randomized, phase 2 study where patients will be randomized to one of the 3 treatment arms (1:1:1). This was disclosed in prior art document D6.

3.4.4 Example 4 (described in paragraphs [0138-0152] of the contested patent) discloses the safety interim results of part 1 of the study of example 3. As doses of 80 mg/m^2 liposomal irinotecan, 60 mg/m^2 oxaliplatin, 2,400 mg/m^2 of 5-fluorouracil and 400 (l+d) leucovorin were not well tolerated (resulting in dose limiting toxicities), the dose of liposomal irinotecan was

reduced to 60 mg/m^2. This example was not present in any of the priority documents whose priority is claimed by the contested patent.

3.4.5 Example 5 (described in paragraphs [0153-155] of the contested patent) describes the marketed product ONIVYDE® (irinotecan liposome injection), which was approved by the FDA on 15 October 2015, i.e., between the first and the second priority dates. The prescribing information of Onivyde is document D4 in this opposition. This example was not present in any of the priority documents whose priority is claimed by the contested patent and is thus a representation of the state of the art.

4. Lack of priority

4.1 <u>The claimed doses and indication</u>

4.1.1 Claim 1 of the first priority application US 62/208,209 of August 21, 2015 reads

1. *A method for treating pancreatic cancer in a human subject who has not previously received chemotherapy to treat the pancreatic cancer, the method comprising: administering to the subject a therapeutically effective amount of MM-398 liposomal irinotecan.*

4.1.2 Therefore, the first priority application was directed to the first-line treatment of pancreatic cancer in general (pancreatic cancer includes also non-adenocarcinomas, such as neuroendocrine tumours, see, e.g., paragraphs [0067-69] of the sixth and the seventh priority documents) with any therapeutically effective amount of liposomal irinotecan. The optional addition of further medicaments was specified in the dependent claims. E.g., in claim 2 the addition of leucovorin and 5-fluorouracil is indicated (i.e. a triple combination), while in claim 4 also oxaliplatin is added (quadruple combination).

4.1.3 Moreover, several dosages for the various medicaments were mentioned in the dependent claims, e.g. two preferred dosages of 60 and 80 g/m^2 for liposomal irinotecan (claim 5) and of 60, 75 and 85 mg/m^2 for oxaliplatin (claim 8). The disease to be treated could be adenocarcinoma of the pancreas (claim 18) at a stage chosen among unresectable, locally advanced or metastatic (claim 19).

4.1.4 On the contrary claim 1 of the contested patent is limited to the treatment of a specific type of cancer (adenocarcinoma) at a specific stage (metastatic) with specific dosages of 4 drugs, in particular with 60 mg/m^2 of liposomal irinotecan.

4.1.5 It goes without saying that different types of pancreatic cancers at difference stages (resectable, locally advanced or metastatic) require different chemotherapy dosages, because patients have different abilities to endure chemotherapy and related side effects: patients with a resectable tumour are generally fitter and are thus able to receive higher dosages (e.g., as neoadjuvant treatment prior to surgery), while patients with metastatic disease are generally frailer and can only endure lower dosages.

4.1.6 It is thus immediately apparent that a number of selections have been performed in relation to the dosages of the various medicaments and the type and stage of the disease to be treated, without any apparent preference for the dosages and the disease stage eventually selected in claim 1 of the contested patent.

4.1.7 On the contrary, the first priority document expressed a clear preference for a <u>different</u> dosage of liposomal irinotecan for treating metastatic adenocarcinoma of the pancreas. The first priority application teaches that such condition should be treated with 80 mg/m^2 of liposomal irinotecan and that said dosage should be reduced to 60 mg/m^2 only if the patient is homozygous for the UGT1A1*28 allele (first priority application, paragraph [0080]). Hence, granted claim 1 represents a new technical teaching that was not disclosed in the first priority application.

4.1.8 In conclusion, no direct and unambiguous disclosure of the claimed dosages for the treatment of the claimed disease and disease stage is disclosed in the first priority application. The same is true for each of the second to the sixth priority applications.

4.2 The dosage regimen

4.2.1 No disclosure of the generic administration frequency of *"a total of once every two weeks"* can be found in the first priority document.

4.2.2 Instead, claim 16 of the first priority document makes reference to an administration of *"liposomal irinotecan, oxaliplatin, leucovorin, and 5-fluorouracil on days 1 and 15 of a 28-day cycle"*. According to the priority document, all 4 drugs should be preferably administered on two specific days of a treatment cycle of a specific length.

This is a much narrower dosage regimen than the one claimed in the contested patent, which instead allows the administration of the 4 drugs on different days, as well as on days other than days 1 and 15 and treatment cycles of different lengths.

4.2.3 Indeed, granted dependent claim 5 specifies that liposomal irinotecan, oxaliplatin and leucovorin are administered on days 1 and 15 of a 28-day treatment cycle, which is thus a more preferred embodiment than the general regimen of once every two weeks.

4.2.4 Granted claim 12, which depends on claim 5(1), specifies that the administration of the 5-fluorouracil is initiated on days 1 and 15 of a 28-day treatment cycle, thus implying that, according to claim 1, 5-fluorouracil can be administered also on different days of the treatment cycle, e.g., on days 2 and 16. This is also consistent with granted claim 5, which does not mention the administration of 5-fluorouracil on days 1 and 15.

4.2.5 One should also note that a treatment cycle of 28 days is not mentioned in granted claim 1, thus allowing for shorter or longer treatment cycles of, e.g., 14, 42 days, 56 days, 70 days, etc... Indeed paragraph [0081] of the first priority application makes reference to a treatment cycle of 2 weeks, i.e., 14 days, with reference to a higher dose of liposomal irinotecan of 80 mg/m^2.

4.2.6 Summing up, all these possibilities are encompassed by granted claim 1, which is thus directed to a substantially broader dosage regimen than the one originally disclosed in the first priority application. The same is true for each of the second to the sixth priority applications.

4.3 MM-398 liposomal irinotecan

4.3.1 Claim 1 of the first priority application makes reference to *"MM-398 liposomal irinotecan"*, while only *"liposomal irinotecan"* is mentioned in claim 1 as granted. This difference is significant.

4.3.2 MM-398 is a liposomal irinotecan formulation marketed in the U.S. under the tradename ONIVYDE® (contested patent, paragraph [0003]). Its US prescribing information is document D4, which reads:

> *"ONIVYDE is a sterile, white to slightly yellow opaque isotonic liposomal dispersion. Each 10 mL single-dose vial contains 43 mg irinotecan free base at a concentration of 4.3 mg/mL. The liposome is a unilamellar lipid bilayer vesicle, approximately 110 nm in diameter, which encapsulates an aqueous space containing irinotecan in a gelated or precipitated state as the sucrose octasulfate salt. The vesicle is composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) 6.81 mg/mL, cholesterol 2.22 mg/mL, and methoxy-terminated polyethylene glycol (MW 2000)-distearoylphosphatidyl ethanolamine (MPEG-2000-DSPE) 0.12 mg/mL. Each mL also contains 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) as a buffer 4.05 mg/mL and sodium chloride as an isotonicity reagent 8.42 mg/mL."*

4.3.3 It is thus clear that MM-398 designates a very specific liposomal formulation of irinotecan. Such formulation contains 43 mg of irinotecan free base encapsulated with sucrose octasulfate, as drug entrapment agent, in a unilamellar bilayer vesicle of a certain size and with a very specific composition. Moreover, the MM-398 formulation contains HEPES and sodium chloride as further excipients.

4.3.4 However, claim 1 of the contested patent is not limited to the specific formulation of MM-398, but encompasses any liposomal irinotecan and is thus not directed to the same invention.

4.3.5 The skilled person would have certainly not considered that the teaching of the first priority document in relation to the choice of the specific liposomal formulation of irinotecan MM-398 could be applied to any liposomal irinotecan. Indeed, the choices of liposome type, composition, size, content of active ingredient, as well as the presence of further excipients are all critical to the stability of the liposomes and thus to their ability of delivering the active ingredient to the disease site.

4.3.6 The skilled person would have understood that the teaching of the dose to be administered could not be generalised to any liposomal formulation of irinotecan, having different stability and different ability of delivering the active ingredient to the disease site, thus necessarily requiring higher or lower effective doses.

4.3.7 The same problem is encountered in each of the priority applications, which disclose only the liposomal MM-398 formulation.

4.4. Conclusion

In conclusion, claim 1 of the contested patent is not entitled to any of the claimed priority dates, but only to its filing date, August 19, 2016. Hence, documents D1 and D4 to D8, published after the first priority date, but before the filing date of the contested patent, belong to the state of the art according to Article 54(2) EPC.

5. Lack of inventive step

5.1 The closest prior art

5.1.1 Document D1 discloses the protocol of a study of nanoliposomal irinotecan (Nal-IRI)-containing regimens in patients with previously untreated, metastatic pancreatic adenocarcinoma (D1, title and page 2, *"Conditions"*, *"First line pancreatic cancer treatment"*). This is the very same study disclosed in examples 3 and 4 of the contested patent. D1 is thus directed to the same purpose of the contested patent, the treatment of metastatic pancreatic adenocarcinoma in patients previously untreated for said disease (D1, title).

5.1.2 D1 discloses that the study will have two experimental arms 1 and 2 and an active comparator arm 3 (D1, page 3, *"Arms and Interventions"*). Experimental arm 1 is particularly interesting, since it involves the administration of nanoliposomal irinotecan, 5-fluorouracil, leucovorin and oxaliplatin, the very same 4 drugs claimed in claim 1 of the contested patent. Therefore, D1 has also the most technical features in common with claim 1.

5.1.3 Hence, D1 qualifies as the closest prior art for claim 1. We note that the choice of the protocol of a clinical study as the closest prior art is fully in line with the approach taken by the Boards of appeal in similar cases (see T 239/16 discussed below in more detail).

5.1.4 Alternatively, conference abstract D6 illustrating the protocol of the clinical trial of D1 (*"NCT02551991"*, D6, last sentence) can be considered as the closest prior art. In addition to D1, D6 teaches that the study will be conducted in two parts. Part 1 is a safety run-in of a regimen including the nanoliposomal formulation of irinotecan MM-398 (Onivyde), 5-fluorouracil, leucovorin and oxaliplatin. The safety run-in will enrol small cohorts of patients following a traditional 3 + 3 dose escalation design to confirm the target dose of oxaliplatin. Part 2 is the efficacy study disclosed in D1 (D6, *"Methods"*).

5.1.5 Hence, also D6 is directed to the same purpose and has the most technical features in common with claim 1 of the contested patent. It thus qualifies as the closest prior art for the alleged invention. In the following we will refer to D1 as the closest prior art for conciseness' sake. However, the same arguments apply starting from D6 instead.

5.2 The objective technical problem

5.2.1 D1 does not disclose that the antineoplastic therapy is administered to the patient once every two weeks, nor the exact dosage of each drug, i.e. 60 mg/m^2 of liposomal irinotecan, 60 mg/m^2 oxaliplatin, 200 mg/m^2 of the (l)-form of leucovorin or 400 mg/m^2 of the (l+d) racemic form of leucovorin, and 2,400 mg/m^2 of 5-fluorouracil. Moreover, D1 does not disclose the results of the clinical study.

5.2.2 As explained in paragraphs 3.2.2 and 3.2.3, above, the dosage regimen of leucovorin (400 mg/m^2 every 14 days) and 5-fluorouracil (2400 mg/m^2 every 14 days) belonged to the common general knowledge and were commonly used in the FOLFIRINOX and mFOLFIRINOX regimens. Therefore, the dosages of leucovorin and 5-fluorouracil as well as the interval of 2 weeks for the administration of the 4 drugs were standard in the art and certainly not inventive. Hence, they should not be taken in consideration in the problem-solution approach.

5.2.3 The dosage regimen of oxaliplatin in the FOLFIRINOX regimen was 85 mg/m^2 every two weeks (D3, paragraph bridging pages 853-854) and the dosage of liposomal irinotecan was 70 mg/m^2 every two weeks (D4, page 1, left-hand column, *"Dosage and administration"*). Therefore, claim 1 is directed to somewhat reduced dosages of these two drugs than those commonly used in other regimens.

5.2.4 The effect of these differences is an effective treatment of metastatic adenocarcinoma of the pancreas. Hence, the objective technical problem solved by the subject-matter of claim 1 can be formulated as the confirmation of a safe treatment of metastatic adenocarcinoma of the pancreas.

5.3 Obviousness

5.3.1 The Boards of Appeal have consistently ruled that the mere fact that a clinical trial for a given indication is ongoing gives the skilled person a reasonable expectation of successful treatment. It thus worthwhile to review the relevant case law and its applicability to the present case.

5.3.2 Decision **T 2506/12** of Board 3.3.07 deals with a combination of ET-743 (Yondelis) and pegylated liposomal doxorubicin (PLD, Doxil) for use in the treatment of cancer in a human patient. Prior-art citations disclosed that a clinical phase I trial of Yondelis (ET-743) in combination with Doxil (PLD) was in progress. The Board ruled that this information gave the skilled person a reasonable expectation of success and the performance of an already designed clinical trial amounted to a routine exercise:

> *"Thus, at the publication date of D2, the information was available that the envisaged combination treatment was considered by pharmaceutical researchers with an expectation of success sufficient to justify a clinical phase I trial. In this context it is pointed out that drug compounds to be used in a clinical trial with human subjects are not selected based on a general "try-and-see" attitude, but based on existing favourable scientific data, for both ethical and economical reasons. Thus a clinical trial is not a mere screening exercise."*
> (T 2506/12, r. 3.10, emphasis added)

> *"The reason why clinical studies are carried out at all is that they have uncertain outcomes. But they are routine tests and the fact that their outcome is uncertain does not in itself turn their results into an invention."*
> (T 2506/12, r. 3.12.2, emphasis added)

5.3.3 Decision **T 239/16** of Board 3.3.01 deals with zoledronic acid or a salt thereof for use in the treatment of osteoporosis by intravenous once-a-year administration. There was no disclosure anywhere in the prior art that the active ingredient was efficacious in any dosage for the claimed indication (T 239/16, IX, page 8, first paragraph and r. 5.2, page 29, third

paragraph). Nevertheless, the Board concluded that the skilled person would have expected all study arms to treat osteoporosis effectively:

> *"The board considers that the mere fact that an active agent selected from the group of bisphosphonates is being tested in a clinical study for the treatment of osteoporosis (as disclosed in document (55)) leads to an expectation of success, due to the fact that clinical studies are based on data obtained by preclinical testing both in vitro and in animals and require authority approval which takes ethical considerations into account. This means in the present case that the skilled person would expect all study arms to treat osteoporosis effectively, unless he was dissuaded from this by the prior art."*
> (T 239/16, r. 6.5, second paragraph)

> *"Clinical trials in humans are planned scientific investigations. They require authority approval, which is only given after a risk/benefit evaluation. For ethical (but also economic) reasons it has to be ensured that research risks are minimised and are reasonable in relation to any potential benefits. Ethical and economical considerations require that the "benefit" will arise with reasonable certainty and will not only "be hoped for"."*
> (T 239/16, r. 6.6, fifth paragraph)

5.3.4 In case T 2506/12 the prior art disclosed only that a phase I clinical trial was ongoing. Phase I trials are mainly directly at establishing the safety of certain treatment. In case T 239/16 a phase II trial was ongoing. Phase II trials test the efficacy of the investigational treatment in a small group of subjects and determine the dosage to be used in subsequent phase III trials.

5.3.5 In the present case, all 4 administered drugs were authorised drugs at the relevant date and a standard treatment for metastatic pancreatic cancer (see section 3.2 above). A similar regimen, the FOLFIRINOX regimen, was the standard treatment for metastatic pancreatic cancer and its safety and tolerability was known. Therefore, the claimed combination was already being investigated in a phase II clinical study (D1, top of page 3, *"Study Design"*).

5.3.6 Thus, at the filing date of the contested patent the claimed combination was in the same clinical phase of the investigational treatment of T 239/16, whose reasoning is thus fully applicable to the present case.

5.3.7 Moreover, the claimed combination was in a more advanced stage of development than the combination of case T 2506/12 (which was in phase I), thus substantially increasing the expectation of success compared to this decided case. Moreover, the investigational treatment of T 2506/12 was made-up of an investigational drug (ET-743, Yondelis) and an approved treatment (liposomal doxorubicin) (T 2506/12, r. 2.9-2.10). In the present case, all 4 studied drugs are drugs already individually approved for the claimed indication. Also for this reason, the expectation of success in the present case is substantially higher than that of the case of T 2506/12.

5.3.8 Since these 4 drugs were both known to be effective as anti-cancer agents in various combinations, in particular in the treatment of metastatic adenocarcinoma of the pancreas, the person skilled in the art would have expected from the disclosure of document D1 that the combination treatment disclosed therein would provide clinical efficacy in the treatment of metastatic adenocarcinoma of the pancreas.

5.3.9 Hence, the person skilled in the art would have performed the clinical trial whose protocol is already disclosed in D1 and confirmed with a reasonable expectation of success the safety and efficacy of the claimed combination for treating metastatic adenocarcinoma of the pancreas. As D1 already discloses the protocol of the trial, no scientific input was needed to design the trial. Although the disclosure of D1 cannot confer absolute certainly of success, a reasonable expectation of success is sufficient to lead to a finding of lack of inventive step.

5.3.10 It is important to note that no particular level of treatment is recited in claim 1, as long as it is effective. There is thus no requirement that the combination treatment is in any way better than the treatment of pancreatic cancer with a different dosage regimen.

The skilled person would have certainly expected that the claimed combination treats pancreatic cancer. As a matter of fact, the efficacy of all four drugs for the treatment of pancreatic cancer belonged to the common general knowledge of the skilled person (see section 3.2 above). The person skilled in the art would have thus expected that also their combination effectively treats pancreatic cancer.

5.3.11 As far as the small differences in the dosages of oxaliplatin (60 instead of 85 mg/m^2) and liposomal irinotecan (60 instead of 70 mg/m^2), it is common practice to reduce the dosing of drugs if dose-limiting toxicities are observed, especially if they are part of a multiple drug dosage regimen, wherein the toxicities of all drugs may add up. This is fully in line with the finding of T 2506/12:

"It is typical for combination treatments that interactions between the drugs may give rise to increased toxicities, which may in many cases be balanced by employing reduced dosages of each drug."
(T 2506/12, r. 3.14, emphasis added)

"it is well known that it is typical for combination therapies in cancer treatment to employ dosages lower than those used in monotherapy of each drug, usually for reasons of safety."
(T 2506/12, r. 4.3, emphasis added)

5.3.12 As a matter of fact, closest prior art document D6 already discloses that the exact dosages of the drugs, especially oxaliplatin, need to be determined in part 1 of the clinical study, a safety run-in following a traditional 3 + 3 dose escalation design. The skilled person would have thus expected that a dose reduction may have been needed.

5.3.13 Moreover, the skilled person is well aware that in combination therapy the overall therapeutic benefit of the drugs in combination is greater than the sum of the effects of the drugs individually and thus that more favourable outcomes can be achieved at a lower dose with equal or increased efficacy (review article D9, page 1045, right-hand column second paragraph, and page 1046, right-hand column, second paragraph).

5.3.14 As concluding remark, we note that the Boards of Appeal have stated several times that finding the optimum dosage is a matter of routine experimentation, which does not require inventive skill (T 1760/08, T 1409/06).

5.3.15 In case T 1409/06, the data in the patent showed that the best antiemetic effects were obtained by i.v. administration of 1 mg and 3 mg granisetron as compared to 0.1 mg disclosed in the prior art, where the effect was less accentuated. The Board of Appeal revoked the opposed European patent for the following reasons:

"The board is of the opinion that the mere determination of the dosage which yields the best effect does not involve an inventive step when, as in the present case, the effect as such is already known or obvious. The person skilled in the art is aware that the intensity of a pharmacological effect depends inter alia on the concentration of the active agent. Finding the optimum dosage is a matter of routine experimentation, which does not require inventive skill." (T 1409/06, r. 3.2.1)

5.3.16 The same applies to the present case: the treatment of metastatic adenocarcinoma of the pancreas with slightly higher dosages of oxaliplatin and liposomal irinotecan belonged to the common general knowledge. No technical effect is achieved by the claimed dose that was not already known or obvious based on the prior art.

5.3.17 In conclusion, claim 1 of the contested patent does not involve an inventive step over any of documents D1 or D6 in the light of the common general knowledge of the skilled person and thus does not fulfil the requirements of Article 56 EPC.

5.5 D3 as the closest prior art

5.4.1 Alternatively, the FOLFIRINOX dosage regimen disclosed in document D3 would be a suitable springboard for the alleged invention.

5.4.2 D3 discloses that the promising results of the FOLFIRINOX dosage regimen a phase II clinical trial were confirmed in a phase III study (D3, sentence bridging pages 853-854). Reference 5 of D3 quoted in relation to the phase II trial is entitled *"Randomized phase II trial comparing FOLFIRINOX (5FU/leucovorin [LV], irinotecan [I] and oxaliplatin [O]) vs gemcitabine (G) as first-line treatment for metastatic pancreatic adenocarcinoma (MPA). First results of the ACCORD 11 trial"* (emphasis added). Since FOLFIRINOX was used as a first line treatment, the treated patients had metastatic pancreatic adenocarcinoma that had not previously been treated with chemotherapy. D3 thus discloses the same indication and patient population of claim 1 of the contested patent.

5.4.3 D3 does not disclose that liposomal irinotecan is administered to the patients. Standard irinotecan is administered instead. Moreover, oxaliplatin is administered at a dosage of 85 mg/m^2 instead of 60 mg/m^2 as in claim 1.

5.4.4 The effect of this difference is not apparent from the contested patent, which does not compare the claimed dosage regimen with that of D3. Therefore, the objective technical problem to be solved in the light of D3 is the provision of an alternative treatment of metastatic adenocarcinoma of the pancreas.

5.4.5 D3 warns the skilled person that the toxicity of the FOLFIRINOX regimen cannot be neglected (D3, page 854, left-hand column, second paragraph). Moreover, the authors of D3 highlight the frequent modification of the FOLFIRINOX regimen:

> *"It may once again be noted that FFX is very frequently modified"*
> (D3, page 859, right-hand column, second paragraph).

5.4.6 The skilled person, starting for D3 and looking for an alternative dosage regime for the first-line treatment of metastatic adenocarcinoma of the pancreas would have thus had a strong incentive in modifying the FOLFIRINOX regimen disclosed therein. In particular, he or she would have known based on his or her common general knowledge that liposomal irinotecan MM-398 is an ideal substitute for the standard irinotecan in the FOLFIRINOX dosage regimen (D5, sentence bridging pages 462-463; D7, page 3005, left-hand column, second paragraph).

5.4.7 Moreover, the person skilled in the art would have certainly consulted document D2, disclosing the results of a phase 3 clinical trial in metastatic pancreatic cancer using MM-398, a novel encapsulation of irinotecan in a long-circulating nanoliposome (D2, *"Introduction"*).

5.4.8 D2 discloses that liposomal irinotecan (80 mg/m^2) was administered prior to 5-fluorouracil (2,400 mg/m^2 over 46 h) and racemic leucovorin (400 mg/m^2 over 30 min) every two weeks (q2w). Overall survival (OS), progression free survival (PFS), time to treatment failure (TTF), and overall response rate (ORR) were significantly improved compared to the same treatment without liposomal irinotecan (D2, *"Results"*).

5.4.9 The skilled person would have thus learned from D2 that liposomal irinotecan is not only a suitable substitution to standard irinotecan, especially when administered in combination with 5-fluorouracil and leucovorin, but even a better alternative to it. He or she would have thus replaced the standard irinotecan in the FOLFIRINOX dosage regimen with the newer liposomal formulation with the reasonable expectation of achieving an alternative treatment of metastatic pancreatic cancer.

5.4.10 Moreover, the skilled person would have adjusted the drug dosages, if necessary, without ingenuity, as explained in paragraphs 5.3.11-16 above.

5.4.11 Hence, claim 1 of the contested patent does not involve an inventive step over document D3 in combination with document D2 and thus does not fulfil the requirements of article 56 EPC.

5.5 Lack of inventive step of the remaining claims

5.5.1 Claim 2 specifies that the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposome vesicles consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethyleneglycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE). This is however the composition of the commercial product Onivyde (D4, page 11, first paragraph). Nothing inventive can be seen in detailing the composition of said product.

5.5.2 Claim 3 specifies that the 5-fluorouracil is administered as an infusion over 46 hours. This is however the standard administration time of 5-fluorouracil (D3, sentence bridging pages 853-854) and cannot be inventive.

5.5.3 Claim 4 specifies that the leucovorin is administered immediately prior to the 5-fluorouracil. This is however the standard administration sequence for these two drugs (D4, page 14, last paragraph). Moreover, no technical effect has been demonstrated by this additional technical feature. Claim 4 is thus not inventive for the same reasons of claim 1.

5.5.4 Claim 5 and 6 specify that the liposomal irinotecan, oxaliplatin and leucovorin are administered on days 1 and 15 of a 28-day treatment cycle. Again, this is the standard administration cycle of the combination of these drugs and no technical effect is attached to these additional technical features. The same considerations illustrated for claim 1 apply to claims 5 and 6.

5.5.5 Claim 7 specifies that the liposomal irinotecan is administered as an infusion over a total of about 90 minutes. This is the administration time specified in the prescribing information of Onivyde (D4, page 1, *"Dosage and administration"*) and cannot be inventive.

5.5.6 Claims 8 and 9 specify that the liposomal irinotecan is administered, followed by administering the oxaliplatin, followed by administering the leucovorin, followed by administering the 5-fluorouracil. However, no technical effect, let alone a surprising one, has been demonstrated by the claimed sequence. Claims 8 and 9 are thus not inventive for the same reasons of claim 1.

5.5.7 Claims 10 and 11 specify that the administration of the oxaliplatin begins 2 hours after completing each administration of the liposomal irinotecan. However, no technical effect, let

alone a surprising one, has been demonstrated by the claimed time interval. The same considerations illustrated for claim 1 apply to claims 10 and 11.

5.5.8 Claim 12 specifies that the administration of the 5-fluorouracil is initiated on days 1 and 15 of a 28-day treatment cycle. It is however commonplace to administer fluorouracil immediately after leucovorin, i.e., on the same day (D4, page 14, last paragraph). Moreover, no technical effect has been demonstrated by this additional technical feature. Claim 12 is thus not inventive for the same reasons of claim 1.

5.5.9 Claim 13 specifies that the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposomes. Claim 14 specifies that the liposomal irinotecan comprises irinotecan encapsulated in liposome vesicles consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethyleneglycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE). The same consideration illustrated for claim 2 apply to these claims, which are not inventive.

6. Conclusion

It has been shown above that the contested patent is not in accordance with the requirements of Article 56 EPC. Therefore, the request to revoke EP 3 337 478 in its entirety is fully justified.

Marco Fachini

Enc.
Documents D1-D9

History of Changes for Study: NCT02551991

Study of Nanoliposomal Irinotecan (Nal-IRI)-Containing Regimens in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma

Latest version (submitted February 23, 2021) on ClinicalTrials.gov

- A study version is represented by a row in the table.
- Select two study versions to compare, One each from columns A and B.
- Choose either the "Merged" or "Side-by-Side" comparison format to specify how the two study versions are to be displayed. The Side-by-Side format only applies to the Protocol section of the study.
- Click "Compare" to do the comparison and show the differences.
- Select a version's Submitted Date link to see a rendering of the study for that version.
- The yellow A/B choices in the table indicate the study versions currently compared below. A yellow table row indicates the study version currently being viewed.
- Hover over the "Recruitment Status" to see how the study's recruitment status changed.
- Study edits or deletions are displayed in ~~red~~.
- Study additions are displayed in *green*.

Study Record Versions

Version	A	B	Submitted Date	Changes
1	◉	◉	September 15, 2015	None (earliest Version on record)
2	○	○	September 28, 2015	Contacts/Locations and Study Status
3	○	○	October 26, 2015	Recruitment Status, Study Status, Contacts/Locations, Outcome Measures and Oversight
4	○	○	November 3, 2015	Study Status and Contacts/Locations
5	○	○	February 17, 2016	Study Status and Contacts/Locations
6	○	○	March 8, 2016	Study Status and Contacts/Locations
7	○	○	March 28, 2016	Contacts/Locations and Study Status
8	○	○	May 31, 2016	Outcome Measures, Study Status and Eligibility
9	○	○	July 28, 2016	Study Status and Contacts/Locations
10	○	○	December 14, 2016	Contacts/Locations and Study Status
11	○	○	April 10, 2017	Study Status, Sponsor/Collaborators and Study Identification
12	○	○	April 18, 2017	Contacts/Locations and Study Status
13	○	○	May 29, 2017	Study Status and Contacts/Locations
14	○	○	July 13, 2017	Contacts/Locations and Study Status
15	○	○	September 29, 2017	Study Status
16	○	○	February 26, 2018	Outcome Measures, Study Status, Study Design, Contacts/Locations, Eligibility and Study Description
17	○	○	May 31, 2018	Outcome Measures, Arms and Interventions, Study Status, Study Design, Eligibility and Study Description
18	○	○	August 23, 2018	Contacts/Locations and Study Status
19	○	○	November 29, 2018	Recruitment Status, Study Status, Contacts/Locations and Study Design
20	○	○	March 27, 2019	Study Status, Eligibility, Outcome Measures, Study Description and Study Identification
21	○	○	September 27, 2019	Study Status
22	○	○	March 30, 2020	Study Status
23	○	○	September 25, 2020	Study Status

Version	A	B	Submitted Date	Changes
24	○	○	October 23, 2020	Study Status and Study Identification
25	○	○	November 25, 2020	Study Status
26	○	○	February 23, 2021	Outcome Measures and Study Status

Compare Comparison Format: ◉ Merged ○ Side-by-Side

Scroll up to access the controls

Study NCT02551991
Submitted Date: September 15, 2015 (v1)

Study Identification

Unique Protocol ID: MM-398-07-02-03

Brief Title: Study of Nanoliposomal Irinotecan (Nal-IRI)-Containing Regimens in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma

Official Title: A Randomized, Open-label Phase 2 Study of Nanoliposomal Irinotecan (Nal-IRI)-Containing Regimens Versus Nab-Paclitaxel Plus Gemcitabine in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma

Secondary IDs:

Study Status

Record Verification: September 2015

Overall Status: Not yet recruiting

Study Start: September 2015

Primary Completion: March 2017 [Anticipated]

Study Completion:

First Submitted: September 10, 2015

First Submitted that Met QC Criteria: September 15, 2015

First Posted: September 16, 2015 [Estimate]

Last Update Submitted that Met QC Criteria: September 15, 2015

Last Update Posted: September 16, 2015 [Estimate]

Sponsor/Collaborators

Sponsor: Merrimack Pharmaceuticals

Responsible Party: Sponsor

Collaborators:

Oversight

U.S. FDA-regulated Drug:

U.S. FDA-regulated Device:

Data Monitoring: Yes

Study Description

Brief Summary: This is an open-label, phase 2 comparative study to assess the safety, tolerability, and efficacy of nal-IRI in combination with other anticancer therapies, compared to nab-paclitaxel + gemcitabine, in patients with advanced pancreatic adenocarcinoma who have not received prior chemotherapy. This study will assess the following regimens:

- nal-IRI + 5-FU/LV + oxaliplatin
- nal-IRI + 5-FU/LV
- nab-paclitaxel + gemcitabine

The study will be conducted in two parts:

1. a safety run-in of the nal-IRI + 5-FU/LV + oxaliplatin regimen, and
2. a randomized, efficacy study of nal-IRI + 5-FU/LV + oxaliplatin, and nal-IRI + 5-FU/LV, versus nab-paclitaxel + gemcitabine.

Detailed Description:

Conditions

Conditions: Pancreatic Cancer

Keywords: Pancreatic cancer
MM-398
Metastatic pancreatic cancer
First line pancreatic cancer treatment

Study Design

Study Type: Interventional

Primary Purpose: Treatment

Study Phase: Phase 2

Interventional Study Model: Parallel Assignment

Number of Arms: 3

Masking: None (Open Label)

Allocation: Randomized

Enrollment: 168 [Anticipated]

Arms and Interventions

Arms	Assigned Interventions
Experimental: Arm 1 nal-IRI + 5-FU/LV + oxaliplatin	Drug: nal-IRI Other Names: • MM-398 Drug: 5 fluorouracil Other Names: • 5-FU Drug: LeucovorinDrug: Oxaliplatin
Experimental: Arm 2 nal-IRI + 5-FU/LV	Drug: nal-IRI Other Names: • MM-398 Drug: 5 fluorouracil Other Names: • 5-FU Drug: Leucovorin
Active Comparator: Arm 3 nab-paclitaxel + gemcitabine	Drug: nab-paclitaxelDrug: Gemcitabine

Outcome Measures

Primary Outcome Measures:

1. Progression Free Survival (PFS) at 24 weeks
   up to 18 months

Eligibility

Minimum Age: 18 Years

Maximum Age:

Sex: All

Gender Based:

Accepts Healthy Volunteers: No

Criteria: Inclusion Criteria:

- Pathologically confirmed adenocarcinoma of the pancreas not previously treated in the metastatic setting

Part 1: Unresectable, locally advanced or metastatic disease; diagnosed within 6 wks prior to enrollment; Part 2: Metastatic disease; diagnosed within 6 wks prior to randomization

- Measurable or non-measurable disease as defined by RECIST v1.1
- ECOG performance of 0 or 1
- Adequate hematological, hepatic, renal and cardiac function

Exclusion Criteria:

- Prior treatment of pancreatic cancer in the metastatic setting with surgery (placement of stent is allowed), radiotherapy, chemotherapy or investigational therapy
- Prior treatment of pancreatic cancer with chemotherapy (radiation sensitizer allowed if ≥ 6 months has elapsed from completion)
- Known metastasis to the central nervous system
- Clinically significant gastrointestinal disorder
- History of any second malignancy in the last 3 years. Patients with prior history of in-situ cancer or basal or squamous cell skin cancer are eligible
- Presence of any contraindications for nal-IRI, irinotecan, 5-FU, leucovorin, oxaliplatin, nab-paclitaxel (part 2 only) or gemcitabine (part 2 only)
- Use of strong CYP3A4 or CYP2C8 inhibitors or inducers (part 2 only)
- Pregnant or breast feeding

Contacts/Locations

Study Officials: Eliel Bayever, MBBCh, MRCP
Study Director
Merrimack Pharmaceuticals

Locations:

IPDSharing

Plan to Share IPD:

References

Citations:

Links:

Available IPD/Information:

Scroll up to access the controls Scroll to the Study top

Annals of Oncology 25 (2): ii105–ii117, 2014
doi:10.1093/annonc/mdu193.3

oral abstracts

O_0003 **NAPOLI-1: RANDOMIZED PHASE 3 STUDY OF MM-398 (NAL-IRI), WITH OR WITHOUT 5-FLUOROURACIL AND LEUCOVORIN, VERSUS 5-FLUOROURACIL AND LEUCOVORIN, IN METASTATIC PANCREATIC CANCER PROGRESSED ON OR FOLLOWING GEMCITABINE-BASED THERAPY**

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Introduction: MM-398 (nal-IRI) is a novel encapsulation of irinotecan in a long-circulating nanoliposome. MM-398 had clinical activity in a Phase 2 study of pts with metastatic pancreatic adenocarcinoma (mPAC) after prior gemcitabine-based therapy.

Methods: Pts with mPAC after prior gemcitabine-based therapy, were randomized 1:1:1 in an open-label study to receive: (A) MM-398 (120 mg/m^2 IV over 90 min) q3w; (B) 5FU (2,000 mg/m^2 over 24 h) plus racemic leucovorin (LV) (200 mg/m^2 over 30 min) x 4w followed by 2w rest; or (C) combination of MM-398 (80 mg/m^2 IV over 90 min) prior to 5FU (2,400 mg/m^2 over 46 h) and racemic LV (400 mg/m^2 over 30 min) q2w. The primary endpoint was OS in arms A and C, each vs. the control arm B.

Results: A total of 417 patients were randomized, of which 398 received treatment. Baseline characteristics were balanced, 61% head of pancreas, and 68% liver metastases. OS, PFS, TTE, and ORR were significantly improved by MM-398 + 5FU/LV vs. 5FU/LV. Median OS was 6.1m (95% CI: 4.8–8.9) and 4.2m (3.3–5.3), respectively, HR = 0.67, p = 0.012; and median PFS 3.1m (2.7–4.2) and 1.5m (1.4–1.8), respectively, HR = 0.55, p < 0.001. MM-398 alone did not demonstrate a statistical improvement in efficacy. Major grade ≥3 AEs in the MM-398 + 5-FU/LV, MM-398 and 5-FU/LV arms were neutrophil count decreased (23.1%, 15.2%, 3%), fatigue (13.7%, 6.1%, 3.7%), diarrhea (12.8%, 21.1%, 4.5%), and vomiting (11.1%, 13.6%, 3.0%), respectively; neutrophil count decreased was based on lab. values [by investigator report, "neutropenia". (14.5%, 5.4%, 0.7%) and "neutrophil count decreased" (10.3%, 8.2%, 0.7%) respectively]. Additional AEs of interest were febrile neutropenia (1.7%, 4.1%, 0%) and sepsis (3.4%, 2.0%, 0.7%) in MM-398 + 5FU/LV, MM-398 and 5FU/LV respectively.

Cancer Medicine

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REVIEW

Pancreatic cancer and FOLFIRINOX: a new era and new questions

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Keywords
Chemotherapy, FOLFIRINOX, genomics, modifications, pancreatic cancer, toxicity

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Cancer Medicine 2015; 4(6):853–863

doi: 10.1002/cam4.433

Abstract

FOLFIRINOX (FFX) was introduced to clinical practice in 2010 following publication of the PRODIGE 4/ACCORD 11 study, which compared this novel regimen to gemcitabine in metastatic pancreatic cancer. Median overall survival, progression-free survival, and objective responses were all superior with FFX and there was improved time to definitive deterioration in quality of life. Despite initial concerns over toxicity, there has been rapid uptake of this regimen, both revolutionizing management and opening the door to innovative research. As experience with FFX has accrued, many questions have arisen including the management of toxicities, the impact of frequent modifications, the optimal number of cycles, integration with other regimens and modalities, interpretation of radiologic and serologic response, utility of molecular signatures, and potential benefit in unique clinical settings such as pre- and postsurgery. This review will closely examine these issues, not only to summarize current knowledge but also to fuel scientific debate.

Introduction

Historical context

Previously published studies have suggested that combination therapy could be an improvement on gemcitabine alone. These include the phase III study of gemcitabine versus gemcitabine plus erlotinib [1], the phase III study of gemcitabine versus gemcitabine plus capecitabine [2], and the phase II study of GTX (gemcitabine, taxotere, and capecitabine) [3, 4]. In the first study, overall survival (OS) (median 6.24 vs. 5.91 months, HR = 0.82, 95% CI = 0.69–0.99; $P = 0.038$), 1-year survival (23% vs. 17%; $P = 0.023$), and progression-free survival (HR = 0.77, 95% CI = 0.64–0.92; $P = 0.004$) were better with gemcitabine plus erlotinib. In the second study, objective response rate (19.1% vs. 12.4%; $P = 0.034$) and progression-free survival (HR = 0.78; 95% CI = 0.66–0.93; $P = 0.004$) favored the combination and there was a trend toward improved OS (7.1 vs. 6.2 months, HR = 0.86, 95% CI = 0.72–1.02; $P = 0.08$). In the GTX study, median progression-free survival of responders was 6.3 months (95% CI = 4.4–10.4 months) and median survival was 11.2 months (95% CI = 8.1–15.1 months). While certainly of interest, the clinical benefit of these regimens was either marginal, of uncertain impact on quality of life, or achieved in very small numbers, resulting in sporadic and unenthusiastic uptake.

FOLFIRINOX

Promising phase II results with FOLFIRINOX (FFX) [5] (oxaliplatin 85 mg/m^2, leucovorin 400 mg/m^2, irinotecan 180 mg/m^2, bolus 5-fluorouracil 5FU) (400 mg/m^2, infu-

sional 5FU 2400 mg/m^2 over 46 h, every 14 days) were confirmed in a sentinel phase III study (PRODIGE 4/ACCORD 11) [6], which randomized patients ≤75 years of age with metastatic pancreatic cancer and an ECOG PS of 0 or 1, to receive either gemcitabine or FFX. With a median follow up of 26.6 months and with 171 patients in each arm (38% of patients had lesions in the head of the pancreas with 14.3% requiring biliary stents), the median survival with FFX was 11.1 months versus 6.8 months for gemcitabine ($P < 0.001$, HR = 0.57, 95% CI = 0.45–0.73). More impressively, 1-year survival was 48.4% versus 20.6%, respectively, and this difference was sustained at 18 months, 18.6% versus 6%. Quality of life measures, equally, strongly favored the FFX group [7].

While toxicity was not inconsequential (45.7% grade 3 or 4 neutropenia, 5.4% febrile neutropenia, 12.7% diarrhea, 9.1% thrombocytopenia, 9.0% sensory neuropathy), oncologists rapidly adopted the FFX regimen following the 2010 ASCO meeting [8]. Many questions have now arisen such as: best management of common and uncommon toxicities; potential impact of adjustments to the original regimen; number of cycles administered for optimal results; innovative strategies in early disease; radiologic and serologic assessment of response; evolving data on integration into overall treatment planning; and utility of molecular profiling.

In order to derive the data used in this review, all relevant papers in Medline, CANCERLIT, and Index Medicus together with meeting abstracts from ASCO, ASTRO, and AACR since 1990, were examined. No ethnic or racial group or gender was excluded. Approximately 65% of discovered references have been included based on relevance, timeliness, and quality of data.

How is Toxicity of FFX Best Managed?

As with usual practice, reduction in individual drug dosing is a standard approach for many of the common complications such as low blood counts, fever, infection, diarrhea, weight loss, and fatigue. However, some problems engendered by FFX are either idiosyncratic, not dose related, or not manageable with simple dose reduction and may require more innovative strategies (Table 1).

If platelet counts are problematic despite dose modifications, then splenectomy, either surgical [9] or by endovascular means using an embolic approach [10], can help in selected patients. The typical phenotype would be someone who is responding to chemotherapy, with a good functional status, but who has isolated thrombocytopenia (<90 × 10^3/μL). In the surgical series, counts increased significantly ($P < 0.01$) with a mean value of 87 × 10^3/μl prior to treatment and 425 × 10^3/μL on discharge (average 3 days later). All patients were able to resume chemotherapy within a median of 11.5 days (range 6–27). The IR procedure could be particularly useful in those either too frail for surgery or for whom surgery is relatively contraindicated (e.g., disease in the splenic hilum or carcinomatosis). Complications of postoperative pain and splenic abscess are limiting factors [11] and relative efficacy is unknown.

Infusion reactions are common and desensitizing protocols may be needed [12]. A significant hypercholinergic response with excess salivation, cramping, and sweating, related to the piperidine structure of irinotecan, which mimics a cholinergic drug when metabolized by esterases to form SN-38, is not unusual [13]. The potentiating role of oxaliplatin is real but not well understood [14]. Slowing of the infusion, aggressive medication with atropine, and a proton pump inhibitor may be required.

The common problems of oral dysesthesia and thick tongue, and the rare complication of total body weakness, near paralysis and even coma from oxaliplatin may be difficult to manage. Slowing the infusion and a warm drink works best for the former, while aggressive correction of serum potassium and calcium prior to, and following, the infusion may resolve the latter [15, 16].

Table 1. Management of FOLFIRINOX toxicity.

Toxicity	Strategy	Concern
Low blood counts, fatigue, diarrhea, mucositis	Decrease doses of one or more of the drugs; lomotil/pegfilgrastim	Decreased efficacy of therapy; bone pain
Low platelet counts despite appropriate dose reduction	Splenectomy—surgical or via interventional radiology	Pain; abscess formation; treatment delay
Acute allergic reaction to oxaliplatin infusion	Desensitization protocol and possible discontinuation	Ineffective to resolve problem; resources
Hypercholinergic reaction with cramping and sweating	Slow infusion rate and premedicate with atropine	Prolonged treatment time; resources
Oral dysesthesia with sense of swollen tongue	Slow infusion rate and warm drink	Prolonged treatment time; anxiety; resources
Weakness, paralysis, and even coma	Maintenance of normal potassium and calcium prior to and during infusion	Patient anxiety; staff anxiety; imperfect results

Do Modifications to the FFX Regimen Matter?

Oncologists in the United States and elsewhere were anxious to use FFX, but initially concerned about toxicity, particularly in patients with lesions in the head of the pancreas and with biliary stents. A Canadian report suggested that there could be considerable toxicity when the regimen is used outside of a clinical study and in community centers [17]. In their series of 46 patients, there were 3 (7%) treatment-related deaths, 54% of patients were hospitalized with sepsis, 33% had neutropenia grade ≥3, 15% had diarrhea grade ≥3, and 4 (9%) patients had febrile neutropenia.

With this scenario in mind, many modifications have been made (Table 2). Initially, physicians removed the bolus of 5FU, which is notably myelosuppressive, with some adding pegfilgrastim 6 mg on day 3 or 4. Commonly referred to as "mFOLFIRINOX," this seems to be the way it is often used today [18]. Historically, a bolus of 5FU has been used in the majority of fluoropyrimidine regimens, together with a more prolonged infusion to maximize total exposure [19]. A Japanese study shows that the bolus contributes significantly to the overall exposure to 5FU via AUC [20]. In addition, 5FU functions differently depending on how it is administered [21] and thus, theoretically, the omission of the bolus could lead to loss of efficacy. Data reported at the 2014 GI ASCO meeting suggest, however, that this may not be the case, and longer follow-up will be needed for clarification [22].

A further dilemma concerns the omission of leucovorin, should the bolus of 5FU be removed. Previous dose-finding studies of infusional 5FU with leucovorin clearly demonstrated that there is considerable synergy, and that omission of leucovorin results in less toxicity [23], suggesting that efficacy could equally be impacted. Absent real data, and given the low cost of leucovorin, it seems reasonable to leave it untouched.

Ohio State physicians reported their experience with limiting irinotecan to 165 mg/m^2 in addition to these changes, in either locally advanced or borderline resectable disease. They concluded that the modified regimen was effective and well tolerated with no episodes of grade 3 or 4 neutropenia/thrombocytopenia, but with 46% of patients requiring a dose reduction for other toxicities [24]. Similarly, physicians at Yale reported that in their hands dose reductions were common (relative dose intensities: oxaliplatin 88%, irinotecan 64%, bolus 5FU 57%, infusional 5FU 100%, compared to oxaliplatin 78%, irinotecan 81%, and 5FU 82%—PRODIGE 4/ACCORD 11) [25]. Despite these modifications, efficacy was comparable to that of the original regimen—response (CR + PR 33% —similar to historical data 31.6%; $P = 0.21$), and toxicity was notably less (grade 3 or 4 neutropenia 6.4%, $P < 0.0001$; fatigue 9.6%, $P < 0.02$).

For frail and elderly patients, additional adjustments have been made. In a series of 19 patients over age 65,

Table 2. FOLFIRINOX dose modifications and results.

Author	Modification	Results/comments
Mahaseth et al. [18]	Drop 5FU bolus	Grade 4 neutropenia 3%
	Add pegfilgrastim 6 mg	Grade 3/4 diarrhea 13%, fatigue 13%
		OS 9.0 months, PFS 8.5 months
Blazer et al. [24]	Drop 5FU bolus	Grade 3/4 neutropenia or thrombocytopenia 0%
	Decrease irinotecan to 165 mg/m^2	46% further dose reductions for other toxicities
	Add pegfilgrastim 6 mg	
Gunturu et al. [25]	Median dose intensity 5FU bolus 57%	Grade 3/4 neutropenia 6.4%
	Median dose intensity oxaliplatin 88%	Grade 3/4 fatigue 9.6%
	Median dose intensity irinotecan 64%	CR plus PR 31.6%
Metges et al. [27]	Median dose intensity 5FU bolus 82%	Grade 3/4 hematologic and neurotoxicity 32%
	Median dose intensity oxaliplatin 78%	Response rate 39%
	Median dose intensity irinotecan 81%	PFS 6.5 months
		OS 10.9 months
Alessandretti et al. [26]	Drop 5FU bolus	Grade 3/4 neutropenia 21% or thrombocytopenia 5%
	Decrease 5FU infusion to 2000 mg/m^2	Grade 3/4 fatigue 15.7%
	Decrease oxaliplatin to 50 mg/m^2	CR plus PR 31.7%
	Decrease irinotecan to 135 mg/m^2	OS and PFS not reached at 4 months
	Add pegfilgrastim 6 mg	
James et al. [22]	Decrease 5FU bolus 25%	Grade 3/4 neutropenia 17% or thrombocytopenia 11.3%
	Decrease irinotecan 25%	Grade 3/4 fatigue 11.3%
	Add pegfilgrastim 6 mg	CR plus PR 29%

the bolus of 5FU was dropped and doses of both oxaliplatin and irinotecan were lowered (5FU 2000 mg/m^2 over 46 h, oxaliplatin 50 mg/m^2, irinotecan 135 mg/m^2) [26]. Grade 3/4 toxicities were reported in 10 patients: nausea/vomiting in one, diarrhea in one, fatigue in three, neutropenia in four, thrombocytopenia in one, and febrile neutropenia in three—all manageable. A follow-up study by the original investigators in the PRODIGE 4/ACCORD 11 study, based on their established criteria, showed that 81% of 242 patients required a dose reduction, but that this did not affect results (response rate 39% vs. 32%, PFS 6.5 vs. 6.4 months and OS 10.9 vs. 11.1 months) [27].

A biologically based refinement, using genotype-derived dosing of irinotecan via UGT1A1, the enzyme that inactivates SN-38 (the active metabolite of irinotecan) showed that those with a *28*28 genotype are at highest risk of severe neutropenia, *1*28 at intermediate risk, and *1*1 at lowest risk [28]. Initial doses of irinotecan could be adjusted accordingly.

A close examination of clinicaltrials.gov confirms that the majority of regimens presently under investigation incorporate some modification of FFX.

How is the Number of Treatment Cycles with FFX Determined?

The optimal number of treatment cycles is not well understood, but the goal of therapy (i.e., curative vs. palliative) is critical in this regard. The disease should be unambiguously defined as either resectable, borderline resectable, locally advanced unresectable, or metastatic. This has implications for ensuring that treatment is not unnecessarily modified, or conversely, that excessive treatment (and toxicity) is not given. This is simplest in a palliative setting, where duration and intensity of treatment is determined by response and quality of life. The median number of cycles in the original PRODIGE 4/ACCORD 11 study was 10, with a range of 1–47 [6]. In locally advanced and borderline resectable disease, it is common to use four cycles of FFX (± chemo/RT) in a neoadjuvant strategy (e.g., ALLIANCE/Intergroup study A021101). This is based on very limited data, and an alternative approach might be to treat to maximal response and/or maximum-tolerated dose. A retrospective study of this strategy in borderline (60%) and locally advanced, unresectable (40%) disease examined outcomes in 18 patients [29]. An R0 resection was ultimately possible in 44% of patients, with a median number of six cycles (range 5–17) prior to surgery. A report on FFX plus chemo/RT in 22 patients with locally advanced, unresectable disease, examined use of an initial four cycles with an additional four cycles prior to chemo/RT, if disease was either stable or improved [30]. A median of eight cycles was administered, with 12 patients taken to the OR and 5 (42%) were able to have an R0 resection. However, three patients developed distant recurrence within 81 days, confirming their dismal prognosis.

Steatohepatitis (irinotecan) and sinusoidal obstructive syndrome (oxaliplatin) are dose-related complications which effect outcome in liver resection for colorectal cancer [31]. A Whipple operation, in and of itself, leads to an increase in hepatic steatosis [32]. Further, a BMI exceeding 25 kg/m^2, diabetes mellitus, and preexisting steatosis all significantly increase the risk of steatohepatitis and postoperative morbidity [33]. These data suggest that the number of cycles be limited to the minimum necessary, as the effects on patients undergoing a Whipple operation are as yet unknown.

Complicating matters further, pancreatic cancer is clearly a heterogeneous disease [34]. Aggressive subsets (if they do respond) may require three or four cycles of therapy before showing a decline in CA 19-9, implying response, and may conceivably require further cycles of chemotherapy prior to surgery.

In locally advanced (arterial encasement) or metastatic disease, initial intensive therapy could be followed by omission of either oxaliplatin or irinotecan (depending on which is more problematic) for continuation of a "maintenance program," as this is strictly palliative therapy. While there are few publications on the efficacy of FOLFOX or FOLFIRI, those that do exist are positive [35–37].

Is Preoperative or Postoperative FFX the Optimal Strategy for Potentially Resectable Disease?

One of the most intriguing questions currently under study is whether FFX will improve on results in the adjuvant therapy of resectable pancreatic cancer. A recent update of the CONKO-001 study shows that median OS is 22.8 months in the gemcitabine group versus 20.2 months in the observation group (HR = 0.76, P = 0.01) [38]. OS at 5 and 10 years is 20.7% versus 12.2% and 10.4% versus 7.7%, respectively – all dismal numbers.

Studies comparing gemcitabine with combination therapy, and even vaccine therapy, have failed to improve on these results [39–41]. There are no data as yet on FFX in the adjuvant setting (PRODIGE 24/ACCORD 24—gemcitabine vs. mFFX; and Marsh et al. four cycles of mFFX pre- and postsurgery, are in progress) (clinicaltrials.gov). The latter approach is intriguing as early systemic treatment, prior to surgical intervention, is attractive for many reasons: better selection of patients for surgery based on the exclusion of those with rapidly progressive disease; better tumor exposure to chemotherapy prior to disrup-

tion of the vasculature; ability to gauge response; better tolerance of chemotherapy prior to debilitating surgery; and increased R0 resections. Furthermore, pancreatic cancer has been shown to be systemic from the earliest stages [42–44] and thus an early systemic approach is not only logical but may also be essential.

Previous studies of neoadjuvant therapy in resectable patients include gemcitabine plus radiation (73/86 were taken to surgery, with 64/86 undergoing successful surgery) [45]; and gemcitabine plus nab-paclitaxel (14/25 completing the planned three cycles, with surgery in 20/25, 19/20 R0) [46]; (9/16 undergoing surgery at the time of reporting, with 8/9 R0 resections) [47].

The University of Michigan reported improved 1- and 3-year OS, lower margin and node positivity, and minimal additional perioperative toxicity in a retrospective review of various neoadjuvant regimens in borderline resectable disease [48]. University of Washington similarly reported almost doubling of OS in a small series of patients with both resectable and borderline resectable disease (neoadjuvant GTX vs. historical controls) [49], and Columbia was able to convert 57% of inoperable patients to operable with 49% R0 resections [50]. Finally, the Medical College of Wisconsin reported on mFFX followed by radiation therapy in borderline resectable disease and found this approach both safe and favorable compared to historical controls [51]. The ALLIANCE/Intergroup A021101 study is examining the feasibility of mFFX for four cycles followed by RT with oral capecitabine in a multi-institutional setting. Gemcitabine is given in the adjuvant space. The primary endpoint is 1-year OS and there are multiple levels of quality control to ensure validity (clinicaltrials.gov).

How Best Can Response to FFX Therapy be Assessed?

Both serologic and radiographic response to therapy has come under increasing scrutiny. CA 19-9 has been used for decades as a serum marker in pancreatic cancer in Lewis antigen-positive individuals [52, 53]. However, this is complicated by the fact that biliary obstruction, pancreatitis, intestinal inflammation, and even elevated blood glucose [54] all lead to an increase in CA 19-9. While there is evidence that there is a difference in outcome between no responders and stable or good responders [55, 56], there are opposing findings suggesting that there may be no correlation [57], and additional data are awaited.

Change in tumor dimensions, as assessed on CT scan and/or MRI, is both challenging to measure and often insignificant [58]. In a study of 129 patients with borderline resectable tumors, post therapy, presurgical imaging suggested that only 1% had been down staged, 78% had no change, and 21% had progressive disease [59]. In fact, 66% were able to undergo resection with 95% R0 resections. Provided the patient has acceptable performance status and no evidence of metastatic disease, even where there is no obvious radiographic response, surgery should proceed as pathology may indicate clear-cut treatment effect [60]. Whether pathologic response has any meaning in the clinical context awaits further clarification, but initial reports suggest that more than 5% viable cells in the final specimen portends a bad outcome [61, 62].

While endoscopic ultrasound can be valuable [63], novel ways of imaging the tumor, such as perfusion imaging [64], dynamic PET scans [65] and routine CT scan derived mass transport parameters, are increasingly being incorporated into investigational algorithms [66].

How is FFX Optimally Combined with Radiation Therapy?

Many protocols in borderline and locally advanced, unresectable disease switch to radiation therapy following initial FFX [51]. However, the precise role of radiation in these settings is the subject of ongoing debate. The LAP 07 study found that in locally advanced disease, chemo/radiation had no effect on OS compared to continued chemotherapy alone (over 40% of patients developed metastatic disease prior to being randomized to radiation or not) in those patients stable after an initial phase of gemcitabine ± erlotinib [67]. Updated results in 2014 suggested less local recurrence in the CRT arm (34% vs. 65%, $P < 0.0001$). The true impact of radiation may not be fully evaluable until systemic disease control improves further. An upcoming study will re-explore this question: the three-arm randomized phase II RTOG 1201 study, which is evaluating systemic chemotherapy alone (gemcitabine plus nab-paclitaxel) versus capecitabine plus standard versus intensified local RT (50.4 Gy vs. 63 Gy) preceded and followed by systemic therapy. Given that FFX is notably more active than gemcitabine in metastatic disease [6], the combination of radiation with FFX deserves to be examined and novel approaches such as SBRT [68, 69], may make it possible to do so.

How Best to Combine FFX with Other Regimens?

Recently, the MPACT study reported on gemcitabine plus nab-paclitaxel (GN) versus gemcitabine in 861 patients with metastatic pancreatic cancer [56]. Median OS was 8.5 versus 6.7 months (HR for death, 0.72; 95% CI = 0.62–0.83; $P < 0.001$), and progression-free survival was 5.5 versus 3.7 months (HR = 0.69, 95% CI = 0.58–0.82; $P < 0.0001$). While less than that of FFX in the

Table 3. Selected current studies using FOLFIRINOX in all stages of pancreatic cancer.

Setting	Study	Regimen	Goal	Opened
Resectable neoadjuvant	NorthShore/University of Chicago Pilot study	mFFX—no 5FU bolus—four cycles pre- and postop	Assess safety and efficacy (R0, ORR, PFS, and OS)	August 2012
Resectable neoadjuvant	Indiana University Phase II study	Standard full dose FFX—four cycles preoperatively	Assess safety and efficacy (Path CR, DFS, OS, ORR)	June 2014
Resectable neoadjuvant	Yale/NCI Phase II study	mFFX—no 5FU bolus—six cycles pre and post op	Assess safety and efficacy (R0, path CR, PFS, and OS)	January 2014
Resectable adjuvant	PRODIGE 24/ACCORD 24 Phase III	mFFX—no 5FU bolus—versus gemcit, each for 24 weeks	DFS, OS, specific survival	February 2012
Resectable adjuvant	Krankenhaus Nordwest Phase II/III	Standard full dose FFX—six cycles pre and postop vs. gemcit postop	Assess safety and efficacy (OS, PFS, R0, path CR)	Opening pending
Resectable adjuvant	Sidney Kimmel Comprehensive Cancer Center Pilot Study	SBRT plus Vaccine (GVAX)/cyclophosphamide then standard full dose FFX—six cycles with GVAX	Toxicity, safety, OS, DFS, TTF	April 2012
Borderline resectable	ALLIANCE A021101 Pilot study	mFFX—no 5FU bolus—four cycles, then RT/cape gemcit postop	Accrual rate, toxicity, CR/PR, completion of all therapy, R0/R1	March 2013
Borderline resectable	Medical University of South Carolina Phase II	mFFX—no 5FU bolus—six cycles then RT/cape	R0/R1 resection (OS, TTF, ORR, path CR) and safety	August 2012
Borderline resectable	University of Maryland Pilot Study	mFFX—no 5FU bolus—four cycles then SBRT	Resectability, DFS, OS, TTR, path CR and safety	September 2013
Locally advanced	UNC LINEBERGER Phase II	Standard full dose FFX	Assess safety and efficacy (OS, PFS, ORR)	September 2012
Locally advanced	Foundation for Liver Research/Erasmus Medical Center Phase II	Standard full dose FFX—four cycles then SBRT	OS, radiologic RR, Resection rate, PFS, Biologic predictive markers	July 2014
Locally advanced	Massachusetts General Hospital/NCI Phase II	Standard full dose FFX—eight cycles plus losartan then proton beam RT	Feasibility, PFS, OS, toxicity, downstaging, gene mutations	March 2013
Metastatic disease	University of Chicago Phase II	Modified FFX—irinotecan dose determined by UGT1A1 status, no 5FU bolus	DLT in course 1; RR, cumulative dose intensity of irinotecan	July 2012
Metastatic disease	Institut Cancerologie de l'Ouest Phase II	Modified FFX—irinotecan dose determined by UGT1A1 status; 5FU dose by DPD expression	Safety, toxicity and efficacy (OS, PFS)	May 2L14
Metastatic disease	Centre Val d'Aurelle—Paul Lamarque Phase I–II	Standard Gemcitabine plus nab-paclitaxel followed by standard fFX	MTD; Phase II dosing; RR	August 2013

ORR, overall response rate; PFS, progression-free survival; OS, overall survival, CR, complete remission; gemcit, gemcitabine; SBRT, stereotactic body radiation therapy; TTF, time-to-treatment failure; cape, capecitabine; TTR, time to response; DLT, dose-limiting toxicity, DPD, dihydropyrimidine dehydrogenase; MTD, maximum-tolerated dose; FFX, FOLFIRINOX; postop, postoperative.

PRODIGE 4/ACCORD 11 study—11.1 months [6], median OS is significant enough to be of major interest, raising the issue of how best to integrate these two regimens in a comprehensive treatment plan. One of the more interesting questions is whether there is synergism, and whether pretreatment with GN would alter the cancer-associated stroma such that FFX would be more effective. A recent phase II study used up to six cycles of GN followed by consolidation with FFX for up to 12 cycles and was deemed feasible [70]. A case report from Germany, reported success with this approach in locally advanced disease [71].

The efficacy of GN following failure of FFX is unknown. In a retrospective study from Yale, 23 patients were so treated with an estimated time-to-treatment failure of 11 weeks, about half of that in first-line GN [72]. Interestingly, dose densities of only 56.9% and 63.5% for nab-paclitaxel and gemcitabine, respectively, were achieved which suggest that alternative dosing schedules should be examined.

Innovative approaches currently under investigation include addition of a Hedgehog inhibitor to FFX [73]; combination of FFX, SBRT, and GVAX as adjuvant therapy; and a combination of FFX and hyperacute vaccine in borderline and locally advanced disease. As we learn more, it is hoped that future study design will be based on biology and molecular profiling of tumors, rather than empiricism or intuition.

How Do We Use Molecular Signatures in Planning FFX Treatment?

There is an increasing interest in the molecular profiling of cancers. Certainly, patients testing positive for a BRCA 1 or BRCA 2 mutation might have increased sensitivity to a platin [74], but this has uncertain practical value. PARP inhibitors might be more effective [75]. From the Pancreatic Cancer Genome Project, we know that pancreatic cancers contain an average of 63 genetic alterations, the majority of which are point mutations [76]. A core set of 12 cellular signaling pathways and processes are defined by these alterations in 67–100% of tumors. KRAS, Hedgehog, Wnt/Notch, SMAD4, and TGF-β signaling pathways are key, with abnormalities of one or more of these pathways in 100% of cancers. The effects on therapy with FFX are as yet unknown.

Candidates for future study include predictors of drug metabolism and toxicity—ERCC1 expression (oxaliplatin) [77], UGT 1A1 genotype (irinotecan) [28], thymidylate synthase expression (5FU) [78], HENT-1 expression (gemcitabine—both positive and negative studies) [79, 80] and SPARC expression—both nab-paclitaxel [81] and gemcitabine [82].

What Important Clinical Studies are Currently Underway in Pancreatic Cancer Using FFX Alone or in Combination?

As a final note, it is relevant to include a table of selected current and ongoing studies using FFX in all stages of pancreatic cancer (Table 3). These studies have been selected from many for their potentially significant impact on the use of this regimen in the future. It may once again be noted that FFX is very frequently modified.

Summary

FFX has had a major impact on the treatment of pancreatic cancer. As experience with this regimen has accrued, and as we have learned how to manage the toxicities, we have been presented with a new set of questions: the effect of frequent modifications; optimal use in all stages of pancreatic cancer; integration with both established and emerging therapies; how to evaluate response; and the incorporation of evolving molecular data. Furthermore, while metastasectomy in pancreatic cancer has historically been fraught with futility and failure, the markedly improved activity of FFX [5, 6] could mean that the time to study surgery plus FFX (in highly selected patients) is near [83, 84]. The next few years should prove to be exciting for all working to improve the outlook for this challenging group of patients.

Conflict of Interest

None declared.

References

1. Moore, M. J., D. Goldstein, J. Hamm, A. Figer, J. R. Hecht, S. Gallinger, et al. 2007. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J. Clin. Oncol. 25:1960–1966.
2. Cunningham, D., I. Chau, D. D. Stocken, J. W. Valle, D. Smith, W. Steward, et al. 2009. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J. Clin. Oncol. 27:5513–5518.
3. Fine, R. L., D. R. Fogelman, S. M. Schreibman, M. Desai, W. Sherman, J. Strauss, et al. 2008. The gemcitabine, docetaxel, and capecitabine (GTX) regimen for metastatic pancreatic cancer: a retrospective analysis. Cancer Chemother. Pharmacol. 61:167–175.
4. Dakik, H. K., D. J. Moskovic, P. J. Carlson, E. P. Tamm, W. Qiao, R. A. Wolff, et al. 2012. The use of GTX as

second-line and later chemotherapy for metastatic pancreatic cancer: a retrospective analysis. Cancer Chemother. Pharmacol. 69:425–430.

5. Ychou, M., F. Desseigne, R. Guimbaud, M. Ducreux, O. Bouche, Y. Becouarn, et al. 2007. Randomized phase II trial comparing FOLFIRINOX (5FU/leucovorin [LV], irinotecan [I] and oxaliplatin [O]) vs gemcitabine (G) as first-line treatment for metastatic pancreatic adenocarcinoma (MPA). First results of the ACCORD 11 trial. ASCO Meeting Abstr. 25(Suppl. 18): 4516.
6. Conroy, T., F. Desseigne, M. Ychou, O. Bouche, R. Guimbaud, Y. Becouarn, et al. 2011. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N. Engl. J. Med. 364:1817–1825.
7. Gourgou-Bourgade, S., C. Bascoul-Mollevi, F. Desseigne, M. Ychou, O. Bouche, R. Guimbaud, et al. 2013. Impact of FOLFIRINOX compared with gemcitabine on quality of life in patients with metastatic pancreatic cancer: results from the PRODIGE 4/ACCORD 11 randomized trial. J. Clin. Oncol. 31:23–29.
8. Bendell, J., S. Britton, M. Green, J. Willey, K. Lemke, J. Marshall. 2011. Immediate impact of the FOLFIRINOX phase III data reported at the 2010 ASCO Annual Meeting on prescribing plans of American oncology physicians for patients with metastatic pancreas cancer. J. Clin. Oncol. 29:2011 (suppl 4; abstr 286).
9. Donahue, T. R., K. K. Kazanjian, W. H. Isacoff, H. A. Reber, and O. J. Hines. 2010. Impact of splenectomy on thrombocytopenia, chemotherapy, and survival in patients with unresectable pancreatic cancer. J. Gastrointest. Surg. 14:1012–1018.
10. Kauffman, C. R., A. Mahvash, S. Kopetz, R. A. Wolff, J. Ensor, and M. J. Wallace. 2008. Partial splenic embolization for cancer patients with thrombocytopenia requiring systemic chemotherapy. Cancer 112:2283–2288.
11. Wang, H. Y., S. C. Shih, S. C. Lin, W. S. Chang, T. E. Wang, F. J. Lin, et al. 2008. Partial splenic embolization: 12-month hematological effects and complications. Hepatogastroenterology 55:1838–1842.
12. Gammon, D., P. Bhargava, and M. J. McCormick. 2004. Hypersensitivity reactions to oxaliplatin and the application of a desensitization protocol. Oncologist 9:546–549.
13. Dodds, H. M., J. F. Bishop, and L. P. Rivory. 1999. More about: irinotecan-related cholinergic syndrome induced by coadministration of oxaliplatin. J. Natl. Cancer Inst. 91:91–92.
14. Krexner, E., A. Stickler, C. Prainer, and J. Finsterer. 2012. Acute, generalised but transient muscle cramping and weakness shortly after first oxaliplatin infusion. Med. Oncol. 29:3592–3593.
15. De Marco, S., E. Squilloni, L. Vigna, M. F. Bertagnolio, and C. N. Sternberg. 2004. Irinotecan chemotherapy associated with transient dysarthria and aphasia. Ann. Oncol. 15:1147–1148.
16. Basso, M., A. Cassano, A. Modoni, D. Spada, N. Trigila, M. Quirino, et al. 2008. A reversible coma after oxaliplatin administration suggests a pathogenetic role of electrolyte imbalance. Eur. J. Clin. Pharmacol. 64:739–741.
17. Amireault, C., J. Beaudet, G. Gaudet, N. Raymond, J-P. Ayoub, R. Letourneau, et al. 2014. FOLFIRINOX in the real-world setting: the multicentric experience of six Canadian institutions. ASCO Meeting Abstr. 32(Suppl. 3): 367.
18. Mahaseth, H., E. Brutcher, J. Kauh, N. Hawk, S. Kim, Z. Chen, et al. 2013. Modified FOLFIRINOX regimen with improved safety and maintained efficacy in pancreatic adenocarcinoma. Pancreas 42:1311–1315.
19. De Gramont, A., M. Krulik, J. Cady, B. Lagadec, J. E. Maisani, J. P. Loiseau, et al. 1988. High-dose folinic acid and 5-fluorouracil bolus and continuous infusion in advanced colorectal cancer. Eur. J. Cancer Clin. Oncol. 24:1499–1503.
20. Tamura, T., A. Kuwahara, K. Kadoyama, M. Yamamori, K. Nishiguchi, T. Inokuma, et al. 2011. Effects of bolus injection of 5-fluorouracil on steady-state plasma concentrations of 5-fluorouracil in Japanese patients with advanced colorectal cancer. Int. J. Med. Sci. 8:406–412.
21. Sobrero, A. F., C. Aschele, and J. R. Bertino. 1997. Fluorouracil in colorectal cancer—a tale of two drugs: implications for biochemical modulation. J. Clin. Oncol. 15:368–381.
22. James, E. S., X. Yao, X. Cong, S. Stein, K. Kaley, C. Hahn, et al. 2014. Interim analysis of a phase II study of dose-modified FOLFIRINOX (mFOLFIRINOX) in locally advanced (LAPC) and metastatic pancreatic cancer (MPC). ASCO Meeting Abstr. 32(Suppl. 3): 256.
23. Leichman, C. G., L. Leichman, C. P. Spears, P. J. Rosen, F. Muggia, S. Jeffers, et al. 1990. Biological modification of protracted infusion of 5 fluorouracil with weekly leucovorin. A dose seeking clinical trial for patients with disseminated gastrointestinal cancers. Cancer Chemother. Pharmacol. 26:57–61.
24. Blazer, M. A., C. S.-Y. Wu, R. M. Goldberg, G. S. Phillips, C. R. Schmidt, P. Muscarella, et al. 2014. Tolerability and efficacy of modified FOLFIRINOX (mFOLFIRINOX) in patients with borderline-resectable pancreatic cancer (BRPC) and locally advanced unresectable pancreatic cancer (LAURPC). ASCO Meeting Abstr. 32(Suppl. 3): 275.
25. Gunturu, K. S., J. R. Thumar, H. S. Hochster, S. Stein, X. Yao, X. Cong, et al. 2012. Single-institution experience with FOLFIRINOX in advanced pancreatic cancer (PC). ASCO Meeting Abstr. 30(Suppl. 4): 330.
26. Alessandretti, M. B., E. P Brandao, C. M. Abrahao, A. R. Lino, R. M. Junior, M. A. Costa, et al. 2013. Safety and efficacy of modified dose-attenuated FOLFIRINOX chemotherapy in patients over 65 years with advanced pancreatic adenocarcinoma. ASCO Meeting Abstr. 31 (Suppl. 15): e15176.

27. Metges, J. P., J. F. Ramee, J.-Y. Douillard, E. Boucher, R. Faroux, V. Guerin-Meyer, et al. 2014. Efficacy and safety of FOLFIRINOX in patients with metastatic pancreatic cancer. ASCO Meeting Abstr. 32(Suppl. 3): 305.
28. Innocenti, F., R. L. Schilsky, J. Ramirez, L. Janisch, S. Undevia, L. K. House, et al. 2014. Dose-finding and pharmacokinetic study to optimize the dosing of irinotecan according to the UGT1A1 genotype of patients with cancer. J. Clin. Oncol. 32:2328–2334.
29. Hosein, P. J., J. Macintyre, C. Kawamura, J. C. Maldonado, V. Ernani, A. Loaiza-Bonilla, et al. 2012. A retrospective study of neoadjuvant FOLFIRINOX in unresectable or borderline-resectable locally advanced pancreatic adenocarcinoma. BMC Cancer 12:199.
30. Faris, J. E., L. S. Blaszkowsky, S. McDermott, A. R. Guimaraes, J. Szymonifka, M. A. Huynh, et al. 2013. FOLFIRINOX in locally advanced pancreatic cancer: the Massachusetts General Hospital Cancer Center experience. Oncologist 18:543–548.
31. Morris-Stiff, G., Y. M. Tan, and J. N. Vauthey. 2008. Hepatic complications following preoperative chemotherapy with oxaliplatin or irinotecan for hepatic colorectal metastases. Eur. J. Surg. Oncol. 34:609–614.
32. Nomura, R., Y. Ishizaki, K. Suzuki, and S. Kawasaki. 2007. Development of hepatic steatosis after pancreatoduodenectomy. AJR Am. J. Roentgenol. 189:1484–1488.
33. Zorzi, D., A. Laurent, T. M. Pawlik, G. Y. Lauwers, J. N. Vauthey, and E. K. Abdalla. 2007. Chemotherapy-associated hepatotoxicity and surgery for colorectal liver metastases. Br. J. Surg. 94:274–286.
34. Samuel, N., and T. J. Hudson. 2012. The molecular and cellular heterogeneity of pancreatic ductal adenocarcinoma. Nat. Rev. Gastroenterol. Hepatol. 9:77–87.
35. Ghosn, M., F. Farhat, J. Kattan, F. Younes, W. Moukadem, F. Nasr, et al. 2007. FOLFOX-6 combination as the first-line treatment of locally advanced and/or metastatic pancreatic cancer. Am. J. Clin. Oncol. 30:15–20.
36. Taieb, J., T. Lecomte, T. Aparicio, A. Asnacios, T. Mansourbakht, P. Artu, et al. 2007. FOLFIRI.3, a new regimen combining 5-fluorouracil, folinic acid and irinotecan, for advanced pancreatic cancer: results of an Association des Gastro-Enterologues Oncologues (Gastroenterologist Oncologist Association) multicenter phase II study. Ann. Oncol. 18:498–503.
37. Son, C. O., S. G. Pai, S. Satti, A. Dornelles, J. S. Bolton, W. C. Conway, et al. 2014. Outcomes of neoadjuvant chemotherapy with FOLFOX/FOLFIRINOX and chemoradiotherapy in borderline resectable pancreatic cancer: Single-institution experience. ASCO Meeting Abstr. 32(Suppl. 3): 327.
38. Oettle, H., P. Neuhaus, A. Hochhaus, J. T. Hartmann, K. Gellert, K. Ridwelski, et al. 2013. Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: the CONKO-001 randomized trial. JAMA 310:1473–1481.
39. Sultana, A., T. Cox, P. Ghaneh, and J. P. Neoptolemos. 2012. Adjuvant therapy for pancreatic cancer. Recent Results Cancer Res. 196:65–88.
40. Hardacre, J. M., M. Mulcahy, W. Small, M. Talamonti, J. Obel, S. Krishnamurthi, et al. 2013. Addition of algenpantucel-L immunotherapy to standard adjuvant therapy for pancreatic cancer: a phase 2 study. J. Gastrointest. Surg. 17:94–100; discussion p 00–1.
41. Weden, S., M. Klemp, I. P. Gladhaug, M. Moller, J. Eriksen, G. Gaudernack, et al. 2011. Long-term follow-up of patients with resected pancreatic cancer following vaccination against mutant K-ras. Int. J. Cancer 128:1120–1128.
42. Haeno, H., M. Gonen, M. B. Davis, J. M. Herman, C. A. Iacobuzio-Donahue, and F. Michor. 2012. Computational modeling of pancreatic cancer reveals kinetics of metastasis suggesting optimum treatment strategies. Cell 148:362–375.
43. Tuveson, D. A., and J. P. Neoptolemos. 2012. Understanding metastasis in pancreatic cancer: a call for new clinical approaches. Cell 148:21–23.
44. Sohal, D. P., R. M. Walsh, R. K. Ramanathan, and A. A. Khorana. 2014. Pancreatic adenocarcinoma: treating a systemic disease with systemic therapy. J. Natl. Cancer Inst. 106: dju011.
45. Evans, D. B., G. R. Varadhachary, C. H. Crane, C. C. Sun, J. E. Lee, P. W. Pisters, et al. 2008. Preoperative gemcitabine-based chemoradiation for patients with resectable adenocarcinoma of the pancreatic head. J. Clin. Oncol. 26:3496–3502.
46. MacKenzie, S., H. Zeh, L. E. McCahill, T. D. Sielaff, N. Bahary, T. F. Gribben, et al. 2013. A pilot phase II multicenter study of nab-paclitaxel (Nab-P) and gemcitabine (G) as preoperative therapy for potentially resectable pancreatic cancer (PC). ASCO Meeting Abstr. 31 (Suppl. 15): 4038.
47. Alvarez-Gallego, R., A. Cubillo, J. Rodriguez-Pascual, Y. Quijano, E. De Vicente, L. Garcia, et al. 2012. Antitumor activity of nab-paclitaxel and gemcitabine in resectable pancreatic cancer. ASCO Meeting Abstr. 30(Suppl. 15): 4040.
48. Minter, R. M., M. U.-S. Feng, M. Al-Hawary, J. Shen, M. J. Schipper, F. Bednar, et al. 2014. Effect of neoadjuvant chemoradiotherapy (nCRT) on survival in patients with borderline resectable (BR) pancreatic adenocarcinoma (PDA) with acceptable peri-operative morbidity. ASCO Meeting Abstr. 32(Suppl. 3): 288.
49. McCormick, K., S. H. Whiting, G. Gyurkey, W.-J. Koh, M. Sinanan, and A. J. Coveler. 2014. Long-term survival of patients receiving multimodality neoadjuvant therapy for resectable or borderline resectable pancreatic ductal adenocarcinoma. ASCO Meeting Abstr. 32(Suppl. 3): 309.
50. Gulati, A. P., S. M. Schreibman, B. Schrope, J. A. Lee, J. Allendorf, J. A. Chabot, et al. 2014. Prospective phase II

study of inoperable pancreatic adenocarcinoma with neoadjuvant gemcitabine, docetaxel, and capecitabine (GTX). ASCO Meeting Abstr. 32(Suppl. 3): 274.

51. Christians, K. K., S. Tsai, A. Mahmoud, P. Ritch, J. P. Thomas, L. Wiebe, et al. 2014. Neoadjuvant FOLFIRINOX for borderline resectable pancreas cancer: a new treatment paradigm? Oncologist 19:266–274.
52. Tian, F., H. E. Appert, J. Myles, and J. M. Howard. 1992. Prognostic value of serum CA 19-9 levels in pancreatic adenocarcinoma. Ann. Surg. 215:350–355.
53. Haglund, C., P. Kuusela, and P. J. Roberts. 1989. Tumour markers in pancreatic cancer. Ann. Chir. Gynaecol. 78:41–53.
54. Benhamou, P. Y., J. P. Vuillez, S. Halimi, G. Meffre, and I. Bachelot. 1991. Influence of metabolic disturbances of diabetes mellitus on serum CA 19-9 tumor marker. Diabete Metab. 17:39–43.
55. Pelzer, U., A. Hilbig, M. Sinn, J. Stieler, M. Bahra, B. Dorken, et al. 2013. Value of carbohydrate antigen 19-9 in predicting response and therapy control in patients with metastatic pancreatic cancer undergoing first-line therapy. Front. Oncol. 3:155.
56. Von Hoff, D. D., T. Ervin, F. P. Arena, E. G. Chiorean, J. Infante, M. Moore, et al. 2013. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N. Engl. J. Med. 369:1691–1703.
57. Hess, V., B. Glimelius, P. Grawe, D. Dietrich, G. Bodoky, T. Ruhstaller, et al. 2008. CA 19-9 tumour-marker response to chemotherapy in patients with advanced pancreatic cancer enrolled in a randomised controlled trial. Lancet Oncol. 9:132–138.
58. Donahue, T. R., W. H. Isacoff, O. J. Hines, J. S. Tomlinson, J. J. Farrell, Y. M. Bhat, et al. 2011. Downstaging chemotherapy and alteration in the classic computed tomography/magnetic resonance imaging signs of vascular involvement in patients with pancreaticobiliary malignant tumors: influence on patient selection for surgery. Arch. Surg. 146:836–843.
59. Katz, M. H., J. B. Fleming, P. Bhosale, G. Varadhachary, J. E. Lee, R. Wolff, et al. 2012. Response of borderline resectable pancreatic cancer to neoadjuvant therapy is not reflected by radiographic indicators. Cancer 118:5749–5756.
60. Dholakia, H.-P., and R. Wild. 2013. Resection of borderline resectable pancreatic cancer after neoadjuvant chemoradiation does not depend on improved radiographic appearance of tumor–vessel relationships. J. Radiat. Oncol. 2:413–425.
61. Zhao, Q., A. Rashid, Y. Gong, and M. Katz. 2012. Pathologic complete response to neoadjuvant therapy in patients with pancreatic ductal adenocarcinoma is associated with a better prognosis. Ann. Diagn. Pathol. 16:29–37.
62. Chun, Y. S. C. H., S. J. Cohen, A. Konski, B. Burtness, C. S. Denlinger, I. Astsaturov, et al. 2011. Significance of pathologic response to preoperative therapy in pancreatic cancer. Ann. Surg. Oncol. 18:3601–3607.
63. De Angelis, C., R. F. Brizzi, and R. Pellicano. 2013. Endoscopic ultrasonography for pancreatic cancer: current and future perspectives. J. Gastrointest. Oncol. 4:220–230.
64. Lu, N., X. Y. Feng, S. J. Hao, Z. H. Liang, C. Jin, J. W. Qiang, et al. 2011. 64-slice CT perfusion imaging of pancreatic adenocarcinoma and mass-forming chronic pancreatitis. Acad. Radiol. 18:81–88.
65. Epelbaum, R., O. Israel, R. Haddad, N. Sikorski, and A. Frenkel. 2010. Dynamic FDG-PET/CT as an indicator of tumor aggressiveness and patient outcome in pancreatic cancer. ASCO Meeting Abstr. 28(Suppl. 15): e14526.
66. Koay, E. J., M. J. Truty, V. Cristini, R. M. Thomas, R. Chen, D. Chatterjee, et al. 2014. Transport properties of pancreatic cancer describe gemcitabine delivery and response. J. Clin. Invest. 124:1525–1536.
67. Hammel, P., F. Huguet, J.-L. Van Laethem, D. Goldstein, B. Glimelius, P. Artru, et al. 2013. Comparison of chemoradiotherapy (CRT) and chemotherapy (CT) in patients with a locally advanced pancreatic cancer (LAPC) controlled after 4 months of gemcitabine with or without erlotinib: final results of the international phase III LAP 07 study. ASCO Meeting Abstr. 31(Suppl. 15): LBA4003.
68. Chuong, M. D., G. M. Springett, J. M. Freilich, C. K. Park, J. M. Weber, E. A. Mellon, et al. 2013. Stereotactic body radiation therapy for locally advanced and borderline resectable pancreatic cancer is effective and well tolerated. Int. J. Radiat. Oncol. Biol. Phys. 86:516–522.
69. Chang, S. T., K. A. Goodman, G. P. Yang, and A. C. Koong. 2007. Stereotactic body radiotherapy for unresectable pancreatic cancer. Front. Radiat. Ther. Oncol. 40:386–394.
70. Ramanathan, R. K., P. Lee, J. E. Seng, S. P. Anthony, P. J. Rosen, R. R. Mena, et al. 2014. Phase II study of induction therapy with gemcitabine and nab-paclitaxel followed by consolidation with mFOLFIRINOX in patients with metastatic pancreatic cancer. ASCO Meeting Abstr. 32 (Suppl. 3): 224.
71. Hartlapp, I., J. Muller, W. Kenn, U. Steger, C. Isbert, M. Schuerlen, et al. 2013. Complete pathological remission of locally advanced, unresectable pancreatic cancer (LAPC) after intensified neoadjuvant chemotherapy. Onkologie 36:123–125.
72. Zhang, Y., H. S. Hochster, S. Stein, and J. Lacy. 2014. Second-line gemcitabine plus nab-paclitaxel (G+A) for advanced pancreatic cancer (APC) after first-line FOLFIRINOX: single institution retrospective review of efficacy and toxicity. ASCO Meeting Abstr. 32(Suppl. 3): 344.
73. Spivak-Kroizman, T. R., G. Hostetter, R. Posner, M. Aziz, C. Hu, M. J. Demeure, et al. 2013. Hypoxia triggers hedgehog-mediated tumor-stromal interactions in pancreatic cancer. Cancer Res. 73:3235–3247.

74. Sonnenblick, A., L. Kadouri, L. Appelbaum, T. Peretz, M. Sagi, Y. Goldberg, et al. 2011. Complete remission, in BRCA2 mutation carrier with metastatic pancreatic adenocarcinoma, treated with cisplatin based therapy. Cancer Biol. Ther. 12:165–168.
75. Leung, K., and M. W. Saif. 2013. BRCA-associated pancreatic cancer: the evolving management. J. Pancreas 14:149–151.
76. Jones, S., X. Zhang, D. W. Parsons, J. C. Lin, R. J. Leary, P. Angenendt, et al. 2008. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321:1801–1806.
77. Reed, E. 2005. ERCC1 and clinical resistance to platinum-based therapy. Clin. Cancer Res. 11:6100–6102.
78. Hu, Y. C., R. A. Komorowski, S. Graewin, G. Hostetter, O. P. Kallioniemi, H. A. Pitt, et al. 2003. Thymidylate synthase expression predicts the response to 5-fluorouracil-based adjuvant therapy in pancreatic cancer. Clin. Cancer Res. 9:4165–4171.
79. Spratlin, J., R. Sangha, D. Glubrecht, L. Dabbagh, J. D. Young, C. Dumontet, et al. 2004. The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Clin. Cancer Res. 10:6956–6961.
80. Poplin, E., H. Wasan, L. Rolfe, M. Raponi, T. Ikdahl, I. Bondarenko, et al. 2013. Randomized, multicenter, phase II study of CO-101 versus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma: including a prospective evaluation of the role of hENT1 in gemcitabine or CO-101 sensitivity. J. Clin. Oncol. 31:4453–4461.
81. Von Hoff, D. D., R. Ramanathan, M. Borad, D. Laheru, L. Smith, T. Wood, et al. 2009. SPARC correlation with response to gemcitabine (G) plus nab-paclitaxel (nab-P) in patients with advanced metastatic pancreatic cancer: a phase I/II study. ASCO Meeting Abstr. 27:4525.
82. Sinn, M., B. V. Sinn, I. K. Striefler, J. L. Lindner, J. M. Stieler, P. Lohneis, et al. 2014. SPARC expression in resected pancreatic cancer patients treated with gemcitabine: results from the CONKO-001 study. Ann. Oncol. 25:1025–1032.
83. Arnaoutakis, G. J., D. Rangachari, D. A. Laheru, C. A. Iacobuzio-Donahue, R. H. Hruban, J. M. Herman, et al. 2011. Pulmonary resection for isolated pancreatic adenocarcinoma metastasis: an analysis of outcomes and survival. J. Gastrointest. Surg. 15:1611–1617.
84. Danschede, F., L. Will, C. von Langsdorf, M. Mohler, P. R. Galle, G. Otto, et al. 2010. Treatment of metachronous and simultaneous liver metastases of pancreatic cancer. Eur. Surg. Res. 44:209–213.

HIGHLIGHTS OF PRESCRIBING INFORMATION
These highlights do not include all of the information needed to use ONIVYDE™ safely and effectively. See full prescribing information for ONIVYDE™

ONIVYDE™ (irinotecan liposome injection), for intravenous use
Initial U.S. Approval: 1996

WARNING: SEVERE NEUTROPENIA and SEVERE DIARRHEA
See full prescribing information for complete boxed warning

- **Fatal neutropenic sepsis occurred in 0.8% of patients receiving ONIVYDE. Severe or life-threatening neutropenic fever or sepsis occurred in 3% and severe or life-threatening neutropenia occurred in 20% of patients receiving ONIVYDE in combination with fluorouracil and leucovorin. Withhold ONIVYDE for absolute neutrophil count below 1500/mm^3 or neutropenic fever. Monitor blood cell counts periodically during treatment (2.2), (5.1).**
- **Severe diarrhea occurred in 13% of patients receiving ONIVYDE in combination with fluorouracil and leucovorin. Do not administer ONIVYDE to patients with bowel obstruction. Withhold ONIVYDE for diarrhea of Grade 2-4 severity. Administer loperamide for late diarrhea of any severity. Administer atropine, if not contraindicated, for early diarrhea of any severity (2.2), (5.2).**

INDICATIONS AND USAGE

ONIVYDE is a topoisomerase inhibitor indicated, in combination with fluorouracil and leucovorin, for the treatment of patients with metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy. (1)

Limitation of Use: ONIVYDE is not indicated as a single agent for the treatment of patients with metastatic adenocarcinoma of the pancreas. (1)

DOSAGE AND ADMINISTRATION

- **Do not substitute ONIVYDE for other drugs containing irinotecan HCl. (2.1)**
- Recommended dose of ONIVYDE is 70 mg/m^2 intravenous infusion over 90 minutes every two weeks. (2.2)
- Recommended starting dose of ONIVYDE in patients homozygous for UGT1A1*28 is 50 mg/m^2 every two weeks. (2.2)
- There is no recommended dose of ONIVYDE for patients with serum bilirubin above the upper limit of normal. (2.2)
- Premedicate with a corticosteroid and an anti-emetic, 30 minutes prior to ONIVYDE. (2.2)

DOSAGE FORMS AND STRENGTHS

Injection: 43 mg/10 mL single dose vial (3)

CONTRAINDICATIONS

Severe hypersensitivity reaction to ONIVYDE or irinotecan HCl. (4, 5.4)

WARNINGS AND PRECAUTIONS

- Interstitial lung disease (ILD): Fatal ILD has occurred in patients receiving irinotecan HCl. Discontinue ONIVYDE if ILD is diagnosed. (5.3)
- Severe hypersensitivity reaction: Permanently discontinue ONIVYDE for severe hypersensitivity reactions. (5.4, 4)
- Embryo-fetal toxicity: Can cause fetal harm. Advise females of reproductive potential of the potential risk to a fetus and to use effective contraception. (5.5, 8.1, 8.3)

ADVERSE REACTIONS

The most common adverse reactions (≥ 20%) of ONIVYDE: diarrhea, fatigue/asthenia, vomiting, nausea, decreased appetite, stomatitis, and pyrexia. The most common laboratory abnormalities (≥ 10% Grade 3 or 4) were lymphopenia and neutropenia. (6)

To report SUSPECTED ADVERSE REACTIONS, contact Merrimack Pharmaceuticals, Inc. at 1-844-441-6225 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

DRUG INTERACTIONS

- Strong CYP3A4 Inducers: Avoid the use of strong CYP3A4 inducers if possible. Substitute non-enzyme inducing therapies at least 2 weeks prior to initiation of ONIVYDE. (7.1)
- Strong CYP3A4 Inhibitors: Avoid the use of strong CYP3A4 or UGT1A1 inhibitors, if possible; discontinue strong CYP3A4 inhibitors at least 1 week prior to starting therapy. (7.2)

USE IN SPECIFIC POPULATIONS

- Lactation: Do not breastfeed. (8.2)

See 17 for PATIENT COUNSELING INFORMATION.

Revised: 10/2015

FULL PRESCRIBING INFORMATION: CONTENTS*

*Sections or subsections omitted from the full prescribing information are not listed

FULL PRESCRIBING INFORMATION

WARNING: SEVERE NEUTROPENIA and SEVERE DIARRHEA

Fatal neutropenic sepsis occurred in 0.8% of patients receiving ONIVYDE. Severe or life-threatening neutropenic fever or sepsis occurred in 3% and severe or life-threatening neutropenia occurred in 20% of patients receiving ONIVYDE in combination with fluorouracil and leucovorin. Withhold ONIVYDE for absolute neutrophil count below 1500/mm^3 or neutropenic fever. Monitor blood cell counts periodically during treatment ***[see Dosage and Administration (2.2) and Warnings and Precautions (5.1)].***

Severe diarrhea occurred in 13% of patients receiving ONIVYDE in combination with fluorouracil and leucovorin. Do not administer ONIVYDE to patients with bowel obstruction. Withhold ONIVYDE for diarrhea of Grade 2-4 severity. Administer loperamide for late diarrhea of any severity. Administer atropine, if not contraindicated, for early diarrhea of any severity ***[see Dosage and Administration (2.2) and Warnings and Precautions (5.2)].***

1 INDICATIONS AND USAGE

ONIVYDE™ is indicated, in combination with fluorouracil and leucovorin, for the treatment of patients with metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy.

Limitation of Use: ONIVYDE is not indicated as a single agent for the treatment of patients with metastatic adenocarcinoma of the pancreas *[see Clinical Studies (14)]*.

2 DOSAGE AND ADMINISTRATION

2.1 Important Use Information

DO NOT SUBSTITUTE ONIVYDE for other drugs containing irinotecan HCl.

2.2 Recommended Dose

Administer ONIVYDE prior to leucovorin and fluorouracil *[see Clinical Studies (14)]*.

- The recommended dose of ONIVYDE is 70 mg/m^2 administered by intravenous infusion over 90 minutes every 2 weeks.
- The recommended starting dose of ONIVYDE in patients known to be homozygous for the UGT1A1*28 allele is 50 mg/m^2 administered by intravenous infusion over 90 minutes. Increase the dose of ONIVYDE to 70 mg/m^2 as tolerated in subsequent cycles.
- There is no recommended dose of ONIVYDE for patients with serum bilirubin above the upper limit of normal *[see Adverse Reactions (6.1) and Clinical Studies (14)]*.

Premedication

Administer a corticosteroid and an anti-emetic 30 minutes prior to ONIVYDE infusion.

2.3 Dose Modifications for Adverse Reactions

Table 1: Recommended Dose Modifications for ONIVYDE

Toxicity NCI CTCAE v4.0[*]	Occurrence	ONIVYDE adjustment in patients receiving 70 mg/m^2	Patients homozygous for UGT1A1*28 without previous increase to 70 mg/m^2
Grade 3 or 4 adverse reactions	Withhold ONIVYDE. Initiate loperamide for late onset diarrhea of any severity. Administer intravenous or subcutaneous atropine 0.25 to 1 mg (unless clinically contraindicated) for early onset diarrhea of any severity. Upon recovery to ≤ Grade 1, resume ONIVYDE at:		
	First	50 mg/m^2	43 mg/m^2
	Second	43 mg/m^2	35 mg/m^2
	Third	Discontinue ONIVYDE	Discontinue ONIVYDE
Interstitial Lung Disease	First	Discontinue ONIVYDE	Discontinue ONIVYDE
Anaphylactic Reaction	First	Discontinue ONIVYDE	Discontinue ONIVYDE

[*] NCI CTCAE v 4.0=National Cancer Institute Common Toxicity Criteria for Adverse Events version 4.0

For recommended dose modifications of fluorouracil (5-FU) or leucovorin (LV), refer to the Full Prescribing Information; refer to Clinical Studies (14).

2.4 Preparation and Administration

ONIVYDE is a cytotoxic drug. Follow applicable special handling and disposal procedures.[1]

Preparation

- Withdraw the calculated volume of ONIVYDE from the vial. Dilute ONIVYDE in 500 mL 5% Dextrose Injection, USP or 0.9% Sodium Chloride Injection, USP and mix diluted solution by gentle inversion.
- Protect diluted solution from light.
- Administer diluted solution within 4 hours of preparation when stored at room temperature or within 24 hours of preparation when stored under refrigerated conditions [2°C to 8°C (36°F to 46°F)]. Allow diluted solution to come to room temperature prior to administration.
- Do NOT freeze.

Administration

- Infuse diluted solution intravenously over 90 minutes. Do not use in-line filters. Discard unused portion.

3 DOSAGE FORMS AND STRENGTHS

Injection: 43 mg/10 mL irinotecan free base as a white to slightly yellow, opaque, liposomal dispersion in a single-dose vial.

4 CONTRAINDICATIONS

ONIVYDE is contraindicated in patients who have experienced a severe hypersensitivity reaction to ONIVYDE or irinotecan HCl.

5 WARNINGS AND PRECAUTIONS

5.1 Severe Neutropenia

ONIVYDE can cause severe or life-threatening neutropenia and fatal neutropenic sepsis. In Study 1, the incidence of fatal neutropenic sepsis was 0.8% among patients receiving ONIVYDE, occurring in one of 117 patients in the ONIVYDE plus fluorouracil/leucovorin (ONIVYDE/5-FU/LV) arm and one of 147 patients receiving ONIVYDE as a single agent. Severe or life-threatening neutropenia occurred in 20% of patients receiving ONIVYDE/5-FU/LV compared to 2% of patients receiving fluorouracil/leucovorin alone (5-FU/LV). Grade 3 or 4 neutropenic fever/neutropenic sepsis occurred in 3% of patients receiving ONIVYDE/5-FU/LV, and did not occur in patients receiving 5-FU/LV.

In patients receiving ONIVYDE/5-FU/LV, the incidence of Grade 3 or 4 neutropenia was higher among Asian patients [18 of 33 (55%)] compared to White patients [13 of 73 (18%)]. Neutropenic fever/neutropenic sepsis was reported in 6% of Asian patients compared to 1% of White patients *[see Clinical Pharmacology (12.3)]*.

Monitor complete blood cell counts on Days 1 and 8 of every cycle and more frequently if clinically indicated. Withhold ONIVYDE if the absolute neutrophil count (ANC) is below 1500/mm^3 or if neutropenic fever occurs. Resume ONIVYDE when the ANC is 1500/mm^3 or above. Reduce ONIVYDE dose for Grade 3-4 neutropenia or neutropenic fever following recovery in subsequent cycles *[see Dosage and Administration (2.2)]*.

5.2 Severe Diarrhea

ONIVYDE can cause severe and life-threatening diarrhea. Do not administer ONIVYDE to patients with bowel obstruction.

Severe or life-threatening diarrhea followed one of two patterns: late onset diarrhea (onset more than 24 hours following chemotherapy) and early onset diarrhea (onset within 24 hours of

chemotherapy, sometimes occurring with other symptoms of cholinergic reaction) *[see Cholinergic Reactions (6.1)]*. An individual patient may experience both early and late-onset diarrhea.

In Study 1, Grade 3 or 4 diarrhea occurred in 13% receiving ONIVYDE/5-FU/LV compared to 4% receiving 5-FU/LV. The incidence of Grade 3 or 4 late onset diarrhea was 9% in patients receiving ONIVYDE/5-FU/LV, compared to 4% in patients receiving 5-FU/LV. The incidence of Grade 3 or 4 early onset diarrhea was 3% in patients receiving ONIVYDE/5-FU/LV, compared to no Grade 3 or 4 early onset diarrhea in patients receiving 5-FU/LV. Of patients receiving ONIVYDE/5-FU/LV in Study 1, 34% received loperamide for late-onset diarrhea and 26% received atropine for early-onset diarrhea. Withhold ONIVYDE for Grade 2-4 diarrhea. Initiate loperamide for late onset diarrhea of any severity. Administer intravenous or subcutaneous atropine 0.25 to 1 mg (unless clinically contraindicated) for early onset diarrhea of any severity. Following recovery to Grade 1 diarrhea, resume ONIVYDE at a reduced dose *[see Dosage and Administration (2.2)]*.

5.3 Interstitial Lung Disease

Irinotecan HCl can cause severe and fatal interstitial lung disease (ILD). Withhold ONIVYDE in patients with new or progressive dyspnea, cough, and fever, pending diagnostic evaluation. Discontinue ONIVYDE in patients with a confirmed diagnosis of ILD.

5.4 Severe Hypersensitivity Reaction

Irinotecan HCl can cause severe hypersensitivity reactions, including anaphylactic reactions. Permanently discontinue ONIVYDE in patients who experience a severe hypersensitivity reaction.

5.5 Embryo-Fetal Toxicity

Based on animal data with irinotecan HCl and the mechanism of action of ONIVYDE, ONIVYDE can cause fetal harm when administered to a pregnant woman. Embryotoxicity and teratogenicity were observed following treatment with irinotecan HCl, at doses resulting in irinotecan exposures lower than those achieved with ONIVYDE 70 mg/m^2 in humans, administered to pregnant rats and rabbits during organogenesis. Advise pregnant women of the potential risk to a fetus. Advise females of reproductive potential to use effective contraception during treatment with ONIVYDE and for one month following the final dose *[see Use in Specific Populations (8.1, 8.3), Clinical Pharmacology (12.1)]*.

6 ADVERSE REACTIONS

The following adverse drug reactions are discussed in greater detail in other sections of the label:

- Severe Neutropenia [see *Warnings and Precautions (5.1) and Boxed Warning]*
- Severe Diarrhea *[see Warnings and Precautions (5.2) and Boxed Warning]*
- Interstitial Lung Disease *[see Warnings and Precautions (5.3)]*

- Severe Hypersensitivity Reactions *[see Warnings and Precautions (5.4)]*

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in clinical trials of ONIVYDE cannot be directly compared to rates in clinical trials of other drugs and may not reflect the rates observed in practice.

The safety data described below are derived from patients with metastatic adenocarcinoma of the pancreas previously treated with gemcitabine-based therapy who received any part of protocol-specified therapy in Study 1, an international, randomized, active-controlled, open-label trial. Protocol-specified therapy consisted of ONIVYDE 70 mg/m^2 with leucovorin 400 mg/m^2 and fluorouracil 2400 mg/m^2 over 46 hours every 2 weeks (ONIVYDE/5-FU/LV; N=117), ONIVYDE 100 mg/m^2 every 3 weeks (N=147), or leucovorin 200 mg/m^2 and fluorouracil 2000 mg/m^2 over 24 hours weekly for 4 weeks followed by 2 week rest (5-FU/LV; N=134) *[see Clinical Studies (14)]*. Serum bilirubin within the institutional normal range, albumin ≥ 3 g/dL, and Karnofsky Performance Status (KPS) ≥ 70 were required for study entry. The median duration of exposure was 9 weeks in the ONIVYDE/5-FU/LV arm, 9 weeks in the ONIVYDE monotherapy arm, and 6 weeks in the 5-FU/LV arm.

The most common adverse reactions (≥ 20%) of ONIVYDE were diarrhea, fatigue/asthenia, vomiting, nausea, decreased appetite, stomatitis, and pyrexia. The most common, severe laboratory abnormalities (≥ 10% Grade 3 or 4) were lymphopenia and neutropenia. The most common serious adverse reactions (≥ 2%) of ONIVYDE were diarrhea, vomiting, neutropenic fever or neutropenic sepsis, nausea, pyrexia, sepsis, dehydration, septic shock, pneumonia, acute renal failure, and thrombocytopenia.

Adverse reactions led to permanent discontinuation of ONIVYDE in 11% of patients receiving ONIVYDE/5-FU/LV; the most frequent adverse reactions resulting in discontinuation of ONIVYDE were diarrhea, vomiting, and sepsis. Dose reductions of ONIVYDE for adverse reactions occurred in 33% of patients receiving ONIVYDE/5-FU/LV; the most frequent adverse reactions requiring dose reductions were neutropenia, diarrhea, nausea, and anemia. ONIVYDE was withheld or delayed for adverse reactions in 62% of patients receiving ONIVYDE/5-FU/LV; the most frequent adverse reactions requiring interruption or delays were neutropenia, diarrhea, fatigue, vomiting, and thrombocytopenia.

Table 2 provides the frequency and severity of adverse reactions in Study I that occurred with higher incidence (≥5% difference for Grades I-4 or ≥2% difference for Grades 3-4) in patients who received ONIVYDE/5-FU/LV compared to patients who received 5-FU/LV.

Table 2: Adverse Reactions with Higher Incidence (≥5% Difference for Grades 1-4* or ≥2% Difference for Grades 3 and 4) in the ONIVYDE/5-FU/LV Arm

Adverse Reaction	ONIVYDE/5-FU/LV N=117		5-FU/LV N=134	
	Grades 1-4 (%)	Grades 3-4 (%)	Grades 1-4 (%)	Grades 3-4 (%)
Gastrointestinal disorders				
Diarrhea	59	13	26	4
Early diarrhea†	30	3	15	0
Late diarrhea‡	43	9	17	4
Vomiting	52	11	26	3
Nausea	51	8	34	4
Stomatitis§	32	4	12	1
Infections and infestations	38	17	15	10
Sepsis	4	3	2	1
Neutropenic fever/neutropenic sepsis•	3	3	1	0
Gastroenteritis	3	3	0	0
Intravenous catheter-related infection	3	3	0	0
General disorders and administration site conditions				
Fatigue/asthenia	56	21	43	10
Pyrexia	23	2	11	1
Metabolism and nutrition disorders				
Decreased appetite	44	4	32	2
Weight loss	17	2	7	0
Dehydration	8	4	7	2
Skin and subcutaneous tissue disorders				
Alopecia	14	1	5	0

* NCI CTCAE v4.0
† Early diarrhea: onset within 24 hours of ONIVYDE administration
‡ Late diarrhea: onset >1 day after ONIVYDE administration
§ Includes stomatitis, aphthous stomatitis, mouth ulceration, mucosal inflammation.
• Includes febrile neutropenia

Cholinergic Reactions: ONIVYDE can cause cholinergic reactions manifesting as rhinitis, increased salivation, flushing, bradycardia, miosis, lacrimation, diaphoresis, and intestinal hyperperistalsis with abdominal cramping and early onset diarrhea. In Study 1, Grade 1 or 2 cholinergic symptoms other than early diarrhea occurred in 12 (4.5%) ONIVYDE-treated patients. Six of these 12 patients received atropine and in 1 of the 6 patients, atropine was administered for cholinergic symptoms other than diarrhea.

Infusion Reactions: Infusion reactions, consisting of rash, urticaria, periorbital edema, or pruritus, occurring on the day of ONIVYDE administration were reported in 3% of patients receiving ONIVYDE or ONIVYDE/5-FU/LV.

Laboratory abnormalities that occurred with higher incidence in the ONIVYDE/5-FU/LV arm compared to the 5-FU/LV arm (≥5% difference) are summarized in the following table.

Table 3: Laboratory Abnormalities with Higher Incidence (≥5% Difference) in the ONIVYDE/5-FU/LV Arm[*#]

Laboratory abnormality	ONIVYDE/5-FU/LV N=117		5-FU/LV N=134	
	Grades 1-4 (%)	Grades 3-4 (%)	Grades 1-4 (%)	Grades 3-4 (%)
Hematology				
Anemia	97	6	86	5
Lymphopenia	81	27	75	17
Neutropenia	52	20	6	2
Thrombocytopenia	41	2	33	0
Hepatic				
Increased alanine aminotransferase (ALT)	51	6	37	1
Hypoalbuminemia	43	2	30	0
Metabolic				
Hypomagnesemia	35	0	21	0
Hypokalemia	32	2	19	2
Hypocalcemia	32	1	20	0
Hypophosphatemia	29	4	18	1
Hyponatremia	27	5	12	3
Renal				
Increased creatinine	18	0	13	0

[*] NCI CTCAE v4.0, worst grade shown.
[#] Percent based on number of patients with a baseline and at least one post-baseline measurement.

7 DRUG INTERACTIONS

7.1 Strong CYP3A4 Inducers

Following administration of non-liposomal irinotecan (i.e., irinotecan HCl), exposure to irinotecan or its active metabolite, SN-38, is substantially reduced in adult and pediatric patients concomitantly receiving the CYP3A4 enzyme-inducing anticonvulsants phenytoin and strong CYP3A4 inducers. Avoid the use of strong CYP3A4 inducers (e.g., rifampin, phenytoin, carbamazepine, rifabutin, rifapentine, phenobarbital, St. John's wort) if possible. Substitute non-enzyme inducing therapies at least 2 weeks prior to initiation of ONIVYDE therapy *[see Clinical Pharmacology (12.3)]*.

7.2 Strong CYP3A4 or UGT1A1 Inhibitors

Following administration of non-liposomal irinotecan (i.e., irinotecan HCl), patients receiving concomitant ketoconazole, a CYP3A4 and UGT1A1 inhibitor, have increased exposure to irinotecan and its active metabolite SN-38. Co-administration of ONIVYDE with other inhibitors of CYP3A4 (e.g., clarithromycin, indinavir, itraconazole, lopinavir, nefazodone, nelfinavir, ritonavir, saquinavir, telaprevir, voriconazole) or UGT1A1 (e.g., atazanavir, gemfibrozil, indinavir) may increase systemic exposure to irinotecan or SN-38. Avoid the use of strong CYP3A4 or UGT1A1 inhibitors if possible. Discontinue strong CYP3A4 inhibitors at least 1 week prior to starting ONIVYDE therapy *[see Clinical Pharmacology (12.3)]*.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

Based on animal data with irinotecan HCl and the mechanism of action of ONIVYDE, ONIVYDE can cause fetal harm when administered to a pregnant woman *[see Clinical Pharmacology (12.1)]*. There are no available data in pregnant women. Embryotoxicity and teratogenicity were observed following treatment with irinotecan HCl, at doses resulting in irinotecan exposures lower than those achieved with ONIVYDE 70 mg/m^2 in humans, administered to pregnant rats and rabbits during organogenesis *[see Data]*. Advise pregnant women of the potential risk to a fetus.

In the U.S. general population, the estimated background risk of major birth defects and miscarriage in clinically recognized pregnancies is 2-4% and 15-20%, respectively.

Data

Animal Data

No animal studies have been conducted to evaluate the effect of irinotecan liposome on reproduction and fetal development; however, studies have been conducted with irinotecan HCl. Irinotecan crosses the placenta of rats following intravenous administration. Intravenous administration of irinotecan at a dose of 6 mg/kg/day to rats and rabbits during the period of organogenesis resulted in increased post-implantation loss and decreased numbers of live fetuses. In separate studies in rats, this dose resulted in an irinotecan exposure of approximately 0.002 times the exposure of irinotecan based on area under the curve (AUC) in patients administered ONIVYDE at the 70 mg/m^2 dose. Administration of irinotecan HCl resulted in structural abnormalities and growth delays in rats at doses greater than 1.2 mg/kg/day (approximately 0.0002 times the clinical exposure to irinotecan in ONIVYDE based on AUC). Teratogenic effects included a variety of external, visceral, and skeletal abnormalities. Irinotecan HCl administered to rat dams for the period following organogenesis through weaning at doses of 6 mg/kg/day caused decreased learning ability and decreased female body weights in the offspring.

8.2 Lactation

Risk Summary

There is no information regarding the presence of irinotecan liposome, irinotecan, or SN-38 (an active metabolite of irinotecan) in human milk, or the effects on the breastfed infant or on milk production. Irinotecan is present in rat milk *[see Data]*.

Because of the potential for serious adverse reactions in breastfed infants from ONIVYDE, advise a nursing woman not to breastfeed during treatment with ONIVYDE and for one month after the final dose.

Data

Radioactivity appeared in rat milk within 5 minutes of intravenous administration of radiolabeled irinotecan HCl and was concentrated up to 65-fold at 4 hours after administration relative to plasma concentrations.

8.3 Females and Males of Reproductive Potential

Contraception

Females

ONIVYDE can cause fetal harm when administered to a pregnant woman *[see Use in Specific Populations (8.1)]*. Advise females of reproductive potential to use effective contraception during treatment with ONIVYDE and for one month after the final dose.

Males

Because of the potential for genotoxicity, advise males with female partners of reproductive potential to use condoms during treatment with ONIVYDE and for four months after the final dose *[see Nonclinical Toxicology (13.1)]*.

8.4 Pediatric Use

Safety and effectiveness of ONIVYDE have not been established in pediatric patients.

8.5 Geriatric Use

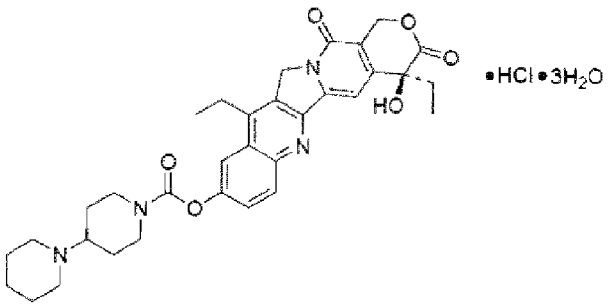
Of the 264 patients who received ONIVYDE as a single agent or in combination with 5-FU and leucovorin in Study 1, 49% were ≥ 65 years old and 13% were ≥ 75 years old. No overall differences in safety and effectiveness were observed between these patients and younger patients.

10 OVERDOSAGE

There are no treatment interventions known to be effective for management of overdosage of ONIVYDE.

11 DESCRIPTION

ONIVYDE is formulated with irinotecan hydrochloride trihydrate, a topoisomerase inhibitor, into a liposomal dispersion for intravenous use. The chemical name of irinotecan hydrochloride trihydrate is (S)-4,11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo1H-pyrano[3',4':6,7]-indolizino[1,2-b]quinolin-9-yl-[1,4'bipiperidine]-1'-carboxylate, monohydrochloride, trihydrate. The empirical formula is $C_{33}H_{38}N_4O_6 \cdot HCl \cdot 3H_2O$ and the molecular weight is 677.19 g/mole. The molecular structure is:



ONIVYDE is a sterile, white to slightly yellow opaque isotonic liposomal dispersion. Each 10 mL single-dose vial contains 43 mg irinotecan free base at a concentration of 4.3 mg/mL. The liposome is a unilamellar lipid bilayer vesicle, approximately 110 nm in diameter, which encapsulates an aqueous space containing irinotecan in a gelated or precipitated state as the sucrose octasulfate salt. The vesicle is composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) 6.81 mg/mL, cholesterol 2.22 mg/mL, and methoxy-terminated polyethylene glycol (MW 2000)-distearoylphosphatidyl ethanolamine (MPEG-2000-DSPE) 0.12 mg/mL. Each mL also contains 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) as a buffer 4.05 mg/mL and sodium chloride as an isotonicity reagent 8.42 mg/mL.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Irinotecan liposome injection is a topoisomerase 1 inhibitor encapsulated in a lipid bilayer vesicle or liposome. Topoisomerase 1 relieves torsional strain in DNA by inducing single-strand breaks. Irinotecan and its active metabolite SN-38 bind reversibly to the topoisomerase 1-DNA complex and prevent re-ligation of the single-strand breaks, leading to exposure time-dependent double-strand DNA damage and cell death. In mice bearing human tumor xenografts, irinotecan liposome administered at irinotecan HCl-equivalent doses 5-fold lower than irinotecan HCl achieved similar intratumoral exposure of SN-38.

12.3 Pharmacokinetics

The plasma pharmacokinetics of total irinotecan and total SN-38 were evaluated in patients with cancer who received ONIVYDE, as a single agent or as part of combination chemotherapy, at doses between 50 and 155 mg/m^2 and 353 patients with cancer using population pharmacokinetic analysis.

The pharmacokinetic parameters of total irinotecan and total SN-38 following the administration of ONIVYDE 70 mg/m^2 as a single agent or part of combination chemotherapy are presented in Table 4.

Table 4: Summary of Mean (±Standard Deviation) Total Irinotecan and Total SN-38

Dose (mg/m^2)	Total Irinotecan					Total SN-38		
	C_{max} [μg/mL] (n=25)	$AUC_{0-\infty}$ [h·μg/mL] (n=23)	$t_{1/2}$ [h] (n=23)	CL [L/h] (n=23)	V_d [L] (n=23)	C_{max} [ng/mL] (n=25)	$AUC_{0-\infty}$ [h·ng/mL] (n=13)	$t_{1/2}$ [h] (n=13)
70	37.2 (8.8)	1364 (1048)	25.8 (15.7)	0.20 (0.17)	4.1 (1.5)	5.4 (3.4)	620 (329)	67.8 (44.5)

C_{max}: Maximum plasma concentration
$AUC_{0-\infty}$: Area under the plasma concentration curve extrapolated to time infinity
$t_{1/2}$: Terminal elimination half-life
CL: Clearance
V_d: Volume of distribution

Over the dose range of 50 to 155 mg/m^2, the C_{max} and AUC of total irinotecan increases with dose. Additionally, the C_{max} of total SN-38 increases proportionally with dose; however, the AUC of total SN-38 increases less than proportionally with dose.

Distribution

Direct measurement of irinotecan liposome showed that 95% of irinotecan remains liposome-encapsulated, and the ratios between total and encapsulated forms did not change with time from 0 to 169.5 hours post-dose. The mean volume of distribution is summarized in Table 4.

Plasma protein binding is <0.44% of the total irinotecan in ONIVYDE.

Elimination

Metabolism

The metabolism of irinotecan liposome has not been evaluated. Irinotecan is subject to extensive metabolic conversion by various enzyme systems, including esterases to form the active metabolite SN-38, and UGT1A1 mediating glucuronidation of SN-38 to form the inactive glucuronide metabolite SN-38G. Irinotecan can also undergo CYP3A4-mediated oxidative metabolism to several inactive oxidation products, one of which can be hydrolyzed by carboxylesterase to release SN-38. In the population pharmacokinetic analysis using the results of a subset with UGT1A1*28 genotypic testing, in which the analysis adjusted for the lower dose administered to patients homozygous for the UGT1A1*28 allele, patients homozygous (N=14) and non-homozygous (N=244) for this allele had total SN-38 average steady-state concentrations of 1.06 and 0.95 ng/mL, respectively.

Excretion

The disposition of ONIVYDE has not been elucidated in humans. Following administration of irinotecan HCl , the urinary excretion of irinotecan is 11 to 20%; SN-38, <1%; and SN-38 glucuronide, 3%. The cumulative biliary and urinary excretion of irinotecan and its metabolites (SN-38 and SN-38 glucuronide), over a period of 48 hours following administration of irinotecan HCl in two patients, ranged from approximately 25% (100 mg/m^2) to 50% (300 mg/m^2).

Specific Populations

Age, Gender, and Renal Impairment:

The population pharmacokinetic analysis suggests that age (28 to 87 years) had no clinically meaningful effect on the exposure of irinotecan and SN-38.

The population pharmacokinetic analysis suggests that gender (196 males and 157 females) had no clinically meaningful effect on the exposure of irinotecan and SN-38 after adjusting for body surface area (BSA).

In a population pharmacokinetic analysis, mild-to-moderate renal impairment had no effect on the exposure of total SN-38 after adjusting for BSA. The analysis included 68 patients with moderate (CLcr 30 - 59 mL/min) renal impairment, 147 patients with mild (CLcr 60 - 89 mL/min) renal impairment, and 135 patients with normal renal function (CLcr > 90 mL/min). There was insufficient data in patients with severe renal impairment (CLcr < 30 mL/min) to assess its effect on pharmacokinetics.

Ethnicity: The population pharmacokinetic analysis suggests that Asians (East Asians, N=150) have 56% lower total irinotecan average steady state concentration and 8% higher total SN-38 average steady state concentration than Whites (N=182).

Hepatic Impairment: The pharmacokinetics of irinotecan liposome have not been studied in patients with hepatic impairment. In a population pharmacokinetic analysis, patients with baseline bilirubin concentrations of 1-2 mg/dL (N=19) had average steady state concentrations for total SN-38 that were increased by 37% compared to patients with baseline bilirubin concentrations of <1 mg/dL (N=329); however, there was no effect of elevated ALT/AST concentrations on total SN-38 concentrations. No data are available in patients with bilirubin >2 mg/dL.

Drug Interactions

In a population pharmacokinetic analysis, the pharmacokinetics of total irinotecan and total SN-38 were not altered by the co-administration of fluorouracil/leucovorin.

Following administration of irinotecan HCl, dexamethasone, a moderate CYP3A4 inducer, does not alter the pharmacokinetics of irinotecan.

In vitro studies indicate that irinotecan, SN-38 and another metabolite, aminopentane carboxylic acid (APC), do not inhibit cytochrome P-450 isozymes.

12.5 Pharmacogenomics

Individuals who are homozygous for the UGT1A1*28 allele are at increased risk for neutropenia from irinotecan HCl. In Study 1, patients homozygous for the UGT1A1*28 allele (N=7) initiated ONIVYDE at a reduced dose of 50 mg/m^2 in combination with 5-FU/LV. The frequency of Grade 3 or 4 neutropenia in these patients [2 of 7 (28.6%)] was similar to the frequency in patients not homozygous for the UGT1A1*28 allele who received a starting dose of ONIVYDE of 70 mg/m^2 [30 of 110 (27.3%)].

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No studies have been performed to assess the potential of irinotecan liposome for carcinogenicity, genotoxicity or impairment of fertility. Intravenous administration of irinotecan hydrochloride to rats once weekly for 13 weeks followed by a 91-week recovery period resulted in a significant linear trend between irinotecan HCl dosage and the incidence of combined uterine horn endometrial stromal polyps and endometrial stromal sarcomas. Irinotecan HCl was clastogenic both in vitro (chromosome aberrations in Chinese hamster ovary cells) and in vivo (micronucleus test in mice). Neither irinotecan nor its active metabolite, SN-38, was mutagenic in the in vitro Ames assay.

Dedicated fertility studies have not been performed with irinotecan liposome injection. Atrophy of male and female reproductive organs was observed in dogs receiving irinotecan liposome injection every 3 weeks at doses equal to or greater than 15 mg/kg, (approximately 3 times the clinical exposure of irinotecan following administration to ONIVYDE dosed at 70 mg/m^2) for a total of 6 doses. No significant adverse effects on fertility and general reproductive performance were observed after intravenous administration of irinotecan HCl in doses of up to 6 mg/kg/day to rats; however, atrophy of male reproductive organs was observed after multiple daily irinotecan HCl doses both in rodents at 20 mg/kg (approximately 0.007 times the clinical irinotecan exposure following ONIVYDE administration at 70 mg/m^2) and in dogs at 0.4 mg/kg (0.0007 times the clinical exposure to irinotecan following administration of ONIVYDE).

14 CLINICAL STUDIES

The efficacy of ONIVYDE was evaluated in Study 1, a three-arm, randomized, open-label trial in patients with metastatic pancreatic adenocarcinoma with documented disease progression, after gemcitabine or gemcitabine-based therapy. Key eligibility criteria included Karnofsky Performance Status (KPS) ≥70, serum bilirubin within institution limits of normal, and albumin ≥3.0 g/dL. Patients were randomized to receive ONIVYDE plus fluorouracil/leucovorin (ONIVYDE/5-FU/LV), ONIVYDE, or fluorouracil/leucovorin (5-FU/LV). Randomization was stratified by ethnicity (White vs. East Asian vs. other), KPS (70-80 vs. 90-100), and baseline albumin level (≥ 4 g/dL vs. 3.0-3.9 g/dL). Patients randomized to ONIVYDE/5-FU/LV received ONIVYDE 70 mg/m^2 as an intravenous infusion over 90 minutes, followed by leucovorin 400 mg/m^2 intravenously over 30 minutes, followed by fluorouracil 2400 mg/m^2 intravenously over 46 hours, every 2 weeks. The ONIVYDE dose of 70 mg/m^2 is based on irinotecan free base (equivalent to 80 mg/m^2 of irinotecan as the hydrochloride trihydrate). Patients randomized to ONIVYDE as a single agent received ONIVYDE 100 mg/m^2 as an intravenous infusion over 90 minutes every 3 weeks. Patients randomized to 5-FU/LV received leucovorin 200 mg/m^2 intravenously over 30 minutes, followed by fluorouracil 2000 mg/m^2 intravenously over 24 hours, administered on Days 1, 8, 15 and 22 of a 6-week cycle. Patients homozygous for the UGT1A1*28 allele initiated ONIVYDE at a reduced dose (50 mg/m^2 ONIVYDE, if given with 5-FU/LV or 70 mg/m^2 ONIVYDE as a single agent). When ONIVYDE was withheld or discontinued for adverse reactions, 5-FU was also withheld or discontinued. When the dose of

ONIVYDE was reduced for adverse reactions, the dose of 5-FU was reduced by 25%. Treatment continued until disease progression or unacceptable toxicity.

The major efficacy outcome measure was overall survival (OS) with two pair-wise comparisons: ONIVYDE versus 5-FU/LV and ONIVYDE/5-FU/LV versus 5-FU/LV. Additional efficacy outcome measures were progression-free survival (PFS) and objective response rate (ORR). Tumor status assessments were conducted at baseline and every 6 weeks thereafter. The trial was initiated as a two-arm study and amended after initiation to include a third arm (ONIVYDE/5-FU/LV). The comparisons between the ONIVYDE/5-FU/LV and the 5-FU/LV arms are limited to patients enrolled in the 5-FU/LV arm after this protocol amendment.

Four hundred seventeen patients were randomized to: ONIVYDE/5-FU/LV (N=117), ONIVYDE (N=151), or 5-FU/LV (N=149). Baseline demographics and tumor characteristics for the 236 patients randomized to ONIVYDE/5-FU/LV or 5-FU/LV (N=119) after the addition of the third arm to the study were a median age of 63 years (range 34-81 years) and with 41% ≥ 65 years of age; 58% were men; 63% were White, 30% were Asian, 3% were Black or African American, and 5% were other. Mean baseline albumin level was 3.97 g/dL, and baseline KPS was 90-100 in 53% of patients. Disease characteristics included liver metastasis (67%) and lung metastasis (31%). A total of 13% of patients received gemcitabine in the neoadjuvant/adjuvant setting only, 55% of patients had 1 prior line of therapy for metastatic disease, and 33% of patients had 2 or more prior lines of therapy for metastatic disease. All patients received prior gemcitabine (alone or in combination with another agent), 54% received prior gemcitabine in combination with another agent, and 13% received prior gemcitabine in combination with nab-paclitaxel.

Study 1 demonstrated a statistically significant improvement in overall survival for the ONIVYDE/5-FU/LV arm over the 5-FU/LV arm as summarized in Table 5 and Figure 1.

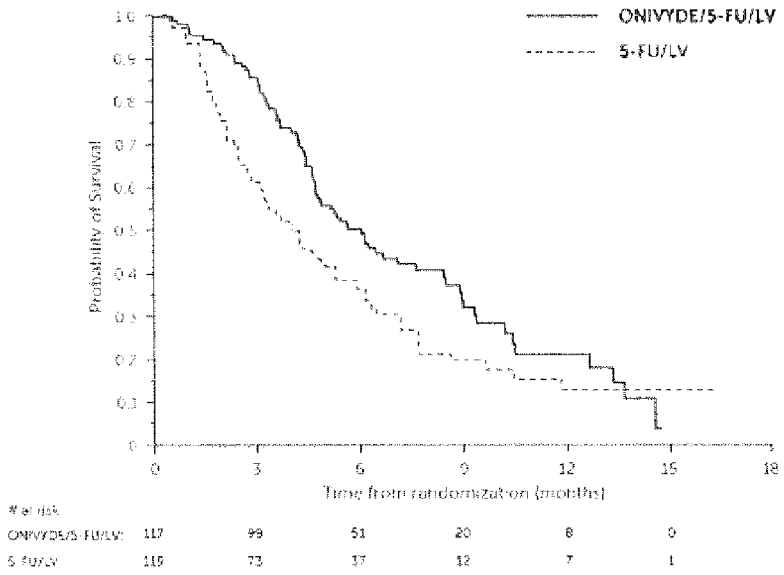
There was no improvement in overall survival for the ONIVYDE arm over the 5-FU/LV arm (hazard ratio=1.00, p-value=0.97 (two-sided log-rank test)).

Table 5: Efficacy Results from Study 1†

	ONIVYDE/5-FU/LV (N=117)	5-FU/LV (N=119)
Overall Survival		
Number of Deaths, n (%)	77 (66)	86 (72)
Median Overall Survival (months)	6.1	4.2
(95% CI)	(4.8, 8.5)	(3.3, 5.3)
Hazard Ratio (95% CI)	0.68 (0.50, 0.93)	
p-value (log-rank test)	0.014	
Progression-Free Survival		
Death or Progression, n (%)	83 (71)	94 (79)
Median Progression-Free Survival (months)	3.1	1.5
(95% CI)	(2.7, 4.2)	(1.4, 1.8)
Hazard Ratio (95% CI)	0.55 (0.41, 0.75)	
Objective Response Rate		
Confirmed Complete or Partial Response n (%)	9 (7.7%)	1 (0.8%)

† 5-FU/LV=5-fluorouracil/leucovorin; CI=confidence interval

Figure 1: Overall Survival



15 REFERENCES

1. OSHA Hazardous Drugs. *OSHA*. http://www.osha.gov/SLTC/hazardousdrugs/index.html

16 HOW SUPPLIED/STORAGE AND HANDLING

How Supplied

ONIVYDE is available in a single-dose vial containing 43 mg irinotecan free base at a concentration of 4.3 mg/mL

NDC: 69171-398-01

Storage and Handling

Store ONIVYDE at 2°C to 8°C (36°F to 46°F). Do NOT freeze. Protect from light.

ONIVYDE is a cytotoxic drug. Follow applicable special handling and disposal procedures.[1]

17 PATIENT COUNSELING INFORMATION

Advise patients of the following:

Severe Neutropenia
Advise patients of the risk of neutropenia leading to severe and life-threatening infections and the need for monitoring of blood counts. Instruct patients to contact their healthcare provider immediately if experiencing signs of infection, such as fever, chills, dizziness, or shortness of breath *[see Warnings and Precautions (5.1)]*.

Severe Diarrhea
Inform patients of the risk of severe diarrhea. Advise patients to contact their healthcare provider if they experience persistent vomiting or diarrhea; black or bloody stools; or symptoms of dehydration such as lightheadedness, dizziness, or faintness *[see Warnings and Precautions (5.2)]*.

Interstitial Lung Disease
Inform patients of the potential risk of ILD. Advise patients to contact their healthcare provider as soon as possible for new onset cough or dyspnea *[see Interstitial Lung Disease (5.3)]*.

Hypersensitivity to irinotecan HCl or ONIVYDE
Advise patients of the potential risk of severe hypersensitivity and that ONIVYDE is contraindicated in patients with a history of severe allergic reactions with irinotecan HCl or ONIVYDE. Instruct patients to seek immediate medical attention for signs of severe hypersensitivity reaction such as chest tightness; shortness of breath; wheezing; dizziness or faintness; or swelling of the face, eyelids, or lips *[see Contraindications (4) and Warnings and Precautions (5.4)]*.

Females and males of reproductive potential

Embryo-fetal toxicity: Inform females of reproductive potential of the potential risk to a fetus, to use effective contraception during treatment and for one month after the final dose, and to inform their healthcare provider of a known or suspected pregnancy *[see Warnings and Precautions (5.5), Use in Specific Populations (8.1, 8.3)]*.

Contraception: Advise male patients with female partners of reproductive potential to use condoms during treatment with ONIVYDE and for four months after the final dose *[see Females and Males of Reproductive Potential (8.3)]*.

Lactation

Advise women not to breastfeed during treatment with ONIVYDE and for one month after the final dose *[see Use in Special Populations (8.2)]*.

Manufactured for:
Merrimack Pharmaceuticals, Inc.
Cambridge, MA 02139

ONIVYDE is a trademark of Merrimack Pharmaceuticals, Inc.

DRUG EVALUATION

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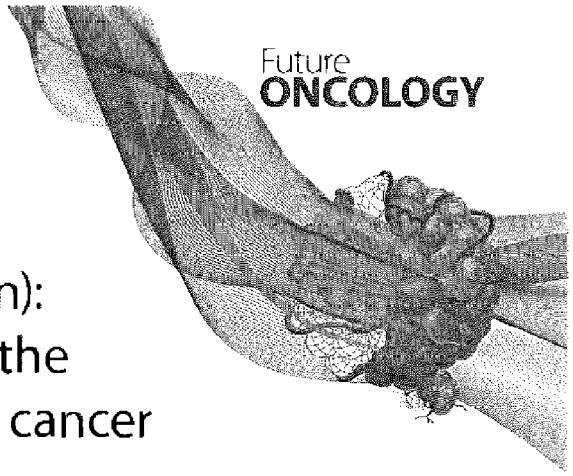

MM-398 (nanoliposomal irinotecan): emergence of a novel therapy for the treatment of advanced pancreatic cancer

Julia Carnevale[1] & Andrew H Ko*[1]

While progress in the treatment of advanced pancreatic cancer has accelerated in recent years, this malignancy continues to have an exceedingly poor prognosis, with no standard of care options beyond front-line chemotherapy. Currently, there are a number of new therapeutic agents in varying stages of clinical development, including molecularly targeted agents, immunotherapies, and modified versions of cytotoxics. MM-398, a novel nanoliposomal formulation of irinotecan, was designed to maximize tumor exposure while minimizing systemic toxicity due to its favorable pharmacokinetic profile. Overall, across multiple clinical trials in multiple disease indications, MM-398 has been shown to have a favorable safety and tolerability profile compared with standard irinotecan. Recent results of the Phase III NAPOLI-1 trial in patients with metastatic pancreatic cancer refractory to gemcitabine-based chemotherapy have shown a significant improvement in overall survival of MM-398 when combined with 5-fluorouracil/leucovorin, compared with 5-fluorouracil/leucovorin alone. This review focuses on the development and pharmacokinetic properties of MM-398, followed by evaluation of its safety and efficacy with a primary emphasis on clinical trials in advanced pancreatic cancer.

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KEYWORDS
• irinotecan • MM-398
• nanoliposomal
• pancreatic cancer • PEP02

Notorious for its late presentation at diagnosis as well as its poor prognosis, pancreatic cancer continues to be one of the most deadly of all human diseases. The estimated incidence rate of pancreatic cancer in the USA is 48,960, with most patients expected to succumb to their disease, even those with early stage disease who are able to undergo resection [1]. Globally, pancreatic cancer is the eighth leading cause of cancer deaths in men (138,100 deaths annually) and the ninth in women (127,900 deaths annually) [2]. While progress in the therapeutic development of this disease has historically taken a slow and painstaking course, littered with numerous negative trial results, over the past 5 years several new treatment options have emerged and research is accelerating in this area. This review will focus on the development and clinical evaluation of MM-398, a novel nanoliposomal irinotecan, which, based on recent clinical results, is now being incorporated into standard treatment paradigms for patients with advanced pancreatic cancer.

Overview of the market: currently available therapeutics

Following the approval of gemcitabine for advanced pancreatic cancer in 1996, there followed well over a decade of numerous negative Phase III clinical trials, most of which compared gemcitabine monotherapy to gemcitabine combined with either a second cytotoxic or a molecularly targeted agent

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in the front-line setting. During this period, only one major randomized study demonstrated an improvement in survival in this patient population [3]; while it did lead to the approval of the EGFR inhibitor erlotinib as an option to add to gemcitabine, the degree of clinical benefit, and hence its widespread usage for this indication, is relatively modest.

This stretch of failed or clinically inconsequential trials changed in 2010, when results of the PRODIGE 4/ACCORD 11 trial demonstrated that the combination of 5-fluorouracil (5-FU), leucovorin (LV), irinotecan and oxaliplatin (FOLFIRINOX) led to a significant improvement in median overall survival (OS; 11.1 vs 6.8 months; hazard ratio [HR]: 0.57; $p < 0.001$) when compared with gemcitabine for advanced pancreatic cancer patients with good performance status in the first-line setting [4]. Subsequently, in 2013, the combination of gemcitabine with nanoparticle albumin-bound paclitaxel (nab-paclitaxel; Abraxane, Celgene, NJ, USA) was also approved as an acceptable regimen for the first-line treatment of advanced pancreatic cancer patients. This approval was based on the results from the Phase III MPACT trial that showed an improvement in median OS of this doublet when compared with gemcitabine monotherapy (8.5 vs 6.7 months; HR: 0.72; $p < 0.001$) [5]. Therefore, for patients with advanced pancreatic cancer and good performance status, FOLFIRINOX and gemcitabine plus nab-paclitaxel both represent viable options in the first-line setting. These two regimens have not been directly compared head-to-head, and absent any predictive biomarker that allows for selection of one versus the other, the choice depends on clinical and practical factors such as age, medical co-morbidities and patient preference.

Upon progression following first-line treatment, the appropriate choice of therapy (for those who remain well enough to continue with some form of active treatment) becomes less clear. Before the introduction of FOLFIRINOX, most studies were designed to assess the efficacy of second-line therapy following progression on gemcitabine or a gemcitabine-based regimen. A prior systematic review of 34 clinical trials evaluating the efficacy of different second-line regimens after gemcitabine-based therapy concluded that there does appear to be a survival advantage of continuing with some form of therapy as opposed to best supportive care [6]. However, whether combination therapy is superior to monotherapy in this setting is uncertain. A randomized Phase III trial from Germany, CONKO-003, showed that oxaliplatin when combined with folinic acid and fluorouracil (OFF) significantly extended median OS (5.9 *vs* 3.3 months; HR: 0.66; log-rank $p = 0.010$) compared with folinic acid and fluorouracil (FF) in patients with gemcitabine-resistant pancreatic cancer [7]. Conversely, a similar trial conducted in Canada called PANCREOX did not find any benefit of FOLFOX over 5-FU/LV alone [8].

Now that both FOLFIRINOX and gemcitabine/nab-paclitaxel are being commonly used as first-line treatment, there is considerable interest in evaluating how well each of these regimens works in patients who have already received the other. Randomized clinical trial data sequencing these regimens one after the other are still lacking, although some small series (primarily retrospective) and case reports do indicate modest efficacy of each in the second-line setting [7,9–13]. Recognizing the limitations of available data, current consensus guidelines [14] recommend that patients who have progressed on first-line gemcitabine-based therapy could be offered a 5-FU-based regimen, albeit without offering clearer direction as to whether and when it should be offered as part of combination therapy. On the other hand, for those who have started with a 5-FU-based regimen such as FOLFIRINOX, a gemcitabine-based regimen would be the next logical step – although again, without certainty as to the magnitude of benefit of using such a sequencing approach.

Overview of the market: competitor compounds in the clinic/late development

Despite the significant clinical advances that have been made recently for patients with advanced pancreatic cancer, overall prognosis remains poor, with only around 2% of patients surviving 5 years [15]. Thankfully, a number of research efforts are ongoing evaluating new therapeutic agents for this disease, including some in later stages of clinical development. For organizational purposes, each of these drugs can be placed into one of four broad categories: cytotoxics, stromal targeting agents, targeted signaling inhibitors and immune modulators, although many may actually mediate effects across multiple of these categories [16].

The two main cytotoxic agents furthest along in clinical development include nanoliposomal irinotecan (MM-398, Merrimack

Pharmaceuticals, MA, USA), the main focus of this article, and evofosfamide (TH-302, Merck/EMD Serono, Rockland, MA, USA). MM-398 is a novel nanoparticle/liposome construct containing irinotecan designed to improve drug delivery to the tumor while minimizing toxic effects to the rest of the body. By changing the pharmacokinetic properties of irinotecan, MM-398 enhances tumor retention and increases the therapeutic window [17]. As will be discussed in detail below, Phase III clinical data has led to the recent approval of this agent by the US FDA that should lead to incorporation of this agent into our treatment algorithms for pancreatic cancer patients. Evofosfamide, meanwhile, is a prodrug of the cytotoxic alkylating agent, bromo-isophosphoramide mustard. This drug is activated under hypoxic conditions, providing a strong rationale for its evaluation in pancreatic cancer, an exceedingly hypoxic tumor. A randomized Phase II study comparing gemcitabine versus gemcitabine plus evofosfamide in patients with advanced pancreatic cancer demonstrated an improvement in median progression-free survival (PFS) with the addition of evofosfamide (5.6 vs 3.6 months; HR: 0.63; p = 0.005) [18], prompting a Phase III study, the MAESTRO trial, for this same patient population, results of which are pending.

Due to the impressive desmoplastic stroma that characterizes most pancreatic cancers, there has been considerable interest in targeting the stroma to alter the biology and the therapeutic accessibility of the tumor. Most advanced in clinical development is an agent termed PEGPH20 (Halozyme, CA, USA), a pegylated form of recombinant human hyaluronidase, which acts by breaking down hyaluronic acid, a major stromal component. Interim analysis of the Phase II HALO-109-202 study, a study of gemcitabine/nab-paclitaxel with or without PEGPH20 in previously untreated stage IV pancreatic cancer patients, showed intriguing early results. In a subset of patients with high levels of hyaluronic acid in their tumor specimens, overall response rate (71 vs 29%) and median PFS (9.2 vs 4.3 months) were significantly improved in the PEGPH20-containing arm [19]. These results have prompted the development of a global Phase III randomized controlled trial of gemcitabine/nab-paclitaxel with or without PEGPH20 specifically in patients with high intratumoral levels of hyaluronic acid, scheduled to start in 2016. Other ongoing studies are evaluating this same agent in combination with different chemotherapy backbones, including FOLFIRINOX.

A number of agents targeting specific signaling pathways that drive the growth, proliferation and survival of pancreatic cancer are also fairly far along in clinical development for advanced pancreatic cancer. Ruxolitinib (Jakafi, InCyte Pharmaceuticals, NY, USA), a small molecule inhibitor of the JAK1 and JAK2 kinases, may act both through direct anti-oncogenic mechanisms as well as by reducing the cytokine burden that contributes to pancreatic cancer-related cachexia. This agent was evaluated in combination with capecitabine in patients who had failed gemcitabine-based chemotherapy in the Phase II RECAP trial. In a preplanned analysis of patients with elevated levels of C-reactive protein (CRP), reflecting high levels of systemic inflammation, median survival was significantly longer in those patients who received the ruxolitinib/capecitabine combination as opposed to capecitabine alone (83 vs 55 days; HR: 0.47; p = 0.01) [20]. Based on these encouraging results, two Phase III trials (JANUS 1 and JANUS 2) have been initiated to evaluate this agent specifically in pancreatic cancer patients with high CRP levels. Another recently opened randomized Phase II trial, this one in the frontline setting, is evaluating MM-141, a bispecific IGFR/HER3 monoclonal antibody, in combination with gemcitabine plus nab-paclitaxel for patients with metastatic pancreatic cancer who have high serum levels of free IGF-1. The use of IGF-1 as a selection biomarker was informed by results from a preceding Phase I trial that showed that patients with higher circulating levels of this growth factor were able to stay on MM-141 approximately twice as long [21].

Finally, while pancreatic cancer has historically been considered an immune-privileged tumor, there have been a number of attempts to stimulate an immune attack in this disease, with different immune modulatory agents currently in various stages of clinical testing. Vaccination-based strategies represent one such approach far along in clinical development. For example, CRS-207 (Aduro Biosciences, Berkeley, CA, USA) is a live-attenuated *Listeria monocytogenes* vaccine vector genetically engineered to express mesothelin, a tumor-associated antigen expressed in the majority of pancreatic cancers. CRS-207 was shown to prolong survival when combined with the cellular vaccine GVAX in a

Phase II trial of patients with chemorefractory metastatic pancreatic cancer, as compared with GVAX alone (median OS: 6.0 vs 3.4 months; HR: 0.4477; p = 0.0057) [22]. These results have led to successor trials comparing this vaccine-based strategy to standard cytotoxic therapy, as well as evaluating it in combination with immune checkpoint blockade (nivolumab). Other immune modulatory agents currently under evaluation in pancreatic cancer include monoclonal antibodies directed against PD-1, PDL-1 and CTLA-4; IDO inhibitors; CD40 agonist antibodies; and Bruton tyrosine kinase inhibitors. One very novel approach still in pilot stages of clinical evaluation in pancreatic cancer (as well as other solid tumors) consists of adoptive T-cell transfer, using a patient's own (autologous) T cells that are genetically engineered to express chimeric antigen receptors (CAR) that then recognize tumor-specific proteins such as mesothelin.

Introduction to MM-398

MM-398 (also known as nal-IRI; previously referred to as PEP02) is a novel nanoparticle/liposome construct containing irinotecan, which has been engineered to optimize drug delivery and retention in the tumor while minimizing systemic toxicity [17]. First approved in 1996, irinotecan is a semisynthetic analog of the natural alkaloid camptothecin that is currently used widely for a variety of solid tumor indications. By stabilizing the complex between topoisomerase I and bound DNA, irinotecan induces stalling of replication forks which ultimately leads to DNA strand breaks and inhibits replication [23,24].

While it has been used in the treatment of many different malignancies, irinotecan has historically been most heavily used in the treatment of colon cancer, typically in combination with 5-FU and LV (FOLFIRI) [25]. Based on results of the PRODIGE 4/ACCORD 11 trial, irinotecan is also frequently used in combination with oxaliplatin, fluorouracil and LV (FOLFIRINOX) as first-line treatment in patients with pancreatic cancer and good performance status [4]. The primary dose limiting toxicities of irinotecan include diarrhea and myelosuppression. By improving accumulation and activation specifically within the tumor, MM-398 was designed to increase the therapeutic window of irinotecan to achieve maximum efficacy while minimizing these toxicities.

Chemistry

Liposomal-based systems have been used to improve the delivery of other cytotoxic agents, such as the example of PEGylated liposomal doxorubicin, which is presently used for the treatment of breast cancer, Kaposi sarcoma and other anthracycline-sensitive malignancies. However, development of liposomal constructs for other chemotherapeutics has proven more challenging. In 2006, Drummond and colleagues developed a new intraliposomal drug stabilization technique to successfully encapsulate irinotecan into liposomal nanoparticles [17]. To achieve this, they used highly charged anions of sucrose octasulfate to trap the irinotecan in the liposome (see **Figure 1**). The triethylammonium component of this salt compound is thought to provide a source of cations that exchange for the influx of the irinotecan molecules. The sucrose octasulfate forms a stable complex with the irinotecan and the triethylammonium crosses the lipid bilayer as triethylamine. This leads to an extremely efficient loading system, ultimately packaging 109,000 drug molecules per particle, which far exceeds liposomal formulations of other chemotherapies [17].

Pharmacokinetics & pharmacodynamics

Irinotecan is a prodrug that is converted by nonspecific carboxylesterases into its active metabolite, SN-38, which is about 100- to 1000-fold more potent [26]. In addition, irinotecan exists in an equilibrium between its active lactone form (acidic conditions) and an inactive carboxylate form (basic conditions). SN-38 is glucuronidated in the liver by UGT1A1 and then cleared by biliary excretion.

Genetic variants of UGT1A1 produce significant variability in the metabolism and excretion of irinotecan, which contributes substantially to interpatient differences in tolerability of this agent, particularly hematologic toxicity (see Pharmacogenomics section below). The liposomal carrier system of MM-398 offers a way to protect irinotecan from premature activation, allowing a longer duration in circulation and improved biodistribution. It is thought that these nanoliposomes passively accumulate preferentially in the tumor due in part to what is known as the enhanced permeability and retention (EPR) effect [27]. The EPR effect in tumors is attributed to a combination of irregular and permeable blood vessels along with an impaired lymphatic system that leads

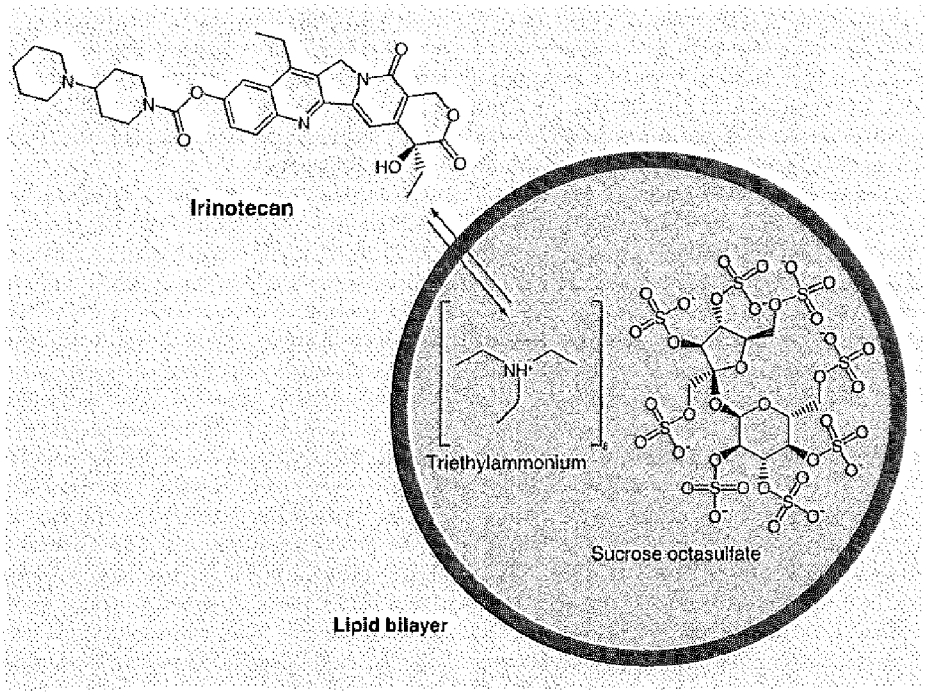

Figure 1. Depiction of exchange of triethylamine for irinotecan, which forms a stable complex with sucrose octasulfate inside the liposome.

to increased cumulative trapping of macromolecules in tumors relative to other tissues. Once deposited in the tumor, liposomes can be taken up by tumor-resident macrophages, which release the bound irinotecan and convert it to active SN-38 [17,28].

Preclinical pharmacokinetic (PK) analyses in rodents demonstrated a significant increase in the half-life of MM-398 in circulation compared with standard irinotecan, by a factor of 40. Standard irinotecan was rapidly cleared from the circulation, with only 2% of the injected dose remaining at 30 min and 35% present in the inactive carboxylate form. By comparison, 23.2% of MM-398 was detectable in circulation at 24 h, with no conversion of the irinotecan to either SN-38 or the carboxylate form in the blood [17]. These preclinical studies suggest that the nanoliposomal carrier could successfully provide protection from lactone hydrolysis or activation to the toxic SN-38 metabolite in circulation, thus increasing circulation time and tumor delivery. Additional studies of breast and colon cancer xenograft models in mice furthered the notion that this novel drug formulation can translate into improved antitumor activity. Specifically, when compared with standard irinotecan, nanoliposomal irinotecan led to greater inhibition of tumor growth and higher rates of complete tumor regressions, with less associated toxicity (transient weight loss representing the primary side effect) [17].

Two published studies in humans have reported on the phamacokinetics of MM-398. In a Phase I study of MM-398 in advanced solid tumor patients [29], the maximal tolerated dose (MTD) was determined to be 120 mg/m^2 every 3 weeks, with the primary toxicities including myelosuppression and diarrhea. In this study, the slow release of irinotecan from MM-398 resulted in a small volume of distribution and a prolonged terminal half-life of total irinotecan in circulation, while the active metabolite of irinotecan, SN-38, demonstrated a lower C_{max} and longer terminal half-life than respective values of these measures reported in the literature for standard irinotecan. Furthermore, the area under the curve (AUC) of SN-38 after 120 mg/m^2 MM-398 was in the same range as that achieved with 300–350 mg/m^2 of standard irinotecan described in the literature, suggesting that MM-398 confers an improved therapeutic window and may be a better choice for combination with other cytotoxic agents.

Meanwhile, a randomized Phase II trial of MM-398 compared with either irinotecan or docetaxel as second-line treatment for patients with locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma [30] afforded the opportunity to directly compare the PK properties of MM-398 with those of standard irinotecan. PK values were analyzed for MM-398 dosed at 120 mg/m^2 and for irinotecan dosed at 300 mg/m^2. The dose-normalized PK results of these PK parameters evaluated are displayed in **Table 1**. Compared with standard irinotecan, MM-398 demonstrated a higher AUC, a lower clearance and smaller volume of distribution for total and encapsulated irinotecan. The active metabolite SN-38 showed a longer mean T_{max} (10.2 vs 2.1 h after infusion of 120 mg/m^2 MM-398 and 300 mg/m^2 irinotecan, respectively). After administration of MM-398, the dose-normalized C_{max} value for the formation of SN-38 was about 50% less than after infusion of standard irinotecan, indicating less premature activation of the prodrug irinotecan in the circulation in its encapsulated form. Additionally, the dose-normalized $AUC_{0-\infty}$ and AUC_{0-t} values of SN-38 following MM-398 administration were 3.3- and five-times higher, respectively, than those seen with standard irinotecan [30]. These favorable PK parameters confirmed that liposomally encapsulated irinotecan can stably circulate in plasma significantly longer than free irinotecan, enabling a slow release of irinotecan over time and increasing the likelihood of exposure of the tumor to the active SN-38 metabolite.

Pharmacogenetics

As discussed above, SN-38 is detoxified by *UGT1A1* to its inactive form SN-38 glucuronide (SN-38G), which is subsequently excreted via bile and urine. More than 60 genetic variants in the promoter region and exon 1 of *UGT1A1* have been identified, most of which are associated with reduced or absent enzymatic activity. This results in higher levels and prolonged exposure to SN-38, and consequently, a higher risk for irinotecan-associated toxicity, including neutropenia and diarrhea. The most well-studied among these *UGT1A1* genetic variants is the *28 polymorphism [31–33], which produces seven repeats of the two-base insertion TA in TATA box in the promoter region of the gene. Individuals who are homozygous for this *UGT1A1*28* allele (also known as 7/7) are more than threefold likely to develop severe neutropenia compared with those with wild genotype (reviewed in [34]).

In the above referenced Phase I trial of MM-398, the study investigators were only able to analyze *UGT1A1* genetic polymorphisms in three study patients. Interestingly, the one

Table 1. Pharmacokinetic values from the Phase II trial by Roy *et al.*

Parameter	Unit	Drug given	Total irinotecan	MM-398/ irinotecan	SN-38	MM-398/ irinotecan
C_{max}	ng/ml	MM-398	60,842	36	9	0.498
		Irinotecan	4265		44	
T_{max}	h	MM-398	2.1	1.31	10.2	4.9
		Irinotecan	1.6		2.1	
AUC_{0-t}	h·ng/ml	MM-398	1,651,508	171	476	3.3
		Irinotecan	24,155		361	
$AUC_{0-\infty}$	h·ng/ml	MM-398	1,812,221	173	879	4.99
		Irinotecan	26,159		440	
Vss	ml/m^2	MM-398	2234	0.0227	NA	NA
		Irinotecan	98,527		NA	NA
CL	ml/h/m^2	MM-398	191	0.0148	NA	NA
		Irinotecan	12,886		NA	NA
$T_{½}$	h	MM-398	21.2	2.75	88.8	3.9
		Irinotecan	7.7		22.8	
$MRT_{0-\infty}$	h	MM-398	30.1	3.58	128.7	5
		Irinotecan	8.4		26	

AUC_{0-t}: Area under the curve at T_{max}; $AUC_{0-\infty}$: Area under the curve extrapolated to infinite time; CL: Clearance; C_{max}: maximum concentration; $MRT_{0-\infty}$: Mean residence time; SN-38: Active metabolite of irinotecan; T½: Half-life; T_{max}: Time at which Cmax is reached; Vss: Volume of distribution at steady state.
Data taken from [30].

Table 2. List of clinical trials evaluating MM-398.

Year	Title	First author	Ref.
2010	Phase I study of liposome irinotecan (PEP02) in combination with weekly infusion of 5-FU/LV in advanced solid tumors	Chen *et al.*	[38]
2012	Phase I study of biweekly liposome irinotecan (PEP02, MM-398) in metastatic colorectal cancer failed on first-line oxaliplatin-based chemotherapy	Chen *et al.*	[39]
2013	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 gastroesophageal junction adenocarcinoma	Roy *et al.*	[30]
2013	A multinational Phase II study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer	Ko *et al.*	[35]
2015	Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients	Chang *et al.*	[29]
2015	PEPCOL: a randomized noncomparative Phase II study of PEP02 (MM-398) or irinotecan in combination with leucovorin and 5-fluorouracil as second-line therapy in patients with unresectable metastatic colorectal cancer – A GERCOR Study	Chibaudel *et al.*	[41]
2015	A Phase I trial of intravenous liposomal irinotecan in patients with recurrent high-grade gliomas	Clarke *et al.*	[42]
2015	Phase III study of MM-398 (nal-IRI), with or without 5-fluorouracil and leucovorin, versus 5-fluorouracil and leucovorin, in metastatic pancreatic cancer (mPAC) previously treated with gemcitabine-based therapy	Wang-Gillam *et al.*	[40]

patient experiencing treatment-related death due to myelosuppression on this study was heterozygous for both UGT1A1*6 and UGT1A1*28, and showed a higher $AUC_{0-\infty}$ and C_{max} of SN-38 (two- to three-fold higher than those of the other three patients treated at the same dose level) [29]. In the Phase II gastric cancer study, UGT1A1 variants were observed to correlate with toxicity; specifically, among patients treated with MM-398, a significantly higher frequency of grade 3–4 neutropenia was seen in patients heterozygous for UGT1A1*6 compared with wild-type patients [30]. A separate nonrandomized Phase II study of MM-398 monotherapy in gemcitabine-resistant metastatic pancreatic cancer patients included analysis of polymorphisms in both UGT1A1 and UGT1A9; however, no correlation was identified with either nonhematologic or hematologic toxicity [35].

It is worth noting a prior PK and dose-finding study performed by Innocenti and colleagues evaluating the MTD and dose-limiting toxicities in patients who were either homozygous or heterozygous carriers of the UGT1A1*28 variant. These results suggested that doses of irinotecan could be individualized based on *UGT1A1* genotype [36]. Despite these and other studies demonstrating differences in PK and toxicity based on *UGT1A1* genotype, there are currently not well-established guidelines on testing for *UGT1A1* polymorphisms in clinical practice. The package insert for irinotecan notes that a reduction in the starting dose of this drug should be considered for patients known to be homozygous for the UGT1A1*28 allele, although the appropriate dose reduction is not known [37]. It is reasonable to expect that similar fairly general recommendations will extend to MM-398 unless and until further larger-scale pharmacogenetic studies with this agent are conducted.

Clinical efficacy

A number of Phase I, II and III clinical trials of MM-398 have been conducted across many different solid tumors (listed in **Table 2**) [29,30,35,38–42]. As noted previously, Chang *et al.* [29] published a multicenter, first-in-human, open-label, Phase I, dose-escalation study of MM-398 in patients with advanced refractory solid tumors, including one patient with pancreatic cancer who had been refractory to gemcitabine and FOLFOX. Best response by RECIST criteria was partial response in 20% of the evaluable study cohort (including the patient with pancreatic cancer); with an overall disease control rate of 50%. The maximum tolerated dose was 120 mg/m^2.

Specific to pancreatic cancer, Ko *et al.* conducted a multinational Phase II study of MM-398 in patients with metastatic pancreatic cancer refractory to gemcitabine-based front-line chemotherapy [35]. Forty patients were enrolled and received MM-398 at a dose of 120 mg/m^2 every 3 weeks, with the option of dose-escalating to 150 mg/m^2 if the first cycle was tolerated well. The study met its primary end point with a 3-month OS rate of 75%, and median PFS and OS was 2.4 and 5.2 months, respectively. An objective response was noted in 7.5% of patients and disease control (partial response + stable disease) in 50%. Additionally, of the patients with an elevated CA19-9 level at baseline, 31.3% showed a decline in this tumor marker by greater

than 50%.

Based on these results, an international randomized Phase III trial called NAPOLI-1 was developed to evaluate the efficacy of MM-398 for this same disease indication, with OS serving as the primary end point [40]. Patients were stratified according to albumin levels (less than or greater than 4.0 g/dl), Karnofsky Performance Status (70–80 vs 90–100) and ethnicity (Caucasian vs east Asian vs other). The study was originally designed with two arms only, comparing MM-398 (120 mg/m^2 every 3 weeks) to a control arm of 5-FU (administered as a weekly 24-h infusion at 2000 mg/m^2) plus LV (200 mg/m^2), for 4 out of 6 weeks. Subsequently, after the first 63 patients were enrolled, a third arm consisting of biweekly MM-398 (80 mg/m^2) plus 46-h infusion of 5-FU (2400 mg/m^2) and LV (400 mg/m^2) was added. The impetus for inclusion of this additional arm stemmed not only from the historical precedent of combining irinotecan with 5-FU in gastrointestinal malignancies, but also from preclinical evidence suggesting a biologic rationale to evaluate liposomal irinotecan in combination with other therapies. Specifically, studies of Irinophore-C (another liposomal irinotecan formulation) in subcutaneous and orthotopic mouse models showed that this agent increased microvessel density and thereby increased delivery of small molecule chemotherapies [43,44]. In a randomized Phase II trial of MM-398 in advanced colorectal cancer (PEPCOL), patients receiving MM-398 in combination with 5-FU and leuvocorin demonstrated not only promising antitumor activity, but also an improved safety profile in comparison to subjects on the irinotecan + 5-FU/LV arm [41].

Overall, the three arms on NAPOLI-1 were well balanced according to age (median age 63 years), sex (54–59% male) and Karnofsky performance status (56% Karnofsky performance status 90–100). In the entire (intention to treat) cohort (n = 417), patients on the MM-398 + 5-FU/LV arm had an improved median survival compared with the 5-FU/LV alone arm (6.1 vs 4.2 months; HR: 0.67; p = 0.0122). Forest plot analyses showed that survival benefit for the MM-398-containing combination was retained across all subgroups, including according to Karnofsky performance status, age, ethnicity, baseline CA19-9 level and line of therapy (second-line or beyond). The combination arm also showed significant improvements in median PFS (3.1 vs 1.5 months; HR: 0.56, p = 0.0001), PFS at 12 weeks (57 vs 26%), objective response rate (16 vs 1%) and CA19-9 decline ≥50% (36 vs 12%). In a subsequent analysis of the per-protocol population (defined as those patients who received greater than 80% of their planned doses during the first 6 weeks of study treatment), improvement in median OS associated with MM-398 + 5-FU/LV was even more pronounced (8.9 vs 5.1 months; HR: 0.47; p = 0.0018). Notably, there was no statistically significant difference in median survival between patients receiving MM-398 monotherapy and those receiving 5-FU/LV alone (4.9 vs 4.2 months; HR: 0.99; p = 0.94). The main efficacy findings of NAPOLI-1 are summarized in Table 3.

Safety & tolerability

The primary adverse effects seen with MM-398, including bone marrow suppression and diarrhea, are similar to those typically associated with irinotecan. In the Phase II trial of MM-398 monotherapy in patients with gemcitabine-refractory metastatic pancreatic cancer, 15% of patients experienced grade 3 or 4 diarrhea and 30% developed grade 3–4 neutropenia (defined as an absolute neutrophil count less than 1000 cells/μl). Importantly, three of the 40 patients on this study had deaths attributable to treatment-related neutropenia [35]. Other notable grade 3–4 side

Table 3. Efficacy results of the Phase III trial, NAPOLI-1, of MM-398 ± 5-fluorouracil/leucovorin compared with 5-fluorouracil/leucovorin alone.

Parameter	MM-398 + 5-FU/LV	5-FU/LV	MM-398
n	117	149	151
Median OS	6.1 months	4.2 months	4.9 months
Median PFS	3.1 months	1.5 months	2.7 months
PFS at 12 weeks	57%	28%	47%
ORR	16%	1%	6%
CA19-9 reduction ≥50%	36%	12%	31%

5-FU: 5-fluorouracil; LV: Leucovorin; ORR: Objective response rate; OS: Overall survival; PFS: Progression-free survival.

Table 4. Adverse events >grade 3 in trials evaluating MM-398.

	Ko *et al.* (2013) [35]	Roy *et al.* (2013) [30]	Chang *et al.* (2015) [29]	Chibaudel *et al.* (2015) [41]	Wang-Gillam *et al.* (2015) [40]	
Study phase	II	II	I	II	III	III
Dosing regimen	MM-398 120 mg/m² q3 weekly	MM-398 120 mg/m² q3 weekly	MM-398 60–180 mg/m² q3 weekly	MM-398 80 mg/m² plus LV 400 mg/m² and 46-h infusion 5-FU 2400 mg/m² q2 weekly	MM-398 80 mg/m² plus LV 400 mg/m² and 46-h infusion 5-FU 2400 mg/m² q2 weekly	MM-398 120 mg/m² q3 weekly
Sample size	40	44	11	28	117	151
Nonhematologic toxicity (%)						
Diarrhea	15	27	33	33	13	21
Nausea	10	11	33	4	8	5
Vomiting	N/A	5	67	4	11	14
Fatigue	20	5	17	N/A	14	6
Hyponatremia	15	N/A	N/A	N/A	3	6
Anorexia	10	7	0	N/A	N/A	N/A
Hematologic toxicity (%)						
Neutropenia	30	7	17	11	20	16
Anemia	15	5	0	N/A	6	7
Thrombocytopenia	N/A	2	N/A	N/A	2	1

5-FU: 5-fluorouracil; LV: Leucovorin; N/A: Not applicable; q2 weekly: Once every 2 weeks; q3 weekly: Once every 3 weeks.

effects included fatigue (20%), anemia (15%), hyponatremia (15%), anorexia (10%) and nausea (10%). Meanwhile, in the Phase III NAPOLI-1 study, grade 3–4 diarrhea was reported in 13% of patients receiving the combination of MM-398 plus 5-FU/LV, and grade 3–4 neutropenia in an additional 20%. Other grade 3–4 toxicities associated with this combination included fatigue (14%), vomiting (11%), anemia (6%) and thrombocytopenia (2%).

In considering the toxicities of MM-398 in the future context in which it will be most commonly used in pancreatic cancer, perhaps the most appropriate comparison would be of (MM-398 + 5-FU/LV) to FOLFIRI. The previously discussed Phase II PEPCOL study, in which patients with advanced colorectal cancer received either MM-398 plus 5-FU/LV or FOLFIRI (including both FOLFIRI-1 and modified FOLFIRI-3 regimens), is instructive in that regard. This study utilized the same dose of 5-FU in all arms, and 80 mg/m² of MM-398 compared with 180 mg/m² total of irinotecan. In terms of grade 3–4 toxicity, lower levels of both neutropenia (10.7 vs 29.6%) and diarrhea (21.4 vs 33.3%) were observed in the MM-398 + 5-FU/LV arm compared with the FOLFIRI arm [41].

Taken together, the safety and toxicity profile of MM-398 appears to be comparable to, if not better than, that of irinotecan. Although administration of MM-398 as monotherapy is associated with substantial cytopenias, including several deaths associated with neutropenic complications in the Phase II pancreatic cancer trial, this does not appear to be as a major issue when using the biweekly dosing schedule of MM-398 with 5-FU/leuvocorin. Therefore, primary prophylaxis with granulocyte colony-stimulating factors is not necessarily indicated, except perhaps for patients with a prior history of neutropenia on prior antineoplastic agents. **Table 4** provides a summary of the primary toxicity profile of MM-398 reported in various clinical studies, both as a single agent and in combination with 5-FU/LV.

Regulatory affairs

In 2011, MM-398 received orphan drug designation from both the US FDA and the EMA for the treatment of advanced pancreatic cancer. Based on the positive results of the NAPOLI-1 trial, in November 2014 the FDA granted MM-398 a Fast Track designation as second-line therapy, in combination with 5-FU and LV, for patients with metastatic pancreatic cancer who have progressed on a gemcitabine-based regimen. Merrimack Pharmaceuticals, in partnership with Baxter International, completed rolling submission of

the New Drug Agreement (NDA) to the FDA in April 2015, where it was subsequently assigned priority review designation. This priority review assignment indicates that the FDA will take action on the marketing application within 6 months from the date of assignment (June 2015). In parallel, the EMA also accepted for review a Marketing Authorization Application (MAA) for the same indication.

In October 2015, MM-398 received official FDA approval, under the name Onivyde, for use in combination with fluorouracil and LV for the treatment of metastatic pancreatic cancer in patients who have previously received gemcitabine-based chemotherapy.

Conclusion

MM-398 is a novel nanoliposomal formulation of irinotecan designed to increase the drug payload to the tumor while minimizing systemic toxicity. There is now evidence from multiple clinical trials demonstrating improvement in the pharmacokinetic properties of MM-398 compared with standard irinotecan. Furthermore, at the maximum tolerated dose MM-398 appears to be as safe as, if not safer than, standard irinotecan. MM-398 is currently being tested across a range of different solid tumors, but has now completed Phase III evaluation in advanced pancreatic cancer. Based on results from the Phase III NAPOLI-1 trial of pancreatic cancer patients refractory to gemcitabine-based therapy, MM-398 monotherapy appears to perform similarly to 5-FU/LV, but significantly better in terms of OS and other clinical outcome measures when administered in combination with 5-FU/LV.

Future perspective

Based on the positive results of NAPOLI-1, MM-398 in combination with 5-FU/LV is the first therapeutic agent to gain approval specifically for second-line use in patients with metastatic pancreatic cancer. This regimen might now be expected to be used commonly in patients who have progressed on gemcitabine plus nab-paclitaxel, a first-line standard of care combination. It is also of interest whether the optimized PK and safety profile of MM-398 over standard irinotecan would make it an ideal substitute for irinotecan in the first-line FOLFIRINOX

EXECUTIVE SUMMARY

Mechanism of action

- MM-398 is a nanoliposomal formulation of irinotecan, designed to maximize tumor delivery and minimize systemic toxicity via optimized pharmacokinetic properties.

Pharmacokinetic properties

- Compared with standard irinotecan, MM-398 demonstrates a significantly increased area under the curve and half-life when both total irinotecan and SN-38 (the active metabolite) are measured in the blood.
- MM-398 also demonstrates a decreased clearance and decreased volume of distribution.
- Overall, this suggests a longer time in circulation and increased tumor exposure.

Clinical efficacy

- MM-398 has now been assessed in Phase I–III clinical trials in advanced pancreatic cancer patients.
- Based on results from the Phase III NAPOLI-1 trial of pancreatic cancer patients refractory to gemcitabine-based therapy, MM-398 significantly improves overall survival when combined with 5-fluorouracil/leucovorin compared with 5-fluorouracil/leucovorin alone.

Safety & tolerability

- The primary adverse effects of standard irinotecan are bone marrow suppression and diarrhea.
- Across studies, it appears that the safety and tolerability of MM-398 compares favorably to standard irinotecan.

Dosage & administration

- The maximum tolerated dose of MM-398 monotherapy has been determined to be 120 mg/m^2 every 3 weeks.
- When given in combination with 5-fluorouracil/leucovorin, MM-398 is dosed at 70 mg/m^2 every 2 weeks (this is the free base equivalent to the 80 mg/m^2 dose of the salt form used in the NAPOLI-1 trial), with 5-fluorouracil dosed at 2400 mg/m^2 (as a continuous infusion over 46 h) and leucovorin at 400 mg/m^2, every 2 weeks.

regimen. This might represent a natural extension of MM-398's role in metastatic pancreatic cancer. Future clinical trials of MM-398, whether in the first-line setting or beyond, should also assess its combinability with other cytotoxics, including gemcitabine and nab-paclitaxel; as well as with novel therapeutics currently in development. It would also be instructive if such trials were to incorporate tumor biomarker evaluation as well as the pharmacodynamic effects of MM-398 on the tumor and its microenvironment. For instance, pre- and post-treatment evaluation of tumor stroma and microvasculature, macrophage composition and resultant small molecule penetrance, would be of great interest given the predicted modulation of MM-398 on these compartments based on preclinical studies.

Financial & competing interests disclosure

AH Ko has previously served as an advisory board member for Merrimack Pharmaceuticals, and currently receives funding support (paid to his institution) from Merrimack for the conduct of pancreatic cancer-specific clinical trials. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2015. *CA Cancer J. Clin.* 65(1), 5–29 (2015).

2 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global Cancer Statistics. *CA Cancer J. Clin.* 61(2), 69–90 (2011).

3 Moore MJ, Goldstein D, Hamm J *et al.* Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a Phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J. Clin. Oncol.* 25(15), 1960–1966 (2007).

4 Conroy T, Desseigne F, Ychou M *et al.* FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N. Engl. J. Med.* 364(19), 1817–1825 (2011).

5 Von Hoff DD, Ervin T, Arena FP *et al.* Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N. Engl. J. Med.* 369(18), 1691–1703 (2013).

6 Rahma OE, Duffy A, Liewehr DJ, Steinberg SM, Greten TF. Second-line treatment in advanced pancreatic cancer: a comprehensive analysis of published clinical trials. *Ann. Oncol.* 24(8), 1972–1979 (2013).

7 Oettle H, Riess H, Stieler JM *et al.* Second-line oxaliplatin, folinic acid, and fluorouracil versus folinic acid and fluorouracil alone for gemcitabine-refractory pancreatic cancer: outcomes from the CONKO-003 trial. *J. Clin. Oncol.* 32(23), 2423–2429 (2014).

8 Gill S, Ko Y-J, Cripps M *et al.* PANCREOX: a randomized Phase 3 study of 5FU/LV with or without oxaliplatin for second-line advanced pancreatic cancer in patients who have received gemcitabine-based chemotherapy. *J. Clin. Oncol.* 32(5s), Abstract 4022 (2014).

9 Assaf E, Verlinde-Carvalho M, Deibaldo C *et al.* 5-fluorouracil/leucovorin combined with irinotecan and oxaliplatin (FOLFIRINOX) as second-line chemotherapy in patients with metastatic pancreatic adenocarcinoma. *Oncology* 80(5–6), 301–306 (2011).

10 Berger AK, Weber TF, Jager D, Springfeld C. Successful treatment with nab-paclitaxel and gemcitabine after FOLFIRINOX failure in a patient with metastasized pancreatic adenocarcinoma. *Onkologie* 36(12), 763–765 (2013).

11 Bertocchi P, Abeni C, Meriggi F *et al.* Gemcitabine plus nab-paclitaxel as second-line and beyond treatment for metastatic pancreatic cancer: a single institution retrospective analysis. *Rev. Recent Clin. Trials* 10(2), 142–145 (2015).

12 Lee MG, Lee SH, Lee SJ *et al.* 5-fluorouracil/leucovorin combined with irinotecan and oxaliplatin (FOLFIRINOX) as second-line chemotherapy in patients with advanced pancreatic cancer who have progressed on gemcitabine-based therapy. *Chemotherapy* 59(4), 273–279 (2013).

13 Portal A, Pernot S, Siauve N *et al.* Sustained response with gemcitabine plus nab-paclitaxel after folfirinox failure in metastatic pancreatic cancer: report of an effective new strategy. *Clin. Res. Hepatol. Gastroenterol.* 38(2), e23–e26 (2014).

14 National Comprehensive Cancer Network. Pancreatic adenocarcinoma: NCCN clinical practice guidelines in oncology (v. 2.2015). www.nccn.org

15 Howlader N NA, Krapcho M, Garshell J *et al.* SEER Cancer Statistics Review, 1975–2012, National Cancer Institute. Bethesda, MD, USA. http://seer.cancer.gov/csr/1975_2012/

16 Ko AH. Progress in the treatment of metastatic pancreatic cancer and the search for next opportunities. *J. Clin. Oncol.* 33(16), 1779–1786 (2015).

17 Drummond DC, Noble CO, Guo Z, Hong K, Park JW, Kirpotin DB. Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. *Cancer Res.* 66(6), 3271–3277 (2006).

•• **Initial report describing drug development of MM-398.**

18 Borad MJ, Reddy SG, Bahary N *et al.* Randomized Phase II trial of gemcitabine plus TH-302 versus gemcitabine in patients with advanced pancreatic cancer. *J. Clin. Oncol.* 33(13), 1475–1481 (2015).

19 Hingorani SR, Harris WP, Hendifar AE *et al.* High response rate and PFS with PEGPH20 added to nab-paclitaxel/gemcitabine in stage IV previously untreated pancreatic cancer patients with high-HA tumors: interim results of a randomized Phase II study. *ASCO Meeting Abstracts* 33(Suppl. 15), 4006 (2015).

20 Hurwitz H, Uppal N, Wagner SA *et al.* Randomized, (mPC) double-blind, Phase II study of ruxolitinib or placebo in combination with capecitabine in patients with metastatic pancreatic cancer for whom therapy with gemcitabine has failed. *J. Clin. Oncol.* 33(34), 4039–4047 (2015).

21 Isakoff SJ, Saleh MN, Lugovskoy A *et al.* First-in-human study of MM-141: a novel tetravalent monoclonal antibody targeting

IGF-1R and ErbB3. *ASCO Meeting Abstracts* 33(Suppl. 3), 384 (2015).

22 Le DT, Wang-Gillam A, Picozzi V *et al.* Safety and survival with GVAX pancreas prime and Listeria Monocytogenes-expressing mesothelin (CRS-207) boost vaccines for metastatic pancreatic cancer. *J. Clin. Oncol.* 33(12), 1325–1333 (2015).

23 Hsiang YH, Hertzberg R, Hecht S, Liu LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J. Biol. Chem.* 260(27), 14873–14878 (1985).

24 Hsiang YH, Lihou MG, Liu LF. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res.* 49(18), 5077–5082 (1989).

25 Tournigand C, Andre T, Achille E *et al.* FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J. Clin. Oncol.* 22(2), 229–237 (2004).

26 Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Res.* 51(16), 4187–4191 (1991).

27 Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.* 46(12 Pt 1), 6387–6392 (1986).

28 Senior JH. Fate and behavior of liposomes *in vivo*: a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst.* 3(2), 123–193 (1987).

29 Chang TC, Shiah HS, Yang CH *et al.* Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients. *Cancer Chemother. Pharmacol.* 75(3), 579–586 (2015).

• **Original dose-finding study of MM-398 (PEP02).**

30 Roy AC, Park SR, Cunningham D *et al.* 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. *Ann. Oncol.* 24(6), 1567–1573 (2013).

31 Biason P, Masier S, Toffoli G. *UGT1A1*28* and other *UGT1A* polymorphisms as determinants of irinotecan toxicity. *J. Chemoth.* 20(2), 158–165 (2008).

32 Innocenti F, Ratain MJ. Irinotecan treatment in cancer patients with *UGT1A1* polymorphisms. *Oncology (Williston Park)* 17(5 Suppl. 5), 52–55 (2003).

33 Toffoli G, Cecchin E, Corona G *et al.* The role of *UGT1A1*28* polymorphism in the pharmacodynamics and pharmacokinetics of irinotecan in patients with metastatic colorectal cancer. *J. Clin. Oncol.* 24(19), 3061–3068 (2006).

34 Palomaki GE, Bradley LA, Douglas MP, Kolor K, Dotson WD. Can *UGT1A1* genotyping reduce morbidity and mortality in patients with metastatic colorectal cancer treated with irinotecan? An evidence-based review. *Genet. Med.* 11(1), 21–34 (2009).

35 Ko AH, Tempero MA, Shan YS *et al.* A multinational Phase 2 study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer. *Br. J. Cancer* 109(4), 920–925 (2013).

• **Phase II trial of MM-398 in gemcitabine-refractory advanced pancreatic cancer that led to pivotal Phase III study for this disease indication.**

36 Innocenti F, Schilsky RL, Ramirez J *et al.* Dose-finding and pharmacokinetic study to optimize the dosing of irinotecan according to the UGT1A1 genotype of patients with cancer. *J. Clin. Oncol.* 32(22), 2328–2334 (2014).

37 Camptosar (irinotecan) package insert. Pfizer (NY, USA). www.accessdata.fda.gov

38 Chen L, Shiah H, Chao T *et al.* Phase I study of liposome irinotecan (PEP02) in combination with weekly infusion of 5-FU/LV in advanced solid tumors. *ASCO Meeting Abstracts* 28(Suppl. 15), e13024 (2010).

39 Chen L-T, Shiah H-S, Lin P-C *et al.* Phase I study of biweekly liposome irinotecan (PEP02, MM-398) in metastatic colorectal cancer failed on first-line oxaliplatin-based chemotherapy. *ASCO Meeting Abstracts* 30(Suppl. 4), 613 (2012).

40 Wang-Gillam A, Li CP, Bodoky G *et al.* Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global randomised, open-label, Phase 3 trial. *Lancet* doi:10.1016/S0140-6736(15)60986-1 (Epub ahead of print) (2015).

•• **Published results of Phase III NAPOLI-1 trial of MM-398 in advanced pancreatic cancer.**

41 Chibaudel B, Maindrault-Goebel F, Andre T *et al.* PEPCOL: a randomized noncomparative Phase II study of PEP02 (MM-398) or irinotecan in combination with leucovorin and 5-fluorouracil as second-line therapy in patients with unresectable metastatic colorectal cancer – a GERCOR Study. *ASCO Meeting Abstracts* 33(Suppl. 3), 751 (2015).

42 Clarke JL, Molinaro AM, Desilva AA *et al.* A Phase I trial of intravenous liposomal irinotecan in patients with recurrent high-grade gliomas. *ASCO Meeting Abstracts* 33(Suppl. 15), 2029 (2015).

43 Baker JH, Lam J, Kyle AH *et al.* Irinophore C, a novel nanoformulation of irinotecan, alters tumor vascular function and enhances the distribution of 5-fluorouracil and doxorubicin. *Clin. Cancer Res.* 14(22), 7260–7271 (2008).

44 Verreault M, Strutt D, Masin D *et al.* Vascular normalization in orthotopic glioblastoma following intravenous treatment with lipid-based nanoparticulate formulations of irinotecan (Irinophore C), doxorubicin (Caelyx®) or vincristine. *BMC Cancer* 11, 124 (2011).

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CANCERS OF THE PANCREAS, SMALL BOWEL, AND HEPATOBILIARY TRACT

A randomized, open-label phase II study of nanoliposomal irinotecan (nal-IRI)-containing regimens versus nab-paclitaxel plus gemcitabine in patients with previously

untreated metastatic pancreatic adenocarcinoma (mPAC).

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Abstract Disclosures

Abstract

TPS482

Background: Two combination chemotherapy regimens have emerged as standard of care options for first-line treatment of mPAC: 5-fluorouracil (5-FU)/leucovorin (LV) + irinotecan + oxaliplatin (FOLFIRINOX), and nab-paclitaxel + gemcitabine. Nal-IRI (MM-398) is a nanoliposomal formulation of irinotecan. In a randomized phase 3 study (NAPOLI-1), of patients with mPAC who had been previously treated with gemcitabine-based therapy, nal-IRI + 5-FU/LV demonstrated its safety and significant clinical activity, increasing overall survival (OS) and progression-free survival (PFS) relative to 5-FU/LV. The goal of this current study is to determine the preliminary safety and

COMPANION ARTICLES

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ARTICLE CITATION

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Reply to M. Uccello et al
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Gauri R Varadhachary et al., JCO Oncology Practice, 2016

Reply to A. Wang-Gillam et al
Davendra P.S. Sohal et al., J Clin Oncol, 2016

PANCREOX: A Randomized Phase III Study of Fluorouracil/Leucovorin With or Without Oxaliplatin for

efficacy of nal-IRI+ + 5-FU/LV with or without oxaliplatin as compared to nab-paclitaxel + gemcitabine in previously untreated patients with mPAC. **Methods:** This open-label, phase 2 comparative study will be conducted in two parts. Part 1 is a safety run-in of a nal-IRI+5-FU/LV + oxaliplatin regimen. The safety run-in will enroll small cohorts of patients following a traditional 3 + 3 dose escalation design to confirm the target dose of oxaliplatin (n = ~6-18). The primary objective of Part 1 is the safety and tolerability of nal-IRI + 5FU/LV + oxaliplatin. Part 2 is a randomized, efficacy study of a nal-IRI + 5-FU/LV + oxaliplatin regimen (Arm 1), the nal-IRI + 5-FU/LV combination that previously demonstrated efficacy in the NAPOLI-1 trial (Arm 2), versus a nab-paclitaxel + gemcitabine control arm (Arm 3) (n = ~156-168). The primary objective of Part 2 is to assess the efficacy of nal-IRI-containing regimens in first-line mPAC patients compared to nab-paclitaxel + gemcitabine using the progression-free survival (PFS) rate at 24 weeks as the primary endpoint. Secondary of part 1 is a PK study and Part 2 secondary endpoints will include OS, PFS, objective response rate (per RECIST, v1.1), decrease in CA19-9 levels and quality of life assessments. Clinical trial information: NCT02551991.

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REVIEW

Onivyde for the therapy of multiple solid tumors

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Abstract: Drug delivery system based on nanobiotechnology can improve the pharmacokinetics and therapeutic index of chemotherapeutic agents, which has revolutionized tumor therapy. Onivyde, also known as MM-398 or PEP02, is a nanoliposomal formulation of irinotecan which has demonstrated encouraging anticancer activity across a broad range of malignancies, including pancreatic cancer, esophago-gastric cancer, and colorectal cancer. This up-to-date review not only focuses on the structure, pharmacokinetics, and pharmacogenetics of Onivyde but also summarizes clinical trials and recommends Onivyde for patients with advanced solid tumors.

Keywords: irinotecan, PEP02, MM-398, Onivyde, drug delivery system, tumor therapy

Introduction

Irinotecan (CPT-11) is a semisynthetic derivative of camptothecin which targets topoisomerase I, and is currently used in the treatment of multiple solid tumors, such as metastatic colorectal cancer (mCRC), small-cell lung cancer, non-small-cell lung cancer, gastric cancer, and cervical cancer.[1–4] The pharmacology of irinotecan is complicated, with extensive metabolic conversions involved in the activation, inactivation, and elimination.[5–7] Irinotecan as a prodrug is converted by nonspecific carboxylesterases into SN-38, which is a 100- to 1,000-fold more active metabolite.[7,8] SN-38 is glucurono-conjugated in the liver, and this metabolite, although inactive, may participate in the enterohepatic cycling of SN-38 after hydrolysis in the intestinal lumen.[9] The SN-38 metabolite is cleared by the biliary route after glucuronidation by uridine diphosphate-glucuronosyltransferase 1A1 (UGT1A1). UGT1A1 activity exhibits a wide intersubject variability, in part related to UGT1A1 gene polymorphisms. Patients homozygous for the UGT1A1*28 allele are at increased risk of developing hematological and/or digestive toxicities.[10] However, hydrolysis of active lactone rings in irinotecan and SN-38 to inactive carboxylate forms occurs in normal physiologic pH.[11] The clinical disadvantage of irinotecan includes acute toxicities, fast elimination of the drug, and risk of diarrhea and neutropenia as the major dose-limiting toxicities (DLTs).[7,8] These properties of the drug contribute to the limitation of its clinical application. Hence, the rationally designed drug delivery system (DDS) represents a strategy to improve the pharmacokinetics and biodistribution of irinotecan while protecting it from premature metabolism and extending the time of its exposure at the site of action. Advances in nanotechnology have revolutionized medicines, especially for tumor therapy. Nano-DDS is capable of passive drug delivery through the enhanced permeability and retention (EPR) effect or active targeting via binding to receptors associated with the tumor, thus improving the pharmacokinetics and therapeutic index of chemotherapeutic agents.[12–16] Due to the perfect biocompatibility, liposome-based drug carrier has become a favorable DDS for various purposes.[7,8] For example, liposomal doxorubicin, and particularly pegylated liposomal doxorubicin, has shown

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significant pharmacologic advantages and an added clinical value over doxorubicin.[17]

Onivyde, also known as MM-398 or PEP02, has been designed and developed as a nanoliposomal formulation of irinotecan, which improves the pharmacokinetics of the drug by increasing drug encapsulation and loading efficiency, protecting the drug in the active lactone configuration, prolonging circulation time, providing sustained release, rerouting the drug from sites of toxicity such as the gastrointestinal tract, increasing tumor accumulation via the EPR effect, and reducing host toxicity.[7,8] Based on the encouraging preclinical and clinical data available for the treatment of a variety of solid tumors, Onivyde was newly approved by the US Food and Drug Administration (FDA) in October 2015 as a combination regimen for patients with gemcitabine-based chemotherapy-resistant metastatic pancreatic cancer. In addition, it is also currently undergoing Phase II/III clinical trials for the therapy of many cancer types, such as pancreatic cancer, esophago-gastric (OG) cancer, and colorectal cancer.[18–21] These clinical trials demonstrate that Onivyde has potential antitumor activity across a broad range of advanced solid tumors.[7,8,18–21]

In this review, the structure, pharmacokinetics, and pharmacogenetics of Onivyde are addressed. Clinical trials are also summarized, and recommendations are made for Onivyde for patients with advanced pancreatic cancer and other cancer types.

Structure

Molecular formula of irinotecan is C33H38N4O6, and chemical formula is 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycampothecin, which is shown in Figure 1A. Onivyde, the nanoliposomal formulation of irinotecan, employs a modified gradient-loading method using sucrose octasulfate with unprecedented drug-loading efficiency and in vivo drug stability.[7] Figure 1B schematically illustrates the structure of Onivyde.

Pharmacokinetics

The pharmacokinetics of nanoliposomal CPT-11 formulated using either TEA-SOS or TEA-Pn was determined in normal female rats, which is summarized in Table 1.[7] Nanoliposomal CPT-11 was associated with significantly longer circulation times and less rapid clearance from the blood than free CPT-11. What is more, to define pharmacokinetics of Onivyde in human, a total of eleven patients were randomized into three dose levels – 60 (one patient), 120 (six patients), and 180 mg/m^2 (four patients) – in a study by Chang et al.[22] The formulation of Onivyde may modify the pharmacokinetic parameters of irinotecan and SN-38 by

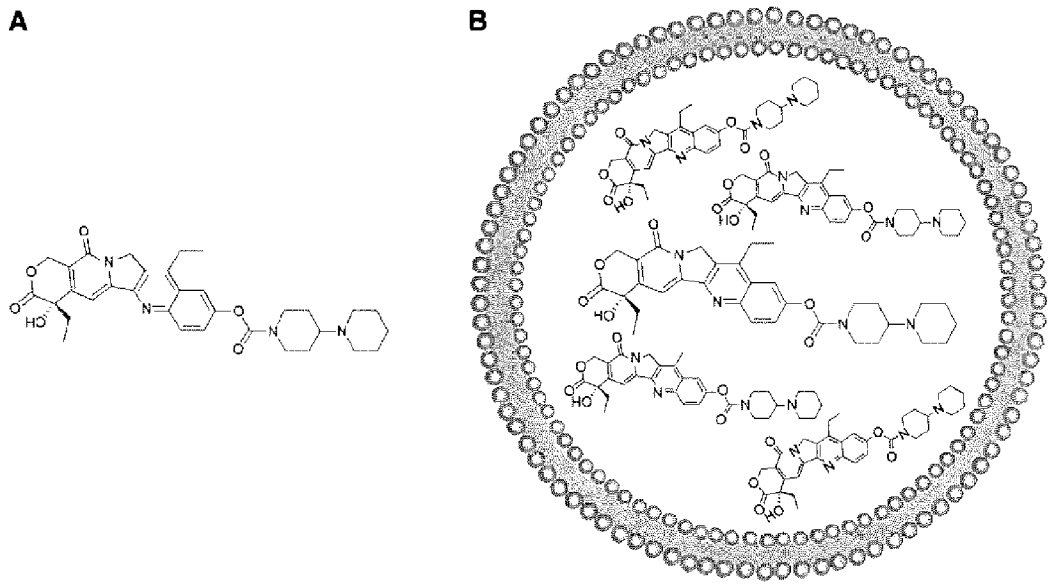

Figure 1 Chemical structure of irinotecan (**A**). Schematic structure of Onivyde: the basic molecule irinotecan forms a nanoscale complex with either poly(phosphate) or sucrose octasulfate in the liposome interior (**B**).

Table 1 Pharmacokinetic variables for free and nanoliposomal CPT-11 in rats

Pharmacokinetic variables	Free CPT-11	Nanoliposomal CPT-11
$t_{1/2}$ (hours)	0.27	6.80
AUC_{∞} (μg h/mL)	6.2	1,407.8
CL (mL/h)	1,609	7.10
V_d (mL)	616.4	69.7
MRT (hours)	0.4	9.8
$t_{1/2}$ of CPT-11 release (hours)	–	14.0

Abbreviations: $t_{1/2}$, terminal half-life; AUC_{∞}, area under the concentration vs time curve in plasma based on the sum of exponential terms; CL, clearance calculated from exponential terms; V_d, volume of distribution; MRT, mean residence time calculated from exponential terms.

liposomal encapsulation. Comparing with the pharmacokinetics after free irinotecan injection, the dose-normalized pharmacokinetics of SN-38 following Onivyde administration was characterized by lower maximum plasma concentration (C_{max}), prolonged terminal half-life ($t_{1/2}$), and higher area under curve (AUC), although with significant interindividual variation. The C_{max}, terminal $t_{1/2}$, and AUC of SN-38 after administration of 120 mg/m^2 of Onivyde were 9.2±3.5 ng/mL, 75.4±43.8 hours, and 710±395 ng·h/mL, respectively. Slow clearance is also an advantage of Onivyde.[23] The levels of CPT-11 and SN-38 following Onivyde administration were persistent and remained in circulation for over 50 hours, whereas the CPT-11 and SN-38 in the plasma were cleared rapidly from circulation within 8 hours after free irinotecan injection.

Pharmacogenetics

Genetic polymorphisms affect toxicity and efficacy of irinotecan, and a pharmacogenetic study showed that a patient with a combined heterozygous genotype of UGT1A1*6/*28 had significantly higher C_{max} and AUC levels of SN-38 after Onivyde administration.[22] In another study, in the group administered with Onivyde, the frequency of grade 3–4 neutropenia was higher among UGT1A1*6 heterozygotes compared with those with wild-type genotype (40% [two out of five] for heterozygotes vs 3% [one out of 30] for wild type, P=0.0220).[18] Patients with combined heterozygous genotype of UGT1A1*6/*28 had severe toxicity leading to death.

Preclinical research

In a series of preclinical studies, Onivyde demonstrated significantly superior anticancer efficacy when compared to free irinotecan in multiple cell lines and xenograft models, including colorectal, gastric, breast, cervical, pancreatic, and lung cancer and glioma, as well as Ewing's sarcoma family of tumors.[7,8,23] At the equivalent dose, free irinotecan showed massive tumor progression, while Onivyde showed enhanced antitumor efficacy.[7,8,23] The striking antitumor activity in the majority of xenograft models showed with Onivyde was significantly correlated with its advantageous pharmacokinetic properties, by which Onivyde achieved higher intratumoral levels of the prodrug irinotecan and its active metabolite SN-38 compared with free irinotecan. Thus, the enhanced accumulation of the active metabolite within the tumor microenvironment could safely improve its antitumor activity with low systemic toxicity.

Clinical trials of Onivyde for the therapy of multiple solid tumors

The efficacy of Onivyde for the therapy of a variety of solid tumors has been investigated in several clinical trials, including various Phase I, II, and III studies on registry, which are summarized in Table 2.

Phase I

In the first-in-human Phase I trial, eleven patients with advanced refractory solid tumors were enrolled to determine the maximum tolerated dose (MTD), DLT, safety

Table 2 Characteristics of the clinical trials of Onivyde for the treatment of multiple solid tumors

Study	Trial	Design	Tumor type	Outcomes
Chang et al[22]	Phase I	Dose escalation	Advanced solid tumors	MTD: 120 mg/m^2 monotherapy at 3-week interval
Roy et al[18]	Phase II	Randomized, three-arm	Esophago-gastric cancer	ORR: 13.6% (Onivyde) vs 6.8% (irinotecan) vs 15.9% (docetaxal)
Chibaudel et al[19]	Phase II	Randomized, noncomparative	Colorectal cancer	ORR: 16.7% (Onivyde + 5-FU/LV) vs 11.5% (irinotecan + 5-FU/LV)
Ko et al[20]	Phase II	Multicenter, open-label, single arm	Pancreatic cancer	mOS: 5.2 months; mPFS: 2.4 months
Von Hoff et al[21]	Phase III	Randomized, open-label	Pancreatic cancer	mOS: 6.1 (Onivyde + 5-FU/LV) vs 4.2 (5-FU/LV) months mPFS: 3.1 (Onivyde + 5-FU/LV) vs 1.5 (5-FU/LV) months

Abbreviations: MTD, maximum tolerated dose; ORR, objective response rate; 5-FU/LV, 5-fluorouracil/leucovorin; mOS, median overall survival; mPFS, median progression-free survival.

profile, pharmacokinetic variables, and antitumor activity of Onivyde.[22] The MTD of Onivyde monotherapy at 3-week interval was 120 mg/m^2, which was the recommended dose for future studies. Myelosuppression and diarrhea were the major DLTs. The toxicity pattern was comparable with that of free-form irinotecan, and the most common toxicity observed in the six patients at the MTD dose level (120 mg/m^2) was diarrhea (100% in all grades, 33% in grade 3–4) and vomiting (83.3% in all grades, 66.7% in grade 3–4). In addition, promising antitumor activities from Onivyde were observed in the patients who were refractory to currently available treatments. Among them, two patients achieved partial response, one with pancreatic cancer who failed several lines of treatment including gemcitabine and 5-fluorouracil/leucovorin (5-FU/LV) alone or in combination with oxaliplatin, and the other with cervical cancer whose tumor relapsed after cisplatin-based concurrent chemoradiotherapy. Another three patients with breast cancer, pancreatic neuroendocrine tumor, and thymic carcinoma had stable disease. The disease control rate was 45.5% for intent-to-treat patients.

Phase II

Onivyde for locally advanced or metastatic gastric or OG junction adenocarcinoma

OG cancer represents a significant global health problem with an estimated one million cases diagnosed every year in the world.[18] There may be clinical remission or disease stabilization in advanced OG cancer patients who receive first-line chemotherapy; however, most will ultimately experience disease progression, and thus, effective second-line chemotherapy is essential.[24] However, currently, there are no standard second-line treatments in this setting.[25,26] Therefore, novel and more effective treatment options are urgently needed to provide survival benefit for patients with advanced OG cancer. Fortunately, the novel liposomal nanocarrier formulation of irinotecan, Onivyde, is a new therapy option providing hope for patients with advanced OG cancer who failed one prior chemotherapy regimen. In a randomized three-arm Phase II study (NCT00813072), Roy et al evaluated the efficacy and safety of single-agent Onivyde compared with irinotecan or docetaxel in the second-line treatment of advanced OG cancer.[18] In this trial, patients with locally advanced/metastatic OG cancer who had failed one prior chemotherapy regimen were randomly divided into three groups: Onivyde 120 mg/m^2, irinotecan 300 mg/m^2, and docetaxel 75 mg/m^2 every 3 weeks. The primary end point was objective response rate (ORR). The secondary end points included progression-free survival (PFS), overall survival (OS), and 1-year survival rate. The outcomes demonstrated that the ORR for Onivyde, irinotecan, and docetaxel was 13.6% (6/44; 95% confidence interval [CI]: 5.2–27.4), 6.8% (3/44; 95% CI: 1.4–18.7), and 15.9% (7/44; 95% CI: 6.6–30.1), respectively. The ORR associated with Onivyde was comparable with docetaxel and numerically greater than that of irinotecan. The median overall survival (mOS) and median progression-free survival (mPFS) were similar in all the three arms. The mOS for Onivyde, irinotecan, and docetaxel was 7.3 (95% CI: 3.84–9.17), 7.8 (95% CI: 4.90–9.20), and 7.7 months (95% CI: 5.32–12.32), respectively. The mPFS was 2.7 (95% CI: 1.54–3.65), 2.6 (95% CI: 1.48–4.34), and 2.7 months (95% CI: 1.41–5.45), respectively. Kaplan–Meier estimates of 1-year survival rates were 21.3%, 30.8%, and 40.4%, respectively, in those three treatment arms. Treatment was well tolerated, and Onivyde was associated with an increased frequency of grade 3–4 diarrhea (27.3% in Onivyde group vs 18.2% in irinotecan group) and nausea, with similar rates of vomiting, neutropenia, and febrile neutropenia compared with irinotecan and docetaxel. These results illustrated that Onivyde is well tolerated and also has a comparable efficacy to docetaxel and irinotecan in patients with locally advanced or metastatic OG cancer, who failed first-line treatment.

Onivyde for mCRC

Colorectal cancer ranks as the third most common cancer worldwide as approximately one million new cases are diagnosed annually.[3,27,28] Chemotherapeutic agents are widely used for patients with mCRC to prolong survival, control symptoms, and improve the quality of life. Irinotecan is one of the key drugs used for the management of mCRC, along with oxaliplatin.[28] These drugs are often combined with 5-FU/LV in regimens such as FOLFIRI (irinotecan, 5-FU, and LV) or FOLFOX (oxaliplatin, 5-FU, and LV), which have exhibited clinical effects and considerably improved OS in patients with mCRC in recent decades.[3,27,28] In a randomized noncomparative Phase II study (NCT01375816), Chibaudel et al evaluated Onivyde or irinotecan in combination with 5-FU/LV as second-line therapy in patients with mCRC who had failed one prior oxaliplatin-based first-line therapy.[19] In the trial, 55 patients were randomly assigned to FUPEP (n=28, Onivyde + 5-FU/LV) or FOLFIRI (n=27, irinotecan + 5-FU/LV). Bevacizumab q2w (5 mg/kg) was allowed in both arms. The primary end point was ORR. The outcomes demonstrated that in the evaluable population (n=50), ORR was 16.7% (n=4/24) and 11.5% (n=3/26) in the FUPEP and FOLFIRI arms, respectively. Most common grade 3–4 adverse events reported in the respective

FUPEP and FOLFIRI arms were diarrhea (21% vs 33%), neutropenia (11% vs 30%), mucositis (11% vs 11%), and alopecia (G2: 25% vs 26%). Hence, Onivyde may provide a new second-line treatment option for mCRC.

Onivyde for gemcitabine-refractory metastatic pancreatic cancer

Pancreatic cancer is well recognized as an extremely challenging disease on multiple fronts, which is characterized by late detection, poor prognosis, and aggressive metastasis.[29,30] Early detection is uncommon with no >15%–20% of the patients being amenable for curative intent surgery at the time of diagnosis.[12] Even in these cases, 5-year overall survival is still only 22%. Systemic chemotherapy based on gemcitabine is the popular regimen for patients with advanced pancreatic cancer, the mOS of whom is generally ~6 months.[12,31] For pancreatic cancer, irinotecan has not been considered as a clinically useful drug. However, since Conroy et al[32] demonstrated that FOLFIRINOX regimen consisting of oxaliplatin, irinotecan, and 5-FU/LV could provide significantly better ORR, PFS, and OS than gemcitabine monotherapy in patients with metastatic pancreatic cancer, irinotecan-included therapy has been receiving increased attention for advanced pancreatic cancer.[33] However, notably and not unexpectedly, this triplet regimen is often hindered by the significant toxicity. Fortunately, the novel nanoliposomal formulation of irinotecan, Onivyde, could present safe therapeutic option. In an international, multicenter, open-label, Phase II trial (NCT00813163), Ko et al evaluated Onivyde monotherapy as second-line treatment for patients with gemcitabine-based chemotherapy-resistant metastatic pancreatic cancer.[20] In the study, a total of 40 patients were enrolled. The primary end point of this trial was 3-month survival rate ($OS_{3\text{-month}}$). Secondary end points included ORR, PFS, clinical benefit response, CA19-9 tumor marker response, and safety profile. Of the 40 treated patients, three patients (7.5%) achieved an objective response, with an additional 17 (42.5%) demonstrating stable disease for a minimum of two cycles. Ten (31.3%) out of 32 patients with an elevated baseline CA19-9 had a 45% biomarker decline. The study met its primary end point with an $OS_{3\text{-month}}$ of 75% and mOS and mPFS of 5.2 and 2.4 months, respectively. The most common severe adverse events included neutropenia (40%), abdominal pain (37.5%), and diarrhea (75%). These results illustrated that Onivyde showed moderate antitumor activity with a manageable side effect profile in patients with metastatic, gemcitabine-refractory pancreatic cancer.

Phase III

As illustrated, Onivyde provides a new treatment option and a new hope for patients with gemcitabine-based chemotherapy-resistant metastatic pancreatic cancer. Further study was carried out. A randomized, open-label, Phase III trial by Von Hoff et al investigated Onivyde, with or without 5-FU/LV, vs 5-FU/LV, in patients with metastatic pancreatic cancer progressed on or following gemcitabine-based therapy (NCT01494506).[21] Patients with metastatic pancreatic cancer after gemcitabine-based therapy were randomized 1:1:1 to receive (A) Onivyde, (B) 5-FU/LV, or (C) combination of Onivyde and 5-FU/LV. The primary end point was OS in arms A and C, each vs the control arm B. The outcomes demonstrated that OS, PFS, ORR, and time to failure were significantly improved by Onivyde + 5-FU/LV compared with 5-FU/LV. mOS was 6.1 months (95% CI: 4.8–8.9) in the Onivyde + 5-FU/LV arm and 4.2 months (95% CI: 3.3–5.3) in the 5-FU/LV arm (hazard ratio [HR] =0.67, P=0.012). mPFS was 3.1 (95% CI: 2.7–4.2) and 1.5 months (95% CI: 1.4–1.8), respectively (HR =0.56, P<0.001). Onivyde alone did not demonstrate a statistical improvement in efficacy compared with 5-FU/LV. The most common grade >3 toxicities seen with the combination of Onivyde and 5-FU/LV were neutropenia (14.5%), fatigue (13.7%), diarrhea (12.8%), and vomiting (11.1%). Given the excellent results obtained in the Phase III trial, the FDA newly approved Onivyde + 5-FU/LV regimen for patients with gemcitabine-based chemotherapy-resistant metastatic pancreatic cancer. An updated analysis of OS, 6- and 12-month survival estimates, and safety was presented in the 2016 Gastrointestinal Cancers Symposium, San Francisco, CA, USA. Onivyde + 5-FU/LV (n=117) retained an OS advantage relative to 5-FU/LV (n=119): 6.2 (95% CI: 4.8–8.4) vs 4.2 months (95% CI: 3.3–5.2) with an unstratified HR of 0.75 (P=0.0417). Six-month survival estimates were 53% (95% CI: 44%–62%) for Onivyde + 5-FU/LV vs 38% (95% CI: 29%–47%) for 5-FU/LV; 12-month survival estimates were 26% (95% CI: 18%–35%) for Onivyde + 5-FU/LV vs 16% (95% CI: 10%–24%) for 5-FU/LV.[34]

Ongoing trials

Currently, there are also multiple ongoing clinical trials investigating antitumor efficiency of Onivyde. For example, a Phase I study which explores Onivyde + cyclophosphamide in pediatric solid tumors is underway in the South Plains Oncology Consortium (NCT02013336). A randomized, open-label, Phase II study of Onivyde-containing regimens vs nab-Paclitaxel + gemcitabine in patients with previously untreated, metastatic pancreatic adenocarcinoma is actively

recruiting (NCT02551991). A study of liposomal irinotecan and veliparib in treating patients with solid tumors that are metastatic or cannot be removed by surgery is yet to recruit (NCT02631733). A dose escalation study of MM-398 + irinotecan in patients with unresectable advanced cancer is also recruiting (NCT02640365). Activity of MM-398, a nanoliposomal irinotecan (nal-IRI), in Ewing's family tumor xenografts has been found associated with high exposure of tumor to drug and high SLFN11 expression, which improves antitumor activity compared with the current clinical formulation of irinotecan.[8] Other liposomal formulations of irinotecan or SN-38 have also been developed, such as IHL-305 (pegylated liposomal irinotecan) and LE-SN-38 (a liposome-encapsulated SN-38). But PEP02 at 120 mg/m^2 showed higher SN-38 exposure than IHL-305 at 160 mg/m^2. Notwithstanding the relatively high SN-38 AUC, unfortunately, LE-SN-38 did not meet the prespecified activity criteria in the Phase II CALGB 80402 study on mCRC patients.[22]

Side effects of Onivyde treatment

Onivyde is generally well tolerated by patients. However, adverse effects also occur, and they are considered manageable. The most frequently observed drug-related adverse events were diarrhea, myelosuppression, vomiting, abdominal pain, asthenia, mucositis, and alopecia.[18–22] Management of these toxicities could include antidiarrheal agents, anticholinergic agents, and dose reduction, interruption, or termination.

Conclusion and future directions for drug development

The potential advantages of Onivyde, a novel nanoliposomal formulation of irinotecan, include improvement of pharmacokinetics and biodistribution, extension of the circulation time, increase in passive targeting and tumor accumulation via EPR effect, and reduction in organ toxicity. Onivyde as a promising agent has demonstrated improved outcomes and a tolerable safety profile in the treatment of a variety of tumor types. Since single anticancer agent has not always been sufficiently effective, the combination of Onivyde with conventional cytotoxic agent and/or recently emergent molecular-targeted agent should also be investigated to improve the clinical outcomes. Moreover, the optimal dosages of Onivyde for such combinations remain to be determined. Besides, predictive biomarkers could identify cancer indications and patient populations with an increased likelihood of Onivyde responsiveness and avoid exposure to useless toxic medicine. Thus, additional studies into biomarkers may be useful in predicting personalized therapeutic response.

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Disclosure

The author reports no conflicts of interest in this work.

References

1. Xiao XG, Xia S, Zou M, et al. The relationship between UGT1A1 gene polymorphism and irinotecan effect on extensive-stage small-cell lung cancer. *Onco Targets Ther*. 2015;8:3575–3583.
2. Liu Y, Luan L, Wang X. A randomized Phase II clinical study of combining panitumumab and bevacizumab, plus irinotecan, 5-fluorouracil, and leucovorin (FOLFIRI) compared with FOLFIRI alone as second-line treatment for patients with metastatic colorectal cancer and KRAS mutation. *Onco Targets Ther*. 2015;8:1061–1068.
3. Suenaga M, Mizunuma N, Matsusaka S, et al. Phase II study of reintroduction of oxaliplatin for advanced colorectal cancer in patients previously treated with oxaliplatin and irinotecan: RE-OPEN study. *Drug Des Devel Ther*. 2015;9:3099–3108.
4. Ryuge S, Jiang SX, Wada M, et al. Long-term disease-free survivor of metastatic large cell neuroendocrine carcinoma of the lung treated with amrubicin and irinotecan. *Drug Des Devel Ther*. 2009;3:213–217.
5. Garcia-Carbonero R, Supko JG. Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. *Clin Cancer Res*. 2002;8(3):641–661.
6. Mathijssen RH, van Alphen RJ, Verweij J, et al. Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). *Clin Cancer Res*. 2001;7(8):2182–2194.
7. Drummond DC, Noble CO, Guo Z, Hong K, Park JW, Kirpotin DB. Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. *Cancer Res*. 2006;66(6):3271–3277.
8. Kang MH, Wang J, Makena MR, et al. Activity of MM-398, nanoliposomal irinotecan (nal-IRI), in Ewing's family tumor xenografts is associated with high exposure of tumor to drug and high SLFN11 expression. *Clin Cancer Res*. 2015;21(5):1139–1150.
9. Rivory LP. Metabolism of CPT-11. Impact on activity. *Ann N Y Acad Sci*. 2000;922:205–215.
10. Etienne-Grimaldi MC, Boyer JC, Thomas F, et al. UGT1A1 genotype and irinotecan therapy: general review and implementation in routine practice. *Fundam Clin Pharmacol*. 2015;29(3):219–237.
11. Akimoto K, Kawai A, Ohya K. Kinetic-studies of the hydrolysis and lactonization of camptothecin and its derivatives, Cpt-11 and Sn-38, in aqueous-solution. *Chem Pharm Bull (Tokyo)*. 1994;42:2135–2138.
12. Tsai CS, Park JW, Chen LT. Nanovector-based therapies in advanced pancreatic cancer. *J Gastrointest Oncol*. 2011;2(3):185–194.
13. Zhang H. Multifunctional nanomedicine platforms for cancer therapy. *J Nanosci Nanotechnol*. 2012;12(5):4012–4018.
14. Zhang H, Wang C, Chen B, Wang X. Daunorubicin-TiO2 nanocomposites as a "smart" pH-responsive drug delivery system. *Int J Nanomedicine*. 2012;7:235–242.
15. Zhang H, Xiong J, Guo L, Patela N, Guang X. Integrated traditional Chinese and western medicine modulator for overcoming the multidrug resistance with carbon nanotubes. *RSC Adv*. 2015;5:71287–71296.
16. Zhang H, Patela N, Xiong J, Ding S. Targeting and noninvasive treatment of hepatocellular carcinoma in situ by ZnO nanorod-mediated concurrent chemoradiotherapy. *RSC Adv*. 2015;5:85720–85729.
17. Tahover E, Patil YP, Gabizon AA. Emerging delivery systems to reduce doxorubicin cardiotoxicity and improve therapeutic index: focus on liposomes. *Anticancer Drugs*. 2015;26(3):241–258.

18. Roy AC, Park SR, Cunningham D, et al. 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. *Ann Oncol*. 2013;24(6):1567–1573.
19. Chibaudel B, Maindrault-Goebel F, André T, et al. PEPCOL: a randomized noncomparative phase II study of PEP02 (MM-398) or irinotecan in combination with leucovorin and 5-fluorouracil as second-line therapy in patients with unresectable metastatic colorectal cancer – a GERCOR Study. *J Clin Oncol*. 2015;33(Suppl 3):Abstr 751.
20. Ko AH, Tempero MA, Shan YS, et al. A multinational phase 2 study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer. *Br J Cancer*. 2013;109(4):920–925.
21. Von Hoff D, Li CP, Wang-Gillam A, et al. Napoli-1: randomized phase 3 study MM-398 (nal-IRI), with or without 5-fluorouracil and leucovorin, versus 5-fluorouracil and leucovorin, in metastatic pancreatic cancer progressed on or following gemcitabine-based therapy. *Ann Oncol*. 2014;25(Suppl 2):ii105–ii106.
22. Chang TC, Shiah HS, Yang CH, et al. Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients. *Cancer Chemother Pharmacol*. 2015;75(3):579–586.
23. Kalra AV, Kim J, Klinz SG, et al. Preclinical activity of nanoliposomal irinotecan is governed by tumor deposition and intratumor prodrug conversion. *Cancer Res*. 2014;74(23):7003–7013.
24. Zhang H. Apatinib for molecular targeted therapy in tumor. *Drug Des Devel Ther*. 2015;9:6075–6081.
25. Wesolowski R, Lee C, Kim R. Is there a role for second-line chemotherapy in advanced gastric cancer? *Lancet Oncol*. 2009;10(9):903–912.
26. Lordick F. Gastrointestinal cancer: salvage chemotherapy in gastric cancer – more than a straw? *Nat Rev Clin Oncol*. 2012;9(6):312–313.
27. Liang RF, Zheng LL. The efficacy and safety of panitumumab in the treatment of patients with metastatic colorectal cancer: a meta-analysis from five randomized controlled trials. *Drug Des Devel Ther*. 2015;9:4471–4478.
28. Suenaga M, Mizunuma N, Matsusaka S, et al. A phase I/II study of biweekly capecitabine and irinotecan plus bevacizumab as second-line chemotherapy in patients with metastatic colorectal cancer. *Drug Des Devel Ther*. 2015;9:1653–1662.
29. Wang C, Zhang H, Chen Y, Shi F, Chen B. Gambogic acid-loaded magnetic Fe(3)O(4) nanoparticles inhibit Panc-1 pancreatic cancer cell proliferation and migration by inactivating transcription factor ETS1. *Int J Nanomedicine*. 2012;7:781–787.
30. Wang C, Zhang H, Chen B, Yin H, Wang W. Study of the enhanced anticancer efficacy of gambogic acid on Capan-1 pancreatic cancer cells when mediated via magnetic Fe3O4 nanoparticles. *Int J Nanomedicine*. 2011;6:1929–1935.
31. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007;25(15):1960–1966.
32. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011;364(19):1817–1825.
33. Vaccaro V, Sperduti I, Milella M. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011;365(8):768–769.
34. Wang-Gillam A, Li CP, Bodoky G, et al. Phase III study of nanoliposomal irinotecan (nal-IRI, MM-398), with or without 5-fluorouracil and leucovorin (5-FU/LV), versus 5-FU/LV in metastatic pancreatic cancer (mPAC) previously treated with gemcitabine-based therapy. *J Clin Oncol*. 2016;34(Suppl 4S):Abstr 417.

CANCER RESEARCH

Experimental and Molecular Therapeutics

Abstract 4830: Preclinical anti-tumor activity of nanoliposomal irinotecan (Nal-IRI, MM-398) + 5-FU + oxaliplatin in pancreatic cancer

Daniel F. Gaddy, Helen Lee, Nancy Paz, Shannon C. Leonard, Ashish Kalra, Ninfa L. Straubinger, Robert M. Straubinger, Bryan M. Gillard, Michael T. Moser, Daryl C. Drummond, Stephan G. Klinz, Bart S. Hendriks, and Jonathan B. Fitzgerald

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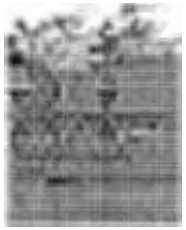
Abstract

Nanoliposomal irinotecan (Nal-IRI, MM-398) recently gained approval in combination with 5-fluorouracil/leucovorin (5-FU/LV) in post-gemcitabine metastatic pancreatic cancer based on results of the Phase 3 NAPOLI-1 trial. Nal-IRI, in combination with 5-FU/LV, improved overall survival in gemcitabine-refractory metastatic PDAC relative to 5-FU/LV alone with a well-defined and manageable toxicity profile in pretreated patients. FOLFIRINOX (5-FU/LV, irinotecan and oxaliplatin) is a chemotherapy regimen active in first-line metastatic PDAC. Herein, we evaluate the preclinical anti-tumor activity of a nal-IRI + 5-FU + oxaliplatin regimen relative to the FOLFIRINOX regimen. Using pancreatic cancer cell lines, we demonstrate enhanced cell death when nal-IRI treatment is simulated using prolonged exposure of SN-38 (the active metabolite of irinotecan) in combination with 5-FU and oxaliplatin. In cell line-derived and patient-derived xenograft models of pancreatic cancer we demonstrate improved anti-tumor activity of nal-IRI relative to exposure-matched doses of unencapsulated



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irinotecan. Further, nal-IRI consistently improved tumor growth inhibition and survival relative to unencapsulated irinotecan in preclinical models, both as a monotherapy and in combination with 5-FU and oxaliplatin. The addition of nal-IRI to 5-FU and/or oxaliplatin did not exacerbate the baseline toxicities of these agents, including weight loss and neutropenia, and tolerability could be further improved by delaying the administration of oxaliplatin to 1 day post-MM-398. These findings illustrate the therapeutic potential of nal-IRI in combination with 5-FU/LV and oxaliplatin and support an ongoing Phase 2 trial (NCT02551991) of this triplet regimen in first-line PDAC.

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Nanotechnology-based combinational drug delivery: an emerging approach for cancer therapy

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Combination therapy for the treatment of cancer is becoming more popular because it generates synergistic anticancer effects, reduces individual drug-related toxicity and suppresses multi-drug resistance through different mechanisms of action. In recent years, nanotechnology-based combination drug delivery to tumor tissues has emerged as an effective strategy by overcoming many biological, biophysical and biomedical barriers that the body stages against successful delivery of anticancer drugs. The sustained, controlled and targeted delivery of chemotherapeutic drugs in a combination approach enhanced therapeutic anticancer effects with reduced drug-associated side effects. In this article, we have reviewed the scope of various nanotechnology-based combination drug delivery approaches and also summarized the current perspective and challenges facing the successful treatment of cancer.

Introduction

Cancer is one of the most devastating diseases and it involves various genetic alterations and cellular abnormalities. This complexity and heterogeneity promotes the aggressive growth of cancer cells leading to significant morbidity and mortality in patients [1,2]. Currently, different chemotherapeutic agents are used effectively for anticancer therapy by targeting specific multiple pathways. However, repeated treatment with these single drug agents can result in resistance to the chemotherapies or development of multi-drug resistance (MDR) [3]. Additional hurdles associated with conventional single drug chemotherapy include limited accessibility of drug to tumor tissues, which therefore requires a higher dose, leading to intolerable cytotoxicity and nonspecific targeting [4–6]. Over the years, combination therapy has been adopted in clinics that have addressed the problems associated with single chemotherapeutic cancer treatment. Combination therapy generally refers to two or more therapeutic agents co-delivered simultaneously or a combination of different therapies, such as chemotherapy, hormone therapy, immunotherapy and radiotherapy. Above all approaches, the co-delivery of different chemotherapeutic agents is the most common combination therapeutic modality in clinical practice regarding effective cancer treatment. By combinations of two or more agents, side-effects associated with high doses of single drugs can be overcome by countering different biological signaling pathways synergistically, enabling a low dosage of each compound or accessing context-specific multi-target mechanisms [7]. Further, using multiple drugs with different molecular targets can modulate the genetic barriers that are responsible for cancer cell mutations, thereby delaying the cancer adaptation process. In addition, the co-delivered drugs can target the same cellular pathways that could function synergistically for higher therapeutic efficacy and higher target selectivity. However, inadequate membrane transport properties, low bioavailability and minimal biodistribution were the common obstacles associated with conventional cancer drug delivery for effective clinical practice. To this end, the preface of nanotechnology makes significant advances in cancer therapy by offering smart drug delivery systems. Nanotechnology is the science that touches every corner of life starting from nanoscale devices and helping in disease diagnosis to nanoparticulate drug delivery systems that finally evade life-threatening diseases such as cancer. Various types of nanoscale drug delivery systems such as liposomes, micelles, dendrimers, nanotubes and nanoparticles (NPs) are the reward of nanotechnology [8,9]. Nanotechnology-based combination drug delivery systems to the cancer tissue offer better pharmacokinetic parameters including more bioavailability to the tumor site by the enhanced permeable retention (EPR) mechanism. Additionally, it can also help to overcome the systemic toxicity toward normal tissue and adverse effects that resulted from conventional

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cancer therapeutic agents. In this review, we discuss different nanotechnology-based combination drug delivery systems and their clinical implication toward cancer therapy.

Advantages of combinational strategies in cancer therapy

At present, combinations of different chemotherapeutic drugs in a chemotherapy regime are an attractive strategy for effective anticancer treatment. In a clinical setting the treated patients were found to fail the experiences of single agent chemotherapy, because it is limited to act on specific cancer survival pathways and showed low response rates and relapse of tumor [3]. The major limiting factors associated with poor prognosis of cancer following single agent chemotherapeutic treatment in cancer patients are: MDR, significant toxicity and undesirable side effects. To improve the therapeutic potential of cancer chemotherapy, it is essential to establish an alternative approaches that could provide a solution to the problems involved in single drug chemotherapy. To this end, much attention has been given to combination approaches for a better long-term prognosis and to decrease side effects associated with high doses of single drug treatment. Unlike single agent therapy, combination therapy can modulate different signaling pathways, maximizing the therapeutic effect by overcoming toxicity and, moreover, can overcome the mechanisms of drug resistance associated with cancer treatment. The use of combination therapy for cancer treatment has been well established in recent years and its advantages applied to cancer therapy are illustrated below.

One of the prime benefits of combination therapies is the potential for providing synergistic effects. In combination therapy the overall therapeutic benefit of the drugs in combination were found to be greater than the sum of the effects of the drugs individually. These advantages have driven drug discovery efforts toward the search for combination therapies. The best drug combination with maximal antitumor efficacy can be calculated by multiple drug effect/combination index (CI) isobologram analysis, an effective way to demonstrate that drugs are working synergistically [10]. The prime mechanism of synergistic effect following combinational drug treatment could act on the same or different signaling pathways to achieve more-favorable outcomes at a lower dose with equal or increased efficacy (Fig. 1a).

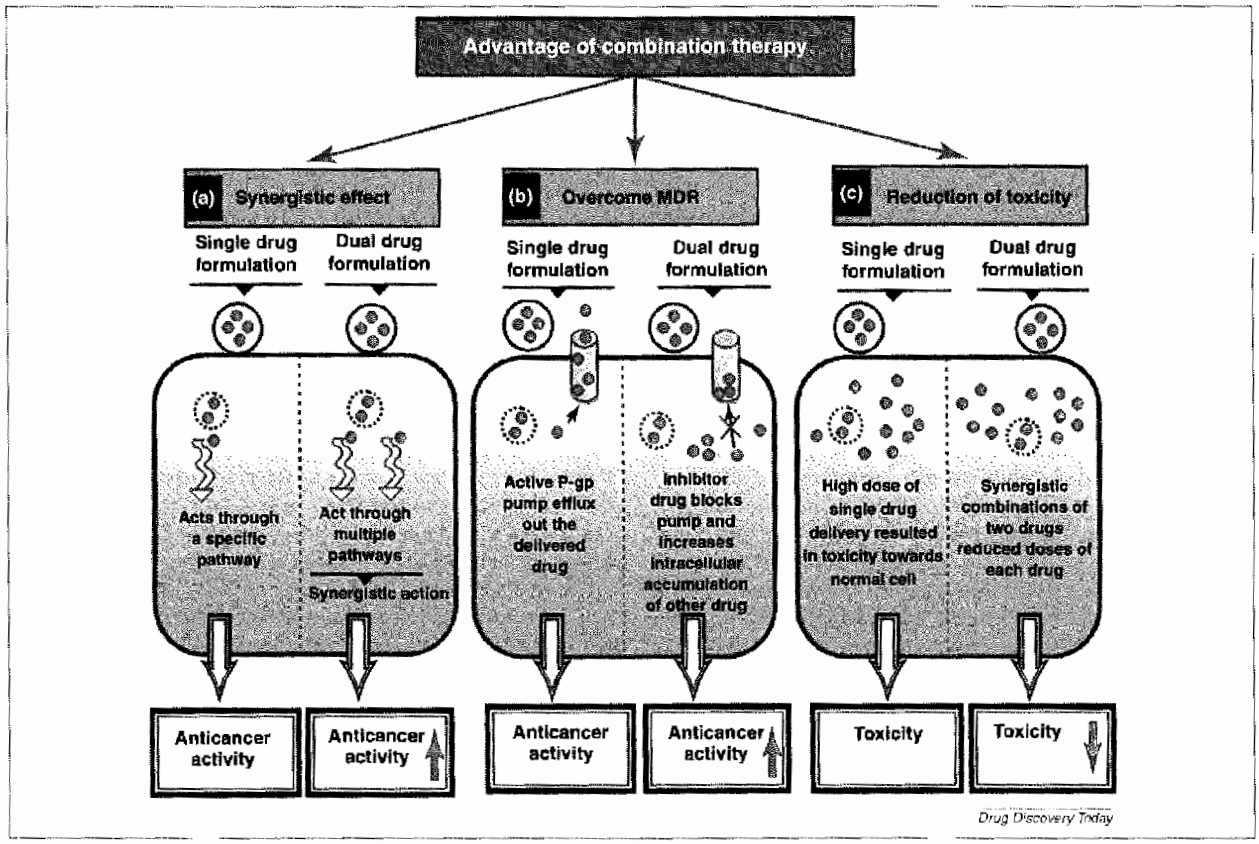

FIGURE 1

Schematic representation depicting various advantages shown by combination drug delivery for cancer therapy. **(a)** Single drug acts through a particular pathway, whereas multiple drugs can show enhanced anticancer activity by acting through several pathways. **(b)** In the case of single drug treatment, MDR proteins such as P-gp efflux drug out of the cell, whereas for dual formulations P-gp inhibitor blocks the role of MDR proteins and increases the intracellular concentration of other co-administered drugs resulting in higher efficacy by overcoming the MDR phenotype. **(c)** High dose is needed for single drug treatment and consequently results in toxicity to the normal cells, whereas treatment with different drug combinations by synergistic action can reduce the dose of each single drug and thereby decrease the toxicity.

For example, in a combination approach, celecoxib (a cyclooxygenase-2 inhibitor) was found to act synergistically with co-treatment of emodin and suppressed the growth of rat cholangiocarcinoma through a mechanism involving enhanced Akt inactivation and increased activation of caspase-9 and -3 [11]. Recently, Chiang *et al.* elucidated the synergistic interaction of RAD001 (an mTOR inhibitor) with gemcitabine or paclitaxel for anticancer treatment in a heterogeneous group of non-Hodgkin lymphoma (NHL) cell lines. They have demonstrated enhanced caspase-dependent apoptosis exerted by gemcitabine or paclitaxel with downregulation of the mTOR signaling pathway due to RAD001 in NHL cell lines following treatment with RAD001 and gemcitabine or paclitaxel in combination [12].

The main reason for the failure of chemotherapy treatment to cure cancers is the ability of tumor cells to become simultaneously resistant to several different anticancer drugs. Many mechanisms are known to contribute to MDR in tumor cells, of which the presence of a multidrug efflux pump is one. Three ABC family members P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1) and breast cancer resistance protein (BCRP) are likely to be the major drug efflux pumps overexpressed in human cancers. These actively pump the chemotherapeutic drugs out from the cell (Fig. 1b) [13]. In this regard, co-treatment with modulators (P-gp inhibitors) is an important insinuation that can interfere with MDR protein (overexpressed in cancer cells) and thereby reverse the MDR effect [14]. Recently, Hubensack *et al.* showed a significant elevated concentration of paclitaxel in the brain domain of nude mice when administrated with elacridar and tariquidar (both are third-generation P-gp inhibitors), caused by downregulation of P-gp expressed at the blood–brain barrier [15]. In another Phase I clinical trial, zosuquidar was co-delivered with daunorubicin and cytarabine to older acute myeloid leukemia patients and the results demonstrated increased anticancer activity experienced by patients overcoming drug resistance [16].

One of the biggest hurdles encountered in cancer therapy includes the problem of dose-dependent toxicity toward the non-cancerous cells because high doses of drug treatment were generally required owing to poor drug accessibility to the tumor site. However, unlike single agent therapy, synergistic combinations of two or more agents proved to overcome toxicity and other side-effects associated with high doses of single drugs by countering biological compensation and enabling reduced dosage of each compound (Fig. 1c). Recently, Dasanu *et al.* demonstrated that combination treatment of two different drugs (i.e. carboplatin and gemcitabine) in a small group of patients with metastatic ovarian cancer resulted in significant antitumor activity with no hematological toxicity [17]. In another study, Bava *et al.* co-administered the herbal drug curcumin with the cytotoxic anticancer drug paclitaxel with a view to reducing the dose-limiting toxicity exerted by paclitaxel on systemic delivery, and they demonstrated

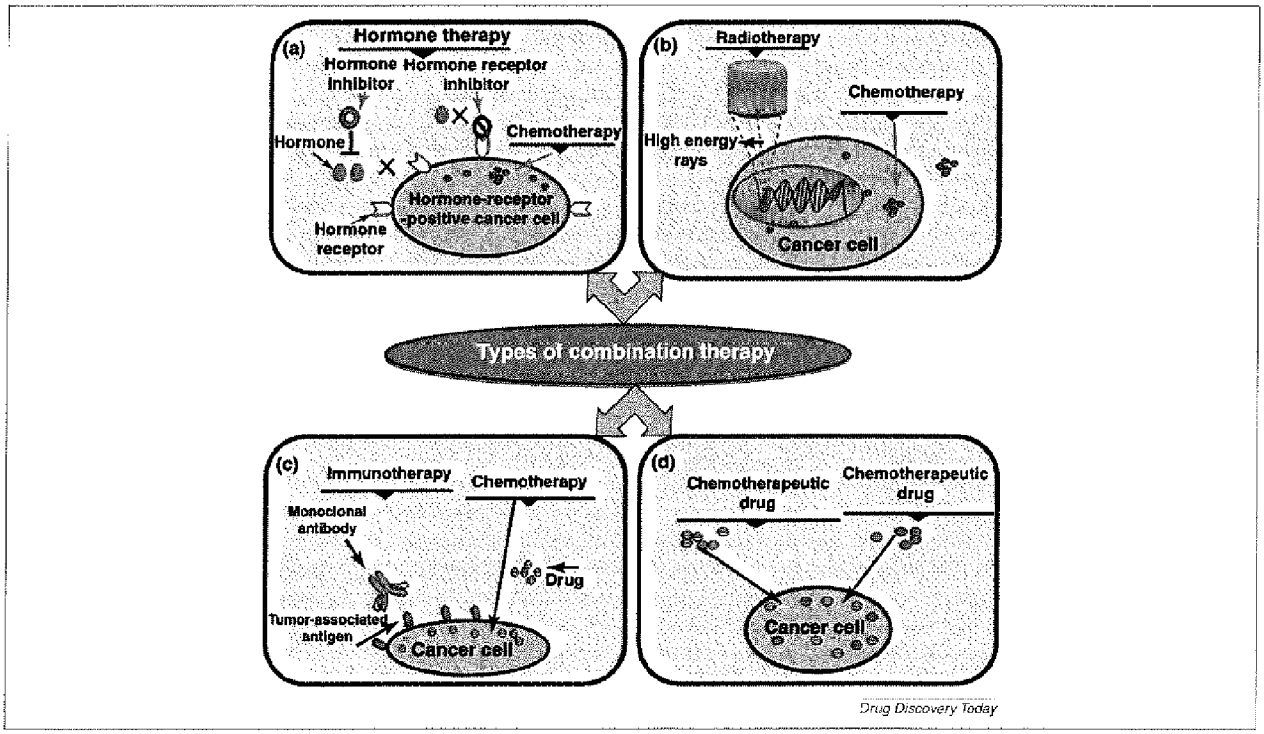

FIGURE 2

Different types of combination therapy for cancer treatment. **(a)** A combination of hormone therapy and chemotherapy can be applied to hormone-receptor-positive cancer cells for better therapeutics. **(b)** Combination of chemotherapy and radiotherapy can be used to enhance the efficacy of the chemotherapeutic drugs. **(c)** Immunotherapy combined with chemotherapy can be used for effective cancer therapy. **(d)** Treatment with different drug combinations can be used to improve the efficacy of the drug in a synergistic mechanism at a lower dose.

that combination therapy not only reduced the dose of paclitaxel but also demonstrated increased anticancer cytotoxic activity compared with paclitaxel alone in HeLa cells [18].

Current combination therapies for cancer treatment

The most common combinational strategies for effective cancer treatment generally involve, for example, the combination of multiple chemotherapeutic agents, chemotherapy with radiotherapy, chemotherapy with hormone therapy and immunotherapy with chemotherapy (Fig. 2).

Hormone therapy can be performed with exogenous administration of specific steroid hormones or drugs that will inhibit hormone production or activity. Usually, prostate and breast cancers are considered as hormone-dependent cancers. Hormone therapy is often given to patients with early stages of breast cancer and it can act either by blocking or downregulating the estrogen receptor or by reducing the estrogen concentration around or within the cancer cell. Aromatase is an enzyme responsible for the production of estrogens. Therefore, inhibitors of aromatase can stop the production of estrogen and have been used intensely for the treatment of breast cancer. Similarly, the use of various hormone receptor inhibitors (such as tamoxifen and raloxifene) can block the overexpressed cancer cell receptor and prevent its activation for consequent uncontrolled proliferation (Fig. 2a). Recent studies have suggested that endocrine therapy can be used with adjuvant bisphosphonate therapy (using zoledronic acid) for the treatment of breast cancer. Zoledronic acid prevents aromatase-inhibitor-associated bone loss in post-menopausal women with breast cancer and it represented a cost-effective treatment for the prevention of bone loss and/or fracture [19].

Combination of chemotherapy and radiotherapy is one of the most promising strategies for current cancer therapeutics. It has been shown to improve patient survival and the locoregional control of various cancers when compared with radiotherapy alone [20]. In the early 1970s, the combination of 5-fluorouracil (5-FU) and mitomycin C with radiotherapy demonstrated improved results for patients with locally advanced stages of anal cancer [21]. Clinical trials have shown that in glioblastoma multiforme the combination of radiotherapy with topotecan could improve patient quality of life – in a Phase I trial topotecan exhibits radiosensitization owing to the synergistic effect [22]. A randomized Phase II clinical trial was performed to examine the combination of radiotherapy and a prostate-specific-antigen-based vaccine in prostate cancer patients and the results have shown that radiotherapy makes the tumor more susceptible to vaccines [23].

Cancer treatment using different monoclonal antibodies is another practiced strategy for the current treatment of cancer. Antibodies have the unique capacity to target and kill tumor cells while simultaneously activating immune effectors to kill tumor cells through the complement cascade or antibody-dependent cellular cytotoxicity (ADCC). This multifaceted antibody-mediated killing mechanism combined with chemotherapy has been taken up by various research groups to achieve enhanced antitumor activity (Fig. 2c).

Combination strategies using different chemotherapeutic drugs were first used in the 1940s and illustrated a remarkable survival improvement in childhood leukemia and Hodgkin's disease. These developments were based on biochemical synergy, tumor cell kinetics, nonoverlapping toxicity, increase of fractional cell kill, noncross-resistant agents or tumor cell resistance [24].

Studies conducted by Tanabe *et al.* have demonstrated that the combinations of mitomycin C and methotrexate showed significant antitumor activity in metastatic breast cancer patients pretreated with anthracycline and taxanes [25]. In another study, Arkenau *et al.* showed that, after curative resection of stage III colon cancer, adjuvant treatment with 5-FU plus leucovorin is generally well tolerated and the long-term follow-up study demonstrated that this combination administered for 12 cycles significantly reduced tumor relapse and improved survival [26]. The other combinations of drugs in cancer therapeutics include a combination of paclitaxel with carboplatin or with vinorelbine for the treatment of nonsmall-cell lung cancer [27]. Among different combination therapies the most practiced and well researched therapy method is co-treatment with multiple chemotherapeutic drugs (Fig. 2d). Therefore, current trends in anticancer research have progressively concentrated on the development of new combinations of anticancer drugs with a view to overcoming the limitations frequently associated with conventional chemotherapy.

Nanotechnology-based combination drug delivery

Although combination chemotherapeutic strategies help to some extent toward the better treatment of cancer, their triumph is largely hindered as a result of the inadequate accessibility of antineoplastic agents to tumor tissue, requiring high doses, rapid abolition, poor solubility and inconsistent bioavailability [24]. Thus, to mitigate the difficulty associated with conventional chemotherapy, there is a call for developing a drug delivery system that could optimize the pharmaceutical action of drugs while reducing toxic side effects. The application of nanotechnology to cancer drug delivery is widely expected to create novel therapeutics for successful cancer treatment. Nanotechnology has a crucial role in cancer therapy regarding the use of different nanocarriers such as liposomes, dendrimers, micelles, carbon nanotubes (CNTs), polymer–drug conjugates and NPs (Fig. 3). Nanocarriers can protect a drug from degradation by evading the reticuloendothelial system (RES) and, thus, a high blood circulation profile enables transport through biological barriers, increasing the availability of drug at the targeted intracellular compartments reducing the toxicity and other side-effects associated with conventional drug delivery. In recent years, several types of nanocarriers loaded with multiple chemotherapeutic agents have been well characterized and have demonstrated improved anticancer activity. Recent application of different types of nanoparticulate drug delivery system for transporting combination drugs is frontier research, and we have illustrated it below as a separate section owing to its remarkable therapeutic value and relevance to the review.

Liposomes

Liposomes are defined as spherical lipid vesicles composed of amphiphilic phospholipids with a bilayer membrane structure having central aqueous space. The closed bilayers are formed by protecting the hydrophobic groups from the aqueous environment while there is simultaneous contact with the aqueous phase

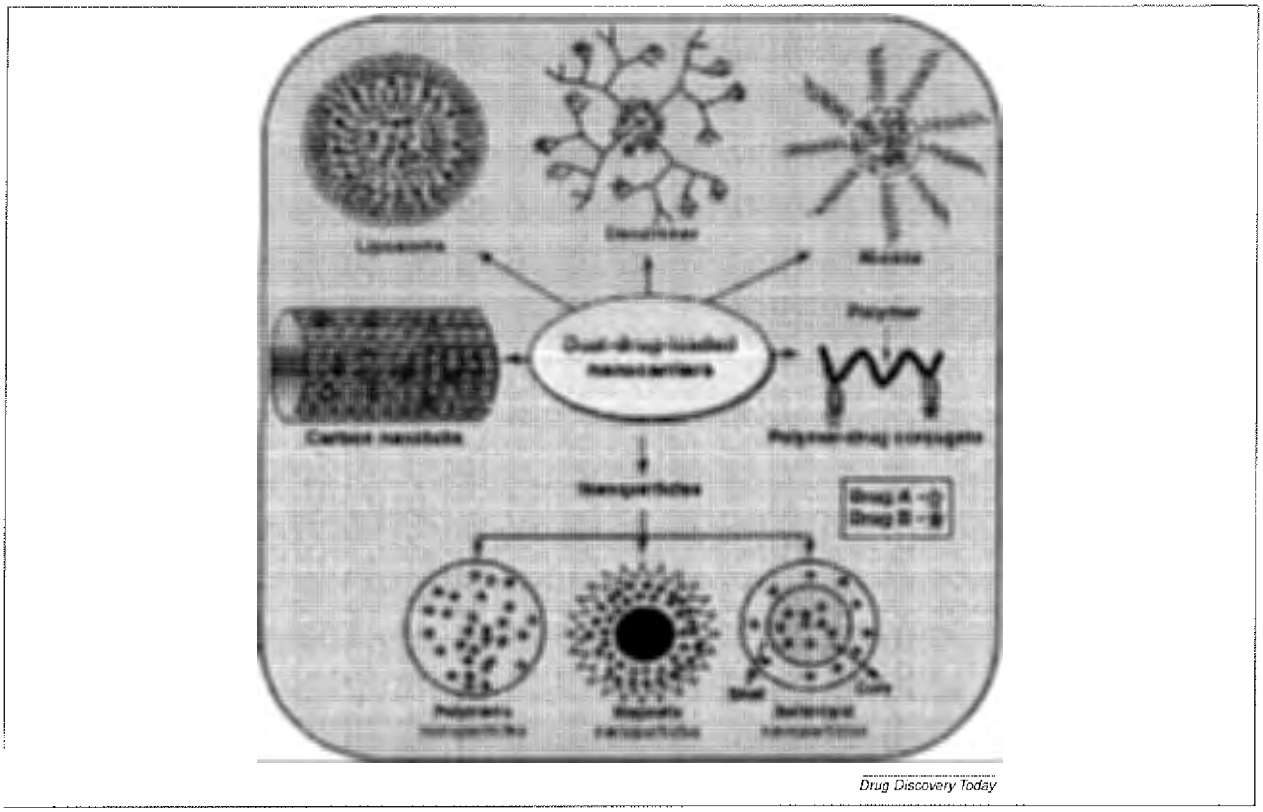

FIGURE 3

Diagrammatic representation of different types of nanocarriers for combination drug delivery. Different types of chemotherapeutic drugs can be loaded into the nanocarriers. Liposome, made up of lipid, can be used as a potent vehicle for co-delivery of multiple anticancer drugs. Dendrimers, macromolecular structures comprising a series of 'branches' around an inner core, are capable of carrying multiple chemotherapeutic drugs at the inner core as well as at the surface. Polymeric micelles are nano colloidal particles with a hydrophobic core, where different types of hydrophobic drugs can be carried. Carbon nanotubes can be used as carriers for delivering multiple anticancer drugs. In polymer–drug conjugates, drugs are covalently bound through a biodegradable linker and can be used as an effective drug delivery vehicle. Nanoparticles are colloidal particles of submicron size where multiple drugs can be carried. Representative examples of different nanoparticles, such as polymeric nanoparticles, magnetic nanoparticles and solid-lipid nanoparticles, are shown here.

from the hydrophilic head group [28]. The current marketed liposomal products used for cancer therapy include the liposomal formulation of doxorubicin (Doxil®), daunorubicin (DaunoXome®), cytarabine (DepoCyt®) and vincristine (Onco-TCS) [8,29]. Recently, a lot of attention has been given to the combination approach that uses liposomes as a delivery vehicle. Regarding combinational delivery, liposome-encapsulated curcumin and resveratrol have been formulated and, upon systemic administration, their chemopreventive effect was exemplified in prostate-specific PTEN knockout mice *in vivo*, as a result of PTEN loss and/or activated p-Akt signaling pathways [30]. In another study, Wu *et al.* formulated a transferrin-conjugated liposome by co-entrapping doxorubicin and varapamil (P-gp inhibitor). They evaluated the effectiveness in doxorubicin-resistant K562 cells and showed enhanced cytotocity caused by overcoming P-gp-mediated multidrug resistance [31]. The ratiometric approach involving the co-delivery of two different drugs with differing molar ratios following systemic administration is also an important factor for improving the therapeutic efficacy of the dual drug in a combination approach for cancer treatment [32]. In this regard, a Phase I study was performed by taking a liposomal formulation entrapped with two different drugs [i.e. irinotecan and floxuridine in the ratio of (1:1)], and the maximum tolerated dose and pharmacokinetic parameters of the liposomal formulation were determined in patients with advanced solid tumors. The results demonstrated the above dual-drug-loaded liposomal formulation was well tolerated showing enhanced anticancer activity in patients [33].

Dendrimers

The word dendrimer was derived from the Greek words *dendron* meaning tree or branch and *meros* meaning part. Dendrimers can be defined as synthetic symmetrical macromolecular compounds consisting of tree-like branches atound an inner core [34]. Currently, these are emerging as effective drug delivery vehicles owing to their nanometer size range and ability to have multiple copies of surface groups for biological recognition processes [35]. Owing to the presence of the highly branched structure, the surfaces can be used as a suitable targeted drug delivery vehicle by conjugating with different ligands. The presence of multivalent branches with cage-like structures has been used as a suitable platform for

simultaneous delivery of hydrophobic and hydrophilic drugs. Taking this into consideration, Tekade *et al.* have formulated dual-drug-loaded dendrimers by co-encapsulating methotrexate (MTX; a hydrophobic drug) and all-*trans* retinoic acid (ATRA; a hydrophilic drug) inside the polyamidoamine (PAMAM) dendrimer and have demonstrated reduced hemolytic toxicity of the dendrimer and enhanced the cytotoxicity profile in HeLa cells, compared with free drug [36].

Polymeric micelle systems

Polymeric micelles are based on block co-polymers with hydrophilic and hydrophobic units that self-assemble in an aqueous environment into structures composed of a hydrophobic core stabilized by a hydrophilic shell [28]. Polymeric micelles have several advantages over other nano drug delivery systems and, owing to their small size, they can extravasate from blood vessels and accumulate in tumor tissue. Currently, many drug-loaded polymeric micelles (either single or dual) for anticancer therapy are under investigation in preclinical studies to improve drug efficacy. Recently, Wang *et al.* investigated the efficiency of the simultaneous and targeted delivery of paclitaxel, along with verapamil, by using a micellar system to overcome MDR and demonstrated enhanced cytotoxicity in drug-resistant tumor cells [37]. Further, Katragadda *et al.* formulated paclitaxel and 17-allylamino-17-desmethoxygeldanamycin (17-AAG)-loaded PEG-distearoylphosphatidylethanolamine/tocopheryl polyethylene glycol 1000 (PEG-DSPE/TPGS) mixed micelles and demonstrated that the dual-drug-loaded mixed micelles effectively blocked the proliferation of human ovarian cancer SKOV-3 cells [38]. In an another study, Shin *et al.* developed block co-polymeric micelles (PEG-b-PLA) by entrapping three different drugs (paclitaxel, 17-AAG and rapamycin), and they have shown the above drug-loaded micelle acting as a three-in-one nanocontainer for solubilizing multiple drugs [39]. They have demonstrated that this micelle consisting of three drugs had a high synergistic effect in MCF-7 and 4T1 breast cancer cell lines so that the above formulation provided a simple and efficacious three-in-one nanomedicine for cancer therapy.

Carbon nanotubes

CNTs are carbon cylinders composed of benzene rings and they have attracted a tremendous amount of attention regarding their use in biomedical applications. In comparison with other nanomaterials, CNTs appear to be more dynamic in their biological application. Application of CNTs for the delivery of drugs to their site of action has become one of the main areas of interest for different research groups. This is mainly because of the characteristics of these materials, including their unique chemical, physical and biological properties, their nanoneedle shape, hollow monolithic structure and their ability to obtain required functional groups on their outer layers [8]. The shape of the CNT enables these materials to enter the cell *via* different methods, such as passive diffusion across the lipid bilayer or endocytosis, whereby the CNT attaches to the surface of the cell and is subsequently engulfed by the cell membrane. Recently, P-gp-antibody-functionalized CNTs loaded with doxorubicin were synthesized and demonstrated that the cytotoxicity induced by doxorubicin to MDR leukemic K562 cells was enhanced significantly up to four-fold more than that of free doxorubicin [40]. In a study by Dhar *et al.*, Pt (IV) complex with a folic acid specifically targets folate-receptor-enriched tumor cells. When conjugated to CNTs it was demonstrated as an effective targeted delivery of platinum-based anticancer agents [41].

Polymer–drug conjugates based on combination therapy

The advantages of polymer–drug conjugates include passive tumor targeting by EPR effect due to its leaky vasculature allows selective extravasation of the conjugate in tumor tissue and retained for longer period due to its poor lymphatic drainage. The other benefits of polymer-drug conjugates are reduced toxicity, overcoming MDR, capability to remove immunostimulatory effects and enhanced solubility and bioavailability of drug conjugate in biological fluids that modulate its pharmacokinetic behaviour [42]. The mechanism of passive tumor targeting by EPR effect to the tumor tissues is shown in Fig. 4. Recently, Lammers *et al.* synthesized HPMA-based polymer–drug conjugates carrying

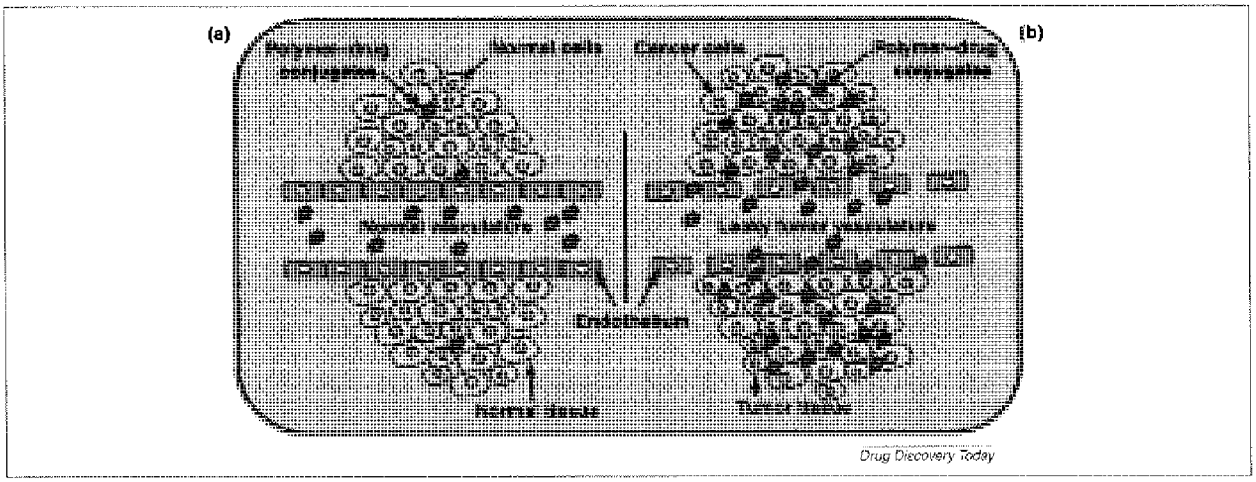

FIGURE 4

(a) Represents the normal vasculature and, owing to the presence of a tight junction, the polymer drug conjugates are not able to extravasate. **(b)** Depicts an enhanced accumulation of polymer–drug conjugates in the tumor owing to the leaky tumor vasculature, leading to an enhanced permeability and retention effect.

gemcitabine, doxorubicin and tyrosinamide, and they demonstrated the combinations effectively kill cancer cells by inhibiting angiogenesis and inducing apoptosis [43]. In an another study, by Verschraegen *et al.*, in a Phase I clinical trial, a poly-L-glutamic acid (PGA)–paclitaxel conjugate combined with platinates-based chemotherapy showed good anticancer activity in refractory patients with advanced solid tumors [44]. Further, HMPA-based polymer conjugates with two drugs, a cytostatic drug doxorubicin and an anti-inflammatory drug dexamethason, were developed by covalently attaching drugs to the polymer backbone – it was demonstrated that the above dual drug polymeric conjugates could be good candidates for cancer therapy [45].

Nanoparticles

Polymeric nanoparticles

NPs are submicron colloidal particles, and a drug is dissolved, entrapped, encapsulated or attached to the NP matrix [28]. In recent years, biodegradable polymeric NPs have attracted considerable attention as potential drug delivery devices in view of their application in the field of cancer. These polymeric NPs can be targeted to specific sites by surface modifications using different ligands to the receptors that are highly overexpressed on the target cancer cells [1]. Our group has developed different polymeric nanoparticulate systems loaded with dual drugs for cancer therapy. Acharya and Sahoo demonstrated the synergistic effect of dual drugs entrapped in polymeric NPs resulting in enhanced cytotoxicity of the combination formulation as compared with the free drugs at a low dose against leukemic K562 cells [46]. Misra and Sahoo demonstrated that the synergistic effect of co-formulation of doxorubicin and curcumin in poly (D,L-lactide-co-glycolide) (PLGA) NPs promotes the cytotoxicity of both the drugs in leukemic K562 cells *in vitro* by overcoming the MDR phenotype [47]. The polymeric NPs can be used as a delivery vehicle with the combination of small interfering (si)RNA and chemotherapy. In this regard, poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) and PEO-modified poly(epsilon-caprolactone) (PEO-PCL) NPs encapsulated with MDR-1 silencing siRNA and paclitaxel significantly enhanced the cytotoxic activity of paclitaxel in resistant SKOV3 cells similar to that observed in drug-sensitive SKOV3 cells [48]. The several dual-drug-loaded nanoparticulate formulations include: (i) epidermal growth factor receptor (EGFR)-targeted polymeric NPs loaded with lonidamine and paclitaxel that showed targeted antitumor activity by downregulating MDR in human breast and ovarian tumor cells [49]; (ii) PLGA NPs entrapped with vincristine and verapamil with moderate MDR reversal activity on MCF-7/ADR cells resistant to vincristine [50]; (iii) targeted co-delivery of paclitaxel and tariquidar loaded in PLGA NPs that demonstrated better inhibition of tumor growth in a mouse model by reversing MDR [51].

Magnetic nanoparticles

Magnetic NPs have been regarded as a promising candidate in biomedical research with special emphasis on magnetic-mediated drug delivery to the cancer tissues as a result of their small size and magnetic properties [52]. Further, magnetic NPs can be surface functionalized with different ligands for targeted drug delivery [53,54]. Currently, many drug-loaded magnetic NPs (either single or dual) have been investigated for effective anticancer therapy to improve drug efficacy. Recently, our group successfully developed dual-drug-loaded magnetic NPs conjugated with Her-2 antibody to increase further the efficiency in human breast cancer cell lines *in vitro* [53]. In another study, Singh *et al.* developed polymer-coated magnetic NPs as a dual-drug-delivery vehicle by encapsulating hydrophilic and hydrophobic anticancer drugs, and Her-2 was used as a targeting moiety for targeted delivery of drugs for breast cancer therapy. They demonstrated enhanced cellular uptake and an improved synergistic effect of dual-drug-loaded magnetic NPs as compared with native drugs [54]. Further, Jain *et al.* formulated multifunctional oleic-acid-coated iron-oxide and Pluronic stabilized magnetic NPs incorporating doxorubicin and paclitaxel either individually or in combination, both for drug delivery and magnetic resonance imaging (MRI) properties. In their study, high synergistic antiproliferative activity resulted in drug combinations incorporated within the magnetic NPs in breast cancer cells and prolonged circulation time of magnetic NPs was observed in mice, which is important for drug delivery and vascular imaging [55].

Solid-lipid nanoparticles

Solid-lipid nanoparticles (SLNPs) are colloidal NPs made from solid lipids (lipids that are solid at room temperature and at body temperature) and stabilized by surfactants. Upon systemic administration SLNPs have shown a longer bioavailability, thereby avoiding clearance *via* RES [52]. Serpe *et al.* have formulated SLNPs carrying cholesteryl-butyrate (a prodrug of butyrate), doxorubin or paclitaxel and evaluated the antiproliferative effect on the human colorectal cancer cell line HT-28. They have shown higher cytotoxicity than the equivalent amount of free-drug treatment as a result of the synergetic effect [56]. Currently, polymer–lipid hybrid NPs (PLN) are an emerging new form of SLNPs. A novel PLN was formulated by encapsulating doxorubicin and a chemosensitizer, GG918 (elacridar), for the treatment of MDR breast cancer cells and the results demonstrated that the doxorubicin and GG918 co-encapsulated PLN formulation showed a greater efficiency when compared with the single drug formulation on a MDR breast cancer cell line [57].

Combinations of different nanocarriers

In addition to the aforementioned nanocarriers being used alone, nanocarriers can also be used in combinations to achieve better therapeutic results in cancer therapy. Currently, multifunctional nanomedicinal platforms are designed for delivering siRNA by encapsulating it in magnetic NPs and complexing it with poly(propyleneimine) generation 5 dendrimers (PPI G5). A cancer-specific targeting moiety (LHRH peptide) was incorporated into superparamagnetic iron oxide – poly(propyleneimine) (SPIO-PPI) G5-siRNA complexes to increase selective internalization by cancer cells. The above example of siRNA delivery systems can increase the *in vivo* antitumor activity of the anticancer drug cisplatin [58]. Further, dendrimer-encapsulated polymeric-micelle-mediated photochemical internalization (PCI) combined with doxorubicin was investigated and the efficacy of photodynamic therapy effects on reversal of drug resistance in drug-resistant MCF-7 cells and a xenograft model was studied [59].

Challenges associated with nanoparticulate drug delivery systems

Although NPs have tremendous potential for effective anticancer drug delivery, their constant exposure on living cells can lead to a

range of adverse effects in humans and animals. Adverse effect of NPs on human health primarily depends on their chemistry, size, shape, agglomeration state and electromagnetic properties [60,61]. In this view, safety concerns are associated with the introduction of NPs to the human body. It will require further detailed toxicological studies before some of the products can be approved for clinical use. Carbon NPs, for example, have been shown to induce lipid peroxidation in the brain cells of fish and pulmonary inflammation in rats [62]. Similarly, silver NPs (AgNPs), which have well-known antimicrobial properties, are extensively used in various medical and general applications. Despite the widespread use of AgNPs, their constant use can cause toxicity at the cellular as well as the gene level [63]. Semiconductor quantum dots (QDs) hold increasing potential for cellular imaging *in vitro* and *in vivo*. However, they show dose-dependent toxicity affecting cell growth and viability. Similarly, CNTs predominantly used as diagnostic and therapeutic tools demonstrated cellular inflammation, epithelioid granulomas, fibrosis in lungs and biochemical/toxicological changes by inducing oxidative stress upon systemic application [64]. Although it is an interesting and emerging field, further studies are required with special focus on generated toxicity before clinical trials take place.

Concluding remarks

Nanotechnology has been developed as a powerful tool for cancer drug delivery. Here, we have discussed the current status of combination drug delivery based nanocarriers as an emerging approach for cancer therapy, as well as the need to develop such combination strategies in terms of targeted therapy. Liposomes, dendrimers, polymeric micelles, CNTs and polymeric NPs can transport several chemotherapeutic agents including siRNA, antiangiogenic agents and chemosensitizers, among others. Therefore, administration of different chemotherapeutic drugs in combination, with a suitable nanocarrier platform could be considered as an emerging approach for the treatment of cancer in near future.

Future perspectives

The ultimate goal of cancer treatment in the modern era is to kill as many cancer cells as possible and leave the healthy cells unaffected at the same time. The current aim is to develop a single dose magic bullet for multiple drug delivery with a suitable potent nanovehicle, thus improving the drug efficiency at a lower dose and the transportation of the drug across physiological barriers present within the tumors, as well as reducing drug-related toxicity. Current development in the field of nanotechnology-based combination therapeutics has illustrated many distinctive characteristics that are not available with traditional chemotherapy. Nanoparticulate dual combinations have an optimal antitumor effect and can be delivered in a more effective way. In the near future, oncologists could develop a suitable nanocarrier or a targeting moiety that, in combination with chemotherapeutic agents, could lead to improved therapeutic outcomes with reduced costs. The advance of emerging techniques involving polymer–drug conjugates and nanotechnology-based vehicles is still going on to attain a better therapeutic regimen. Lastly, we envisage that multi-therapeutic nanocarriers will be investigated that entrap multiple drugs. Managing the precise dosage of drug and systematic release of the drugs from the carrier system for the treatment of several cancers with different molecular mechanisms is considered as an inevitable step for future cancer therapeutics.

References

1 Das, M. *et al.* (2009) Ligand-based targeted therapy for cancer tissue. *Expert Opin. Drug Deliv.* 6, 285–304
2 Mohanty, C. *et al.* (2011) Receptor mediated tumor targeting: an emerging approach for cancer therapy. *Curr. Drug Deliv.* 8, 45–58
3 Persidis, A. (1999) Cancer multidrug resistance. *Nat. Biotechnol.* 17, 94–95
4 Parveen, S. and Sahoo, S.K. (2006) Nanomedicine: clinical applications of polyethylene glycol conjugated proteins and drugs. *Clin. Pharmacokinet.* 45, 965–988
5 Parveen, S. and Sahoo, S.K. (2008) Polymeric nanoparticles for cancer therapy. *J. Drug Target.* 16, 108–123
6 Sahoo, S.K. *et al.* (2007) The present and future of nanotechnology in human health care. *Nanomedicine* 3, 20–31
7 Lebar, J. *et al.* (2009) Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nat. Biotechnol.* 27, 659–666
8 Misra, R. *et al.* (2010) Cancer nanotechnology: application of nanotechnology in cancer therapy. *Drug Discov. Today* 15, 842–850
9 Sahoo, S.K. *et al.* (2008) Nanotechnology in ocular drug delivery. *Drug Discov. Today* 13, 144–151
10 Chou, T.C. (2010) Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* 70, 440–446
11 Lai, G.H. *et al.* (2003) Celecoxib acts in a cyclooxygenase-2-independent manner and in synergy with emodin to suppress rat cholangiocarcinoma growth *in vitro* through a mechanism involving enhanced Akt inactivation and increased activation of caspases-9 and -3. *Mol. Cancer Ther.* 2, 265–271
12 Chiang, C.T. *et al.* (2010) Combinations of mTORC1 inhibitor RAD001 with gemcitabine and paclitaxel for treating non-Hodgkin lymphoma. *Cancer Lett.* 298, 195–203
13 Lage, H. (2008) An overview of cancer multidrug resistance: a still unsolved problem. *Cell Mol. Life Sci.* 65, 3145–3167
14 Robert, J. and Jarry, C. (2003) Multidrug resistance reversal agents. *J. Med. Chem.* 46, 4805–4817
15 Hubensack, M. *et al.* (2008) Effect of the ABCB1 modulators elacridar and tariquidar on the distribution of paclitaxel in nude mice. *J. Cancer Res. Clin. Oncol.* 134, 597–607
16 Lancet, J.E. *et al.* (2009) A phase I trial of continuous infusion of the multidrug resistance inhibitor zosuquidar with daunorubicin and cytarabine in acute myeloid leukemia. *Leuk. Res.* 33, 1055–1061
17 Dasanu, C.A. *et al.* (2010) Carboplatin-gemcitabine in the therapy of advanced ovarian cancer: dose reduction consideration. *J. Oncol. Pharm. Pract.* 16, 63–66
18 Bava, S.V. *et al.* (2005) Sensitization of taxol-induced apoptosis by curcumin involves down-regulation of nuclear factor-kappaB and the serine/threonine kinase Akt and is independent of tubulin polymerization. *J. Biol. Chem.* 280, 6301–6308
19 Logman, J.F. *et al.* (2010) Economic evaluation of zoledronic acid for the prevention of osteoporotic fractures in postmenopausal women with early-stage breast cancer receiving aromatase inhibitors in the UK. *Ann. Oncol.* 21, 1529–1536
20 Pauwels, B. *et al.* (2005) Combined modality therapy of gemcitabine and radiation. *Oncologist* 10, 34–51
21 Nigro, N.D. *et al.* (1974) Combined therapy for cancer of the anal canal: a preliminary report. *Dis. Colon Rectum* 17, 354–356
22 DeAngelis, L.M. *et al.* (2002) Combination chemotherapy and radiotherapy for primary central nervous system lymphoma: Radiation Therapy Oncology Group Study 93-10. *J. Clin. Oncol.* 20, 4643–4648
23 Gulley, J.L. *et al.* (2005) Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. *Clin. Cancer Res.* 11, 3353–3362
24 Greco, F. and Vicent, M.J. (2009) Combination therapy: opportunities and challenges for polymer-drug conjugates as anticancer nanomedicines. *Adv. Drug Deliv. Rev.* 61, 1203–1213

25 Tanabe, M. *et al.* (2009) Possible use of combination chemotherapy with mitomycin C and methotrexate for metastatic breast cancer pretreated with anthracycline and taxanes. *Breast Cancer* 16, 301–306

26 Arkenau, H.T. *et al.* (2003) 5-Fluorouracil plus leucovorin is an effective adjuvant chemotherapy in curatively resected stage III colon cancer: long-term follow-up results of the adjCCA-01 trial. *Ann. Oncol.* 14, 395–399

27 Berhoune, M. *et al.* (2008) Therapeutic strategy for treatment of metastatic non-small cell lung cancer. *Ann. Pharmacother.* 42, 1640–1652

28 Sahoo, S.K. and Labhasetwar, V. (2003) Nanotech approaches to drug delivery and imaging. *Drug Discov. Today* 8, 1112–1120

29 Hu, C.M. *et al.* (2010) Nanoparticle-assisted combination therapies for effective cancer treatment. *Therapeutic Deliv.* 1, 323–334

30 Narayanan, N.K. *et al.* (2009) Liposome encapsulation of curcumin and resveratrol in combination reduces prostate cancer incidence in PTEN knockout mice. *Int. J. Cancer* 125, 1–8

31 Wu, J. *et al.* (2007) Reversal of multidrug resistance by transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil. *J. Pharm. Pharm. Sci.* 10, 350–357

32 Dicko, A. *et al.* (2010) Use of nanoscale delivery systems to maintain synergistic drug ratios *in vivo*. *Expert Opin. Drug Deliv.* 7, 1329–1341

33 Batist, G. *et al.* (2009) Safety, pharmacokinetics, and efficacy of CPX-1 liposome injection in patients with advanced solid tumors. *Clin. Cancer Res.* 15, 692–700

34 Dykes, G. (2001) Dendrimers: a review of their appeal and applications. *J. Chem. Technol. Biotechnol.* 76, 903–918

35 Menjoge, A.R. *et al.* (2010) Dendrimer-based drug and imaging conjugates: design considerations for nanomedical applications. *Drug Discov. Today* 15, 171–185

36 Tekade, R.K. *et al.* (2009) Exploring dendrimer towards dual drug delivery: pH responsive simultaneous drug-release kinetics. *J. Microencapsul.* 26, 287–296

37 Wang, F. *et al.* (2011) Synergistic effect of folate-mediated targeting and verapamil-mediated P-gp inhibition with paclitaxel-polymer micelles to overcome multi-drug resistance. *Biomaterials* 32, 9444–9456

38 Katragadda, U. *et al.* (2011) Multi-drug delivery to tumor cells via micellar nanocarriers. *Int. J. Pharm.* 419, 281–286

39 Shin, H.C. *et al.* (2011) A 3-in-1 polymeric micelle nanocontainer for poorly water-soluble drugs. *Mol. Pharm.* 8, 1257–1265

40 Li, R. *et al.* (2010) P-glycoprotein antibody functionalized carbon nanotube overcomes the multidrug resistance of human leukemia cells. *ACS Nano* 4, 1399–1408

41 Dhar, S. *et al.* (2008) Targeted single-wall carbon nanotube-mediated Pt(IV) prodrug delivery using folate as a homing device. *J. Am. Chem. Soc.* 130, 11467–11476

42 Maeda, H. *et al.* (2001) Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. *J. Control. Release* 74, 47–61

43 Lammers, T. *et al.* (2009) Simultaneous delivery of doxorubicin and gemcitabine to tumors in vivo using prototypic polymeric drug carriers. *Biomaterials* 30, 3466–3475

44 Verschraegen, C.F. *et al.* (2009) A phase I and pharmacokinetic study of paclitaxel poliglumex and cisplatin in patients with advanced solid tumors. *Cancer Chemother. Pharmacol.* 63, 903–910

45 Krakovicova, H. *et al.* (2009) HPMA-based polymer conjugates with drug combination. *Eur. J. Pharm. Sci.* 37, 405–412

46 Acharya, S. and Sahoo, S.K. (2011) Sustained targeting of Bcr-Abl + leukemia cells by synergistic action of dual drug loadec nanoparticles and its implication for leukemia therapy. *Biomaterials* 32, 5643–5662

47 Misra, R. and Sahoo, S.K. (2011) Coformulation of doxorubicin and curcumin in poly(D,L-lactide-co-glycolide) nanoparticles suppresses the development of multidrug resistance in K562 cells. *Mol. Pharm.* 8, 852–866

48 Yadav, S. *et al.* (2009) Evaluations of combination MDR-1 gene silencing and paclitaxel administration in biodegradable polymeric nanoparticle formulations to overcome multidrug resistance in cancer cells. *Cancer Chemother. Pharmacol.* 63, 711–722

49 Milane, L. *et al.* (2010) Development of EGFR-targeted polymer blend nanocarriers for combination paclitaxel/lonidamine delivery to treat multi-drug resistance in human breast and ovarian tumor cells. *Mol. Pharm.* 8, 185–203

50 Song, X.R. *et al.* (2009) Reversion of multidrug resistance by co-encapsulation of vincristine and verapamil in PLGA nanoparticles. *Eur. J. Pharm. Sci.* 37, 300–305

51 Patil, Y. *et al.* (2009) Nanoparticle-mediated simultaneous and targeted delivery of paclitaxel and tariquidar overcomes tumor drug resistance. *J. Control. Release* 136, 21–29

52 Parveen, S. *et al.* (2012) Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine* 8, 147–166

53 Dilnawaz, F. *et al.* (2010) Dual drug loaded superparamagnetic iron oxide nanoparticles for targeted cancer therapy. *Biomaterials* 31, 3694–3706

54 Singh, A. *et al.* (2011) Composite polymeric magnetic nanoparticles for co-delivery of hydrophobic and hydrophilic anticancer drugs and MRI imaging for cancer therapy. *ACS Appl. Mater. Interfaces* 3, 842–856

55 Jain, T.K. *et al.* (2008) Magnetic nanoparticles with dual functional properties: drug delivery and magnetic resonance imaging. *Biomaterials* 29, 4012–4021

56 Serpe, L. *et al.* (2004) Cytotoxicity of anticancer drugs incorporated in solid lipid nanoparticles on HT-29 colorectal cancer cell line. *Eur. J. Pharm. Biopharm.* 58, 673–680

57 Wong, H.L. *et al.* (2006) A mechanistic study of enhanced doxorubicin uptake and retention in multidrug resistant breast cancer cells using a polymer–lipid hybrid nanoparticle system. *J. Pharmacol. Exp. Ther.* 317, 1372–1381

58 Taratula, O. *et al.* (2011) Multifunctional nanomedicine platform for cancer specific delivery of siRNA by superparamagnetic iron oxide nanoparticles-dendrimer complexes. *Curr. Drug Deliv.* 8, 59–69

59 Lu, H.L. *et al.* (2011) Dendrimer phthalocyanine-encapsulated polymeric micelle-mediated photochemical internalization extends the efficacy of photodynamic therapy and overcomes drug-resistance *in vivo*. *J. Control. Release* 155, 458–464

60 Longmire, M. *et al.* (2008) Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. *Nanomedicine (Lond.)* 3, 703–717

61 Longmire, M.R. *et al.* (2011) Biologically optimized nanosized molecules and particles: more than just size. *Bioconjug. Chem.* 22, 993–1000

62 Stern, S.T. and McNeil, S.E. (2008) Nanotechnology safety concerns revisited. *Toxicol. Sci.* 101, 4–21

63 Asare, N. *et al.* (2012) Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells. *Toxicology* 291, 65–72

64 Fenoglio, I. *et al.* (2012) Thickness of multiwalled carbon nanotubes affects their lung toxicity. *Chem. Res. Toxicol.* 25, 74–82

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Notice of opposition to a European patent

I. Patent opposed

Patent No.	EP3337478
Application No.	EP16758337.6
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	12 August 2020
Title of the invention	DRUG COMBINATION COMPRISING LIPOSOMAL IRINOTECAN, OXALIPLATIN, 5-FLUOROURACIL AND LEUCOVORIN FOR TREATING METASTATIC PANCREATIC CANCER

II. Proprietor of the patent

first named in the patent specification	Ipsen Biopharm Ltd.
Opponent's or representative's reference	RSC/R80463OP

III. Opponent

Name	Generics [UK] Limited
Address:	Building 4 Trident Place Mosquito Way Hatfield Hertfordshire AL10 9UL United Kingdom
State of residence or of principal place of business	United Kingdom
Multiple opponents (see additional sheet)	☐

IV. Authorisation

1. Representative

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Additional representative(s) on additional sheet/see authorisation ☐

Authorisation(s)

is/are enclosed ☐

has/have been registered under No. ☐

V. Opposition is filed against

the patent as a whole ☒

claim(s) No(s).

VI. Grounds for opposition:

Opposition is based on the following grounds:

(a) the subject-matter of the European patent opposed is not patentable (Art. 100(a) EPC) because:

- it is not new (Art. 52(1); Art. 54 EPC) ☐
- it does not involve an inventive step (Art. 52(1); Art. 56 EPC) ☒
- patentability is excluded on other grounds, namely articles ☐

(b) the patent opposed does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art (Art. 100(b) EPC; see Art. 83 EPC). ☐

(c) the subject-matter of the patent opposed extends beyond the content of the application/of the earlier application as filed (Art. 100(c) EPC, see Art. 123(2) EPC). ☐

VII. Facts (Rule 76(2)(c) EPC)

presented in support of the opposition are submitted herewith on an attached document ☒

VIII. Other requests:

- Oral proceedings are hereby requested auxiliarily.

IX. Evidence presented

D10	Other evidence	FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer, N Engl J Med 364: 1817- 1825, 2011 original file name: D10.pdf attached as: Other-evidence-1.pdf
D11	Other evidence	Impact of FOLFIRINOX compared with gemcitabine on quality of life in patients with metastatic pancreatic cancer original file name: D11.pdf attached as: Other-evidence-2.pdf
D12	Other evidence	A multinational phase 2 study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) original file name: D12.pdf attached as: Other-evidence-3.pdf
D13	Other evidence	Lipidic nanparticle CPT-11 in a bioluminescent orthotopic pancreas cancer model, AACR Annual Meeting, Apr 14-18, 2007 original file name: D13.pdf attached as: Other-evidence-4.pdf
D14	Other evidence	Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients, Cancer Chemother Pharmacol, 2015 Mar;75(3) original file name: D14.pdf attached as: Other-evidence-5.pdf
D15	Other evidence	Phase I study of liposome irinotecan (PEP02) in combination with weekly infusion of 5-FU/LV in advanced solid tumors original file name: D15.pdf attached as: Other-evidence-6.pdf
D16	Other evidence	Modified FOLFIRINOX regimen with improved safety and maintained efficacy in pancreatic adenocarcinoma, Pancreas. 2013 Nov;42(8): original file name: D16.pdf attached as: Other-evidence-7.pdf
D17	Other evidence	Development of a Highly Active Nanoliposomal Irinotecan Using a Novel Intraliposomal Stabilization Strategy, Cancer Res 2006; 66 original file name: D17.pdf attached as: Other-evidence-8.pdf

X. **Payment**

Method of payment	Debit from deposit account

The European Patent Office is hereby authorised, to debit from the deposit account with the EPO any fees and costs indicated in the fees section below.

Currency:	EUR
Deposit account number:	28050051
Account holder:	Elkington and Fife LLP

Refunds

Any refunds should be made to EPO deposit account:	28050051
Account holder:	Elkington and Fife LLP

Fees	Factor applied	Fee schedule	Amount to be paid
010 Opposition fee	1	815.00	815.00
Total:		**EUR**	**815.00**

A	**Forms**	Details:	System file name:
A-1	Form for notice of opposition		ep-oppo.pdf

B	**Attached document files**	Original file name:	System file name:
B-1	1. Facts and arguments	2021-05-12-EP 3 337 478 Statement of opposition_FINAL 1.pdf	OPPO.pdf
B-2	1. Any annexes (other than citation) to an opposition letter - Covering Letter	2021-05-12-R80463OP-EPO Opposition Letter.pdf	OTHER-1.pdf

C	**Attached evidence files**	Original file name:	System file name:
C-1	1. Other evidence	D10.pdf	Other-evidence-1.pdf
C-2	2. Other evidence	D11.pdf	Other-evidence-2.pdf
C-3	3. Other evidence	D12.pdf	Other-evidence-3.pdf
C-4	4. Other evidence	D13.pdf	Other-evidence-4.pdf
C-5	5. Other evidence	D14.pdf	Other-evidence-5.pdf

C-6	6. Other evidence	D15.pdf	Other-evidence-6.pdf
C-7	7. Other evidence	D16.pdf	Other-evidence-7.pdf
C-8	8. Other evidence	D17.pdf	Other-evidence-8.pdf

Signature of opponent or representative

Place	**GB**
Date:	**12 May 2021**
Signed by:	**Richard Cooke 10750**
Association:	**Elkington and Fife LLP**
Representative name:	**Richard COOKE**
Capacity:	**(Representative)**

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Our ref RSC/HJK/R80463OP

12 May 2021

ONLINE FILING

Dear Sirs

Re: European Patent No. 3337478 (Application No. 16758337.6)
Ipsen Biopharm Ltd.
Opponent: Generics [UK] Limited

We enclose a notice of opposition against the above patent.

The opposition fee is being paid from our deposit account. If either the fee is insufficient or the fee is not received, please debit any necessary funds from our deposit account (Elkington and Fife LLP: 2805.0051).

Yours faithfully
Elkington and Fife LLP

Richard Cooke

Enc: Notice of Opposition

ELKINGTON + FIFE

European patent no. 3 337 478
(application no. 16758337.6)
Ipsen Biopharm Ltd
Opponent: Generics [UK] Limited

Notice of Opposition

1. Requests

(01) We request revocation of the patent in its entirety. If the opposition division intends to reach any other decision, we request oral proceedings.

2. Documents

(02) We refer to D1 to D9 in the consolidated list of documents published following the opposition filed by Sandoz AG, and enclose new documents D10 to D17. A copy of each newly cited document is enclosed.

3. Lack of inventive step over D1 and D6 (Articles 56 and 100(a) EPC)

(03) We endorse the arguments of Sandoz AG that the patent is not entitled to a relevant priority date and lacks an inventive step over D1 and D6.

4. Lack of inventive step over D3, D10 or D11 (Articles 56 and 100(a) EPC)

4.1 The closest prior art and the distinguishing features

(04) The aim of the patent is to provide a treatment of metastatic pancreatic cancer (see paragraph [0002] of the patent).

(05) The patent claims a medical use of a liposomal irinotecan quadruple-combination as an antineoplastic therapy wherein the combination, disease and dosage regimen are defined as follows:

Combination:	60 mg/m^2 liposomal irinotecan, 60 mg/m^2 oxaliplatin, 200 mg/m^2 (l)-leucovorin or 400mg/m^2 (l/d)-leucovorin 2400mg/m^2 5-fluorouracil
Disease:	Metastatic adenocarcinoma of the pancreas
Patient:	Previously untreated
Regimen:	Once every two weeks

(06) D3 (Pancreatic cancer and FOLFIRINOX: a new era and new questions) explains that FOLFIRINOX, a quadruple combination consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin, relative to gemcitabine provides improved median overall survival time, progression-free survival and objective responses, as well as improved time to deterioration in quality of life (Abstract).

(07) D10 (FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer; reference 6 in D3) describe a phase 2-3 trial comparing the combination therapy, FOLFIRINOX, with single agent gemcitabine for use in treating metastatic pancreatic cancer as a first line treatment i.e. in previously untreated patients (see title).

(08) D10 explains that the treatment was successful, and was the first therapy to show a statistically and clinically significant survival advantage over the single agent gemcitabine, the previously used standard for the treatment of metastatic pancreatic adenocarcinoma (page 1822):

> "In this study, FOLFIRINOX was an effective first-line treatment option for patients with metastatic pancreatic adenocarcinoma and good ECOG performance status. The median overall survival was significantly prolonged, with an increase of 4.3 months in the FOLFIRINOX group as compared with the gemcitabine group (11.1 vs 6.8 months)."

(09) D11 (Impact of FOLFIRINOX Compared With Gemcitabine on Quality of Life in Patients With Metastatic Pancreatic Cancer: Results From the PRODIGE 4/ACCORD 11 Randomized Trial; reference 7 in D3) explains that FOLFIRINOX improved quality of life i.e. QoL (page 23):

> "FOLFIRINOX significantly reduces QoL impairment compared with gemcitabine in patients with metastatic pancreatic cancer. Furthermore, baseline QoL scores improved estimation of survival probability when added to baseline clinical and demographic variables."

(10) The patent accepts that FOLFIRINOX was the "standard of care for first-line treatment of metastatic pancreatic cancer" (paragraph [0003] of the patent).

(11) Accordingly, D3, D10 and D11 each discloses a quadruple combination comprising the same four active ingredients for treating the same disease in the same patient as the patent, and therefore are reasonable starting points for the assessment of inventive step.

(12) The regimen disclosed in D3, D10 and D11 involves the following doses of each component in the quadruple combination (D10, page 1817, methods):

Component	Dose (mg/m^2)	
	D10	*Claim 1*
Irinotecan	180	60 (liposomal)
Oxaliplatin	85	60
Leucovorin	400	400
5-Fluorouracil	400 bolus and then 2400 infusion	2400

(13) Therefore, the distinguishing features are:

- the use of liposomal irinotecan; and
- the doses of irinotecan, oxaliplatin and 5-fluorouracil.

4.2 The objective technical problem

(14) The patentee has not provided any evidence comparing the treatment defined by the claim with FOLFIRINOX as disclosed by D3, D10 and D11.

(15) Therefore, the objective technical problem is the provision of an alternative treatment of metastatic adenocarcinoma of the pancreas in patients not having previously received chemotherapy for treating the said condition.

4.3 Obviousness of the solution

4.3.1 The use of liposomal irinotecan

(16) Liposomal irinotecan, and its combinations with 5-fluorouracil and leucovorin, were known at the priority date to have improved antitumor activity compared to its non-liposomal form.

D12 and D13

(17) D12 (A multinational phase 2 study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer) describes a phase II study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic cancer.

(18) D12 explains that pre-clinical data had shown an improved antitumor activity of PEP02 which led to the conduction of this clinical trial:

> "Preclinical *in vivo* efficacy data have shown improved antitumor activity of PEP02 over the equivalent dose of free irinotecan in multiple established human tumour xenograft mouse models, including brain, colon and pancreatic cancers."

(19) The preclinical data referred to in D12 is D13 ("Hann"; Lipidic nanoparticle CPT-11 in a bioluminescent orthotopic pancreas cancer model), which explains that an improvement in a pancreatic xenograft model was provided by liposomal delivery:

> "We conclude that nanoparticle-mediated delivery of CPT-11 via nanoliposomal CPT-11 or anti-EGFR immunoliposomal CPT-11 greatly enhances antitumor efficacy in the orthotopic COLO357 pancreatic xenograft model. This therapeutic approach offers potential advantages for pancreatic cancer treatment, and this type of model system may be useful in preclinical evaluation."

(20) D12 explains that liposomal irinotecan provided improved pharmacokinetics, and tumor bio-distribution (page 921):

> "PEP02 (also known as MM-398) is irinotecan sucrosofate encapsulated in a liposome drug delivery system. This stable nanoliposomal formulation has been shown in preclinical studies to improve pharmacokinetics and tumour bio-distribution of both irinotecan and its active metabolite SN-38, when compared with the free form of the drug, with less accumulation in many of the target organs associated with toxic side effects."

(21) The results of the phase II clinical trial in D12 further support the potential of PEP02, as summarised in the final paragraph of p924, encouraging a person skilled in the art to further explore its potential in the first-line treatment of advanced pancreatic cancer (APC):

> "The results of this clinical trial are encouraging enough to warrant moving ahead with a larger study in a similar patient population, currently ongoing as an international randomised phase 3 trial called NAPOLI-1 (clinicaltrial.gov. ID: NCT01494506, EudraCT Number: 2011-004687-30). Additional studies may explore this drug's potential role in the first-line settings and as part of combination regimens for APC." (Emphasis added).

(22) We note that D12 cites D6 (Conroy et al 2011) and explains that irinotecan in the FOLFIRINOX regimen is better than gemcitabine as a first line treatment (page 923, bottom of right-hand column):

> "Specific to pancreatic cancer, irinotecan represents a component of the FOLFIRINOX regimen that has recently demonstrated superior activity to gemcitabine in the front-line setting ..."

D14

(23) D14 (Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients) explains that the liposomal formulation of irinotecan had been shown to provide improved pharmacokinetics and anti-tumor activity (paragraph bridging the columns on page 580):

> "PEP02 is a novel nanoparticle formulation of irinotecan sucrosofate encapsulated with polyethylene glycolated liposome. The coupling of high molecular weight polyethylene-glycol (PEG) on the surface of PEP02 can effectively protect it from circulating protein binding and subsequent phagocytosis of the reticuloendothelial system to further enhance its circulation time. In preclinical animal studies, PEP02 showed improved preclinical pharmacokinetic properties and anti-tumor activity (in house data) [4]."

(24) D14 also explains that the lower toxicity of the liposomal form makes it more appropriate for use in combinations (page 584, left-hand column):

> "The lower toxicity profile potentially makes PEP02 a better agent to combine with other cytotoxic agents, i.e., 5-fluorouracil and folinic acid, and/or targeted agents, i.e., bevacizumab or cetuximab for advanced colorectal cancer."

(25) Although the main aim of D14 was not to investigate antitumor efficacy, the conclusion drawn from this trial was that further investigations using liposomal-irinotecan should be carried out (page 584, right-hand column):

> "Promising anti-tumor activities that were observed in the patients who were refractory to available treatments warrant further clinical investigations."

Summary

(26) Starting from D3, D10 or D11 and in order to solve the problem of providing an alternative treatment, the skilled person would use the liposomal form of irinotecan. The skilled person was aware that this form had improved properties (e.g. D12, D13 and D14).

4.3.2 The doses of irinotecan, oxaliplatin and 5-fluorouracil

(27) D14 determined the maximum tolerated dose (MTD) when administered every three weeks to be 120 mg/m^2 (top of left-hand column of page 580):

> "The MTD of PEP02 monotherapy at 3-week interval is 120 mg/m^2, which will be the recommended dose for future studies."

(28) As explained by Sandoz AG, the dose utilised in combination therapies is generally lower (T 2506/12, r 3.14 and 4.3).

(29) In D15, the phase I study of PEP02 in combination with 5-fluorouracil and leucovorin, the maximum tolerated dose was found to be lower than 120 mg/m^2 after analysing cohorts of patients treated at 60, 80, 100 and 120 mg/m^2, which as discussed above had a good tumour response in two patients:

> "The MTD of PEP02 in combination with HDFL given every-3-week is 80 mg/m^2."

(30) D12 (A multinational phase 2 study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer) also suggests the use of lower doses (page 921):

> "Owing to concerns of excess toxicity, primarily asthenia, observed in US patients at the starting dose of 120 mg m^{-2}, the protocol was subsequently amended during the second stage of the study to permit a lower starting dose at 100 mg m^{-2}."

(31) We fully agree with Sandoz AG that the discovery of the optimum dose for providing a known or obvious therapeutic effect is routine (T 1409/06, r 3.2.1).

(32) The dose claimed in the patent is within those previously studied, and the skilled person would select that dose without invention.

(33) The slightly different dose of oxaliplatin at 60 mg/m^2 rather than 85 mg/m^2 is also an obvious modification that would be easily derived in view of the common general knowledge. In this regard, we note that the patent discloses the two doses at an equal level of preference (paragraph [0005]):

> "A total of 60, 75 or 85 mg/m^2 oxaliplatin can be administered on each day the liposomal irinotecan is administered."

(34) In the prior art, an additional 400 mg/m^2 5-fluoururacil was given as a bolus prior to the administration of 2400 mg/m^2 5-fluoururacil as claimed.

(35) However, at the priority date it was already known that the bolus 5-fluoururacil contributes to the toxicity of FOLFIRINOX. D16 (Modified FOLFIRINOX regimen with improved safety and maintained efficacy in pancreatic adenocarcinoma) explains that removing the 5-FU bolus to create a modified FOLFIRINOX regimen was advantageous:

> "Modified FOLFIRINOX has an improved safety profile with maintained efficacy in metastatic pancreatic cancer."

(36) We note that the patent accepts that removing the bolus 5-FU was advantageous:

> "Yet due to toxicity, modified FOLFIRINOX regimens are often used (e.g. elimination of the 5-FU bolus) with unknown effects on the efficacy and safety of modified schedules."

(37) Therefore, it would be obvious to a person skilled in the art to remove the additional 400 mg/m^2 5-fluoururacil bolus from the regimen.

4.3.3 Summary

(38) The skilled person was aware that liposomal irinotecan provided improved therapy. The doses defined by the patent represent minor modifications to the doses used in the prior art, and could be determined by the skilled person without invention. Therefore the patent lacks an inventive step.

4.4 The other claims

(39) Claim 2 includes all the features of claim 1 and defines a known liposomal formulation of irinotecan (D17).

(40) Claim 3 claims the standard infusion time of 5-fluoururacil.

(41) Claim 4 defines an arbitrary order of administration of 5-fluoururacil and leucovorin.

(42) Claim 5 and 6 require administration on days 1 and 15 i.e. treatment every two weeks which does not distinguish the prior art.

(43) Claim 7 defines an arbitrary infusion time.

(44) Claim 8 and 9 define an arbitrary order of administration.

(45) Claims 10 and 11 define an arbitrary time between administration of oxiplatin and liposomal irinotecan.

(46) Claim 12 requires administration on days 1 and 15 i.e. treatment every two weeks which does not distinguish the prior art.

(47) Claims 13 and 14 refer to the same formulation as claim 2.

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12 May 2021

Annex: Document list

EPO	CITATIONS-ENTGEGENHALTUNGEN-CITATIONS	P	1
D1	Protocol of the clinical trial NCT02551991 entitled "Study of Nanoliposomal Irinotecan (Nal-IRI)-Containing Regimens Versus Nab-paclitaxel Plus Gemcitabine in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma", version 1 of September 15, 2015		D1
D2	D. Von Hoff et al., Ann. Oncol. 2014, 25(2), ii105–ii117		D2
D3	R.D.W. Marsh et al., Cancer Med 2015, 4(6), 853–863		D3
D4	Onivyde prescribing information, October 2015		D4
D5	J. Carnevale et al., Fut. Oncol. 2016, 12(4), 453-464		D5
D6	A. Dean et al., J. Clin. Oncol. 2016, 34, 4(Supp 1), Abstract TPS482, presented at the 2016 ASCO Gastrointestinal Cancers Symposium in San Francisco, CA, on January 22, 2016		D6
D7	H. Zhang et al., Onco Targets Ther. 2016, 9, 3001-3007		D7
D8	D.F. Gaddy et al., Cancer Res. 2016, 76, 14(Supp), Abstract 4830, presented at the AACR 107th Annual Meeting in New Orleans, LA, on April 16-20, 2016		D8
D9	P. Parhi et al., Drug Discov. Today 2012, 17(17/18), 1044		D9

D10 FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer, N Engl J Med 364: 1817– 1825, 2011

D11 Impact of FOLFIRINOX compared with gemcitabine on quality of life in patients with metastatic pancreatic cancer: results from the PRODIGE 4/ACCORD 11 randomized trial, J Clin Oncol, 2013 Jan 1; 31(1):23-9

D12 A multinational phase 2 study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer, Br J Cancer, 2013 Aug 20;109(4):920-5

D13 Lipidic nanoparticle CPT-11 in a bioluminescent orthotopic pancreas cancer model, AACR Annual Meeting, Apr 14-18, 2007; Los Angeles, CA, A5648

D14 Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients, Cancer Chemother Pharmacol, 2015 Mar;75(3):579-86

D15 Phase I study of liposome irinotecan (PEP02) in combination with weekly infusion of 5-FU/LV in advanced solid tumors, Journal of Clinical Oncology, 28, 15

D16 Modified FOLFIRINOX regimen with improved safety and maintained efficacy in pancreatic adenocarcinoma, Pancreas. 2013 Nov;42(8):1311-5

D17 Development of a Highly Active Nanoliposomal Irinotecan Using a Novel Intraliposomal Stabilization Strategy, Cancer Res 2006; 66: (6). March 15, 2006

ORIGINAL ARTICLE

FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer

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ABSTRACT

BACKGROUND

Data are lacking on the efficacy and safety of a combination chemotherapy regimen consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) as compared with gemcitabine as first-line therapy in patients with metastatic pancreatic cancer.

METHODS

We randomly assigned 342 patients with an Eastern Cooperative Oncology Group performance status score of 0 or 1 (on a scale of 0 to 5, with higher scores indicating a greater severity of illness) to receive FOLFIRINOX (oxaliplatin, 85 mg per square meter of body-surface area; irinotecan, 180 mg per square meter; leucovorin, 400 mg per square meter; and fluorouracil, 400 mg per square meter given as a bolus followed by 2400 mg per square meter given as a 46-hour continuous infusion, every 2 weeks) or gemcitabine at a dose of 1000 mg per square meter weekly for 7 of 8 weeks and then weekly for 3 of 4 weeks. Six months of chemotherapy were recommended in both groups in patients who had a response. The primary end point was overall survival.

RESULTS

The median overall survival was 11.1 months in the FOLFIRINOX group as compared with 6.8 months in the gemcitabine group (hazard ratio for death, 0.57; 95% confidence interval [CI], 0.45 to 0.73; P<0.001). Median progression-free survival was 6.4 months in the FOLFIRINOX group and 3.3 months in the gemcitabine group (hazard ratio for disease progression, 0.47; 95% CI, 0.37 to 0.59; P<0.001). The objective response rate was 31.6% in the FOLFIRINOX group versus 9.4% in the gemcitabine group (P<0.001). More adverse events were noted in the FOLFIRINOX group; 5.4% of patients in this group had febrile neutropenia. At 6 months, 31% of the patients in the FOLFIRINOX group had a definitive degradation of the quality of life versus 66% in the gemcitabine group (hazard ratio, 0.47; 95% CI, 0.30 to 0.70; P<0.001).

CONCLUSIONS

As compared with gemcitabine, FOLFIRINOX was associated with a survival advantage and had increased toxicity. FOLFIRINOX is an option for the treatment of patients with metastatic pancreatic cancer and good performance status. (Funded by the French government and others; ClinicalTrials.gov number, NCT00112658.)

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N Engl J Med 2011;364:1817-25.

Pancreatic adenocarcinoma was the fourth leading cause of death from cancer in the United States in 2010,[1] and it carries a grim prognosis: the 5-year survival rate is 6% in Europe and the United States.[1,2] Gemcitabine became the reference regimen for advanced pancreatic cancer after a randomized trial showed significant improvement in the median overall survival as compared with fluorouracil administered as an intravenous bolus (5.6 vs. 4.4 months, P=0.002).[3] In the subsequent phase 3 trials of single-agent gemcitabine,[4] the median overall survival ranged from 5.0 to 7.2 months. The combination of gemcitabine with a variety of cytotoxic and targeted agents has generally shown no significant survival advantage as compared with gemcitabine alone.[4] Some studies have suggested a significant benefit associated with gemcitabine-based cytotoxic combinations in patients with good performance status.[5-7]

Irinotecan has some clinical activity against advanced pancreatic cancer.[8,9] Preclinical studies have indicated that irinotecan has synergistic activity when it is administered before fluorouracil and leucovorin.[10-13] Oxaliplatin has clinical activity against pancreatic cancer only when combined with fluorouracil.[14] Oxaliplatin and irinotecan show synergistic activity in vitro.[15] Given the relative absence of overlapping toxic effects among fluorouracil, leucovorin, irinotecan, and oxaliplatin, a regimen combining these agents was studied in a phase 1 trial and showed responses in patients with advanced pancreatic cancer.[16] Accordingly, we conducted a phase 2 study of the FOLFIRINOX regimen (oxaliplatin, irinotecan, fluorouracil, and leucovorin) involving 46 patients with good performance status and advanced pancreatic cancer; this regimen was associated with encouraging efficacy and grade 3 or 4 neutropenia in half the patients.[17] These results prompted the initiation of a phase 2-3 trial to further explore FOLFIRINOX as compared with single-agent gemcitabine as first-line treatment in patients with metastatic pancreatic cancer.

METHODS

PATIENTS

Patients were eligible to be included in the study if they were 18 years of age or older and had histologically and cytologically confirmed, measurable metastatic pancreatic adenocarcinoma that had not previously been treated with chemotherapy. Other inclusion criteria were an Eastern Cooperative Oncology Group (ECOG) performance status score of 0 or 1 (with 0 indicating that the patient is fully active and able to carry on all predisease activities without restriction and 1 that the patient is restricted in physically strenuous activity but is ambulatory and able to carry out work of a light or sedentary nature [e.g., light housework or office work])[18] and adequate bone marrow (granulocyte count, ≥1500 per cubic millimeter; and platelet count, ≥100,000 per cubic millimeter), liver function (bilirubin ≤1.5 times the upper limit of the normal range), and renal function.

Exclusion criteria were an age of 76 years or older, endocrine or acinar pancreatic carcinoma, previous radiotherapy for measurable lesions, cerebral metastases, a history of another major cancer, active infection, chronic diarrhea, a clinically significant history of cardiac disease, and pregnancy or breast-feeding.

STUDY DESIGN AND OVERSIGHT

This multicenter, randomized, phase 2–3 trial was conducted at 15 centers during phase 2 and expanded to 48 centers during phase 3. Patients were randomly assigned to receive FOLFIRINOX or gemcitabine within 1 week after enrollment. Randomization was performed centrally in a 1:1 ratio with stratification according to center, performance status (0 vs. 1), and primary tumor localization (the head vs. the body or tail of the pancreas).

The study was approved by the Lorraine ethics committee. All patients provided written informed consent. An independent data and safety monitoring committee supervised the collation of efficacy and safety data. The trial was conducted according the Declaration of Helsinki, the Good Clinical Practice guidelines of the International Conference on Harmonization, and relevant French and European laws and directives. The study was designed and the first draft of the manuscript was prepared by the first author, with writing assistance from an employee of the sponsor, Unicancer, and in cooperation with the other authors. Data were collected at the headquarters of the French anticancer centers (Unicancer, the study sponsor) and analyzed by the statistician, who vouches for the accuracy of the data. Oxaliplatin and irinotecan were donated by Sanofi-Aventis and Pfizer, respectively; these drug manufacturers had no role in the design of the study, in the accrual or analysis of the data, or in the preparation of the manuscript.

The protocol, including the statistical analysis plan, is available with the full text of this article at NEJM.org. The first author vouches for the fidelity of the study to the protocol.

TREATMENT

Gemcitabine, at a dose of 1000 mg per square meter of body-surface area, was delivered by 30-minute intravenous infusion weekly for 7 weeks, followed by a 1-week rest, then weekly for 3 weeks in subsequent 4-week courses. FOLFIRINOX consisted of oxaliplatin at a dose of 85 mg per square meter, given as a 2-hour intravenous infusion, immediately followed by leucovorin at a dose of 400 mg per square meter, given as a 2-hour intravenous infusion, with the addition, after 30 minutes, of irinotecan at a dose of 180 mg per square meter, given as a 90-minute intravenous infusion through a Y-connector. This treatment was immediately followed by fluorouracil at a dose of 400 mg per square meter, administered by intravenous bolus, followed by a continuous intravenous infusion of 2400 mg per square meter over a 46-hour period every 2 weeks. In the gemcitabine group, a cycle was also defined as a 2-week interval. Six months of chemotherapy was recommended for patients who had a response. Patients were followed every 3 months until death.

In the event of predefined toxic events, protocol-specified treatment modifications were permitted (see the Supplementary Appendix, available at NEJM.org). Doses of gemcitabine were reduced by 25% if the granulocyte count decreased to 500 to 999 per cubic millimeter or if the platelet count was 50,000 to 100,000 per cubic millimeter. In case of grade 2, 3, or 4 neutropenia or thrombocytopenia, FOLFIRINOX administration was delayed until recovery and doses were reduced. Filgrastim was not recommended as primary prophylaxis, but it could be considered for high-risk patients.

ASSESSMENTS

At the start of every cycle, the patient's status was assessed according to his or her medical history, complete physical examination by a physician, ECOG performance status, and complete blood counts and blood chemical tests. Baseline evaluations also included measurement of the serum carbohydrate antigen 19-9 level, a computed tomographic (CT) evaluation, and assessment of the patient's quality of life with the use of the European Organization for Research and Treatment of Cancer (EORTC) quality-of-life core questionnaire (QLQ-C30, version 3.0).[19]

EORTC QLQ-C30 questionnaires were to be completed every 2 weeks. Safety assessments were performed before each cycle with the use of the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0).[20] Tumors were measured every 2 months.

Patients discontinued the study in the event of unacceptable toxic effects or evidence of progressive disease, or at their request. Tumor response was determined according to the Response Evaluation Criteria in Solid Tumors (see the Supplementary Appendix).[21] Independent review of CT scans was performed at the end of phase 2 of the study. Overall survival and progression-free survival were calculated from the date of randomization until the date of death and the date of documentation of disease progression or death in patients without disease progression, respectively.

STATISTICAL ANALYSIS

The primary efficacy end point for the phase 2 analysis was tumor response, and the secondary end point was safety. The trial was planned to continue as a phase 3 study if more than 11 responses were observed in the first 40 patients who were randomly assigned to the FOLFIRINOX group. Patients from the phase 2 analysis were included in the phase 3 analysis. The primary end point for the phase 3 analysis was overall survival. Secondary end points were progression-free survival, tumor response, safety, and quality of life. The statistical considerations are detailed in the Sample Size Determination section in the Supplementary Appendix.

All analyses were performed on an intention-to-treat basis. Qualitative variables were compared with the use of the chi-square test or Fisher's test, quantitative variables with the use of Student's t-test or a nonparametric (Wilcoxon) test, and survival data with the use of a stratified log-rank test. All these comparisons were adjusted for stratification factors. All tests were two-sided, with a P value of less than 0.05 considered to indicate statistical significance. Data are presented with 95% confidence intervals, calculated with the use of standard methods based on a binomial distribution. All analyses were performed with the use of Stata software, version 10.

Overall survival and progression-free survival were estimated with the use of the Kaplan–Meier method.[22] A Cox proportional-hazards model was

Table 1. Demographic and Baseline Characteristics of Patients in the Intention-to-Treat Population.*

Characteristic	FOLFIRINOX (N=171)	Gemcitabine (N=171)
Age — yr		
Median	61	61
Range	25–76	34–75
Sex — no. (%)		
Male	106 (62.0)	105 (61.4)
Female	65 (38.0)	66 (38.6)
ECOG performance status score — no. (%)		
0	64 (37.4)	66 (38.6)
1	106 (61.9)	105 (61.4)
2	1 (0.6)	0
Pancreatic tumor location — no. (%)		
Head	67 (39.2)	63 (36.8)
Body	53 (31.0)	58 (33.9)
Tail	45 (26.3)	45 (26.3)
Multicentric	6 (3.5)	5 (2.9)
Biliary stent — no. (%)		
Yes	27 (15.8)	22 (12.9)
No	144 (84.2)	149 (87.1)
No. of metastatic sites involved		
Median	2	2
Range	1–6	1–6
Level of carbohydrate antigen 19-9 — no./total no. (%)		
Normal	24/164 (14.6)	23/165 (13.9)
Elevated, <59×ULN	72/164 (43.9)	65/165 (39.4)
Elevated, ≥59×ULN	68/164 (41.5)	77/165 (46.7)
Unknown	7/171 (4.1)	6/171 (3.5)
No. of measurable metastatic sites — no. of patients/total no. (%)		
Liver	149/170 (87.6)	150/171 (87.7)
Pancreas	90/170 (52.9)	91/171 (53.2)
Lymph node	49/170 (28.8)	39/171 (22.8)
Lung	33/170 (19.4)	49/171 (28.7)
Peritoneal	33/170 (19.4)	32/171 (18.7)
Other	18/170 (10.6)	29/171 (17.0)

* ECOG denotes Eastern Cooperative Oncology Group; FOLFIRINOX oxaliplatin, irinotecan, fluorouracil, and leucovorin; and ULN upper limit of the normal range.

used to estimate the hazard ratios. Hazard ratios indicating the effects of prognostic factors on the risk of death were calculated and are shown in a forest plot.[23] The interaction test was used to assess the heterogeneity of treatment effects for subgroup analyses.[24]

Analysis of the QLQ-C30 questionnaires was performed in accordance with the EORTC guidelines.[25] The preplanned analysis centered on the scales that are usually most affected in patients with pancreatic cancer: the Global Health Status and Quality of Life scale and scales for fatigue, pain, physical functioning, emotional functioning, and role functioning.[26] The other QLQ-C30 domains were only examined in an exploratory manner. Time to definitive deterioration in quality of life, with the use of a 10-point minimal clinically important difference,[27,28] was analyzed with the use of the Kaplan–Meier method and the log-rank test.

RESULTS

CHARACTERISTICS OF THE PATIENTS

Between December 2005 and October 2009, a total of 342 patients from 48 French centers were enrolled in the study. The database was closed for final analysis on April 16, 2010. The intention-to-treat population included 171 patients in each group, and the safety population (all patients who received treatment) included 167 patients in the FOLFIRINOX group and 169 patients in the gemcitabine group (Fig. I in the Supplementary Appendix). There were similar numbers of patients with minor violations of eligibility criteria in the FOLFIRINOX and gemcitabine groups (8 and 7, respectively).

Demographic and baseline disease characteristics of the patients were similar in the two treatment groups (Table 1), but there were fewer measurable target lung metastases in the FOLFIRINOX group than in the gemcitabine group (19.5% vs. 28.7%, P=0.05).

The median number of treatment cycles administered was 10 (range, 1 to 47) in the FOLFIRINOX group and 6 (range, 1 to 26) in the gemcitabine group (P<0.001). More patients in the gemcitabine group had disease progression before 12 cycles (6 months) (79.9%, vs. 54.6% in the FOLFIRINOX group; P<0.001). The median relative dose intensities of fluorouracil, irinotecan, oxaliplatin, and gemcitabine were 82%, 81%, 78%, and 100%, respectively.

EFFICACY

Response to Therapy

A total of 88 patients were recruited between January 2005 and November 2006 during phase 2 of this study. The confirmed response rate, according to the investigators, was 31.8% (14 of 44 pa-

tients) in the FOLFIRINOX group and 11.3% (5 of 44 patients) in the gemcitabine group. Independent review confirmed an objective response rate of 34.1% (in 15 patients) in the FOLFIRINOX group. Since the primary objective of phase 2 was met, the trial proceeded to phase 3. All patients in phase 2 continued treatment, and data on these patients are fully reported in the phase 3 efficacy and safety results.

The response to therapy in the phase 3 trial is summarized in Table 2. The objective response rate was 31.6% (95% confidence interval [CI], 24.7 to 39.1) in the FOLFIRINOX group and 9.4% (95% CI, 5.4 to 14.7) in the gemcitabine group (P<0.001). In both groups, after 12 cycles, chemotherapy could be discontinued in patients with a response or stable disease; in 7.6% of the patients in the FOLFIRINOX group and 7.0% of those in the gemcitabine group, the same regimen was reintroduced with the use of a stop-and-go strategy.

Table 2. Objective Responses in the Intention-to-Treat Population.*

Variable	FOLFIRINOX (N=171)	Gemcitabine (N=171)	P Value
Response — no. (%)			
Complete response	1 (0.6)	0	
Partial response	53 (31.0)	16 (9.4)	
Stable disease	66 (38.6)	71 (41.5)	
Progressive disease	26 (15.2)	59 (34.5)	
Could not be evaluated	25 (14.6)	25 (14.6)	
Rate of objective response†			<0.001
No. (%)	54 (31.6)	16 (9.4)	
95% CI	24.7–39.1	5.4–14.7	
Rate of disease control‡			<0.001
No. (%)	120 (70.2)	87 (50.9)	
95% CI	62.7–76.9	43.1–58.6	
Response duration — mo			0.57
Median	5.9	3.9	
95% CI	4.9–7.1	3.1–7.1	

* CI denotes confidence interval, and FOLFIRINOX oxaliplatin, irinotecan, fluorouracil, and leucovorin.
† The rate of objective response was defined as the percentage of patients who had a complete response or partial response.
‡ The rate of disease control was defined as the percentage of patients who had a complete response, partial response, or stable disease.

Survival

The median duration of follow-up was 26.6 months (95% CI, 20.5 to 44.9). The overall survival analysis was based on 273 deaths among the 342 patients (79.8%). The median overall survival was 11.1 months (95% CI, 9.0 to 13.1) in the FOLFIRINOX group as compared with 6.8 months (95% CI, 5.5 to 7.6) in the gemcitabine group (hazard ratio for death, 0.57; 95% CI, 0.45 to 0.73; P<0.001) (Fig. 1A). Overall survival rates at 6, 12, and 18 months were 75.9%, 48.4%, and 18.6%, respectively, in the FOLFIRINOX group as compared with 57.6%, 20.6%, and 6.0%, respectively, in the gemcitabine group.

Synchronous metastases, a low baseline albumin level (<3.5 g per deciliter), hepatic metastases, and an age of more than 65 years were identified as independent adverse prognostic factors for overall survival (see the Supplementary Appendix). The hazard ratio for death with FOLFIRINOX treatment, adjusted for these variables, was significant (adjusted hazard ratio, 0.54; 95% CI, 0.41 to 0.73; P<0.001). Results were similar when adjusted according to the presence or absence of pulmonary metastases. The effect of FOLFIRINOX was homogeneous in all subgroups (Fig. 2).

The analysis of progression-free survival was based on 317 events among 342 patients (92.7%). The median progression-free survival was 6.4 months (95% CI, 5.5 to 7.2) in the FOLFIRINOX group as compared with 3.3 months (95% CI, 2.2 to 3.6) in the gemcitabine group (hazard ratio for disease progression, 0.47; 95% CI, 0.37 to 0.59; P<0.001) (Fig. 1B). Progression-free survival rates at 6, 12, and 18 months were 52.8%, 12.1%, and 3.3%, respectively, in the FOLFIRINOX group as compared with 17.2%, 3.5%, and 0%, respectively, in the gemcitabine group.

SECOND-LINE THERAPY

Second-line therapy was administered in 80 patients in the FOLFIRINOX group and in 85 patients in the gemcitabine group. No difference in median survival was noted between the groups (4.4 months in each group) from the introduction of second-line therapy. The most common second-line regimens were as follows: in the FOLFIRINOX group, gemcitabine (in 82.5% of the patients) or a gemcitabine-based combination (in 12.5%), and in the gemcitabine group, a combination of fluorouracil, leucovorin, and oxaliplatin (FOLFOX) (in 49.4%); gemcitabine plus oxaliplatin (in 17.6%); a regimen of fluorouracil and leucovorin plus cisplatin every 2 weeks (in 16.5%); and FOLFIRINOX (in 4.7%).

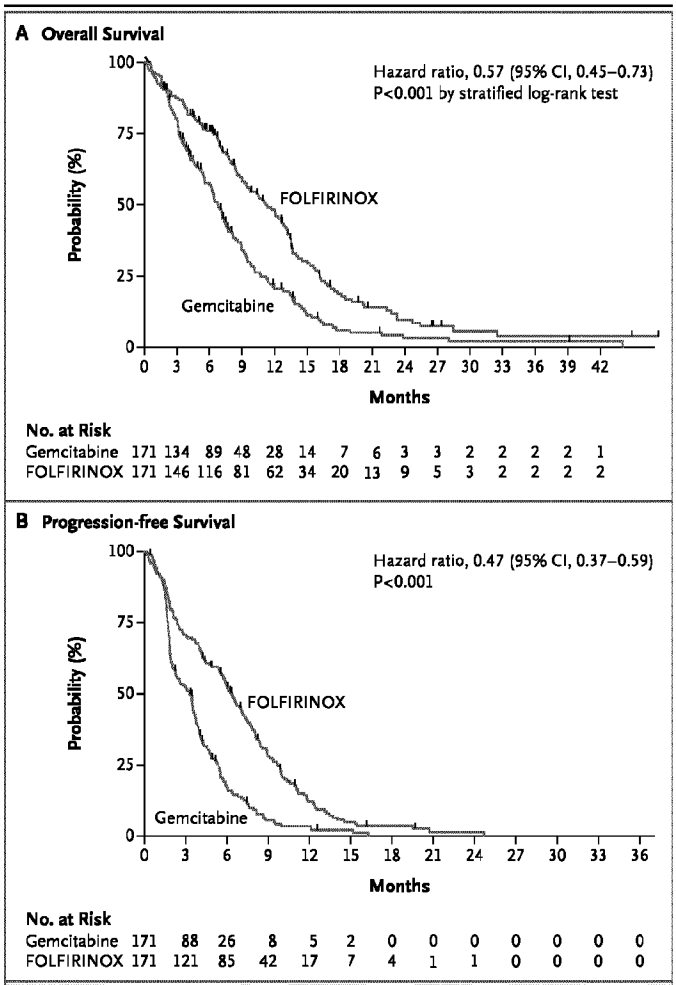

Figure 1. Kaplan–Meier Estimates of Overall Survival and Progression-free Survival, According to Treatment Group.

Panel A shows overall survival; the median was 11.1 months in the group receiving FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin). Panel B shows progression-free survival; the median was 6.4 months in the FOLFIRINOX group and 3.3 months in the gemcitabine group.

ADVERSE EVENTS

Two patients died from treatment-related cause: one from febrile neutropenia in the FOLFIRINOX group and one from cardiac decompensation in the gemcitabine group. Treatment-related grade 3 or 4 adverse events occurring in more than 5% of patients in either treatment group are summarized in Table 3. Incidences of grade 3 or 4 neutropenia, febrile neutropenia, thrombocytopenia, diarrhea, and sensory neuropathy were significantly higher in the FOLFIRINOX group, whereas the incidence of grade 3 or 4 elevated alanine aminotransferase levels was significantly higher in the gemcitabine group. Grade 2 alopecia occurred in 11.4% of patients in the FOLFIRINOX group and in 1.2% of patients in the gemcitabine group (P<0.001). No cholangitis was observed. In both groups, the hematologic toxicity and the risk of infection were similar with or without placement of a biliary stent. Filgrastim was administered in 42.5% of patients who received FOLFIRINOX and in 5.3% of patients who received gemcitabine (P<0.001).

QUALITY OF LIFE

The proportion of patients with QLQ-C30 questionnaires that could be evaluated at baseline was 95.3% in the FOLFIRINOX group and 95.9% in the gemcitabine group. No significant differences between the groups were noted at baseline in the QLQ-C30 scales or single items. Subsequently, the rate of compliance with completion of the QLQ-C30 questionnaire was high: 78.2% in the FOLFIRINOX group and 77.4% in the gemcitabine group. No significant differences were noted between the groups in the Global Health Status and Quality of Life scale or in the individual domains, except that the FOLFIRINOX group had higher scores for diarrhea during the first eight cycles.

At 6 months, 31% of the patients in the FOLFIRINOX group had a definitive decrease in the scores on the Global Health Status and Quality of Life scale versus 66% in the gemcitabine group (hazard ratio, 0.47; 95% CI, 0.30 to 0.70; P<0.001) (Fig. II in the Supplementary Appendix). Significant increases in the time until definitive deterioration in the quality of life were also noted in the FOLFIRINOX group for all functional and symptom scales and with respect to appetite loss, dyspnea, and constipation. Time to a definitive decrease in the scores that were associated with diarrhea, insomnia, or financial difficulties caused by a physical condition or medical treatment did not differ significantly between regimens.

DISCUSSION

In this study, FOLFIRINOX was an effective first-line treatment option for patients with metastatic pancreatic adenocarcinoma and good ECOG performance status. The median overall survival was significantly prolonged, with an increase of 4.3 months in the FOLFIRINOX group as compared with the gemcitabine group (11.1 vs. 6.8 months).

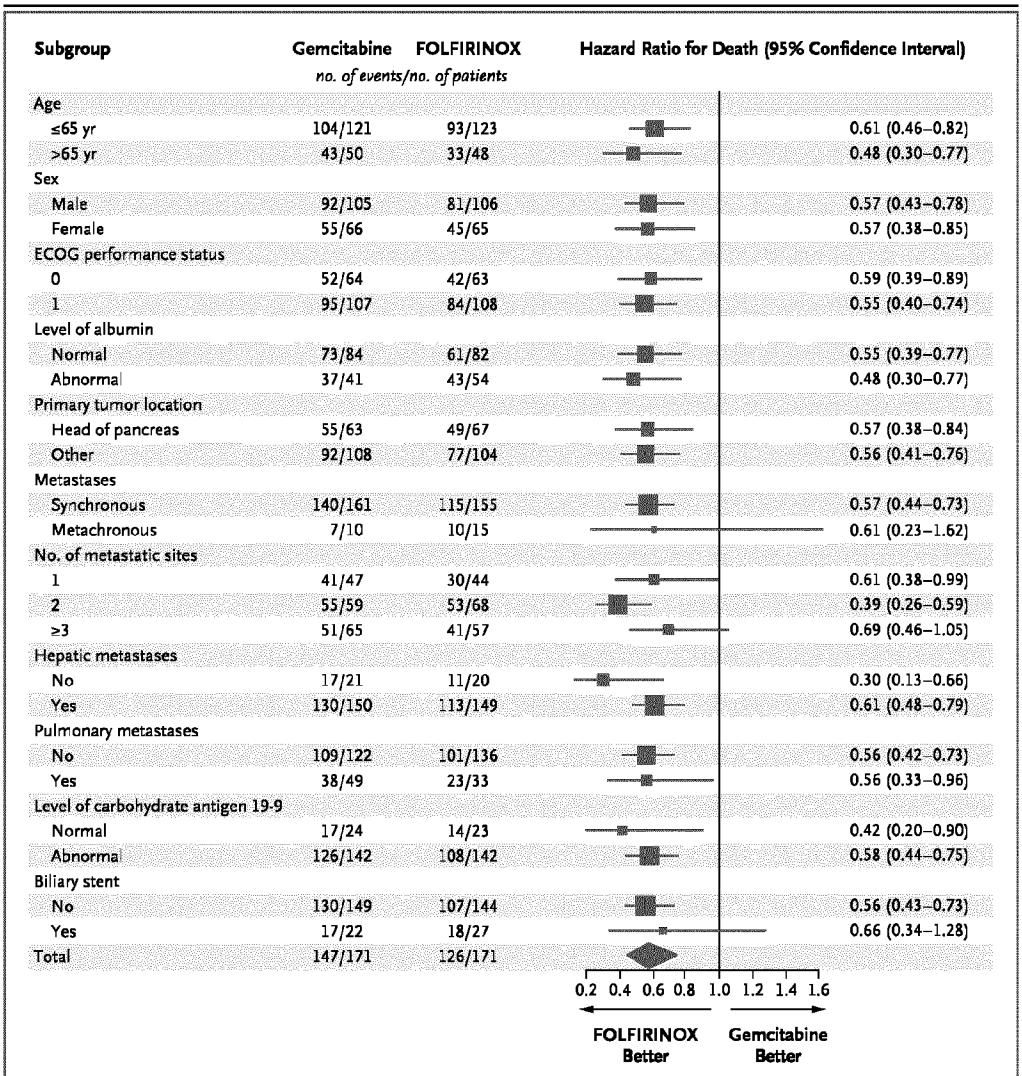

Figure 2. Forest Plot of the Treatment Effect on Overall Survival in Subgroup Analyses.

The Eastern Cooperative Oncology Group (ECOG) grades the status of patients with respect to activities of daily living, with 0 indicating that the patient is fully active and able to carry on all predisease activities without restriction and 1 that the patient is restricted in physically strenuous activity but is ambulatory and able to carry out work of a light or sedentary nature (e.g., light housework or office work). The sizes of the squares are proportional to the sizes of the subgroups. Horizontal lines represent 95% confidence intervals. The position of each square represents the point estimate of the treatment effect.

Single-agent gemcitabine is the current standard of care,[4,29] but the addition of cytotoxic and targeted agents to gemcitabine has almost invariably provided no significant survival improvement,[4] despite an improvement in response rates in some trials.[30-34] Conversely, one phase 3 trial involving 569 patients with locally advanced or metastatic cancer showed a significant prolongation of overall survival with the combination of erlotinib and gemcitabine as compared with gemcitabine alone (hazard ratio for death, 0.82; 95% CI, 0.69 to 0.99; P=0.04). However, the magnitude of the improvement in median overall survival was modest, at 0.33 months (6.24 vs. 5.91 months).[35]

Recently, a phase 3 trial involving 543 patients with advanced pancreatic cancer showed that the combination of capecitabine and gemcitabine as

Table 3. Most Common Grade 3 or 4 Adverse Events Occurring in More Than 5% of Patients in the Safety Population.*

Event	FOLFIRINOX (N=171)	Gemcitabine (N=171)	P Value
	no. of patients/total no. (%)		
Hematologic			
Neutropenia	75/164 (45.7)	35/167 (21.0)	<0.001
Febrile neutropenia	9/166 (5.4)	2/169 (1.2)	0.03
Thrombocytopenia	15/165 (9.1)	6/168 (3.6)	0.04
Anemia	13/166 (7.8)	10/168 (6.0)	NS
Nonhematologic			
Fatigue	39/165 (23.6)	30/169 (17.8)	NS
Vomiting	24/166 (14.5)	14/169 (8.3)	NS
Diarrhea	21/165 (12.7)	3/169 (1.8)	<0.001
Sensory neuropathy	15/166 (9.0)	0/169	<0.001
Elevated level of alanine aminotransferase	12/165 (7.3)	35/168 (20.8)	<0.001
Thromboembolism	11/166 (6.6)	7/169 (4.1)	NS

* Events listed are those that occurred in more than 5% of patients in either group. NS denotes not significant.

compared with gemcitabine alone resulted in an increased response rate (19.1% vs. 12.4%, P=0.03) and improved progression-free survival (hazard ratio for disease progression, 0.78; 95% CI, 0.66 to 0.93; P=0.04), as well as a trend toward improvement in overall survival (hazard ratio for death, 0.86; 95% CI, 0.72 to 1.02; P=0.08).[31] The median survival among patients who received capecitabine plus gemcitabine was 7.1 months, versus 6.2 months among patients who received gemcitabine alone. The authors performed a meta-analysis of their study and two similar but smaller studies. These results showed a significant survival benefit with gemcitabine plus capecitabine as compared with gemcitabine alone (hazard ratio, 0.86; 95% CI, 0.75 to 0.98; P=0.02). The efficacy results obtained with gemcitabine in our study are in line with the results of these studies, as well as the findings in other trials of single-agent gemcitabine in patients with advanced pancreatic cancer.[4,29]

The patient-selection criteria in our study were more rigorous than those in previous studies. Patients had to have metastatic disease and a good performance status (ECOG status score of 0 or 1). Only 38% of our patients had carcinoma of the pancreatic head — a lower rate than in previous trials (52 to 70%).[6,31,32] This difference may be related to the exclusion of patients with a high bilirubin level, because of the increased risk of irinotecan-induced toxicity.[8] As a result of this exclusion criterion, the proportion of enrolled patients with biliary stents was low (14.3%). Cholangitis is a common complication of biliary stenting, and although it did not occur in any of the patients in our study, careful monitoring of the bilirubin level is required when irinotecan is administered in patients with biliary drainage.

The safety profile of FOLFIRINOX was less favorable than that of gemcitabine. FOLFIRINOX was associated with a higher incidence of grade 3 or 4 neutropenia, febrile neutropenia, thrombocytopenia, diarrhea, and sensory neuropathy, as well as grade 2 alopecia. Despite the higher incidence of adverse events associated with the FOLFIRINOX regimen, a significant increase in the time to definitive deterioration of the quality of life was observed in the FOLFIRINOX group as compared with the gemcitabine group.

In conclusion, our findings suggest that FOLFIRINOX is a first-line option for patients with metastatic pancreatic cancer who are younger than 76 years and who have a good performance status (ECOG 0 or 1), no cardiac ischemia, and normal or nearly normal bilirubin levels.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60:277-300.
2. Sant M, Allemani C, Santaquilani M, Knijn A, Marchesi F, Capocaccia R. EUROCARE-4: survival of cancer patients diagnosed in 1995-1999: results and commentary. Eur J Cancer 2009;45:931-91.
3. Burris HA III, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997;15:2403-13.
4. Di Marco M, Di Cicilia R, Macchini M, et al. Metastatic pancreatic cancer: is gemcitabine still the best standard treatment? Oncol Rep 2010;23:1183-92.
5. Herrmann R, Bodoky G, Ruhstaller T, et al. Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group. J Clin Oncol 2007;25:2212-7.
6. Heinemann V, Quietzsch D, Gieseler F, et al. Randomized phase III trial of gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer. J Clin Oncol 2006;24:3946-52.
7. Heinemann V, Boeck S, Hinke A, Labianca R, Louvet C. Meta-analysis of randomized trials: evaluation of benefit from gemcitabine-based combination chemotherapy applied in advanced pancreatic cancer. BMC Cancer 2008;8:82.
8. Ueno H, Okusaka T, Funakoshi A, et al. A phase II study of weekly irinotecan as first-line therapy for patients with metastatic pancreatic cancer. Cancer Chemother Pharmacol 2007;59:447-54.
9. Wagener DJT, Verdonk HER, Dirix LY, et al. Phase II trial of CPT-11 in patients with advanced pancreatic cancer: an EORTC early clinical trials group study. Ann Oncol 1995;6:129-32.
10. Azrak RG, Cao S, Slocum HK, et al. Therapeutic synergy between irinotecan and 5-fluorouracil against human tumor xenografts. Clin Cancer Res 2004;10: 1121-9.
11. Mans DR, Grivicich I, Peters GJ, Schwartsmann G. Sequence-dependent growth inhibition and DNA damage formation by the irinotecan-5-fluorouracil combination in human colon carcinoma cell lines. Eur J Cancer 1999;35:1851-61.
12. Mullany S, Svingen PA, Kaufmann SH, Erlichman C. Effect of adding the topoisomerase I poison 7-ethyl-10-hydroxycamptothecin (SN-38) to 5-fluorouracil and folinic acid in HCT-8 cells: elevated dTTP pools and enhanced cytotoxicity. Cancer Chemother Pharmacol 1998;42:391-9.
13. Pavillard V, Formento P, Rostagno P, et al. Combination of irinotecan (CPT11) and 5-fluorouracil with an analysis of cellular determinants of drug activity. Biochem Pharmacol 1998;56:1315-22.
14. Ducreux M, Mitry E, Ould-Kaci M, et al. Randomized phase II study evaluating oxaliplatin alone, oxaliplatin combined with infusional 5-FU, and infusional 5-FU alone in advanced pancreatic carcinoma patients. Ann Oncol 2004;15:467-73.
15. Zeghari-Squalli N, Raymond E, Cvitkovic E, Goldwasser F. Cellular pharmacology of the combination of the DNA topoisomerase I inhibitor SN-38 and the diaminocyclohexane platinum derivative oxaliplatin. Clin Cancer Res 1999;5:1189-96.
16. Ychou M, Conroy T, Seitz JF, et al. An open label phase I study assessing the feasibility of the triple combination: oxaliplatin plus irinotecan plus leucovorin/5-fluorouracil every 2 weeks in patients with advanced solid tumors. Ann Oncol 2003;14: 481-9.
17. Conroy T, Paillot B, François E, et al. Irinotecan plus oxaliplatin and leucovorin-modulated fluorouracil in advanced pancreatic cancer — a Groupe Tumeurs Digestives of the Fédération Nationale des Centres de Lutte Contre le Cancer study. J Clin Oncol 2005;23:1228-36.
18. Oken MM, Creech RH, Tormey DC, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol 1982;5:649-55.
19. Aaronson NK, Ahmedzai S, Bergman B, et al. The European Organization for Research and Treatment of Cancer QLQ-C30: a quality-of-life instrument for use in international clinical trials in oncology. J Natl Cancer Inst 1993;85:365-76.
20. Common Terminology Criteria for Adverse Events v3.0 (CTCAE). Bethesda, MD: Cancer Therapy Evaluation Program, 2006. (http://ctep.cancer.gov/protocol Development/electronic_applications/ docs/ctcaev3.pdf.)
21. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors: European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205-16.
22. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457-81.
23. Introduction and methods. In: Early Breast Cancer Trialists' Collaborative Group. Treatment of early breast cancer. Vol. 1. Worldwide evidence 1985–1990. Oxford, England: Oxford University Press, 1990.
24. Gail M, Simon R. Testing for qualitative interactions between treatment effects and patient subsets. Biometrics 1985;41: 361-72.
25. Fayers P, Aaronson N, Bjordal K, Groenvold M, Curran D, Bottomley A. EORTC QLQ-C30 scoring manual. Brussels: European Organisation for Research and Treatment of Cancer, 2001.
26. Fayers P, Weeden S, Curran D. EORTC QLQ-C30 reference values. Brussels: European Organisation for Research and Treatment of Cancer, 1998.
27. Osoba D, Rodrigues G, Myles J, Zee B, Pater J. Interpreting the significance of changes in health-related quality-of-life scores. J Clin Oncol 1998;16:139-44.
28. Bonnetain F, Dahan L, Maillard E, et al. Time until definitive quality of life score deterioration as a means of longitudinal analysis for treatment trials in patients with metastatic pancreatic adenocarcinoma. Eur J Cancer 2010;46:2753-62.
29. Hidalgo M. Pancreatic cancer. N Engl J Med 2010;362:1605-17. [Erratum, N Engl J Med 2010;363:298.]
30. Colucci G, Giuliani F, Gebbia V, et al. Gemcitabine alone or with cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic carcinoma: a prospective, randomized phase III study of the Gruppo Oncologia dell'Italia Meridionale. Cancer 2002;94:902-10.
31. Cunningham D, Chau I, Stocken DD, et al. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J Clin Oncol 2009;27: 5513-8.
32. Louvet C, Labianca R, Hammel P, et al. Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. J Clin Oncol 2005;23:3509-16.
33. Oettle H, Richards D, Ramanathan RK, et al. A phase III trial of pemetrexed plus gemcitabine versus gemcitabine in patients with unrespectable or metastatic pancreatic cancer. Ann Oncol 2005;16: 1639-45. [Erratum, Ann Oncol 2006;17: 535.]
34. Rocha Lima CM, Green MR, Rotche R, et al. Irinotecan plus gemcitabine results in no survival advantage compared with gemcitabine monotherapy in patients with locally advanced or metastatic pancreatic cancer despite increased tumor response rate. J Clin Oncol 2004;22: 3776-83.
35. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 2007;25:1960-6.

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Impact of FOLFIRINOX Compared With Gemcitabine on Quality of Life in Patients With Metastatic Pancreatic Cancer: Results From the PRODIGE 4/ACCORD 11 Randomized Trial

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See accompanying editorial on page 3

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ABSTRACT

Purpose
To compare the quality of life (QoL) of patients receiving oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) or gemcitabine as first-line chemotherapy and to assess whether pretreatment QoL predicts survival in patients with metastatic pancreatic cancer.

Patients and Methods
Three hundred forty-two patients with performance status 0 or 1 were randomly assigned to receive FOLFIRINOX (oxaliplatin, 85 mg/m^2; irinotecan, 180 mg/m^2; leucovorin, 400 mg/m^2; and fluorouracil, 400 mg/m^2 bolus followed by 2,400 mg/m^2 46-hour continuous infusion, once every 2 weeks) or gemcitabine 1,000 mg/m^2 weekly for 7 of 8 weeks and then weekly for 3 of 4 weeks. QoL was assessed using European Organisation for the Research and Treatment of Cancer Quality of Life Questionnaire C30 every 2 weeks.

Results
Improvement in global health status (GHS; $P < .001$) was observed in the FOLFIRINOX arm and improvement in emotional functioning ($P < .001$) was observed in both arms, along with a decrease in pain, insomnia, anorexia, and constipation in both arms. A significant increase in diarrhea was observed in the FOLFIRINOX arm during the first 2 months of chemotherapy. Time until definitive deterioration ≥ 20 points was significantly longer for FOLFIRINOX compared with gemcitabine for GHS, physical, role, cognitive, and social functioning, and six symptom domains (fatigue, nausea/vomiting, pain, dyspnea, anorexia, and constipation). Physical functioning, constipation, and dyspnea were independent significant prognostic factors for survival with treatment arm, age older than 65 years, and low serum albumin.

Conclusion
FOLFIRINOX significantly reduces QoL impairment compared with gemcitabine in patients with metastatic pancreatic cancer. Furthermore, baseline QoL scores improved estimation of survival probability when added to baseline clinical and demographic variables.

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INTRODUCTION

We have previously reported the efficacy and safety of the oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) regimen versus gemcitabine as first-line therapy among patients with metastatic pancreatic adenocarcinoma (mPAC).[1] The results showed significantly longer overall survival (OS) but increased toxicity with FOLFIRINOX. Health-related quality of life (QoL) is paramount to patients with mPAC because most have high levels of symptoms when diagnosed and because life expectancy is usually short. Using a validated instrument, the European Organization for the Research and Treatment of Cancer QoL Questionnaire C30 (EORTC QLQ-C30) version 3.0,[2] we prospectively assessed longitudinal QoL as a secondary end point of the PRODIGE (Partenariat de Recherche en Oncologie Digestive) /ACCORD (Actions Concertées dans les Cancers Colo-Rectaux et Digestifs) phase III trial.

We have, in part, previously reported the results of the QoL analysis based on EORTC QLQ-C30 scales that are usually most affected in

patients with pancreatic cancer (global health status [GHS], fatigue, pain, physical functioning (PF), emotional functioning, and role functioning); other domains were examined in an exploratory manner.[1] Time until definitive deterioration (TUDD) of the QoL domains was analyzed for a 10-point minimal clinically important difference (MCID).

The aim of our article is to provide detailed analysis of QoL data for all domains. We extended the analysis to include TUDD using a more stringent and clinically relevant 20-point MCID cutoff.[3] Furthermore, we performed a multivariate analysis to determine the prognostic value of baseline QoL measures for survival.

PATIENTS AND METHODS

Trial Design

Adult patients with measurable mPAC, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) score of 0 or 1, and no prior chemotherapy were eligible for inclusion in this open-label, multicenter, randomized, phase II/III trial. Detailed inclusion and exclusion criteria, study design, and protocol have been published previously.[1]

Gemcitabine 1,000 mg/m^2 was infused weekly for 7 weeks, followed by a 1-week rest period, and then once per week for 3 weeks of a 4-week cycle. FOLFIRINOX consisted of oxaliplatin 85 mg/m^2 as a 2-hour intravenous infusion immediately followed by leucovorin 400 mg/m^2 administered as a 2-hour intravenous infusion and, after 30 minutes, with irinotecan 180 mg/m^2 as a 90-minute intravenous infusion; this was followed by a fluorouracil 400 mg/m^2 intravenous bolus and then continuous intravenous infusion of 2,400 mg/m^2 over 46 hours once every 2 weeks. Six months of chemotherapy was recommended for patients who had a response. Details of treatment have been published previously.[1]

The cutoff date for the current QoL analysis was April 16, 2010, the date used for the original efficacy and safety assessment.[1]

QoL Measurement

QoL was measured using EORTC QLQ-C30, version 3.0,[2] which is a validated, cancer-specific instrument designed for prospective clinical trials. The EORTC QLQ-C30 questionnaire evaluates five functions (physical, role, cognitive, emotional, and social), nine symptoms (fatigue, pain, nausea and vomiting, dyspnea, loss of appetite, insomnia, constipation, diarrhea, and financial difficulties) and the GHS/QoL. This questionnaire adequately covers the main problems and symptoms presented by patients with mPAC. Its psychometric properties and high acceptability rate in the French language have been confirmed.[4]

Questionnaires were to be completed at baseline before random assignment and every 2 weeks until progression. Questionnaires were completed in the clinic before interaction with health care personnel. Because of the multitude of QoL evaluations, we simplified the number of observations to calculate compliance to include baseline, day 15, day 30, and months 2, 4, 6, 8, and 10.

The description of QoL scores are therefore reported for these respective time points, whereas all QoL questionnaires were used for longitudinal analyses. Responses to the questionnaire were transformed into 0 to 100 scores, with a high score implying a high level of symptoms or a high level of functioning or global QoL.

Statistical Analysis

Analysis of findings from the EORTC QLQ-C30 questionnaires followed EORTC guidelines.[5] All analyses were performed in the intent-to-treat population. Comparison of the scores between treatment arms was performed with the Kruskal-Wallis test. Comparison between the baseline and the end of treatment was performed using the Sign rank test. Internal consistency of the questionnaire was estimated by using Cronbach's alpha for each dimension and visit. Values greater than .7 are generally regarded as acceptable, greater than .8 as good, and greater than .9 as excellent.[6] An additional longitudinal analysis was performed based on a survival analysis method.[7] This involves survival methods in which the definitive deterioration of QoL score is considered as an event.

Table 1. Baseline Demographic and Clinical Characteristics of Patients in the Intent-to-Treat Population

Characteristic	FOLFIRINOX (n = 171) No. of Patients	%	Gemcitabine (n = 171) No. of Patients	%
Age, years				
Median	61		61	
Range	25-76		34-75	
Sex				
Male	106	62	105	61
Female	65	38	66	39
ECOG performance status				
0	64	37	66	39
1	106	62	105	61
2	1	< 1	0	0
Pancreatic tumor location				
Head	67	39	63	37
Body	53	31	58	34
Tail	45	26	45	26
Multicentric	6	4	5	3
Metastatic sites involved				
Median	2		2	
Range	1-6		1-6	
Measurable metastatic sites*				
Liver	149	88	150	88
Pancreas	90	53	91	53
Lymph node	49	29	39	23
Lung	33	19	49	29
Peritoneal	33	19	32	19
Other	18	11	29	17

NOTE. Reproduced with permission from Conroy et al.[1]
Abbreviations: ECOG, Eastern Cooperative Oncology Group; FOLFIRINOX, oxaliplatin/irinotecan/fluorouracil/leucovorin.
*Denominator 170 for the FOLFIRINOX arm.

Osoba et al[3] showed that a mean change of 5 to 10 points in a QLQ-C30 score corresponds to a "little" change in QoL, a change of 10 to 20 points corresponds to a "moderate" change, and a change of more than 20 points corresponds to a "large" change. We used this definition when appropriate to indicate the clinical relevance of any differences observed. For a given baseline score, QLQ-C30 score was considered definitive deterioration if this score decreased by more than 10 points (or 20 points) as compared with the score at baseline, without later improvement superior to 10 points (or 20 points) as compared with baseline or without any available score. Thus, TUDD for QoL scores was defined as the time from randomization to the first observation of a definitive deterioration of QLQ-C30 score. Similarly, TUDD was reanalyzed by integrating death as an event. TUDD was analyzed with the use of the Kaplan-Meier method; the log-rank test was used to compare treatment arms.

Multivariate analysis was performed to determine baseline prognostic factors for OS, following the method previously published in an EORTC meta-analysis.[8] OS was calculated from the date of random assignment to the date of death, with living patients censored at the last date of follow-up before the cutoff for analysis. We investigated the prognostic value of QoL scores in complementing the prognostic model based on clinical data. Synchronous metastases, a low baseline albumin (< 3.5 g/dL), liver metastases, age older than 65 years, and treatment arm were previously identified as independent prognostic factors for OS.[1] Initially, each baseline QLQ-C30 dimension was tested in a univariate model to identify prognostic variables for OS ($P < .05$) and was added to a Cox model stratified by ECOG score (0 v 1) and primary tumor localization (head v other sites) containing clinical data. A proportional hazards assumption was verified using Schoenfeld residuals. The predictive power of the models identified was assessed according to Harrell's discrimination C-index.[9] The Harrell's C-index is a probability of concordance between

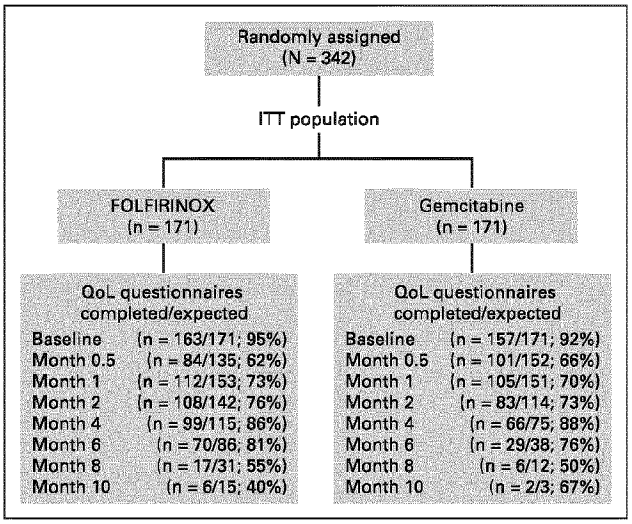

Fig 1. CONSORT diagram for European Organisation for the Research and Treatment of Cancer Quality of Life Questionnaires C30 completed for the total number of eligible patients at different time points in each treatment group. FOLFIRINOX, oxaliplatin/irinotecan/fluorouracil/leucovorin; ITT, intent to treat; QoL, quality of life.

predicted and observed survival, a value of 1 indicates perfect concordance of the score and outcome, whereas a value of .5 indicates only chance agreement.[10] All analyses were performed with STATA software, version 11.0 (STATA, College Station, TX).

RESULTS

Patient Characteristics

A total of 342 patients were enrolled between December 2005 and October 2009; the intent-to-treat population comprised 171 patients in each arm. The median follow-up was 26.6 months (95% CI, 20.5 to 44.9). Baseline demographic and disease characteristics were similar for both treatment arms (Table 1), except there were fewer measurable lung metastases in the FOLFIRINOX arm compared with the gemcitabine arm.

Baseline compliance with completion of QLQ-C30 questionnaires was high: 163 questionnaires (95%) and 157 questionnaires (92%) in the FOLFIRINOX and gemcitabine arms, respectively, and there was no difference between the patients who completed baseline QoL forms and those who did not (Figure 1). Despite the fact that ECOG PS was 0 or 1 at inclusion according to the physician, 30 patients (18.4%) in the FOLFIRINOX arm and 26 patients (16.6%) in the gemcitabine arm answered "quite a bit" (11.6%) or "very much" (6%) at the baseline item "Do you need to stay in bed or a chair during the day?". These patients had a worse prognosis than the overall population with a median OS of 6.7 months and 4.6 months in the FOLFIRINOX and the gemcitabine arms, respectively. In the FOLFIRINOX group, the survival difference between patients who answered they have no need to rest and those who needed to rest is significant, with median survival of 12.6 months and 6.7 months, respectively ($P = .03$). In the gemcitabine arm, the median survival in these groups is not significantly different, 6.7 versus 4.6 months.

Compliance for questionnaire completion over time in surviving patients varied between 40% to 95%. The rate of assessable questionnaires decreased after the 6-month treatment period, as expected. The rates of missing questionnaires were comparable between both treatments during follow-up.

There was no statistically significant difference between treatment arms with respect to baseline QLQ-C30 QoL domains or single items (Appendix Table A1 [online-only]). Functional scores were high and the most marked symptoms were anorexia, fatigue, pain, insomnia, and constipation.

Table 2. Univariate Cox Analysis According to 10-Point and 20-Point MCID to Calculation of TUDD for QLQ-C30 Domain Scores

	10-Point Deterioration					20-Point Deterioration				
	No. of Events					No. of Events				
Domain	FOLFIRINOX (n = 163)	Gemcitabine (n = 157)	HR	95% CI	P	FOLFIRINOX (n = 163)	Gemcitabine (n = 157)	HR	95% CI	P
Global health status	32	42	2.3	1.4 to 3.7	< .001	13	32	4.7	2.3 to 9.5	< .001
Physical functioning	47	59	1.9	1.3 to 2.8	.001	27	37	2.2	1.3 to 3.6	.001
Role functioning	44	59	2.2	1.5 to 3.4	< .001	27	43	2.7	1.6 to 4.4	< .001
Emotional functioning	18	26	2.9	1.6 to 5.6	< .001	14	14	2.1	1.0 to 4.5	.057
Cognitive functioning	30	49	3.0	1.9 to 4.8	< .001	11	16	2.6	1.2 to 5.6	.015
Social functioning	42	54	2.1	1.4 to 3.1	< .001	23	40	2.7	1.6 to 4.7	< .001
Fatigue	52	62	1.9	1.3 to 2.7	.001	36	49	2.4	1.5 to 3.8	< .001
Nausea/vomiting	40	53	2.1	1.4 to 3.2	< .001	19	30	2.8	1.5 to 5.0	< .001
Pain	27	36	2.7	1.6 to 4.6	< .001	12	22	3.7	1.7 to 7.7	< .001
Dyspnea	32	38	2.3	1.4 to 3.8	< .001	32	38	2.3	1.4 to 3.8	< .001
Insomnia	20	15	1.4	0.7 to 2.9	.300	20	15	1.4	0.7 to 2.9	.300
Loss of appetite	24	28	1.9	1.1 to 3.4	.022	24	28	1.9	1.1 to 3.4	.022
Constipation	18	21	2.0	1.0 to 3.8	.033	18	21	2.0	1.0 to 3.8	.033
Diarrhea	37	32	1.5	0.9 to 2.5	.086	37	32	1.5	0.9 to 2.5	.086
Financial difficulties	22	8	0.6	0.2 to 1.4	.214	22	8	0.6	0.2 to 1.4	.214

NOTE. For global health status and functional scales, a high score indicates a better function. For symptoms and financial difficulties, a high score indicates more symptoms or more difficulties.

Abbreviations: FOLFIRINOX, oxaliplatin/irinotecan/fluorouracil/leucovorin; HR, hazard ratio; MCID, minimal clinically important difference; QLQ-C30, European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire C30; TUDD, time until definitive deterioration.

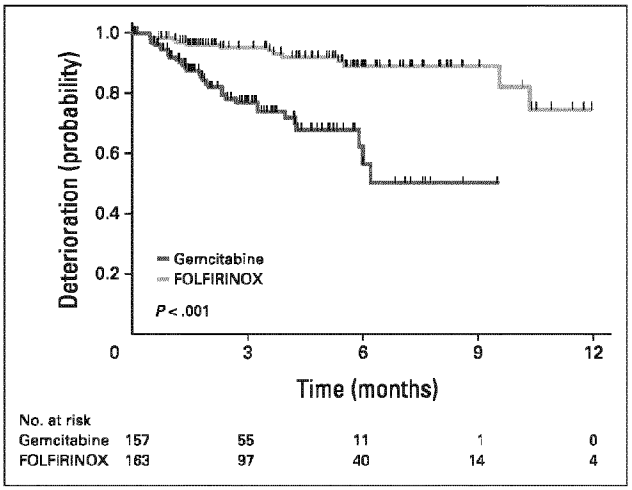

Fig 2. Kaplan-Meier plot for time until definitive deterioration more than 20 points for European Organisation for the Research and Treatment of Cancer Quality of Life Questionnaire C30 global health status/quality of life. FOLFIRINOX, oxaliplatin/irinotecan/fluorouracil/leucovorin.

Longitudinal Analysis

Cronbach's alpha of all domains was calculated and was more than .85, confirming the internal consistency of the questionnaire in this population. When comparing the treatment arms, there did not appear to be any difference in the change in QLQ-C30 domains over time, except for diarrhea. The change in GHS over time was similar when comparing the treatment arms (Appendix Fig A1A [online-only]). GHS scores improved ($P < .001$) in the FOLFIRINOX arm and emotional functioning scores improved ($P < .001$) in both arms between baseline and the end of treatment (6 months). Similarly, a decrease in four symptom scores (pain: FOLFIRINOX, $P < .001$; gemcitabine, $P = .01$; insomnia: FOLFIRINOX, $P < .001$; gemcitabine, $P = .005$; anorexia: FOLFIRINOX, $P < .001$; gemcitabine $P = .02$; and constipation: FOLFIRINOX, $P = .002$; gemcitabine, $P = .02$) was found for both treatments. Patients experienced more diarrhea on the FOLFIRINOX arm than on the gemcitabine arm during the first 2 months of treatment (Appendix Fig A1N; $P = .009$). The other functional and symptom domains are presented in Appendix Figure A1. The percentage of patients with a worsening or improvement of ≥ 10 points each scores are also listed in Appendix Table A2. A moderate improvement of GHS score (≥ 10 points) was found in 30.1% and 18.5% of the patients, in the FOLFIRINOX and gemcitabine arms, respectively. Improvement in fatigue and dyspnea scores was significantly associated with treatment response in both arms, as were the scores for GHS, pain, and insomnia in the FOLFIRINOX arm.

Time Until Definitive Deterioration Analysis

TUDD was determined according to a MCID of at least 10 or 20 points in QLQ-C30 scores for the different domains in both arms (Table 2). TUDD ≥ 10 points was significantly longer for the FOLFIRINOX versus the gemcitabine arm for GHS/QoL, all five functional domains, and six symptom domains (fatigue, nausea/vomiting, pain, dyspnea, anorexia, and constipation). In general, significance was maintained for TUDD ≥ 20 points, except for the emotional functioning domain. The TUDD ≥ 10 points (hazard ratio [HR], 2.3; 95% CI, 1.4 to 3.7; $P < .001$) and ≥ 20 points (HR, 4.7; 95% CI, 2.3 to 9.5; $P < .001$) for GHS score were longer in the FOLFIRINOX arm. Median TUDD of GHS/QoL score was not reached in the FOLFIRINOX arm unlike the gemcitabine arm (Fig 2). Similar results were observed with the TUDD including death as an event (results not shown).

Prognostic Factors for Survival

Univariate Cox analysis revealed the following QLQ-C30 domains as significant prognostic factors for OS for the intent-to-treat population including both treatment arms: PF (HR, 0.88; 95% CI, 0.82 to 0.94; $P < .001$); role functioning (HR, 0.94; 95% CI, 0.90 to

Table 3. Multivariate Analysis of Overall Survival

Variable	Model 0*			Model 1†			Model 2‡			Model 3§		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Baseline clinical and demographic characteristics												
Treatment arm	0.54	0.41 to 0.72	< .001	0.49	0.36 to 0.66	< .001	0.45	0.33 to 0.62	< .001	0.50	0.37 to 0.68	< .001
Age > 65 years	1.45	1.06 to 1.99	.023	1.51	1.09 to 2.09	.012	1.63	1.16 to 2.27	.018	1.55	1.12 to 2.15	.009
Low serum albumin	1.85	1.38 to 2.49	< .001	1.71	1.25 to 2.34	< .001	1.62	1.17 to 2.23	< .001	1.68	1.23 to 2.29	< .001
Synchronous metastases	0.41	0.21 to 0.77	.002							0.69	0.35 to 1.37	.277
Liver metastases	1.62	1.03 to 2.55	.028	1.69	1.05 to 2.71	.022	1.94	1.19 to 3.16	.010	1.60	0.99 to 2.59	.045
Baseline QLQ-C30 domain score												
Physical functioning				0.91	0.84 to 0.99	.039	0.88	0.81 to 0.96	.010	0.91	0.84 to 0.99	.040
Constipation				1.06	1.01 to 1.11	< .001	1.06	1.01 to 1.12	< .001	1.06	1.01 to 1.11	< .001
Dyspnea				1.07	1.00 to 1.14	.001	1.10	1.02 to 1.18	< .001	1.06	1.00 to 1.14	.001
Pain							0.93	0.87 to 0.99	.063			

Abbreviations: HR, hazard ratio; QLQ-C30, European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire C30.
*Model 0: Base model includes only clinical and demographic characteristics.
†Model 1: Final model for backward selection using the five significant clinical and demographic variables (Model 0) plus the six QLQ-C30 domains that were significant on univariate analysis (physical functioning, role functioning, fatigue, constipation, dyspnea, and loss of appetite).
‡Model 2: Final model for forward selection using the variables selected by backward selection (Model 1) plus the nine QLQ-C30 domains that were not significant on univariate analysis (global health status, emotional functioning, cognitive functioning, social functioning, nausea/vomiting, pain, insomnia, diarrhea, and financial difficulties).
§Model 3: Final model excluding pain because of interaction and forcing the variable of the time of appearance of hepatic metastases.

0.98; $P = .005$); fatigue (HR, 1.07; 95% CI, 1.02 to 1.13; $P = .007$); constipation (HR, 1.06; 95% CI, 1.02 to 1.11; $P = .001$); dyspnea (HR, 1.08; 95% CI, 1.03 to 1.13; $P = .004$); and anorexia (HR, 1.06; 95% CI, 1.02 to 1.11; $P = .001$). These six univariate QLQ-C30 domains were added to the base model (model 0) that included the significant clinical and demographic prognostic variables for OS (treatment arm, age > 65 years, low serum albumin, synchronous metastases, and liver metastases) for backward selection (model 1) and forward selection (model 2; Table 3). Only PF, constipation, and dyspnea remained significant after the backward and forward selection procedures. The presence of liver metastases no longer remained significant after the addition of QLQ-C30 domains in the model. However, the presence of liver metastases was forced as a prognostic factor in the final model (model 3) to better measure the contribution of QoL to the clinical model as prognostic factors.

To complete the analysis of prognostic factors for QLQ-C30 data, we determined the Harrell predictive C-index. Two models were compared: a model with the five significant clinical and demographic prognostic variables and another model with the same variables plus the significant QLQ-C30 domains (PF, constipation, and dyspnea). The predictive value added to the three QLQ-C30 domains to the clinical model improved the predictive power by 7.5%, as the predictive power changed from 0.66 to 0.71. Survival curves in different subgroups for baseline QLQ-C30 scores by tercile for PF, constipation, and dyspnea are shown in Figure 3. Severely compromised baseline PF in the lower tercile (ie, less than 33.3 points) seemed to have the most dramatic negative impact on OS, with a median OS of 2.1 months versus 9 months for patients with PF score of more than 66.6 (Fig 3A). The GHS score at baseline and the early improvement of GHS score (within the first 1 or 2 months) are not predictive of a better OS (data not shown).

DISCUSSION

In our original trial, we demonstrated that the FOLFIRINOX regimen is an effective first-line treatment option for patients with mPAC with good PS.[1] Median OS (11.1 *v* 6.8 months; HR, 0.57; 95% CI, 0.45 to 0.73; $P < .001$) and progression-free survival (6.4 *v* 3.3 months; HR, 0.47; 95% CI, 0.37 to 0.59; $P < .001$) were significantly prolonged in patients receiving FOLFIRINOX compared with those receiving gemcitabine, which was considered to be a clinically meaningful improvement.[11-15] However, the safety of FOLFIRINOX was less favorable than gemcitabine, producing higher incidences of neutropenia, febrile neutropenia, thrombocytopenia, diarrhea, and sensory neuropathy. It is therefore important to analyze the impact of treatment on QoL in relation to patient outcome.

A potential source of bias is the proportion of missing QoL forms over the assessment period. However, there was no difference in the rate of missing data between the two arms with a good compliance rate until 6 months. After 6 months, the population was reduced as a result of progressive disease and end of treatment period.

As expected, there were no notable differences between treatment arms over time for EORTC QLQ-30 domains with the exception of diarrhea, which seemed to be negatively affected in the FOLFIRINOX arm during the first 2 months of treatment. However, TUDD ≥ 10 points was significantly longer in the FOLFIRINOX arm for all QLQ-C30 domains (except insomnia, diarrhea, and financial difficulties). Significance was maintained for a TUDD ≥ 20 points for the

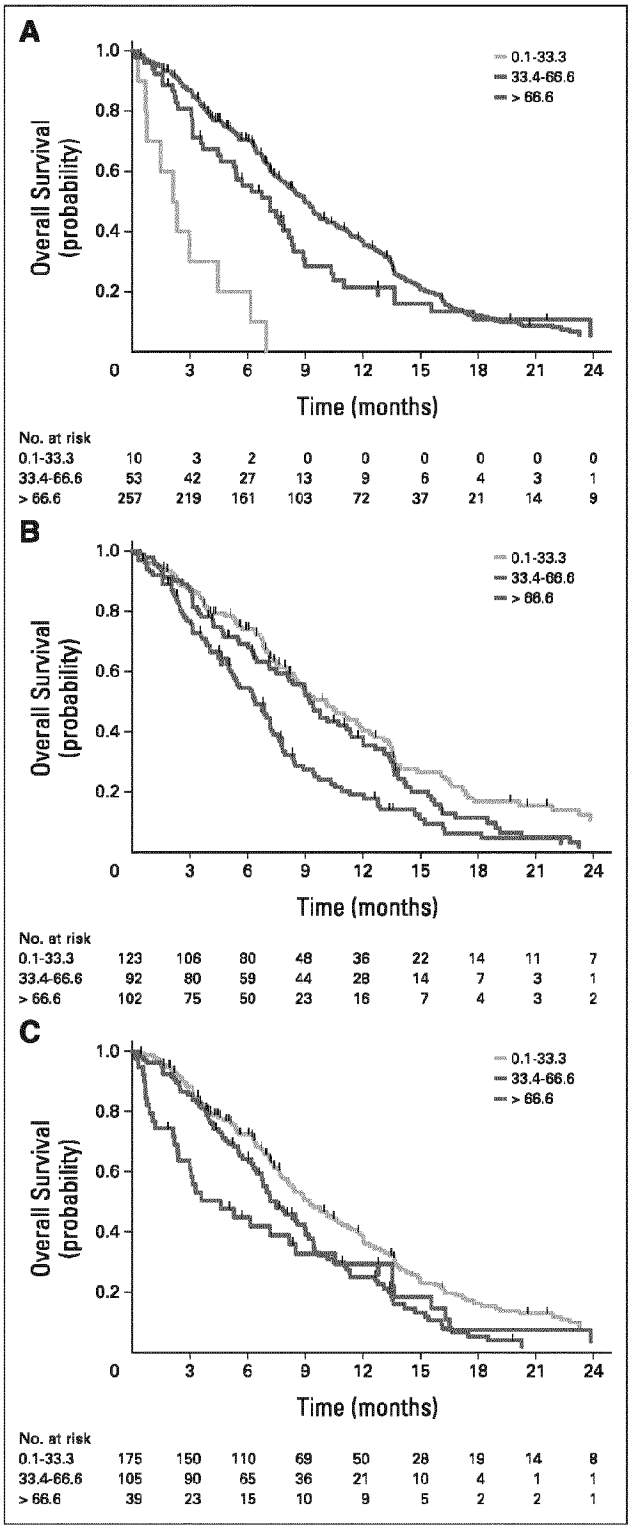

Fig 3. Kaplan-Meier overall survival plots according to baseline tercile point score of significant prognostic European Organisation for the Research and Treatment of Cancer Quality of Life Questionnaire C30 domains (A) physical functioning, (B) constipation, and (C) dyspnea.

aforementioned QLQ-C30 domains except emotional functioning. The longer TUDD ≥ 10 points (HR, 2.3; 95% CI, 1.4 to 3.7; $P < .001$) and ≥ 20 points (HR, 4.7; 95% CI, 2.3 to 9.5; $P < .001$) for GHS score in the FOLFIRINOX arm versus the gemcitabine arm showed a more powerful result when using the more stringent MCID cutoff. The results for a TUDD ≥ 20 points confirm a clinically meaningful prolongation of QoL in patients treated with FOLFIRINOX as compared with gemcitabine despite its less favorable safety.

Another aim of this study was to determine the prognostic value of baseline QLQ-C30 domain scores with respect to survival. This analysis revealed three significant QLQ-C30 domains (PF, constipation, and dyspnea) when added to a model including significant clinical and demographic prognostic variables (treatment arm, age > 65 years, low serum albumin, synchronous metastases, and hepatic metastases). Severely impaired baseline PF seemed to impart the strongest negative impact on OS. This result is particularly important considering that this population was selected on good PS (ECOG 0 or 1) according to the physician's assessment. However, 56 patients (17.4%) wrote in their baseline QoL questionnaire that they needed to stay in bed or a chair during the day. These patients had a worse prognosis, and this result suggests that the PF score of the EORTC QLQ-C30 is a more comprehensive measure of QoL than PS and has a strong prognostic value. The highest prognostic value of patient self-assesment of QoL compared with physician rating of PS has been reported in other studies.[8,16-17]

To our knowledge, there have been no other studies that have determined the impact of baseline QLQ-C30 QoL measures on survival outcome specifically in mPAC, although other QoL measures have been analyzed occasionally in smaller and/or less homogeneous populations. In a meta-analysis of randomized trials including 7,417 patients with cancer who filled out baseline QLQ-C30 questionnaires,[8] multivariate analysis revealed that PF, pain, and anorexia were significant prognostic variables in addition to age, gender, and distant metastases, but not WHO PS. A literature review[18] detailed the relationship between QoL and survival in cancer. Global QoL, functional domains, appetite loss, fatigue, and pain scores were the most prognostic indicators for OS. However, none of the studies identified in this review or our own review of the literature involved homogeneous populations with pancreatic cancer with baseline EORTC QLQ-C30 evaluation. The prognostic values of other QoL scales have been studied in patients with pancreatic cancer. In 55 patients,[19] the health and physical subscale of the Ferrans and Powers QoL Index was shown to be a marginally significant predictor of survival after controlling for the stage at diagnosis. In 86 patients with cachexia,[20] baseline fatigue and PF scores predicted OS using various QoL measures, but multivariate analysis was not performed. The prognostic value of various QoL indicators (including global linear-analog self-assessment indicators) was recently reported in 319 patients with advanced pancreatic cancer; baseline pain and tiredness were independent prognostic factors for OS but less so than baseline carbohydrate antigen 19-9.[21]

In conclusion, our analysis confirms that FOLFIRINOX significantly reduces QoL impairment compared with gemcitabine in patients with mPAC. Furthermore, multivariate analysis showed the additional prognostic value of baseline EORTC QLQ-C30 measures when added to clinical and demographic variables to predict survival among patients with mPAC. Whether or not baseline PF QoL score should be used as a stratification factor in further clinical trials has to be assessed prospectively.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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REFERENCES

1. Conroy T, Desseigne F, Ychou M, et al: FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364:1817-1825, 2011
2. Aaronson NK, Ahmedzai S, Bergman B, et al: The European Organization for Research and Treatment of Cancer QLQ-C30: A quality-of-life instrument for use in international clinical trials in oncology. J Natl Cancer Inst 85:365-376, 1993
3. Osoba D, Rodrigues G, Myles J, et al: Interpreting the significance of changes in health-related quality-of-life scores. J Clin Oncol 16:139-144, 1998
4. Conroy T, Mercier M, Bonneterre J, et al: French version of FACT-G: Validation and comparison with other cancer-specific instruments. Eur J Cancer 40:2243-2252, 2004
5. Fayers P, Aaronson N, Boral K, et al: EORTC QLQ-C30 Scoring Manual. Brussels, Belgium, European Organisation for Research and Treatment of Cancer, 2001
6. Cronbach LJ: Coefficient alpha and the internal structure of tests. Psychometrika 16:297-334, 1951
7. Bonnetain F, Dahan L, Maillard E, et al: Time until definitive quality of life score deterioration as a means of longitudinal analysis for treatment trials in patients with metastatic pancreatic cancer. Eur J Cancer 46:2753-2762, 2010
8. Quinten C, Coens C, Mauer M, et al: Baseline quality of life as a prognostic indicator of survival: A meta-analysis of individual patient data from EORTC clinical trials. Lancet Oncol 10:865-871, 2009
9. Harrell FE Jr, Lee KL, Mark DB: Multivariable prognostic models: Issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med 15:361-387, 1996
10. Harrel F: Regression Modeling Strategies: With Applications to Linear Models, Logistic Regression and Survival Analysis. New York, NY, Springer, 2001
11. Kris MG, Benowitz SI, Adams S, et al: Clinical cancer advances 2010: Annual report on progress

against cancer from the American Society of Clinical Oncology. J Clin Oncol 28:5327-5347, 2010

12. Kim R: FOLFIRINOX: A new standard treatment for advanced pancreatic cancer? Lancet Oncol 12:8-9, 2011

13. Ko AH: FOLFIRINOX: A small step or a great leap forward? J Clin Oncol 29:3727-3729, 2011

14. Saif MW, Chabot J: Chemotherapy: Metastatic pancreatic cancer—Is FOLFIRINOX the new standard? Nat Rev Clin Oncol 8:452-453, 2011

15. Vaccaro V, Sperduti I, Milella M: FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 365:768-769, 2011 (author reply 769)

16. Efficace F, Bottomley A, Coens C, et al: Does a patient's self-reported health-related quality of life predict survival beyond key biomedical data in advanced colorectal cancer? Eur J Cancer 42:42-49, 2006

17. Gotay CC, Kawamoto CT, Bottomley A, et al: The prognostic significance of patient-reported outcomes in cancer clinical trials. J Clin Oncol 26:1355-1363, 2008

18. Montazeri A: Quality of life as prognostic indicators of survival in cancer patients: An overview of the literature from 1982 to 2008. Health Qual Life Outcomes 7:102, 2009

19. Lis CG, Gupta D, Grutsch JF: Patient satisfaction with quality of life as a predictor of survival in pancreatic cancer. Int J Gastrointest Cancer 37:35-44, 2006

20. Robinson DW Jr, Eisenberg DF, Cella D, et al: The prognostic significance of patient-reported outcomes in pancreatic cancer cachexia. J Support Oncol 6:283-290, 2008

21. Bernhard J, Dietrich D, Glimelius B, et al: Estimating prognosis and palliation based on tumor marker CA 19-9 and quality of life indicators in patients with advanced pancreatic cancer receiving chemotherapy. Br J Cancer 103:1318-1324, 2010

FULL PAPER

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Keywords: irinotecan sucrosofate; liposome; pancreatic cancer; second-line; gemcitabine-refractory

A multinational phase 2 study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer

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Background: PEP02, also known as MM-398, is a novel nanoliposomal irinotecan that has improved pharmacokinetics and tumour bio-distribution of the free drug. This phase 2 study evaluated PEP02 monotherapy as second-line treatment for pancreatic cancer.

Methods: Patients who had metastatic pancreatic adenocarcinoma, Karnofsky performance status $\geqslant 70$, and had progressed following gemcitabine-based therapy were eligible. Intravenous injection of PEP02 120 mg m^{-2} was given every 3 weeks. Simon 2-stage design was used. The primary objective was 3-month survival rate ($OS_{3\text{-month}}$).

Results: A total of 40 patients were enrolled. The most common severe adverse events included neutropenia, abdominal pain, asthenia, and diarrhoea. Three patients (7.5%) achieved an objective response, with an additional 17 (42.5%) demonstrating stable disease for a minimum of two cycles. Ten (31.3%) of 32 patients with an elevated baseline CA19-9 had a $>50\%$ biomarker decline. The study met its primary end point with an $OS_{3\text{-month}}$ of 75%, with median progression-free survival and overall survival of 2.4 and 5.2 months, respectively.

Conclusion: PEP02 demonstrates moderate antitumour activity with a manageable side effect profile for metastatic, gemcitabine-refractory pancreatic cancer patients. Given the limited treatment options available to this patient population, a phase 3 trial of PEP02 (MM-398), referred to as NAPOLI-1, is currently underway.

Therapeutic options for patients with advanced pancreatic cancer (APC) range from gemcitabine monotherapy to multiple-drug regimens, depending on age, performance status, comorbid conditions, and patient and physician preference. Recently, results of a phase 3 clinical trial from France (PRODIGE 4/ACCORD 11) demonstrated the superiority of FOLFIRINOX (biweekly infusional 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin) over gemcitabine in the first-line treatment of metastatic pancreatic

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cancer, with improvements in response rate, progression-free survival, and overall survival, albeit with greater toxicity (Conroy *et al*, 2011).

Beyond first-line therapy, options for metastatic pancreatic cancer become less clear, as patients often demonstrate rapid clinical deterioration and are no longer suitable candidates for additional treatment beyond best supportive care. One co-operative group trial reported that only 45% of patients with APC went on to receive additional therapy following progression on front-line study treatment (Schrag *et al*, 2007). A number of small prospective single-arm studies have evaluated both cytotoxic and/or targeted agents in the setting of gemcitabine-refractory disease, generally demonstrating low response rates and progression-free survival of a few months at best (Burris *et al*, 2005; Boeck *et al*, 2007; Kulke *et al*, 2007; Ko *et al*, 2008, 2010; Oh *et al*, 2010; O'Reilly *et al*, 2010). Results from a randomised German trial for the second-line treatment of APC (CONKO-003) suggested a weekly regimen called OFF (oxaliplatin, 5-FU given as a 24-hour infusion, and folinic acid) may improve patient outcomes in patients refractory to gemcitabine (Pelzer *et al*, 2008, 2011). At present, however, there is no recognised standard of care in this setting.

PEP02 (also known as MM-398) is irinotecan sucrosofate encapsulated in a liposome drug delivery system. This stable nanoliposomal formulation has been shown in preclinical studies to improve pharmacokinetics and tumour bio-distribution of both irinotecan and its active metabolite SN-38 when compared with the free form of the drug, with less accumulation in many of the target organs associated with toxic side effects. PEP02 also demonstrated increased efficacy and tolerable toxicity when compared with free irinotecan in an orthotopic pancreatic cancer mouse model (Hann *et al*, 2007). The favourable pharmacokinetics of irinotecan and SN-38 after PEP02 was confirmed in the first-in-human phase 1 trial for refractory solid tumours, in which the maximum tolerated dose of PEP02 given every 3 weeks was determined as 120 mg m^{-2} (Chen *et al*, 2008). This non-randomised phase 2 trial, conducted in the United States and Taiwan, sought to establish the efficacy and toxicity of single-agent PEP02 in patients with metastatic pancreatic cancer after progression on first-line gemcitabine-based therapy.

PATIENTS AND METHODS

Trial design and patients. This trial was an international, multicenter, open-label, phase 2 study of PEP02 (liposome encapsulated irinotecan, PharmaEngine Inc, Taipei, Taiwan) in patients with gemcitabine-based chemotherapy failure metastatic pancreatic adenocarcinoma.

Patients with histologically confirmed adenocarcinoma of the exocrine pancreas refractory to gemcitabine-based (either alone or in combination) systemic chemotherapy, including those with disease progression within 6 months after post operative adjuvant therapy, were eligible. Prior treatment with irinotecan was not allowed. Further inclusion criteria were age $\geqslant$18 years, Karnofsky performance status of $\geqslant$50 (subsequently amended to $\geqslant$70 to ensure patient safety and to be consistent with the eligibility criteria of other clinical trials for this same patient population), with extrapancreatic metastases diagnosed either radiographically or by biopsy confirmation, and adequate bone marrow and hepatic functions within 1 week before commencing treatment (absolute neutrophil count $\geqslant 1.5 \times 10^3$ ml^{-1}, platelets $\geqslant 100 \times 10^3$ ml^{-1}, serum bilirubin within upper limit of normal (ULN), transaminase $\leqslant 2.5 \times$ ULN ($\leqslant 5 \times$ ULN in patients with liver metastases). All prior major surgery, radiotherapy (except palliative), or investigational drug therapy, had to be ceased at least 4 weeks and all treatment-related toxicities had to be resolved to no greater than grade 1 before enrolment. Patients with central nervous system metastases, pregnancy, uncontrolled active infection, another primary malignancy within the past 5 years except curatively treated non-melanoma skin cancer or cervical carcinoma *in situ*, or other concomitant serious diseases, were excluded.

All patients gave written informed consent. The trial was approved by the independent ethics committee of each participating institute, and performed in accordance with the International Conference on Harmonization Good Clinical Practice guidelines, Good Clinical Laboratory Practice, and the Declaration of Helsinki. The trial was also registered with clinical trials.gov identifier NCT00813163.

Treatment and assessments. PEP02 at a dose of 120 mg m^{-2} was diluted in 500 ml of 5% dextrose and delivered as a 90-min intravenous infusion every 21 days. Infusion time was allowed to be prolonged for acute infusion-associated reactions or any other clinical needs. Premedication included dexamethasone and a serotonin antagonist. Prophylactic anticholinergic agent was not given unless an acute cholinergic reaction was observed during a prior cycle of treatment. Imodium, growth factor support, and anticoagulation (warfarin or low-molecular heparin) were allowable per protocol as clinically indicated, but not for primary prophylaxis. Detailed history evaluation, vital signs recording, physical examination, complete blood count with differential classification, and blood biochemistry tests were performed weekly during the first treatment cycle and before the start of each treatment cycle thereafter. Toxicity was recorded according to the National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 3.0.

Dose adjustments in PEP02 were made according to toxicities observed with each treatment cycle. The protocol allowed, at the discretion of the treating physician, escalation of PEP02 to 150 mg m^2 beginning with cycle no. 2 in patients who did not experience drug-related toxicities worse than grade 1. The development of grade 3 or 4 diarrhoea, grade 4 or febrile neutropenia, or any other grade 3 or 4 toxicity required a dose reduction of study drug in 20 mg m^{-2} decrements, to a lowest dose level permissible of 80 mg m^{-2}, with no subsequent dose re-escalation allowed. The treatment was continued until evidence of disease progression, unacceptable toxicity, treatment delay for $>$2 weeks, patient withdrawal of consent, or death.

Imaging studies, preferably using computed tomography, were performed at baseline and after every two cycles of chemotherapy to evaluate tumour response, which was determined according to the RECIST version 1.0 guidelines. All complete and partial responses required confirmation by two consecutive observations no less than 4 weeks apart. CA19-9 was measured before each cycle of treatment, and CA19-9 tumour marker response (defined as a decrease of $\geqslant$50% of CA 19-9 in relation to baseline level at least once during the treatment period, in patients with baseline values above the ULN) was determined. Patient diaries were dispensed to collect pain information (including pain intensity and morphine consumption). Patients' survival status was tracked at the 90th day after the start of PEP02 treatment (cycle 1, day 1) and every 2 months after withdrawal. The date of death was recorded.

Statistical analysis. The primary end point of this study was 3-month survival rate ($OS_{3\text{-month}}$). Secondary end points included other clinical efficacy variables (objective tumour response, progression-free and overall survival, clinical benefit response (as defined in Burris *et al*, 1997), CA19-9 tumour marker response), and safety profile. A randomised phase 3 trial by the German CONKO-study group (Pelzer *et al*, 2011) reported a median survival of 2.3 months in patients receiving best supportive care after front-line gemcitabine-based therapy, with a $OS_{3\text{-month}}$ of $\sim$35%. Thus, for the current study, we used as the null hypothesis (H_0) and alternative hypothesis (H_a) a $OS_{3\text{-month}}$ of 40% and 65%, respectively. The study used an optimal Simon 2-stage design.

With a significance level of $\alpha = 0.05$ and a type 2 error $\beta = 0.10$, if 8 of the first 16 patients enrolled in the first stage reached the 3-month survival time-point, an additional 23 patients would be enrolled in the second stage. At least 21 of the 39 patients were required to survive 3 months or longer to allow rejection of the null hypothesis. A safety stopping rule would be invoked if six or more patients in the first stage experienced grade 3 or 4 diarrhoea.

Descriptive statistics were used for all efficacy variables, with the primary analysis population being the per protocol population (defined as study participants who met all inclusion/exclusion criteria and did not significantly deviate from the study protocol). The frequencies of patients with adverse events were summarised by body system and by major adverse event codes (system/organ/class).

RESULTS

Patient characteristics. Baseline patient characteristics are shown in Table 1. A total of 40 patients were enrolled for the study between March 2009 and September 2010, with an approximately even distribution between US and Taiwanese sites. The majority of patients (60%) had a Karnofsky Performance Score of 90–100 and 77.5% had received a prior gemcitabine-based combination, as opposed to monotherapy, as their first-line regimen. The duration of front-line therapy ranged from 1 to 24 months.

Drug delivery and adverse events. Patients received a mean of 5.875 treatment cycles (range, 1–28 cycles; median 2.5 cycles). Owing to concerns of excess toxicity, primarily asthenia, observed in US patients at the starting dose of 120 mg m^{-2}, the protocol was subsequently amended during the second stage of the study to permit a lower starting dose at 100 mg m^{-2}. In total, 27 of 40 patients (67.5%) on the study were able to be maintained at a dose of 120 mg m^{-2} throughout their entire treatment course, whereas 11 (27.5%) required or initiated therapy at reduced doses. Eleven patients (27.5%) received at least eight treatment cycles. The majority of patients (75%) discontinued study treatment due to disease progression.

The most common toxicities observed during study treatment are shown in Table 2. As expected, gastrointestinal and haematologic toxicities were the most common types seen, as well as fatigue and abdominal pain; these latter symptoms may have been related either to study treatment or to the underlying cancer. In total, 26 patients (65%) experienced at least one treatment-emergent adverse event categorised as grade 3 or higher. Of note, six patients died within 30 days of the last dose of study treatment. Of these, three were attributed to disease progression; the other three were due to respiratory failure, aspiration pneumonia, and sepsis, all in the setting of neutropenia.

Efficacy. Efficacy results are shown in Table 3. Half of the patients (50%) had evidence of disease control (objective response plus stable disease for more than two cycles), including three patients (7.5%) who achieved a confirmed objective response. Fourteen of the 17 patients with stable disease as their best response demonstrated disease stability for at least four cycles (35% of the entire cohort). A waterfall plot (Figure 1) demonstrates best

Table 1. Patient demographics and baseline characteristics

Characteristic	*n* = 40
Sex, *n* (%)	
Male/female	19 (47.5)/21 (52.5)
Age, mean (range) years	58.8 (39–82)
Study site, *n* (%)	
Taiwan/USA	22 (55)/18 (45)
Ethnicity, *n* (%)	
Asian/Caucasian	25 (62.5)/15 (37.5)
Karnofsky performance status, *n* (%)	
100 90 80 70	7 (17.5) 17 (42.5) 6 (15.0) 10 (25.0)
Prior treatment, *n* (%)	
Chemotherapy Radiotherapy Surgery	40 (100) 10 (25.0) 17 (42.5)
First-line chemotherapy and duration in months	
Gemcitabine monotherapy, *n* (%)/median (range) Gemcitabine-based combination, *n* (%)/median (range)	9 (22.5)/2 (1.5–24) 31 (77.5)/6 (1–16)
With elevated CA19-9, *n*	32
Baseline clinical benefit parameters, *n* (%)	
Pain intensity ⩾20 (out of 100) Morphine consumption ⩾10 mg per day	17 (42.5) 14 (35.0)

Table 2. (A) Treatment-emergent adverse events (all grades) occurring in 10% or greater of study patients. (B) Treatment-emergent grades 3–4 advance events occurring in 10% or greater of study patients

A

Adverse event, all grades	*N* (%)
Diarrhoea	30 (75%)
Fatigue	25 (62.5%)
Nausea	24 (60%)
Anorexia	23 (57.5%)
Vomiting	23 (57.5%)
Alopecia	17 (42.5%)
Neutropenia	16 (40%)
Leucopenia	15 (37.5%)
Abdominal pain	15 (37.5%)
Weight decreased	15 (37.5%)
Anaemia	13 (32.5%)

B

Adverse event, grades 3–4	*N* (%)
Neutropenia	12 (30%)
Leucopenia	10 (25%)
Abdominal pain	6 (15%)
Fatigue/asthenia	8 (20%)
Anaemia	6 (15%)
Hyponatremia	6 (15%)
Diarrhoea	6 (15%)
GGT elevated	5 (12.5%)
Anorexia	4 (10%)
Nausea	4 (10%)

Table 3. Efficacy data

Best tumour response ($n=40$)	**N (%)**
Partial response	3 (7.5%)
Stable disease	17 (42.5%)[a]
Disease Progression	10 (25.0%)
Non-evaluable[b]	10 (25.0%)
Disease control (PR+SD) rate	20 (50.0%)
Survival	**Months**
Progression-free survival (median)	2.4
Overall survival (median)	5.2
Proportion of patients alive at:	**N (%)**
Three months	30 (75%)
Six months	17 (42.5%)
Twelve months	10 (25%)
Clinical benefit response ($n=25$ evaluable)	5 (20%)
CA19-9 decline >50% ($n=32$ with elevated level at baseline)	10 (31.3%)

Abbreviations: PR = partial response; SD = Stable disease.
[a]Including eight patients with minor response.
[b]Non-evaluable patients for tumor response included those patients with non-measurable disease at baseline or in whom at least one post treatment radiographic evaluation was not performed.

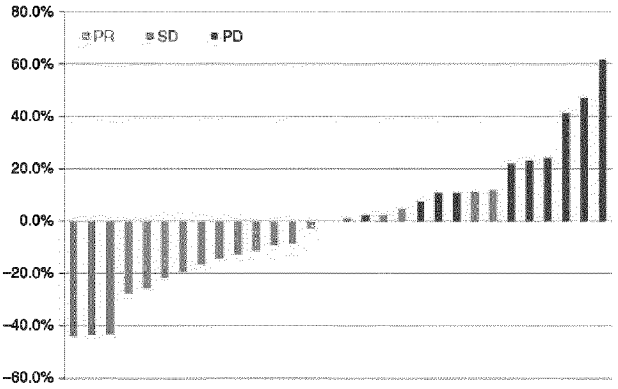

Figure 1. Maximum % change from baseline in sum of target lesion diameters (evaluable patients only, $n=30$). Abbreviations: PD = disease progression; PR = partial response; SD = stable disease.

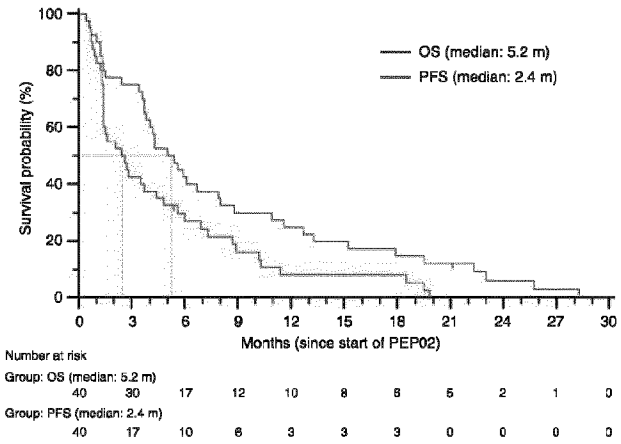

Figure 2. Kaplan–Meier curves of overall and progression-free survival. Abbreviations: m = months; OS = overall survival; PFS = progression-free survival.

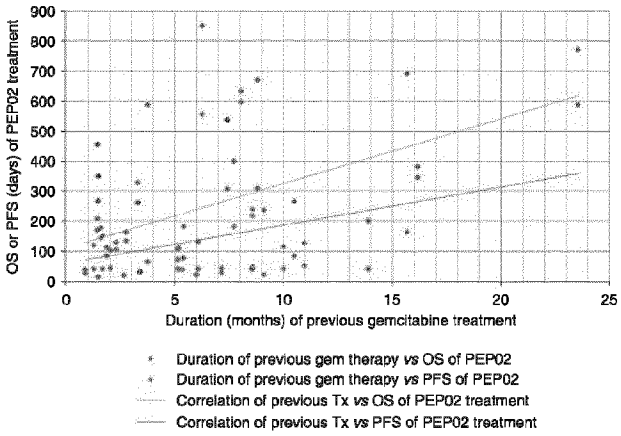

Figure 3. Overall survival (OS) and progression-free survival (PFS) relative to the duration of prior gemcitabine-based therapy. Abbreviations: Gem = gemcitabine; Tx = treatment.

tumour response observed in evaluable study patients. Ten (31.3%) of 32 patients with elevated baseline CA19-9 had >50% biomarker decline, and 5 (20%) of 25 CBR-evaluable patients achieved significant clinical benefit. Median progression-free and overall survival was 2.4 and 5.2 months, respectively (Figure 2). These indicators of antitumour activity are also listed in Table 3. Notably, the study met its primary end point with 75% of patients surviving at least 3 months, including 25% reaching the 1-year mark. Two patients were still alive as of July 2012. Survival outcomes for patients receiving PEP02 showed a modest positive correlation with the duration of prior gemcitabine-based therapy (Figure 3).

DISCUSSION

There is a relative paucity of published studies evaluating the safety and efficacy of chemotherapy regimens in patients with APC who have progressed following first-line therapy. An inherent selection bias is at work in non-randomised trials, as those patients who are well enough to consider salvage treatment may have more favourable tumour biology and a longer survival independent of choice of therapy. Conversely, design of a randomised study in this setting is challenging due to lack of agreement regarding the appropriate selection of control arm; a comparator arm of best supportive care alone, although perhaps appropriate in many cases, is not an appealing option to patients. Results from one of the largest studies conducted to date for the second-line treatment of APC (CONKO-003) randomised 165 patients to receive a weekly regimen called OFF or 5-FU/folinic acid alone (Pelzer *et al*, 2008). Patients receiving the oxaliplatin-containing combination demonstrated significantly improved outcomes in terms of both progression-free survival (13 *vs* 9 weeks, $P=0.012$) and overall survival (26 *vs* 13 weeks, $P=0.014$), leading to the adoption of this regimen (or slight variations thereof) as a *de facto* standard of care in the salvage setting.

Irinotecan is a topoisomerase 1 inhibitor that is currently used to treat the colorectal, gastric, lung, uterine, cervical, and ovarian cancers. At higher doses, the drug causes severe diarrhoea and myelosuppression, which is recognised as its dose-limiting toxicity. Specific to pancreatic cancer, irinotecan represents a component of the FOLFIRINOX regimen that has recently demonstrated superior activity to gemcitabine in the front-line setting (Conroy *et al*,

2011), and has also been evaluated as part of combination regimens for refractory disease in several studies (Ko *et al*, 2008; Yoo *et al*, 2009; Gebbia *et al*, 2010; Oh *et al*, 2010; Assaf *et al*, 2011; Zaniboni *et al*, 2012). A recently reported phase 2 trial performed by the Italian Group for the Study of Gastrointestinal Tract Cancer (GISCAD) showed that FOLFIRI produced median progression-free and overall survival rates of 3.2 and 5 months, respectively, in the second-line treatment of APC (Zaniboni *et al*, 2012).

PEP02 is irinotecan encapsulated in a liposome drug delivery system. Liposome drug formulations may reduce the toxicity of an encapsulated agent to healthy tissue while maintaining, or increasing, its antitumour potency. The therapeutic benefits of liposome encapsulated anticancer drugs such as daunorubicin, doxorubicin, and cytarabine are well-established. Preclinical *in vivo* efficacy data have shown improved antitumour activity of PEP02 over the equivalent dose of free irinotecan in multiple established human tumour xenograft mouse models, including brain, colon, and pancreatic cancers (Hann *et al*, 2007). In previous phase 1 studies, PEP02 either alone or in combination with 5-FU/leucovorin demonstrated prolonged disease control in five of seven (71%) patients with gemcitabine-refractory APC (Chen *et al*, 2008, 2010).

On these bases, the current non-randomised phase 2 trial was conducted to establish the preliminary efficacy and safety of PEP02 in the second-line setting for patients with metastatic pancreatic cancer. Recognising the aforementioned limitations that accompany a single-arm study design, PEP02 did show clear evidence of antitumour activity in a subset of patients in whom no standard of care therapy otherwise exists. In addition, although its efficacy profile appears similar to that seen with FOLFIRI in the GISCAD trial for the same patient population, PEP02 may offer advantages in its relative ease of administration as monotherapy without the requirement of an infusion pump. However, it should also be acknowledged that although PEP02 was generally well-tolerated in most patients, with manageable and predictable toxicities, the majority of subjects did experience at least one grade 3 or higher adverse event. In addition, there were three patient deaths that occurred within 30 days of the last dose of study treatment relating to complications of neutropenia. These findings highlight the need to be particularly vigilant with PEP02 (or any cytotoxic therapy, for that matter) in such a fragile patient population, and may support the use of preemptive growth factor support in select patients. Pharmacogenetic testing for polymorphisms in genes relating to the metabolism of PEP02, including UGT1A1 and UGT1A9, was performed on 28 patients; no correlation with either haematologic or non-haematologic toxicity was observed (data not shown).

Although analysis of germline polymorphisms from peripheral blood samples was possible on all study patients, there were not adequate tumour tissue samples available to look for intratumoural molecular biomarkers of potential predictive significance. Such correlative studies represent one of the 'holy grails' that are often attempted to be embedded within pancreatic cancer clinical trials; however, due to scant archived samples and the difficulties in subjecting this patient population to prospective tissue biopsies for research purposes, they continue to present a tremendous challenge in this disease. This obstacle is magnified all the more so in the salvage treatment setting.

The results of this clinical trial are encouraging enough to warrant moving ahead with a larger study in a similar patient population, currently ongoing as an international randomised phase 3 trial called NAPOLI-1 (clinicaltrial.gov. ID: NCT01494506, EudraCT Number: 2011-004687-30). Additional studies may explore this drug's potential role in the first-line setting and as part of combination regimens for APC. Moreover, given the emergence of FOLFIRINOX as a front-line standard in patients with good performance status, the utility of PEP02 in irinotecan-pretreated patients, alone or in combination with gemcitabine, also merits further investigation.

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CONFLICT OF INTEREST

LTC is a consultant and has received honorarium from PharmaEngine. CGY and YWW are employees and hold stock of PharmaEngine, the makers of PEP02. AHK, MAT, YSS, WCS, YLL, ED and AO declares no conflict of interest.

REFERENCES

Assaf E, Verlinde-Carvalho M, Delbaldo C, Grenier J, Sellam Z, Pouessel D, Bouaita L, Baumgaertner I, Sobhani I, Tayar C, Paul M, Culine S (2011) 5-fluorouracil/leucovorin combined with irinotecan and oxaliplatin (FOLFIRINOX) as second-line chemotherapy in patients with metastatic pancreatic adenocarcinoma. *Oncology* **80**: 301–306.

Boeck S, Weigang-Kohler K, Fuchs M, Kettner E, Quietzsch D, Trojan J, Stotzer O, Zeuzem S, Lordick F, Kohne CH, Kroning H, Steinmetz T, Depenbrock H, Heinemann V (2007) Second-line chemotherapy with pemetrexed after gemcitabine failure in patients with advanced pancreatic cancer: a multicenter phase II trial. *Ann Oncol* **18**: 745–751.

Burris 3rd HA, Rivkin S, Reynolds R, Harris J, Wax A, Gerstein H, Mettinger KL, Staddon A (2005) Phase II trial of oral rubitecan in previously treated pancreatic cancer patients. *Oncologist* **10**: 183–190.

Burris 3rd HA, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD (1997) Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* **15**: 2403–2413.

Chen LT, Chang TC, Cheng AL, Yang CH, Shiah HS, Chang JY, Yeh G (2008) Phase I study of liposome encapsulated irinotecan (PEP02) in advanced solid tumour patients. *J Clin Oncol* **26**: 2565 (abstract).

Chen LT, Shiah HS, Chao TY, Hsieh RK, Chen G, Chang JY, Yeh G (2010) Phase I study of liposome irinotecan (PEP02) in combination with weekly infusion of 5-FU/LV in advanced solid tumors. *J Clin Oncol* **28**: e13024 (abstract).

Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardiere C, Bennouna J, Bachet JB, Khemissa-Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M (2011) FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* **364**: 1817–1825.

Gebbia V, Maiello E, Giuliani F, Borsellino N, Arcara C, Colucci G (2010) Irinotecan plus bolus/infusional 5-Fluorouracil and leucovorin in patients with pretreated advanced pancreatic carcinoma: a multicenter experience of the Gruppo Oncologico Italia Meridionale. *Am J Clin Oncol* **33**: 461–464.

Hann B, Peth K, Wang D, Gysin S, Li S, Kullberg E, Hom Y, Goldman M, Tempero M, Park J (2007) Lipidic nanoparticle CPT-11 in a bioluminescent orthotopic pancreas cancer model *American Association of Cancer Research Annual Meeting* (abstract 5648).

Ko AH, Venook AP, Bergsland EK, Kelley RK, Korn WM, Dito E, Schillinger B, Scott J, Hwang J, Tempero MA (2010) A phase II study of bevacizumab plus erlotinib for gemcitabine-refractory metastatic pancreatic cancer. *Cancer Chemother Pharmacol* **66**: 1051–1057.

Ko AH, Dito E, Schillinger B, Venook AP, Bergsland EK, Tempero MA (2008) Excess toxicity associated with docetaxel and irinotecan in patients with metastatic, gemcitabine-refractory pancreatic cancer: results of a phase II study. *Cancer Invest* **26**: 47–52.

Kulke MH, Blaszkowsky LS, Ryan DP, Clark JW, Meyerhardt JA, Zhu AX, Enzinger PC, Kwak EL, Muzikansky A, Lawrence C, Fuchs CS (2007) Capecitabine plus erlotinib in gemcitabine-refractory advanced pancreatic cancer. *J Clin Oncol* **25**: 4787–4792.

Oh SY, Kim HJ, Kim TH, Lee GW, Kim HG, Jeong CY, Kwon HC, Kang JH (2010) Pilot study of irinotecan/oxaliplitin (IROX) combination chemotherapy for patients with gemcitabine- and 5-fluorouracil- refractory pancreatic cancer. *Invest New Drugs* **28**: 343–349.

O'Reilly EM, Niedzwiecki D, Hall M, Hollis D, Bekaii-Saab T, Pluard T, Douglas K, Abou-Alfa GK, Kindler HL, Schilsky RL, Goldberg RM (2010) A Cancer and Leukemia Group B phase II study of sunitinib malate in patients with previously treated metastatic pancreatic adenocarcinoma (CALGB 80603). *Oncologist* **15**: 1310–1319.

Pelzer U, Schwaner I, Stieler J, Adler M, Seraphin J, Dorken B, Riess H, Oettle H (2011) Best supportive care (BSC) versus oxaliplatin, folinic acid and 5-fluorouracil (OFF) plus BSC in patients for second-line advanced pancreatic cancer: A phase III-study from the German CONKO-study group. *Eur J Cancer* **47**: 1676–1681.

Pelzer U, Kubica K, Stieler J, Schwaner I, Heil G, Gorner M, Molle M, Hilbig A, Dorken B, Riess H, Oettle H (2008) A randomized trial in patients with gemcitabine refractory pancreatic cancer. Final results of the CONKO 003 study. *J Clin Oncol* **26**: 4508 (abstract).

Schrag D, Archer L, Wang X, Romanus D (2007) A patterns-of-care study of post-progression treatment among patients with advanced pancreas cancer after gemcitabine therapy on Cancer and Leukemia Group B study #80303. *J Clin Oncol* **25**: 4524 (abstract).

Yoo C, Hwang JY, Kim JE, Kim TW, Lee JS, Park DH, Lee SS, Seo DW, Lee SK, Kim MH, Han DJ, Kim SC, Lee JL (2009) A randomised phase II study of modified FOLFIRI.3 vs modified FOLFOX as second-line therapy in patients with gemcitabine-refractory advanced pancreatic cancer. *Br J Cancer* **101**: 1658–1663.

Zaniboni A, Aitini E, Barni S, Ferrari D, Cascinu S, Catalano V, Valmadre G, Ferrara D, Veltri E, Codignola C, Labianca R (2012) FOLFIRI as second-line chemotherapy for advanced pancreatic cancer: a GISCAD multicenter phase II study. *Cancer Chemother Pharmacol* **69**: 1641–1645.

CANCER RESEARCH

Experimental and Molecular Therapeutics

Lipidic nanoparticle CPT-11 in a bioluminescent orthotopic pancreas cancer model

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Abstract

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Efforts to treat pancreas cancer have been hampered by a lack of effective therapeutic options as well as predictive preclinical models. We hypothesized that nanoparticle agents encapsulating potent cytotoxic compounds may be useful against pancreatic cancer; and that antibody-targeted versions directed against EGFR may further increase efficacy against EGFR-overexpressing pancreatic cancers. We applied a liposome-based drug loading and stabilization technique to generate nanoliposomal CPT-11, a novel lipidic nanoparticle agent containing the prodrug CPT-11 (irinotecan) that has entered clinical trials. In addition, Fab' fragments of C225 were conjugated to nanoliposomal CPT-11 to generate EGFR-targeted immunoliposomal CPT-11.

Another objective of this study was to develop a bioluminescent-based orthotopic xenograft model of pancreas cancer with EGFR-overexpression to test this therapeutic approach. COLO357, a human pancreatic cell line, was passaged multiple times in vivo to generate the sub-line L3.6pl. This cell line was subsequently modified by lentiviral transduction to generate a firefly luciferase-expressing cell line, L3.6pl-T. L3.6pl-T cells were injected, during surgery, directly into the pancreas of a nude mouse to generate a tumor xenograft. Following ip administration of luciferin, animals were immediately imaged using a Xenogen IVIS 100 bioluminescent imager, and subsequently imaged at weekly intervals. Tumor burden was quantified by measuring luminescence. The signal was quantified by defining regions of interest (ROIs) and measuring photons/sec/str.

Agents evaluated in this model included EGFR-targeted immunoliposomal CPT-11, nanoliposomal CPT-11, free drug or vehicle control. All treatments were administered i.v. by tail vein beginning at 7 days post- tumor implantation and continued weekly for a total of three treatments. Both nanoliposomal CPT-11 and immunoliposomal CPT-11 showed potent antitumor activity, including durable tumor regressions, and were markedly superior to the equivalent dose of free drug. While both nanoparticle constructs were highly potent, the immunoliposome agent appeared to provide more prolonged duration of responses than the non-targeted version. Systemic toxicity was not observed with any treatment.

We conclude that nanoparticle-mediated delivery of CPT-11 via nanoliposomal CPT-11 or anti-EGFR immunoliposomal CPT-11 greatly enhances antitumor efficacy in the orthotopic COLO357 pancreatic xenograft model. This therapeutic approach offers potential advantages for pancreatic cancer treatment, and this type of model system may be useful in preclinical evaluation.

Footnotes

- 98th AACR Annual Meeting-- Apr 14-18, 2007; Los Angeles, CA

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ORIGINAL ARTICLE

Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients

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Abstract
Purpose To define the dose-limiting toxicity (DLT), maximum tolerated dose (MTD) and pharmacokinetics (PK) of PEP02, a novel liposome-encapsulated irinotecan, in patients with advanced refractory solid tumors.
Methods Patients were enrolled in cohorts of one to three to receive escalating dose of PEP02 in a phase I trial. PEP02, from 60 to 180 mg/m^2, was given as a 90-min intravenous infusion, every 3 weeks.
Results A total of 11 patients were enrolled into three dose levels: 60 (one patient), 120 (six patients) and 180 mg/m^2 (four patients). DLT was observed in three patients, one at 120 mg/m^2 (grade 3 catheter-related infection) and two at 180 mg/m^2 (grade 4 neutropenia lasting for >3 days in one, grade 4 hematological toxicities and grade 4 diarrhea in the other). MTD was determined as 120 mg/m^2. Comparing with those after free-form irinotecan in the literature, the dose-normalized PK of SN-38 (the active metabolite) after PEP02 was characterized by lower C_{max}, prolonged terminal half-life and higher AUC but with significant inter-individual variation. One patient who died of treatment-related toxicity had significantly higher C_{max} and AUC levels of SN-38 than those of the other three patients at 180 mg/m^2. Post hoc pharmacogenetic study showed that the patient had a combined heterozygosity genotype

T. C. Chang and H. S. Shiah have contributed equally to this work.

PEP02 is designated as MM-398 by Merrimack Pharmaceuticals, Inc. (Cambridge, MA, USA).

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of *UGT1A1*6/*28*. Two patients had objective tumor response.

Conclusions PEP02 apparently modified the PK parameters of irinotecan and SN-38 by liposome encapsulation. The MTD of PEP02 monotherapy at 3-week interval is 120 mg/m^2, which will be the recommended dose for future studies.

Keywords Irinotecan sucrosofate · Liposome · PEP02 · MM-398 · Pharmacokinetics · *UGT1A1* gene

Introduction

It has been shown that topoisomerase-I (Topo I) is overexpressed in several cancer types, including breast, lung and colorectal cancers [1]. Irinotecan (CPT-11) is a water-soluble semisynthetic inhibitor of Topo I derived from camptothecin—a plant (*Camptotheca acuminata*) alkaloid, which can be converted to a more potent metabolite, SN-38, by carboxylesterase primarily in the liver. SN-38 can also be inactivated through glucuronidation by UDP-glucuronosyl transferase 1A1 (*UGT1A1*) to form SN-38G, which is mainly eliminated via biliary excretion. Both CPT-11 and SN-38 can bind to Topo I-DNA complex to interfere with the re-ligation of Topo I-induced single-strand DNA breaks and produce double-strand DNA damage during DNA synthesis [2]. Of them, SN-38 is approximately 100 to 1,000 times more potent than the CPT-11 as a Topo I inhibitor. Unfortunately, the metabolic conversions contribute to notable heterogeneities in both toxicity and efficacy of CPT-11, which lead to a rather narrow therapeutic index.

A liposome is a bilayer membrane spherical drug carrier vesicle that enables slow release of encapsulated drug so as to (1) lower drug elimination to prolong systemic circulation time, (2) lower maximum plasma concentration (C_{max}) to reduce drug-associated side effects and (3) preferentially pass through the relatively large vascular pore openings in tumors to enhance its local accumulation in tumor tissue [3]. It has been known that both CPT-11 and SN-38 exist in a pH-dependent equilibrium between an inactive carboxylate form and an active lactone form after intravenous injection, and an acidic pH circumstance, for example in tumor microenvironment, will promote the formation of the active lactone form. Therefore, a liposome-encapsulated formulation will theoretically be able to shift the equilibrium toward more active lactone form formation within tumor tissue to enhance the treatment efficacy of CPT-11.

PEP02 is a novel nanoparticle formulation of irinotecan sucrosofate encapsulated with polyethylene glycolated liposome. The coupling of high molecular weight polyethylene-glycol (PEG) on the surface of PEP02 can effectively protect it from circulating protein binding and subsequent phagocytosis of the reticuloendothelial system to further enhance its circulation time. In preclinical animal studies, PEP02 showed improved preclinical pharmacokinetic properties and anti-tumor activity (in house data) [4]. Herein, we report the results of the first-in-human, phase I trial for PEP02 in patients with refractory advanced solid tumors. The objectives were to identify the maximum tolerated dose (MTD), dose-limiting toxicities (DLT) and safety profile, and to characterize the variables of pharmacokinetics (PK) of PEP02 administered as 90-min infusion every 3 weeks.

Methods

Trial design and patients

This trial was a multi-center, first-in-human, open-label, phase I, dose-escalation study of PEP02 (liposome-encapsulated irinotecan, PharmaEngine, Inc., Taipei, Taiwan), in patients with advanced refractory solid tumors. Patients with histologically confirmed advanced solid tumors that were refractory to standard systemic chemotherapy were eligible. Further inclusion criteria were age ≥20 years, Eastern Cooperative Oncology Group (ECOG) performance score of 0 or 1, life expectancy of more than 12 weeks and adequate bone marrow, hepatic and renal functions within 1 week before commencing treatment (hemoglobin ≥ 10 g/dL, absolute neutrophil count ≥1.5 × 10^3/mL, platelets ≥ 100 × 10^3/mL, serum bilirubin within normal limit, ALT ≤ 2.5× upper limit of normal, creatinine within normal limit). All prior active treatments, including major surgery, chemotherapy, radiotherapy (except palliative) or endocrine therapy, had to be ceased at least 4 weeks, and all treatment-related toxicities had to be resolved to no greater than grade 1 before enrollment. Patients with central nervous system metastases, pregnancy, uncontrolled active infection or other concomitant serious diseases and who had previously received irinotecan were excluded. All patients gave written informed consent. The trial was approved by the independent ethics committee of each participating institute and the Department of Health, Executive Yuan, Taiwan, and performed in accordance with International Conference on Harmonization Good Clinical Practice guidelines, Good Clinical Laboratory Practice and the Declaration of Helsinki.

Treatment and assessment

PEP02 was diluted in 500 ml of 5 % dextrose and delivered as a 90-min intravenous infusion, every 21 days. Infusion time was allowed to be prolonged for acute infusion-associated reactions or any other clinical needs. Pre-medication

included dexamethasone and serotonin-antagonist. Prophylactic anticholinergic agent was not given unless acute cholinergic reaction was observed in prior cycle of treatment. Anti-diarrhea agents were given according to the guideline of American Society of Clinical Oncology. After the infusion of PEP02, vital signs including blood pressure, pulse rate, respiratory rate and body temperature were monitored every 15 min for 3 h. Detailed history evaluation, vital signs recording, physical examination, complete blood count with differential classification and blood biochemistry tests were performed before treatment and weekly throughout treatment. Toxicity was recorded according to the National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 3.0, and a DLT was defined as any of the following events: grade 4 hematological toxicity lasting for longer than 3 days; febrile neutropenia; or >grade 3 non-hematological toxicity (except nausea and vomiting).

The starting dose of PEP02 was 60 mg/m^2 (level I) based on the 1/10 of the LD10 in mice (the dose lethal to 10 % tested mice) and then would be escalated by 100 % (120 mg/m^2, level II), 50 % (180 mg/m2, level III), 33 % (240 mg/m^2, level IV), 25 % (300 mg/m^2, level V), 16.7 % (350 mg/m^2, level VI), and 11.4 % (400 mg/m^2, level VIII), subsequently. The study was in a modified patient cohort accelerated titration design, in which single-patient cohorts for dose levels I–II, two-patient cohorts for levels III–IV and three-patient cohorts for level V or above would be recruited until any DLT was observed in the first cycle [5]. If a patient experienced any DLT, then additional patients would be recruited into that cohort. Dose escalation would be stopped if two or more of the patients experienced any DLT, and the prior dose level would be considered as the MTD. A minimum of six patients were required to be tested at the dose level defined as the MTD.

For patients who experienced grade 4 neutropenia and/or ≥grade 3 non-hematological toxicity, the dose of PEP02 would be reduced by one dose level in their subsequent cycle of treatment. Patients would receive PEP02 for a maximum of six courses, or until the presence of disease progression, unacceptable toxicity, treatment delay for ≥2 weeks, or patient's refusal or death. Patients with tumor response or stable disease after six cycles of treatment could receive further PEP02 therapy in a compassionate use expansion program.

Imaging studies consisting of computed tomography of the abdomen and/or chest were performed before and after every two courses of chemotherapy to evaluate tumor response, which was determined according to the RECIST version 1.0 guidelines [6]. All complete and partial responses required confirmation by two consecutive observations no <4 weeks apart. Patients with a rapid objective or symptomatic progression before the next course of treatment were considered to have progressive disease (PD).

Pharmacogenetic sampling and analyzing

Pharmacokinetic testing was done during the first course of PEP02 administration. Blood samples were collected before treatment, during the infusion at 30 and 60 min, at the end of infusion, and after infusion at 1, 2, 3, 6, 9, 12, 24, 48, 72 and 168 h. Plasma levels of encapsulated irinotecan, total irinotecan and SN-38 were determined by validated LC/MS/MS analytical methods. Pharmacokinetic parameters of individual data sets were analyzed by a non-compartmental model using WinNonlin Professional version 4.1 (Pharsight Corporation, Menlo Park, CA).

Peak concentration in plasma (C_{max}) and the time to achieve C_{max} (T_{max}) were determined directly by a visual analysis of the individual observed plasma concentration versus time curve data. Area under the plasma concentration–time curve from time zero to infinity ($AUC_{0\to\infty}$) was determined by the trapezoidal rule and extrapolated to infinity, which was estimated by the last quantifiable concentration divided by the terminal elimination rate constant λ_Z (K_{el}). λ was determined by a simple log-linear regression based on the last three points of plasma concentration. Total clearance of drug from plasma (Cl) was determined by the dose divided by the AUC. Plasma terminal elimination half-life ($t_{1/2}$) was calculated by dividing λ into the natural logarithm of two. Mean residence time from time zero to infinity ($MRT_{0\to\infty}$) was calculated from the area under the first moment curve from time zero to infinity ($AUMC_{0\to\infty}$) divided by $AUC_{0\to\infty}$. Volume of distribution at steady state (V_{ss}) was determined by MRT × Cl.

Statistical analysis

The association between discrete variables was assessed using Fisher's exact test. The two-tailed Wilcoxon rank sum test was used for the comparison of pharmacokinetic parameters. A value of $p < 0.05$ was considered statistically significant.

Results

Patient characteristics, dose escalation, DLT and MTD

Between January 2005 and August 2005, a total of 11 patients (median age 47, range 41–67 and ECOG PS of 0 or 1) were enrolled. The demographics and baseline characteristics of all patients are listed in Table 1. These patients were enrolled into three dose levels, with 1, 6 and 4 patients in dose level I, II and III, respectively (Table 2). Initially, none of the first two patients who were separately enrolled at dose level I and level II experienced a DLT. In dose level III (180 mg/m^2), because one of the three patients (Patient

Table 1 Patient characteristics

Characteristic	Patients, *n* (%)
Patients enrolled	11
Age (years)	
Median	47
Range	41–67
Sex	
Male	1 (9)
Female	10 (91)
ECOG performance status	
0	6 (55)
1	5 (45)
Tumor type	
Cervical cancer	4 (36)
Breast cancer	2 (18)
Neuroendocrine tumor	2 (18)
Pancreatic cancer	1 (9)
Non-small cell lung cancer	1 (9)
Thymic carcinoma	1 (9)
Previous treatment	
Surgery	9 (82)
Radiotherapy	8 (73)
Chemotherapy	11 (100)

Table 2 Dose-escalation schedule and enrolled patient number

Dose level	Dose (mg/m^2)	Proposed patient numbers	Actual patient numbers
I	60	1	1
II	120	1	6
III	180	2	4
IV	240	2	0
V	300	3	0
VI	350	3	0
VII	400	3	0

#103) developed a DLT (grade 4 neutropenia lasting for longer than 3 days), the study cohort was expanded. The first additionally enrolled patient (Patient #203) also had DLTs (grade 4 febrile neutropenia, grade 4 thrombocytopenia with bleeding event and grade 4 diarrhea) so that dose escalation was stopped and five more patients were enrolled at dose level II (120 mg/m^2). Among the total of 6 patients at dose level II, only one patient (Patient #205) experienced a DLT (grade 3 catheter-related infection); thus, 120 mg/m^2 was determined to be the MTD.

Drug delivery and adverse events

A total of 40 courses of chemotherapy were delivered, with a median of four courses per patient (range, 1–6 courses). Treatment delay and dose modification were required in 5 (45.5 %) and 2 (18.2 %) patients, respectively. Of the latter two patients, one had dose reduction from 180 to 120 mg/m^2 and to 60 mg/m^2 because of hematological toxicities, and the other from 120 to 60 mg/m^2 due to grade 3 nausea and vomiting. Table 3 shows the drug-related adverse events experienced in at least two patients. The most common toxicity observed in the six patients at the MTD dose level (120 mg/m^2) was diarrhea (100 % in all grades, 33 % in grade 3/4) and vomiting (83.3 % in all grades, 66.7 % in grade 3/4). There was no anaphylactic allergic or severe infusion reaction occurred in this phase I study. Only one (#301) patient experienced chest tightness after 30 min of infusion during cycle 2 treatment, but corresponding vital signs were stable. No other infusion reactions were reported on the following cycle.

There was one treatment-related death at dose level III (180 mg/m2). A 67-year-old female patient (Patient #203) with poorly differentiated neuroendocrine tumor (small cell carcinoma) of the pancreas developed severe watery diarrhea and neutropenic fever (WBC and ANC of 360 and 4/mm^3, respectively) 8 days after her first dosing of PEP02. Despite empiric antibiotics, granulocyte-colony stimulating factor (G-CSF) and anti-diarrhea therapy, she died of septic shock, disseminated intravascular coagulopathy and acute respiratory distress syndrome 7 days later. The event was likely related to the alterations of PEP02 PK secondary to the presence of combined heterozygosity of irinotecan metabolism-related genetic polymorphisms of *UGT1A1* as described later.

Pharmacokinetic and exploratory pharmacogenetic studies

The PK of PEP02 is listed in Table 4 and graphed in Fig. 1a, b. The PK parameters of CPT-11 PEP02 dosing, i.e., after 120 mg/m^2, were characterized by slow clearance (mean = 0.0591 L/m^2/h), small volume of distribution (mean = 1.8 L/m^2 $\cong$ plasma volume) and prolonged terminal half-life (mean = 29.5 h). In addition, the plasma concentration–time profile of encapsulated irinotecan (PEP02) in each patient matched approximately with that of total irinotecan (Fig. 2). The results suggest that the release of irinotecan from liposomes occurred slowly over time. The C_{max}, terminal $t_{1/2}$ and AUC of SN-38 after 120 mg/m^2 of PEP02 were 9.2 ± 3.5 ng/mL, 75.4 ± 43.8 h and 710 ± 395 ng*h/mL, respectively. However, the correlations between C_{max} or $AUC_{0-\infty}$ of SN-38 and PEP02 doses were weak (r^2 = 0.423 for C_{max} vs. PEP02 dose; r^2 = 0.0652 for $AUC_{0-\infty}$ vs. PEP02 dose). The elimination of SN-38 was much slower and presented larger inter-individual variability than those of PEP02 and irinotecan. With the small number of patients and interpatient variability, it is difficult to conclude the dose-proportionality PK

Table 3 Drug-related adverse events

	60 mg/m^2 $N = 1$		120 mg/m^2 $N = 6$		180 mg/m^2 $N = 4$	
	All grade N (%)	G 3/4 N (%)	All grade N (%)	G 3/4 N (%)	All grade N (%)	G 3/4 N (%)
Diarrhea	1 (100)	0 (0)	6 (100)	2 (33.3)	4 (100)	1 (25)
Vomiting	1 (100)	0 (0)	5 (83.3)	4 (66.7)	2 (50)	2 (50)
Nausea	1 (100)	0 (0)	4 (66.7)	2 (33.3)	2 (50)	1 (25)
Alopecia	0 (0)	NA	3 (50)	NA	3 (75)	NA
Fatigue	1 (100)	0 (0)	3 (50)	1 (16.7)	1 (25)	0 (0)
Leukopenia	0 (0)	0 (0)	2 (33.3)	1 (16.7)	2 (50)	2 (50)
Neutropenia	0 (0)	0 (0)	2 (33.3)	1 (16.7)	2 (50)	2 (50)
Weight decreased	1 (100)	0 (0)	2 (33.3)	0 (0)	1 (25)	0 (0)
Dizziness	0 (0)	0 (0)	2 (33.3)	0 (0)	0 (0)	0 (0)
Anemia	0 (0)	0 (0)	1 (16.7)	0 (0)	1 (25)	0 (0)
Anorexia	0 (0)	0 (0)	1 (16.7)	0 (0)	1 (25)	0 (0)
Electrolyte imbalance	0 (0)	0 (0)	1 (16.7)	0 (0)	1 (25)	0 (0)

of CPT-11 between the three dose levels. However, higher AUC was observed at higher dose levels.

The $AUC_{0-\infty}$ and C_{max} of CPT-11 and SN-38 of the patient who died of treatment-related complications (Patient #203) were investigated as shown in Fig. 3a, b. The $AUC_{0-\infty}$ and C_{max} of SN-38 of patient #203 were 2–3 folds higher than those of the other three patients receiving the same dose of treatment. To explore the potential genetic background for the differences, the irinotecan metabolism-related genetic polymorphisms of patients receiving 180 mg/m^2 were determined after the approval of IRB. Three of the four patients with available stocked peripheral blood mononuclear cells were included in the pharmacogenetic study, which showed that the patient (Patient #203) who died of treatment-related toxicity had combined heterozygosity of *UGT1A1*6/*28* (Table 5).

Efficacy

The best tumor response was partial response in two out of 11 intent-to-treat (ITT) patients (18.2 %) or out of ten response evaluable patients (20 %). One patient with pancreatic cancer who failed to several lines of treatment including gemcitabine and infusional 5FU/LV alone or in combination with oxaliplatin had PEP02 at the dose of 180 mg/m^2, and the other one with cervical cancer whose tumor relapsed after cisplatin-based concurrent chemoradiotherapy had PEP02 at the dose of 120 mg/m^2. Another three patients with breast cancer, pancreatic neuroendocrine tumor and thymic carcinoma in each had stable disease. Therefore, the disease control rate was 45.5 % for ITT population or 50 % for response evaluable patients. At the MTD dose of 120 mg/m^2, 5 out of 6 patients were evaluable for tumor response. One patient (#205) with squamous cell carcinoma of the lung who was early off-studied because of prolonged treatment interruption secondary to DLT (grade 3 catheter-related infection) was excluded from response evaluation. The response rate and disease control rate of evaluable patients were 20 and 60 %, respectively. However, the unevaluable patients (#205) received five additional courses of PEP02 at 120 mg/m^2 after adequate infection control and achieved a partial response under the compassionate use program.

Discussion

The current study has established the MTD, safety profile, PK and preliminary efficacy of PEP02, a nanoliposomal formulation of irinotecan, in patients with refractory advanced cancer. Myelosuppression and diarrhea were the major DLTs, and 120 mg/m^2 was defined as the MTD. The toxicity pattern seems to be comparable with that of free-form irinotecan [7, 8]. Of note, in the absence of prophylactic atropine administration, there was only one episode of grade 1 acute cholinergic syndrome (abdominal pain) observed in this study, as compared to the occurrence in 9 of 23 patients who received free irinotecan ≥240 mg/m^2 in a phase I trial [9]. It has been shown that irinotecan may inhibit acetyl-cholinesterase to enhance parasympathetic discharge, and the frequency and severity of cholinergic syndrome are likely irinotecan concentration dependent [10, 11].

In the current study, pharmacokinetic analysis demonstrated that the plasma concentration–time profile of PEP02 (encapsulated irinotecan) in each patient matched approximately with that of total irinotecan, indicating that the release of free-form irinotecan from the nanoliposomes occurred slowly over time. We were not able to measure

Table 4 Pharmacokinetic parameters of PEP02 at each dose level

	Dose level (mg/m^2)	C_{max} (μg /mL) CPT-11(ng / mL) SN-38	T_{max} (hr)	$AUC_{0-169.5}$ (hr-μg/mL) CPT-11 (hr-ng/mL) SN-38	$AUC_{0-\infty}$ (hr-μg/mL) CPT-11 (hr-ng/mL) SN-38	V_{ss} (L/m^2)	Cl (L/hr/m2)	$t_{1/2}$ (hr)	$MRT_{0-\infty}$ (hr)
Total CPT-11	60, $N = 1$	31.8	1.5	222	223	3.56	0.269	28.7	13.2
	120, $N = 6$	79.4 ± 13.9	2.5 ± 1.1	2,835 ± 1,817	2,963 ± 1,947	1.8 ± 0.771	0.0591 ± 0.0367	29.5 ± 17.2	38.6 ± 19.5
	180, $N = 4$	102 ± 17.6	1.75 ± 0.5	1,945 ± 1,029	1,963 ± 1,035	1.97 ± 0.342	0.119 ± 0.0703	22.3 ± 11.5	20.5 ± 9.47
SN-38	60, $N = 1$	2.58	3.6	38.4	NC	NA	NA	NC	NC
	120, $N = 6$	9.20 ± 3.50	21.9 ± 26.3	710 ± 395	997 ± 680	NA	NA	75.4 ± 43.8	109.0 ± 54.4
	180, $N = 4$	14.3 ± 6.16	21.0 ± 9.0	1,159 ± 969	1,425 ± 1,134	NA	NA	58.0 ± 32.8	90.9 ± 43.1

Mean ± SD; C_{max} peak concentration in plasma; T_{max} time to achieve peak plasma concentration; $AUC_{0-169.5}$, $AUC_{0-\infty}$ area under the plasma concentration–time curve from time zero to 169.5 h and infinity, respectively; V_{ss} volume of distribution at steady state; $t_{1/2}$ plasma terminal elimination half-life; *Cl* total clearance of drug from plasma; $MRT_{0-\infty}$ mean residence time from time zero to infinity; *NC* not calculated because there was no distinct terminal log-linear phase for the λ_z determination; *NA* not available

plasma level of free CPT-11 directly because it was below the lower detection limit of the LC/MS/MS assay. The slow release of irinotecan from PEP02 resulted in small volume of distribution (mean = 1.8 $L/m^2 \cong$ plasma volume), slow clearance and prolonged terminal half-life of circulating total irinotecan, and a favorable PK of its active metabolite, SN-38. Comparing the PK of SN-38 in this study with the published studies following administration of 125 mg/m^2 free-form irinotecan in the literature, the PK parameters of SN-38 after 120 mg/m^2 of PEP02 showed lower C_{max} (9.2 ± 3.5 vs 26.3 ± 11.9 ng/mL), longer terminal $t_{1/2}$ (75.4 ± 43.8 vs 10.4 ± 3.1 h) and higher AUC (710 ± 395 vs 229 ± 108 ng*h/mL) [12, 13]. The AUC of SN-38 after 120 mg/m^2 PEP02 was roughly comparable with that achievable with 300–350 mg/m^2 of "conventional" irinotecan in the literature. The lower toxicity profile potentially makes PEP02 a better agent to combine with other cytotoxic agents, i.e., 5-fluorouracil and folinic acid, and/or targeted agents, i.e., bevacizumab or cetuximab for advanced colorectal cancer. However, the optimal dosages of PEP02 for such combinations remain to be determined.

The correlations between the C_{max} or $AUC_{0-\infty}$ of SN-38 and doses of PEP02 were weak in this phase I study. The elimination of SN-38 was slow and exhibited significant inter-individual variation after administration of PEP02. The reason for such inter-individual variation in kinetic behavior of SN-38 after PEP02 administration is not yet fully explored, but pharmacogenetic variability of irinotecan metabolism-related enzymes is likely to be involved. The presence of the *UGT1A1*28* allele has been shown to cause a 70 % reduction in the expression of UGT, the enzyme responsible for glucuronidation of SN-38 into inactive SN-38 glucuronide (SN-38G). This reduction leads to increased exposure of patients to the cytotoxic metabolite, SN-38 [14, 15]. Clinically, patients with either heterozygous *UGT1A1*1/*28* or homozygous *UGT1A1*28/*28* genotypes are more prone to severe irinotecan-associated toxicity, notably grade 3–4 neutropenia and/or diarrhea [15]. Based on these findings, the

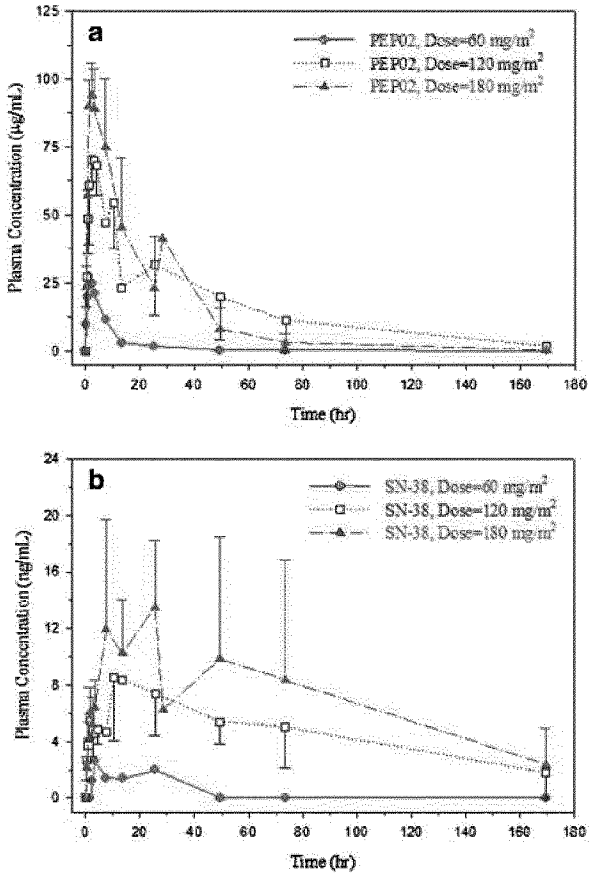

Fig. 1 Plasma concentration–time profiles of **a** encapsulated CPT-11 (PEP02) and **b** SN-38 at 60, 120 and 180 mg/m^2 dose level of PEP02

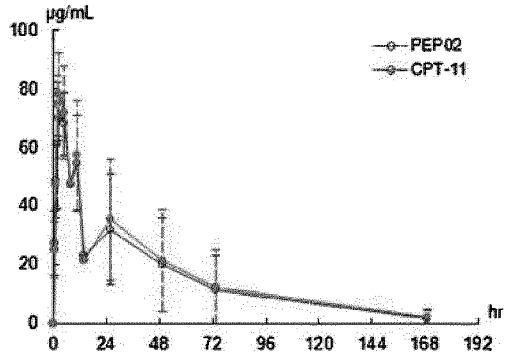

Fig. 2 Plasma concentration–time profiles of encapsulated CPT-11 (PEP02) and total CPT-11 at 120 mg/m^2 dose level of PEP02

US Food and Drug Administration revised the label of irinotecan and recommended that patients who are known to be homozygous for the *UGT1A1*28* allele should receive a reduced initial dose of irinotecan to minimize the risk of significant toxicity [16]. However, ethnic differences in *UGT1A1* allele frequencies are well established, and the Asian population is known to have a lower frequency of the *UGT1A1*28* allele (13.9 % vs 33.4 % in Caucasians) but a significantly higher frequency of *UGT1A1*6* (13.0 % vs 0.5 % in Caucasians) [15]. It has been reported that patients with a combined heterozygosity of *UGT1A1*6* and **28* were more prone to develop toxicities after irinotecan injection, as happened in one of our patients [17, 18]. Comparing the PK of SN-38 in the four patients receiving 180 mg/m^2 of PEP02, the C_{max} and $AUC_{0-\infty}$ of SN-38 of the patient (#203) who died of grade 4 diarrhea, neutropenia and infection were almost threefold higher than in the other three patients.

Other liposome formulations of irinotecan or SN-38 have also been developed. IHL-305 (pegylated liposomal irinotecan) has been identified in its MTD at every 4 week and every 2 week schedule as 160 and 80 mg/m^2, respectively, in a phase I study [19]. The $AUC_{0-\infty}$ of SN-38 at 160 mg/m^2 was 360 ± 370 ng*h/mL, and one PR and two SD were observed among the 60 patients recruited. PEP02 at 120 mg/m^2 showed higher SN-38 exposure than IHL-305 at 160 mg/m^2. LE-SN38 is a liposome-encapsulated SN-38, which had been developed in phase II stage. The MTD of LE-SN38 was identified in its phase I study as 35 mg/m^2 every 3 weeks for both the *UGT1A1*28* wild-type and heterozygous patients [20]. The $AUC_{0-\infty}$ of SN-38 at the MTD for the wild-type and heterozygous patients were 1,751.8 and 3,493.6 ng*h/mL, respectively. Notwithstanding LE-SN38 has relatively high SN-38 AUC, unfortunately, it did not meet the pre-specified activity criteria in its phase II CALGB 80402 study for mCRC patients [21].

Although antitumor activity was not the primary endpoint in this phase I trial, two patients with partial response and three patients with stable disease were observed out of 11 ITT patients. Notably, one patient (#205) who developed non-drug-related toxicity after the first course and received five additional courses under compassionate use program also had partial response. Several researches investigating the efficacy of PEP02 with or without other anticancer drugs are currently ongoing. In conclusion, the MTD of PEP02 given every 3 weeks is 120 mg/m^2, and major treatment-related DLTs are myelosuppression and diarrhea. Promising anti-tumor activities that were observed in the patients who were refractory to available treatments warrant further clinical investigations.

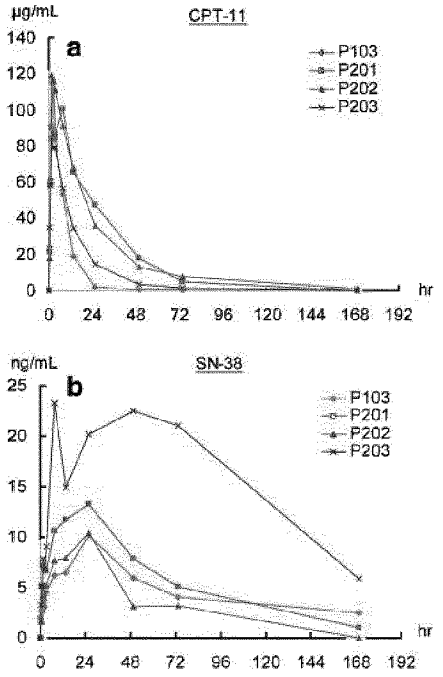

Fig. 3 Plasma concentration–time profiles of **a** total CPT-11 and **b** SN-38 in subjects at 180 mg/m^2 dose level of PEP02

Table 5 Pharmacokinetic parameters of SN-38 and pharmacogenetic data of patients received 180 mg/m^2 of PEP02

Patient unique number	$AUC_{0-\infty}$ (hr-ng/mL)	C_{max} (ng /mL)	$t_{1/2}$ (hr)	UGT1A1*6	UGT1A1*28
201	906	13.3	41.4	W/W	W/W
202	549	10.4	28.2	W/W	W/W
203	3,084	23.3	59.0	V/W	V/W
103	1,159	10.2	104	ND	ND

V variant; *W* wild type

ND not done

Acknowledgments This study was supported by PharmaEngine, Inc., Taipei, Taiwan. We thank the patients and their families who participated in this phase I study and also thank the medical and nursing staff of the investigational sites for the care and support of the patients in this study.

Conflict of interest T. C. Chang has received an honorarium from PharmaEngine for an advisory board. H. S. Shiah, C. H. Yang, K. H. Yeh and A. L. Cheng, J. Y. Chang, N. J. Chiang report no conflict of interests. B. N. Shen, Y. W. Wang and C. G. Yeh are full-time employees of PharmaEngine. L. T. Chen has received an honorarium from PharmaEngine for an advisory board.

Ethical standard All patients gave written informed consent prior their inclusion in the study. The trial was approved by the independent ethics committee of each participating institute and the Department of Health, Executive Yuan, Taiwan, and performed in accordance with International Conference on Harmonization Good Clinical Practice guidelines, Good Clinical Laboratory Practice and the Declaration of Helsinki.

References

1. Costin D, Potmesil M (1994) Preclinical and clinical development of camptothecins. Adv Pharmacol 29B:51–72
2. Iyer L, King CD, Whitington PF, Green MD, Roy SK, Tephly TR, Coffman BL, Ratain MJ (1998) Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. J Clin Invest 101:847–854
3. Zamboni WC, Jung LL, Egorin MJ, Hamburger DR, Joseph E, Jin R, Strychor S, Ramanathan RK, Eiseman JL (2005) Relationship between plasma exposure of 9-nitrocamptothecin and its 9-aminocamptothecin metabolite and antitumor response in mice bearing human colon carcinoma xenografts. Clin Cancer Res 11:4867–4874
4. Drummond DC, Noble CO, Guo Z, Hong K, Park JW, Kirpotin DB (2006) Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. Cancer Res 66:3271–3277
5. Simon R, Freidlin B, Rubinstein L, Arbuck SG, Collins J, Christian MC (1997) Accelerated titration designs for phase I clinical trials in oncology. J Natl Cancer Inst 89:1138–1147
6. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Glabbeke MV, Oosterom ATV, Christian MC, Gwyther SG, European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada (2000) New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 92:205–216
7. Cunningham D, Pyrhönen S, James RD, Punt CJ, Hickish TF, Heikkila R, Johannesen TB, Starkhammar H, Topham CA, Awad L, Jacques C, Herait P (1998) Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet 352:1413–1418
8. Fuchs CS, Moore MR, Harker G, Villa L, Rinaldi D, Hecht JR (2003) Phase III comparison of two irinotecan dosing regimens in second-line therapy of metastatic colorectal cancer. J Clin Oncol 21:807–814
9. Rowinsky EK, Grochow LB, Ettinger DS, Sartorius SE, Lubejko BG, Chen TL, Rock MK, Donehower RC (1994) Phase I and pharmacological study of the novel topoisomerase I inhibitor 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11) administered as a ninety-minute infusion every 3 weeks. Cancer Res 54:427–436
10. Harel M, Hyatt JL, Brumshtein B, Morton CL, Yoon KJ, Wadkins RM, Silman I, Sussman JL, Potter PM (2005) The crystal structure of the complex of the anticancer prodrug 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin (CPT-11) with Torpedo californica acetylcholinesterase provides a molecular explanation for its cholinergic action. Mol Pharmacol 67:1874–1881
11. Blandizzi C, De Paolis B, Colucci R, Lazzeri G, Baschiera F, Del Tacca M (2001) Characterization of a novel mechanism accounting for the adverse cholinergic effects of the anticancer drug irinotecan. Br J Pharmacol 132:73–84
12. Rothenberg ML, Kuhn JG, Burris HA 3rd, Nelson J, Eckardt JR, Tristan-Morales M, Hilsenbeck SG, Weiss GR, Smith LS, Rodriguez GI (1993) Phase I and pharmacokinetic trial of weekly CPT-11. J Clin Oncol 11:2194–2204
13. Rivory LP, Haaz MC, Canal P, Lokiec F, Armand JP, Robert J (1997) Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. Clin Cancer Res 3:1261–1266
14. Bosma PJ, Chowdhury JR, Bakker C, Gantla S, de Boer A, Oostra BA, Lindhout D, Tytgat GN, Jansen PL, Elferink RP, Chowdhury NR (1995) The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. N Engl J Med 333:1171–1175
15. Palomaki GE, Bradley LA, Douglas MP, Kolor K, Dotson WD (2009) Can UGT1A1 genotyping reduce morbidity and mortality in patients with metastatic colorectal cancer treated with irinotecan: an evidence-based review. Genet Med 11:21–34
16. Camptosar brand of irinotecan hydrochloride injection [package insert]. Pfizer, 2012
17. Araki K, Fujita K, Ando Y, Nagashima F, Yamamoto W, Endo H, Miya T, Kodama K, Narabayashi M, Sasaki Y (2006) Pharmacogenetic impact of polymorphisms in the coding region of the UGT1A1 gene on SN-38 glucuronidation in Japanese patients with cancer. Cancer Sci 97:1255–1259
18. Minami H, Sai K, Saeki M, Saito Y, Ozawa S, Suzuki K, Kaniwa N, Sawada J, Hamaguchi T, Yamamoto N, Shirao K, Yamada Y, Ohmatsu H, Kubota K, Yoshida T, Ohtsu A, Saijo N (2007) Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. Pharmacogenet Genomics 17:497–504
19. Infante JR, Keedy VL, Jones SF, Zamboni WC, Chan E, Bendell JC, Lee W, Wu H, Ikeda S, Kodaira H, Rothenberg ML, Burris HA III (2012) Phase I and pharmacokinetic study of IHL-305 (PEGylated liposomal irinotecan) in patients with advanced solid tumors. Cancer Chemother Pharmacol 5:699–705
20. Kraut EH, Fishman MN, Lorusso PM, Gordon MS, Rubin EH, Haas A, Fetterly GJ, Cullinan P, Dul JL, Steinberg JL (2005) Final results of a phase I study of liposome encapsulated SN-38 (LE-SN38): safety, pharmacogenomics, pharmacokinetics, and tumor response [abstract 2017]. ASCO annual meeting
21. Ocean AJ, Niedzwiecki D, Atkins JN, Parker B, O'Neil BH, Lee JW, Wadler S, Goldberg RM (2008) LE-SN38 for metastatic colorectal cancer after progression on oxaliplatin: results of CALGB 80402 [abstract 4109]. ASCO annual meeting

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DEVELOPMENTAL THERAPEUTICS-CLINICAL PHARMACOLOGY AND IMMUNOTHERAPY

Phase I study of liposome irinotecan (PEP02) in combination with weekly infusion of 5-FU/LV in advanced solid tumors.

L. Chen, H. Shiah, T. Chao, R. K. Hsieh, G. Chen, J. Chang...

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Abstract

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Background: PEP02 is a novel nanoparticle liposome formulation of irinotecan (CPT-11) that has improved PK and tumor biodistribution of CPT-11 and its active metabolite-SN38 with encouraging safety and tumor response in preclinical studies and a single-agent phase I study. The study is to define the DLT, MTD, and PK of PEP02 when in combination with high-dose

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fluorouracil/leucovorin (HDFL) in patients (pt) with advanced solid tumors.

Methods: Pts who had failed to standard chemotherapy, ECOG PS 0-1 and adequate organ functions, no prior CPT-11, were eligible. PEP02 was given as 90 mins i.v. infusion on D1 in combination with 24-hr infusion of 5FU (2,000 mg/m^2)/ LV (200 mg/m^2) on D1 and D8, every 3 weeks. Cohorts of 3-6 pts were treated at 60, 80, 100, and 120 mg/m^2. PK and PGx samples were collected.

Results: A total of 16 pts were enrolled, with 3, 6, 5, and 2 at 60, 80, 100, and 120 mg/m^2. DLTs were observed in 4 pts, including 2 each at 100 and 120 mg/m^2 dose levels. DLTs were mainly G3 diarrhea and G4 hematologic toxicities. MTD was determined as 80 mg/m^2. Grade 3 or above adverse events at the MTD dose and all dose levels were 10.6% and 18.4%, respectively. The PK of total CPT-11 after PEP02 (at 80 mg/m^2) in combination with HDFL was characterized by low clearance (mean = 116.4 mL/m^2/hr) and small volume of distribution (mean = 2.93 L/m^2, similar to plasma volume) as did of PEP02 monotherapy study. Compared to the PK of SN-38 after 250 mg/m^2 of CPT-11 (in combination with capecitabine, Ann Oncol 2005; 16: 1123-32), the C_{max} after 80 mg/m^2 of PEP02 was lower (7.98 ± 4.39 *vs* 62.0 ± 37.4 ng/mL), but the $AUC_{0\rightarrow t}$ was similar (354.77 ± 145.35 *vs* 396 ± 247 ng×h/mL). The correlation of UGT1A family with PK and toxicity was not observed. However, the only subject with the coexistence of two variants of *UGT1A1*6* and **28* had higher dose-normalized $AUC_{SN\text{-}38}$and experienced DLT. The best response of 15 evaluable pts was PR in 2 (gastric cancer and breast cancer) and SD in 9.

Conclusions: The MTD of PEP02 in combination with HDFL given every-3-week is 80 mg/m^2. The observation of tumor response in two heavily pre-treated patients suggests the combination deserves further exploration in advanced solid tumor patients who are refractory to standard therapy.

Author Disclosure

Employment or Leadership Position	Consultant or Advisory Role	Stock Ownership	Hc	Yes / No	ıert	Other Remuneration
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Modified FOLFIRINOX regimen with improved safety and maintained efficacy in pancreatic adenocarcinoma

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PMID: 24152956 DOI: 10.1097/MPA.0b013e31829e2006

Abstract

Objectives: FOLFIRINOX (5-fluorouracil [5-FU], oxaliplatin, and irinotecan) as compared with gemcitabine in pancreatic cancer (PC) has superior activity and increased toxicity. The bolus 5-FU contributes to the toxicity. We hypothesized that the elimination of bolus 5-FU and use of hematopoietic growth factor will improve the safety profile without compromising the activity of FOLFIRINOX.

Methods: Sixty patients with PC treated with modified FOLFIRINOX (no bolus 5-FU) were reviewed. Patients were divided into metastatic or nonmetastatic (locally advanced or borderline resectable) disease. Toxicity, response rate, progression-free survival, and overall survival were evaluated.

Results: Nonmetastatic and metastatic disease were present in 24 (40%) and 36 (60%) patients, respectively. The incidence of grade 4 neutropenia, grade 3/4 diarrhea, and fatigue were 3%, 13%, and 13%, respectively. Response rate was 30%. The median progression-free survival for nonmetastatic disease was 13.7 months (95% confidence interval [CI], 9.6-24.6 months), and that for metastatic disease was 8.5 months (95% CI, 3.7-11.0 months), respectively. The median overall survival for nonmetastatic disease was 17.8 months (95% CI, 9.9 months to not estimable), and that for metastatic disease was and 9.0 months (95% CI, 7.1 months to not estimable), respectively.

Conclusions: Modified FOLFIRINOX has an improved safety profile with maintained efficacy in metastatic PC. Modified FOLFIRINOX has promising activity in nonmetastatic disease.

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Development of a Highly Active Nanoliposomal Irinotecan Using a Novel Intraliposomal Stabilization Strategy

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Abstract

Liposome formulations of camptothecins have been actively pursued because of the potential for significant pharmacologic advantages from successful drug delivery of this important class of anticancer drugs. We describe nanoliposomal CPT-11, a novel nanoparticle/liposome construct containing CPT-11 (irinotecan) with unprecedented drug loading efficiency and *in vivo* drug retention. Using a modified gradient loading method featuring a sterically hindered amine with highly charged, multivalent anionic trapping agents, either polymeric (polyphosphate) or nonpolymeric (sucrose octasulfate), liposomes were capable of entrapping CPT-11 at extremely high drug-to-lipid ratios (>800 g CPT-11/mol phospholipid) and retaining encapsulated drug *in vivo* with a half-life of drug release in the circulation of 56.8 hours. CPT-11 was also protected from hydrolysis to the inactive carboxylate form and from metabolic conversion to SN-38 while circulating. The maximum tolerated dose in normal mice was determined to be 80 mg/kg for free CPT-11 and >320 mg/kg for nanoliposomal CPT-11. Nanoliposomal CPT-11 showed markedly superior efficacy when compared with free CPT-11 in human breast (BT474) and colon (HT29) cancer xenograft models. This study shows that intraliposomal stabilization of CPT-11 using a polymeric or highly charged, nonpolymeric polyanionic trapping agent results in a markedly active antitumor agent with low toxicity. (Cancer Res 2006; 66(6): 3271-7)

Introduction

Liposome-based systems have been used to enhance efficacy and/or ameliorate toxicity of certain drugs (1, 2). Thus far, the most successful approach has involved constructs engineered for long circulation times, combined with stable encapsulation of the active compound within the liposome; this allows liposomes to accumulate at sites of cancer, followed by intratumoral drug release. An example is PEGylated liposomal doxorubicin (3), which has received Food and Drug Administration approval for cancer treatment. However, the successful case of liposomal anthracyclines has not yet been matched by liposome constructs containing other anticancer drug classes, although recent progress has been made with vincristine (4–6) and certain camptothecin analogues (7–9). One of the key reasons for this has been the technical facility with which anthracyclines can be stably encapsulated in the liposome interior using remote-loading methodologies (10, 11), giving rise to stable liposome formulations that have been difficult to replicate with other classes of drugs.

CPT-11 {irinotecan; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycampothecin} is a water-soluble camptothecin derivative currently used in cancer chemotherapy. The pharmacology of CPT-11 is complex, with extensive metabolic conversions involved in the activation, inactivation, and elimination of the drug (12, 13). CPT-11 is a prodrug that is converted by nonspecific carboxylesterases into a 100- to 1,000-fold more active metabolite, SN-38 (14). SN-38 is cleared via glucuronidation, for which major pharmacogenetic differences have been shown (15), and biliary excretion. In addition, CPT-11 and other camptothecins exist in a pH- and serum protein-dependent equilibrium between an active lactone form of the drug (predominant under acidic conditions) and an inactive carboxylate form (predominant at neutral or basic pH; ref. 16). These drug properties contribute to the marked heterogeneities in efficacy and toxicity observed clinically with CPT-11 (12, 17). Hence, drug carrier technologies represent a rational strategy to improve the pharmacokinetics and biodistribution of CPT-11 while protecting it from premature metabolism.

In this report, we describe a novel intraliposomal drug stabilization technology for encapsulation of CPT-11 into long-circulating liposome-based nanoparticles with high drug load and high *in vivo* stability, matching or surpassing previous liposomal drugs. This was achieved using polymeric or nonpolymeric highly charged anions, polyphosphate or sucrose octasulfate, as intraliposomal trapping agents in conjunction with a high-pK_a polyalkylamine gradient. The approach also allowed for preservation of the drug in its active lactone form within the liposome interior, protecting it from hydrolysis as well as premature conversion to SN-38. Here we use the term "nanoliposomal drug" to describe a nanoparticle consisting of a lipid bilayer scaffold encapsulating a nanoscale drug complex or aggregate that facilitates *in vivo* drug retention.

Materials and Methods

Liposome Preparation and Drug Loading

Solutions of triethylammonium salts of a linear poly(phosphate) (TEA-Pn, 13-18 phosphate units; Sigma Corp., St. Louis, MO) and sucrose octasulfate (TEA_8SOS) were prepared from commercially obtained sodium salts (Toronto Research Chemicals, Inc., North York, Ontario, Canada) by ion-exchange chromatography on the Dowex 50Wx8-200 resin in the H^+ form, immediately followed by titration with neat triethylamine. Residual sodium in either solution, as determined by potentiometry using a Na^+-selective electrode, was <1% of the cation content. Phosphate content was determined by inorganic phosphate assay following acid hydrolysis and was adjusted to 0.55 mol/L for TEA-Pn (osmolality, 430-480 mmol/kg). The TEA concentration was calculated from the amount of added TEA and was

Note: Z. Guo is presently at First Affiliated Hospital of Jinan University, Guangzhou, P.R. China.

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adjusted to 0.65 mol/L for TEA_8SOS solution (osmolality, 480-530 mmol/kg). The final pH for both solutions was 5.5 to 6.0.

Distearoylphosphatidylcholine (3 mol. parts), methoxypoly(ethylene)glycol (PEG2000)-derivatized distearoylphosphatidylethanolamine (0.015 mol. parts; Avanti Polar Lipids, Alabaster, AL), and cholesterol (2 mol. parts; Calbiochem, La Jolla, CA) were combined in ~ 50% (w/v) ethanolic solution and mixed with 10 volumes of the solution of TEA-Pn or TEA_8SOS at 60°C to 65°C. For pharmacokinetic studies, a nonexchangeable lipid label, [^{3}H] cholesteryl hexadecyl ether (Perkin-Elmer, Boston, MA), was added to the lipids in the amount of 0.5 mCi/mmol phospholipid. The lipid suspension was extruded 15 times through two stacked polycarbonate membranes (Nucleopore, Corning-Costar, Acton, MA) with 0.08-μm pore size using argon pressure at 60°C to 65°C. The extruded liposomes were 88 to 95 nm in diameter by dynamic light scattering.

Unentrapped triethylammonium polyanions were removed by chromatography on a Sepharose CL-4B size exclusion column eluted with HEPES-buffered dextrose (5 mmol/L HEPES, 5% dextrose, pH 6.5). CPT-11·HCl (kindly provided by TTY Biopharmaceuticals, Taipei, Taiwan) was added to the liposomes at a ratio of 500 g CPT-11/mol phospholipid and the pH adjusted to 6.5. The resulting solution was heated to 60°C for 30 minutes and then quenched on ice for 15 minutes. Unencapsulated CPT-11 was subsequently removed using a Sephadex G-75 column eluted with HEPES-buffered saline (5 mmol/L HEPES, 145 mmol/L NaCl, pH 6.5). The loading efficiency was determined in all preparations by quantitating both drug and phospholipid and comparing the resulting drug/phospholipid ratio to its input value. CPT-11 was determined spectrophotometrically at 372 nm in acid/methanol (20 volume % 0.5 mol/L phosphoric acid/80 volume % methanol). Phospholipid was quantitated using a standard phosphate assay (18).

Pharmacokinetic Studies

Female Sprague-Dawley rats (190-210 g) with indwelling central venous catheters were injected with a 0.2 to 0.3 mL bolus of ^{3}H-CHE-labeled CPT-11 liposomes (10 mg/kg). Blood samples (0.2-0.3 mL) were drawn at various times postinjection using a heparin-treated syringe. The withdrawn blood volume was replaced using PBS. Blood samples were diluted with 0.3 mL of ice-cold PBS containing 0.04% EDTA, weighed, and centrifuged. Plasma was assayed for CPT-11 [by fluorometry or high-performance liquid chromatography (HPLC)] and for liposome label (scintillation radioactivity counting). The percent of drug remaining in the liposomes was calculated by dividing the drug/lipid ratio in the blood samples by that of the injected liposomes (taken as 100%). Because free CPT-11 is cleared at a much faster rate than liposomes (Fig. 3*A*), a change in the CPT-11-to-liposomal lipid ratio was indicative of drug leakage from the carrier. Noncompartmental pharmacokinetics data analysis was done using PK Solutions 2.0 software (Summit Research Services, Montrose, CO).

Drug Stability and Metabolism Studies

Liposomal and free CPT-11 were administered i.v. at a dose of 25 mg/kg in female albino rats (180-220 g) as above, and blood samples were withdrawn at intervals up to 48 hours. The blood samples were mixed with ice-cold PBS containing 0.04% EDTA and quickly centrifuged. The plasma was assayed for CPT-11, SN-38, and their carboxylate forms by HPLC using a modification of the method of Warner and Burke (19). Briefly, samples were extracted with 400 μL of ice-cold methanol by vortexing and centrifugation at 14,100 × *g* for 5 minutes. The mobile phase consisted of 3% triethylammonium acetate pH 5.5 (solution A) and acetonitrile (solution B) delivered at 1.0 mL/min in a linear gradient of 20 volume % A to 50 volume % B in 14 minutes. The eluted products were detected by fluorescence with an excitation at 375 nm and emission at 500 nm. The retention times were 5.3 minutes (CPT-11 carboxylate), 6.8 minutes (SN-38 carboxylate), 9.3 minutes (CPT-11), and 11.0 minutes (SN-38).

Conversion of CPT-11 to SN-38 was assayed in macrophages isolated from the peritoneum of female NCR *nu/nu* mice and plated at a density of 150,000 cells per well in a 12-well plate. After 24 hours, nanoliposomal CPT-11 was added to macrophages at a concentration of 10 μg CPT-11/mL and incubated for 24 hours in RPMI 1640 with 10% FCS. At indicated times, the medium was removed and the cells washed twice with Hanks buffered saline. The cells were treated with 0.2 mL of 1% Triton X-100 at room temperature for 5 minutes and solubilized in 0.8 mL of 80 volume % methanol/20 volume % 0.1 mol/L H_3PO_4 with shaking for an additional 5 minutes. The cell debris was removed by centrifugation at 13,000 rpm for 10 minutes and the supernatant was assayed by HPLC as described above.

Acute Toxicity Studies

The maximum tolerated dose following single i.v. administration was evaluated in healthy female Swiss Webster mice following a protocol adapted from the protocol communicated by the National Cancer Institute (NCI) Developmental Therapeutics Program. Briefly, in the first range-seeking step, the drug was administered via the tail vein in groups of two mice, beginning with the dose of 60 mg/kg CPT-11 and continuing with the dose escalation factor of 1.8 until acute mortality or terminal morbidity (within 1 day postinjection) was observed in any animal. The second range-seeking step was similarly done using a dose escalation factor of 1.15 and starting with the highest dose at which no mortality or terminal morbidity was observed (the highest tolerated dose) in the first step. Finally, in a validation step, a group of five mice were injected at the highest tolerated dose achieved in the second step and followed for up to 11 days for signs of general health daily and body weight twice a week. If during the observation period there was no mortality, irreversible (terminal) morbidity, or weight loss in excess of 15% of the preinjection body weight, the administered dose was considered the acute single injection maximum tolerated dose.

Antitumor Efficacy Studies

BT474 tumor model. NCR *nu/nu* athymic female mice (4-6 weeks old; Taconic Farms, Germantown, NY) were s.c. implanted at the base of tail with 60-day sustained release 0.72-mg 17β-estradiol pellets (Innovative Research of America, Inc., Sarasota, FL). Two days later, 2×10^7 BT474 human breast cancer cells were implanted s.c. in the upper back area as a 0.1-mL suspension. Tumor growth was measured by caliper along the largest (length) and smallest (width) axes twice a week. Tumor volumes were calculated using the following formula (20): tumor volume = [(length) × (width)^2] / 2. At day 13 posttumor implantation (mean tumor volume, 200 mm^3), animals were randomized to three treatment groups of 13 to 15 animals per group and treated via i.v. (tail vein) injection as described in the text. The study was continued until day 60, which also represented the duration of estrogen supplementation. Animals were weighed twice weekly. If tumors reached 20% of the mouse body weight, the animals were euthanized.

HT29 tumor model. NCR *nu/nu* athymic male mice (6-week-old, weight >16 g; Charles River, Wilmington, MA) were injected s.c. in the right flank with 0.1-mL suspensions containing 5×10^6 HT-29 human colon cancer cells. Eleven days later (mean tumor volume, 150-350 mm^3), mice were randomized to six treatment groups of 11 animals per group. Starting on day 13, the animals received four tail vein injections at intervals of 4 days of various treatments as described in the text.

Results

Preparation of nanoliposomal CPT-11. A proposed novel process using a polyalkylammonium salt of a polymeric (polyphosphate) or nonpolymeric (sucrose octasulfate) highly charged multivalent anion as intraliposomal trapping agents resulted in improvement of both the encapsulation efficiency and the *in vivo* stability of the liposome-encapsulated weakly basic, amphipathic drug CPT-11. The process may involve the formation of an intraliposomal drug-polyanion complex (Fig. 1). Sucrose octasulfate is a high-charge density molecule with one strongly acidic, negatively charged sulfate group per 1.5 carbon atoms. The triethylammonium component of the salt assists drug loading as well, ensuring the charge neutrality of the liposome interior by allowing the efflux of cations accompanying the influx of the drug and possibly by formation of a self-perpetuating pH gradient to provide a driving force for progressive drug accumulation (10).

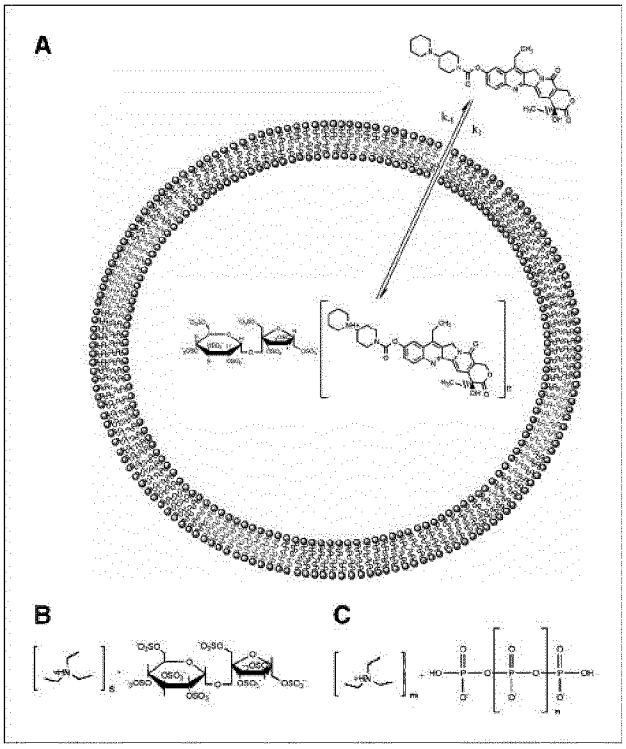

Figure 1. *A*, schematic depicting the intraliposomal stabilization strategy for CPT-11 using polyanionic trapping agents. The basic molecule CPT-11 forms a nanoscale complex with either poly(phosphate) or sucrose octasulfate in the liposome interior, thus stabilizing the liposomal formulation to increase drug retention while in circulation. Chemical structures of the triethylammonium salts of the polyanionic liposome trapping agents, poly(phosphate) (*B*) and sucrose octasulfate (*C*).

To minimize the treatment-associated lipid burden, encapsulation of CPT-11 was attempted up to drug-to-lipid ratios far exceeding the usual ratios achievable by traditional transmembrane-gradient drug loading techniques (Fig. 2). Remarkably, we found that CPT-11 encapsulation in liposomes was quantitative up to 800 g CPT-11/mol phospholipid. The final molar ratio of drug-to-phospholipid corresponds to 1.36:1 for liposomes loaded at 800 g CPT-11/mol phospholipid or 109,000 drug molecules per particle. This represents a 10- to 20-fold improvement over other liposomal formulations, including anthracyclines (3) or camptothecins lurtotecan (8) and SN-38 (21). We hypothesize that the high loading capacity of triethylammonium sucrose octasulfate liposomes is due to the formation of a stable complex between the drug and polyanion whereas the displaced triethylammonium ion dissociates and traverses the lipid bilayer as triethylamine, ensuring that the loading process continues until all added drug is encapsulated or the charge stoichiometry is achieved between the added drug and the liposomally encapsulated anion (Fig. 1).

Pharmacokinetics of nanoliposomal CPT-11. The pharmacokinetics of nanoliposomal CPT-11 formulated using either TEA-SOS or TEA-Pn were determined in normal female rats. Free CPT-11 was rapidly cleared from the circulation with $t_{1/2}$ = 0.27 hours (Fig. 3*A*). Liposome encapsulation was associated with significantly longer circulation times than free drug (Fig. 3*A* and *B*). This was especially true for liposomes loaded with TEA-SOS gradients, with blood half-lives for lipid and CPT-11 of 12.0 and 10.7 hours, respectively (Table 1).

Whereas both liposome constructs displayed long circulation for the lipid component, drug associated with TEA-SOS liposomes unexpectedly showed less rapid clearance from the blood than with TEA-Pn liposomes (Fig. 3*A* and *B*). This likely reflects that the $t_{1/2}$ of CPT-11 release from TEA-Pn liposomes was 14 hours, significantly shorter than that for TEA-SOS liposomes with a $t_{1/2}$ of CPT-11 release of 56.8 hours.

Drug stability of free and nanoliposomal CPT-11. *In vivo*, CPT-11 undergoes transformation to its more active metabolite, SN-38, and both molecules are also subject to inactivation by hydrolysis of the lactone forms to the respective carboxylate forms (Fig. 4*A* and *B*). Liposome encapsulation and delivery markedly altered these bioconversions in rats. Free CPT-11 was rapidly cleared from circulation, with only 2% of the injected dose remaining at 30 minutes and 35% of this present in the carboxylate form (Fig. 4*C*). In contrast, nanoliposomal CPT-11 showed both prolonged circulation, with 23.2% of injected dose still remaining at 24 hours, and drug protection, with no detectable conversion of CPT-11 to either SN-38 or the carboxylate form of CPT-11 (Fig. 4*D*). Thus, the high-charge density polyanionic nanoliposomal matrix provided a chaperone for the stably entrapped prodrug CPT-11, improving its pharmacokinetics and preventing its inactivation or premature conversion to the toxic metabolite SN-38.

Once deposited in tumors, liposomes are known to be taken up avidly by tumor-resident macrophages (22). To determine if macrophages could metabolically activate drug from nanoliposomal CPT-11, an *ex vivo* assay using macrophages isolated from the peritoneum of nude mice was done. Incubation of nanoliposomal CPT-11 with macrophages showed no detectable conversion to SN-38 at 24 hours but 100% conversion to SN-38 by 72 hours. This time course suggested that at least 24 hours was required for macrophage-mediated disruption of the liposome, drug release, and conversion to SN-38.

Acute toxicity of nanoliposomal CPT-11. The acute toxicity of free and nanoliposomal CPT-11 was determined in normal Swiss Webster mice using an NCI-based protocol. The maximum tolerated dose of free CPT-11 was 80 mg/kg whereas the maximum

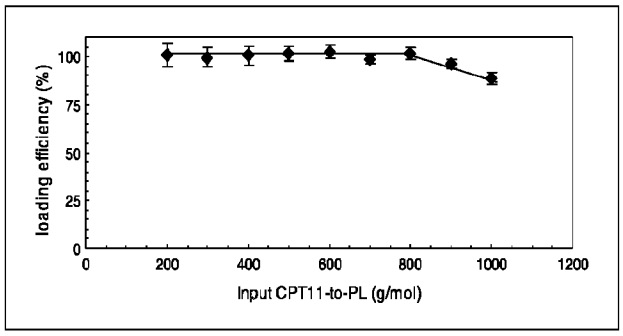

Figure 2. Liposomal loading efficiency as a function of input CPT-11-to-phospholipid (*PL*) ratio. Distearoylphosphatidylcholine/cholesterol/methoxypoly(ethylene)glycol (PEG2000)-derivatized distearoylphosphatidylethanolamine (3:2:0.015) liposomes were loaded with CPT-11 as described in Materials and Methods. The resulting CPT-11-to-phospholipid ratio following loading was determined by quantitating both CPT-11 and phospholipid in the resulting purified liposomal CPT-11 formulation, and the loading efficiency by comparing this ratio to the input ratio.

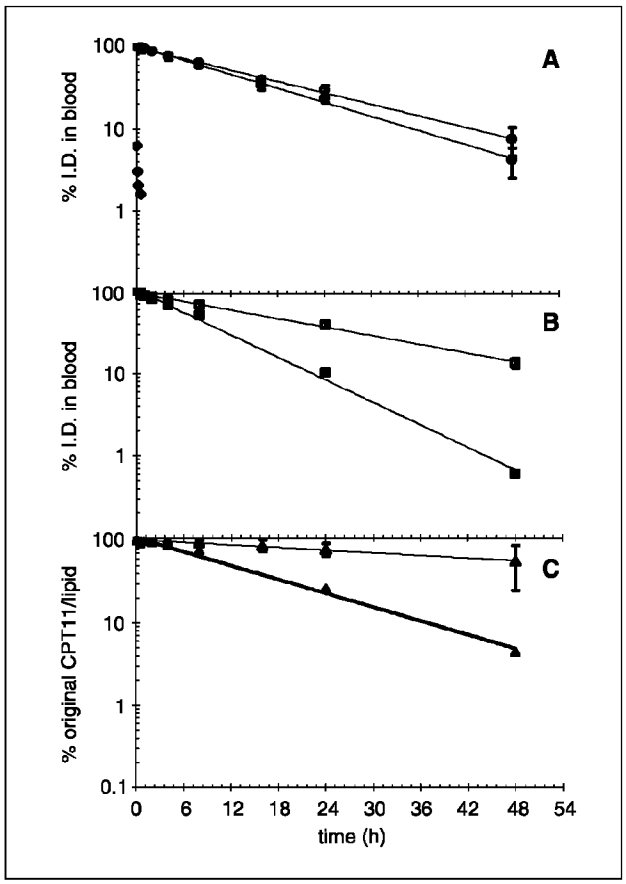

Figure 3. Pharmacokinetics of nanoliposomal CPT-11 in rats. Nanoliposomal CPT-11 prepared using either triethylammonium sucrose octasulfate (*A*) or poly(phosphate) (*B*) was administered i.v. in 9-week-old female Sprague-Dawley rats (body weight ~200 g) with indwelling central venous catheters at a dose of 10 mg CPT-11/kg (17.6 μmol phospholipid/kg). Free CPT-11 was administered i.v. as a bolus injection at 25 mg/kg (*A*, ◆). Plasma was sampled at the indicated times and analyzed for drug and liposomal lipid content. *Points*, % of injected dose (% *I.D.*) of lipid (○, □) or drug (●, ■, ◆). *C*, drug retention was calculated as percent of original drug associated with liposomal lipid at each time point for the poly(phosphate) (▲) and sucrose octasulfate (△) formulations.

tolerated dose of nanoliposomal CPT-11 formulated using a TEA-SOS gradient was not achieved even at the highest administered dose of 324 mg CPT-11/kg. A dose of >324 mg CPT-11/kg was impossible to administer because of concentration and injection volume limitations. Therefore, nanoliposomal CPT-11 delivery reduced drug toxicity in the mouse by at least 4-fold.

Efficacy of nanoliposomal CPT-11 in the BT474 breast cancer model. Treatment using nanoliposomal CPT-11, formulated using the TEA-Pn loading strategy, was evaluated in the BT474 breast tumor xenograft model (Fig. 5*A*). Free CPT-11 was clearly efficacious in this model with noticeable inhibition of tumor growth. However, treatment with nanoliposomal CPT-11 provided further advantage with dramatic regressions in tumor volumes and 100% cures of mice (defined as no residual tumor at study end).

Treatment-related toxicities were not observed. There was a slight decrease in mean body weight by 3.3% on the final treatment day in the animals receiving liposomal CPT-11; this decrease was not statistically significant compared with pretreatment weight ($P = 0.274$, Student's t test). All other weight measurements were within the expected range.

Efficacy of liposomal CPT-11 in the HT29 colon cancer model. In the HT29 colon tumor xenograft model, free CPT-11 again showed efficacy, albeit modest (Fig. 5*B*). However, both nanoliposomal CPT-11 formulations showed pronounced antitumor effects, including tumor regression during treatment followed by prolonged absence of tumor regrowth. Indeed, at 42 days postimplantation, all nanoliposomal CPT-11 treatments seemed to be equivalent and maximally efficacious.

With continued observation, tumor regrowth was observed beginning on day 47 postimplantation. At this point, all control and free CPT-11-treated mice had been sacrificed due to excessive tumor growth. Based on regrowth rates, treatment with TEA-SOS liposomes was more efficacious than TEA-Pn liposomes administered at the same CPT-11 dose. Furthermore, treatment with either liposome type at 50 mg/kg dose was more efficacious than at 25 mg/kg. In an analysis of cure rates, no mice receiving control or free CPT-11 were cured. Mice receiving TEA-Pn liposomal drug at 50 mg/kg per injection, despite initial tumor regressions, showed eventual regrowth. In the two groups receiving 25 mg/kg of either liposome formulation, one animal (9.1%) from each group was tumor-free at study end. In the group receiving 50 mg/kg of the TEA-SOS liposome formulation, 4 animals (36.4%) showed no regrowth and remained tumor-free.

Animals receiving free CPT-11, but not any of the nanoliposomal CPT-11 preparations, showed morbidity (loss of alertness, humped posture, ruffled fur, decreased mobility) for 1 hour after drug injection. Animals receiving free CPT-11 also lost 6% of weight

Table 1. Pharmacokinetic variables for free and nanoliposomal CPT-11 in rats

Formulation	$t_{1/2}$ (h)	AUC_∞ (μg h/mL)	CL (mL/h)	V_d (mL)	MRT (h)	$t_{1/2}$ CPT-11 release (h)
Free CPT-11	0.27	6.2	1,609	616.4	0.4	—
Ls-CPT-11 [TEA-Pn]	6.80	1,407.8	7.10	69.7	9.8	14.0
Ls-CPT-11 [TEA-SOS]	10.7	2,134.4	4.69	72.3	15.4	56.8

NOTE: The data used to calculate the pharmacokinetic variables for CPT-11 when formulated either in the free form or liposomal form refer to the actual drug concentrations measured in the blood that were then used to calculate the %ID values found in the corresponding curves for Fig. 3*B*.
Abbreviations: AUC_∞, area under the concentration versus time curve in plasma based on the sum of exponential terms; MRT, mean residence time calculated from exponential terms; CL, clearance calculated from exponential terms; V_d, volume of distribution.

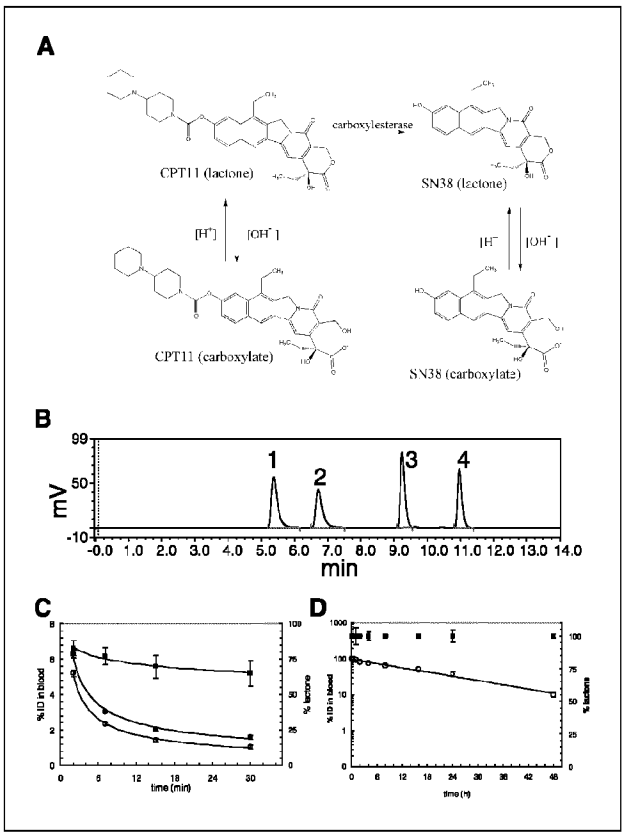

Figure 4. Drug stability of free and nanoliposomal CPT-11. *A*, CPT-11 and SN-38 exist in a pH-dependent equilibrium between closed lactone and open carboxylate configurations. CPT-11 is converted to its more active metabolite, SN-38, by carboxylesterases. *B*, HPLC chromatogram showing the separation of these species: CPT-11 carboxylate (*peak 1*), SN-38 carboxylate (*peak 2*), CPT-11 lactone (*peak 3*), and SN-38 lactone (*peak 4*). The *in vivo* drug stability of free (*C*) and nanoliposomal (*D*) CPT-11 was evaluated following single i.v. bolus administration at a dose of 25 mg CPT-11/kg in 9-week-old female Sprague-Dawley rats (body weight ~200 g). Levels of total CPT-11 (●) and CPT-11 lactone (○) in the blood were determined by HPLC analysis and expressed as percent of initial CPT-11 dose. *Right y axis*, percentage of CPT-11 in the lactone form is plotted as a function of time (■).

during treatment and did not recover, probably because of the effects of the growing tumor. Animals receiving nanoliposomal CPT-11 formulations experienced transient weight loss of 5% (at 25 mg/kg) or 9% (at 50 mg/kg) between the second and third injections as compared with pretreatment values; however, weights recovered following completion of treatment.

Discussion

Liposome delivery has been shown to improve the pharmacokinetic profile and widen the therapeutic index of certain anticancer drugs, especially the anthracycline class (1, 2). Improved efficacy is in part a result of passive targeting to tumor sites based on the enhanced permeability and retention (EPR) effect (23). To fully exploit this process, drug carriers must be engineered to retain drug while circulating, thereby preventing premature drug release before accumulating in the tumor but still allowing for release of drug once in the vicinity of the tumor. Antibody-targeted nanoparticles, such as immunoliposomes against HER2 (24) or epidermal growth factor receptor (25), represent another strategy for more efficient drug delivery to tumor cells.

Gradient-based drug loading technologies, in which electrochemical gradients drive the accumulation of drugs in the liposome interior, represent a key advance in liposome research (11, 26). This approach was further refined when transmembrane gradients of ammonium ion were proposed to form a self-sustaining pH-gradient that can load drugs inside liposomes (10). However, weakly basic anthracyclines represented the only drug class that afforded slow *in vivo* release rates when loaded using gradients involving common anionic counterions, such as sulfate or citrate. With other drug classes, gradient-based loading has been achieved with variable efficiency. To stabilize other cationic drugs against premature escape from liposomes, the use of pre-entrapped polyanionic polymers was proposed (9, 27).

In the present study, we used a drug loading transmembrane gradient system with two components, a substituted ammonium and a poly(anionic) trapping agent of either polymeric (polyphosphate) or nonpolymeric (sucrose octasulfate) nature. The use of polymeric polyanions such as heparin or dextran sulfate to improve liposomal drug retention has been reported (9, 27). Polyphosphate was effective in stabilizing intraliposomal CPT-11

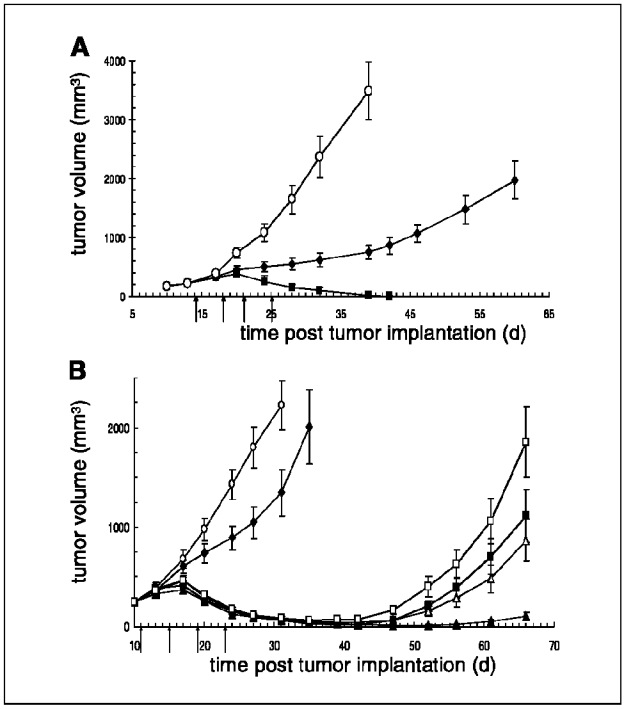

Figure 5. Antitumor efficacy of nanoliposomal CPT-11 in tumor xenograft models. *A*, BT474 breast cancer cells were implanted s.c. in nude mice along with estrogen pellets. When tumors were well established and had reached mean volumes of 200 mm³, the following treatments were initiated: control (○), drug- and liposome-free vehicle only; free CPT-11 (♦); or nanoliposomal CPT-11 stabilized with TEA-Pn (■). Free and nanoliposomal CPT-11 were injected at 50 mg CPT-11/kg/dose i.v. twice per week for four doses (*arrows*). *B*, HT-29 colon cancer cells were implanted s.c. in nude mice. When tumors were well established and had reached mean volumes of 150 to 350 mm³, the following treatments were administered: control (saline; ○); free CPT-11, 50 mg/kg/dose (♦); nanoliposomal CPT-11 using TEA-Pn, 25 mg/kg/dose (□); nanoliposomal CPT-11 using TEA-Pn, 50 mg/kg/dose (■); nanoliposomal CPT-11 using TEA-SOS, 25 mg/kg/dose (△); and nanoliposomal CPT-11 using TEA-SOS, 50 mg/kg/dose (▲).

against *in vivo* release, having the added advantage of being more readily biodegradable than dextran sulfate. However, polyanionic polymers such as heparin and dextran sulfate have notable anticoagulant activity and, in the case of dextran sulfate, toxic to Kupffer cells (28). The undefined chemical nature of many functionalized polymers may also contribute to variability in *in vivo* properties. Unexpectedly, we observed that a highly charged, nonpolymeric anion, such as sucrose octasulfate, provided even better drug retention than a polyanionic polymer, resulting in outstanding *in vivo* drug encapsulation stability. Sucrose octasulfate is a product of exhaustive esterification of sucrose, using chlorosulfonic acid or sulfur trioxide in pyridine or methylpyridine, and is a known pharmaceutical ingredient, the basic aluminum salt (Sucralfate) of which is widely used to treat gastric hyperacidity (29). Compared with dextran sulfate, sucrose octasulfate is chemically well defined; it does not have known anticoagulant or antimacrophage activity (29) and its salts can be produced in pure crystalline form ensuring less interlot variability.

The concept of nanoparticle delivery of camptothecins is very attractive based on potential advantages, including overcoming the solubility limitations of this class, protecting drug in the active lactone configuration, rerouting of drug from sites of toxicity such as the gastrointestinal tract, prolonging circulation time, increasing tumor accumulation via the EPR effect, and providing sustained release for a so-called metronomic effect. Using a novel intraliposomal stabilization technology, we have developed a nanoliposomal CPT-11 featuring drug loading efficiency and drug payload ($>10^5$ per particle) in far excess of that previously reported for this type of encapsulation; this agent showed marked *in vivo* retention of CPT-11 during long circulation times while simultaneously protecting the drug from lactone hydrolysis or premature activation. Compared with free CPT-11, this liposome-based nanoparticle reduced host toxicity in rats by >4-fold and greatly increased antitumor efficacy in animal models. In a separate study, we showed similar improvements in efficacy and host toxicity when nanoliposomal CPT-11 was administered locally to brain tumors using convection-enhanced delivery (30).

Previously reported liposomal camptothecin preparations have shown increased efficacy but not necessarily improved toxicity when compared with free drug (8, 9, 31). Other examples have shown prolonged circulation (32, 33), but not to the extent observed for the TEA-SOS-stabilized liposomes described here. In addition, a liposomal version of SN-38 is cleared even more rapidly with an AUC_∞ that seems to be at least 2 orders of magnitude less than that observed for nanoliposomal CPT-11 (34).

Another aspect of nanoliposomal CPT-11 is that it delivers a prodrug. Cytotoxic drugs encapsulated in liposomes are normally unable to act on their therapeutic targets or cause toxicity until they can be released from the confines of the carrier, and thus liposomal drug delivery can itself be regarded as a prodrug strategy. Hence, in this dual prodrug strategy, liposome delivery of CPT-11 chaperones the camptothecin until it reaches tumor sites where the prodrug can then be activated locally. Although local activation of CPT-11 to SN-38 has yet to be shown, carboxylesterases have a widespread distribution in different tumor types (35–37) and are active in macrophages, the principal scavenger of liposomes. Indeed, we observed that nanoliposomal CPT-11 was completely converted to SN-38 by macrophages after 72-hour incubation. We hypothesize that nanoliposomal CPT-11 may be acted on by tumor-resident macrophages, which convert drug to SN-38 with subsequent diffusion to nearby tumor cells. Alternatively, CPT-11 may be activated directly by tumor cells following release from its liposome carrier.

We conclude that nanoliposomal CPT-11 generated by novel intraliposomal drug stabilization resulted in advantageous pharmacologic properties with increased efficacy and reduced host toxicity *in vivo*. The drug-loading and stabilization technologies used for CPT-11 may also be broadly applicable to other weakly basic anticancer drugs as we have recently shown using a novel histone deacetylase inhibitor, LAQ824 (38). Nanoliposomal CPT-11 may provide a robust and useful nanoparticle-based treatment for cancer.

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References

1. Drummond DC, Meyer OM, Hong K, Kirpotin DB, Papahadjopoulos D. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev 1999;51:691–743.
2. Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. Science 2004;303:1818–22.
3. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res 1994; 54:987–92.
4. Krishna R, Webb MS, St Onge G, Mayer LD. Liposomal and nonliposomal drug pharmacokinetics after administration of liposome-encapsulated vincristine and their contribution to drug tissue distribution properties. J Pharmacol Exp Ther 2001;298:1206–12.
5. Allen TM, Newman MS, Woodle MC, Mayhew E, Uster PS. Pharmacokinetics and anti-tumor activity of vincristine encapsulated in sterically stabilized liposomes. Int J Cancer 1995;62:199–204.
6. Webb MS, Harasym TO, Masin D, Bally MB, Mayer LD. Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models. Br J Cancer 1995;72:896–904.
7. Tardi P, Choice E, Masin D, Redelmeier T, Bally M, Madden TD. Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models. Cancer Res 2000;60:3389–93.
8. Emerson DL, Bendele R, Brown E, et al. Antitumor efficacy, pharmacokinetics, and biodistribution of NX 211: a low-clearance liposomal formulation of lurtotecan. Clin Cancer Res 2000;6:2903–12.
9. Colbern GT, Dykes DJ, Engbers C, et al. Encapsulation of the topoisomerase I inhibitor GL147211C in pegylated (STEALTH) liposomes: pharmacokinetics and antitumor activity in HT29 colon tumor xenografts. Clin Cancer Res 1998;4:3077–82.
10. Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphiphathic weak bases. Biochim Biophys Acta 1993;1151: 201–15.
11. Madden TD, Harrigan PR, Tai LCL, et al. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. Chem Phys Lipids 1990;53:37–46.
12. Garcia-Carbonero R, Supko JG. Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. Clin Cancer Res 2002;8:641–61.
13. Mathijssen RH, van Alphen RJ, Verweij J, et al. Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). Clin Cancer Res 2001;7:2182–94.
14. Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res 1991;51:4187–91.
15. Lee W, Lockhart AC, Kim RB, Rothenberg ML. Cancer pharmacogenomics: powerful tools in cancer chemotherapy and drug development. Oncologist 2005; 10:104–11.
16. Akimoto K, Kawai A, Ohya K. Kinetic studies of the hydrolysis and lactonization of camptothecin and its derivatives, CPT-11 and SN-38, in aqueous solution. Chem Pharm Bull 1994;42:2135–8.
17. Vanhoefer U, Harstrick A, Achterrath W, Cao S,

Seeber S, Rustum YM. Irinotecan in the treatment of colorectal cancer: clinical overview. J Clin Oncol 2001;19:1501–18.
18. Bartlett GR. Phosphorous assay in column chromatography. J Biol Chem 1959;234:466–8.
19. Warner DL, Burke TG. Simple and versatile high-performance liquid chromatographic method for the simultaneous quantitation of the lactone and carboxylate forms of camptothecin anticancer drugs. J Chromatogr B Biomed Sci Appl 1997;691:161–71.
20. Geran RI, Greenberg NH, Mcdonald MM, Shumacher AM, Abbot BJ. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother Rep 1972;3:1–88.
21. Zhang JA, Xuan T, Parmar M, et al. Development and characterization of a novel liposome-based formulation of SN-38. Int J Pharm 2004;270:93–107.
22. Senior JH. Fate and behaviour of liposomes *in vivo*: a review of controlling factors. CRC Crit Rev Ther Drug Carrier Syst 1987;3:123–93.
23. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS. Cancer Res 1986;46:6387–92.
24. Park JW, Hong K, Kirpotin DB, et al. Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery. Clin Cancer Res 2002;8:1172–81.
25. Mamot C, Drummond DC, Noble CO, et al. Epidermal growth factor receptor-targeted immunoliposomes significantly enhance the efficacy of multiple anticancer drugs *in vivo*. Cancer Res 2005;65:11631–8.
26. Nichols JW, Deamer DW. Catecholamine uptake and concentration by liposomes maintaining pH gradients. Biochim Biophys Acta 1976;455:269–71.
27. Zhu G, Oto E, Vaage J, et al. The effect of vincristine-polyanion complexes in STEALTH liposomes on pharmacokinetics, toxicity and anti tumor activity. Cancer Chemother Pharmacol 1996;39:138–42.
28. Liu D, Mori A, Huang L. Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. Biochim Biophys Acta 1992;1104:95–101.
29. Fisher RS. Sucralfate: a review of drug tolerance and safety. J Clin Gastroenterol 1981;3:181–4.
30. Noble CO, Krauze MT, Drummond DC, et al. Novel nanoliposomal CPT-11 infused by convection-enhanced delivery in intracranial tumors: pharmacology and efficacy. Cancer Res 2006;66:2801–6.
31. Messerer CL, Ramsay EC, Waterhouse D, et al. Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer. Clin Cancer Res 2004;10:6638–49.
32. Sadzuka Y, Hirotsu S, Hirota S. Effect of liposomalization on the antitumor activity, side-effects and tissue distribution of CPT-11. Cancer Lett 1998;127:99–106.
33. Sadzuka Y, Hirotsu S, Hirota S. Effective irinotecan (CPT-11)-containing liposomes: intraliposomal conversion to the active metabolite SN-38. Jpn J Cancer Res 1999;90:226–32.
34. Pal A, Khan S, Wang YF, et al. Preclinical safety, pharmacokinetics and antitumor efficacy profile of liposome-entrapped SN-38 formulation. Anticancer Res 2005;25:331–41.
35. Xu G, Zhang W, Ma MK, McLeod HL. Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. Clin Cancer Res 2002;8:2605–11.
36. van Ark-Otte J, Kedde MA, van der Vijgh WJ, et al. Determinants of CPT-11 and SN-38 activities in human lung cancer cells. Br J Cancer 1998;77:2171–6.
37. Guichard S, Terret C, Hennebelle I, et al. CPT-11 converting carboxylesterase and topoisomerase activities in tumour and normal colon and liver tissues. Br J Cancer 1999;80:364–70.
38. Drummond DC, Marx C, Guo Z, et al. Enhanced pharmacodynamic and antitumor properties of a histone deacetylase inhibitor encapsulated in liposomes or ErbB2-targeted immunoliposomes. Clin Cancer Res 2005;11:3392–401.

Development of a Highly Active Nanoliposomal Irinotecan Using a Novel Intraliposomal Stabilization Strategy

Daryl C. Drummond, Charles O. Noble, Zexiong Guo, et al.

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Phase I/II Study of Bi-weekly Irinotecan plus Cisplatin in the Treatment of Advanced Gastric Cancer

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Abstract. *Objectives: To conduct a phase I/II study of irinotecan with cisplatin to establish a recommended dose, and assess the safety, efficacy and feasibility of this regimen in unresectable advanced or recurrent gastric cancer. Patients and Methods: In the phase I portion of the study, patients received a fixed dose of cisplatin (30 mg/m^2) with escalating doses of irinotecan, ranging from 30 mg/m^2 to 70 mg/m^2, on days 1 and 15. In the phase II portion of the study, 40 patients were evaluated for response and safety at the recommended dose. Results: Eighteen patients were enrolled in the phase I study. Dose-limiting toxicity (diarrhea and neutropenia) appeared at the irinotecan dose of 70 mg/m^2. Therefore, the recommended irinotecan dose was 60 mg/m^2. In the phase II study, 40 patients received cisplatin (30 mg/m^2) plus irinotecan (60 mg/m^2). Twenty-five out of 40 patients had received prior chemotherapy. The median number of cycles was 3.5. The response rate was 32.5% (13/40) overall, and 53.3% (8/15) in patients without prior chemotherapy. The median time to tumor progression (TTP) was 162 days. The median survival time was 288 days. Four patients (10%) developed grade 4 neutropenia and 3 patients (7.5%) developed grade 4 anemia. The only observed non-hematological toxicity at grade 3 or higher was diarrhea, seen in 2.5% (1/40) of the patients. Conclusion: Bi-weekly administration of irinotecan and cisplatin is safe and active for the management of unresectable advanced or recurrent gastric cancer.*

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In 2001, 49,958 patients died from gastric cancer in Japan, making gastric cancer the second deadliest cancer, surpassed only by lung cancer. Deaths from gastric cancer account for 16.6% of all cancer deaths. In a comparison of age-adjusted mortality rate from gastric cancer in 28 countries, Japan led all other nations for both men and women, indicating that the number of gastric cancer patients is greater in Japan than in any other country (1).

Chemotherapy is recognized as an effective treatment method for advanced gastric cancer (2, 3). However although phase III comparative studies have been carried out, the standard-of-care chemotherapy regimen has not yet been established (4). Thus, new, more effective therapies are desired.

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) is a semi-synthetic compound derived from a plant alkaloid camptothecin, extracted from *Camptotheca acuminata* (5, 6).

Unlike conventional anticancer drugs, irinotecan inhibits DNA topoisomerase I (7, 8). In Japan, a late phase II multicenter study of irinotecan demonstrated efficacy in patients with advanced gastric cancers.The response rate was 23.3% (14/60). The recommended dose for irinotecan monotherapy in Japan was 100 mg/m^2 weekly or 150 mg/m^2 bi-weekly.

Currently, ongoing studies combine irinotecan with other agents to improve antitumor effects (9). The combination of irinotecan and cisplatin has been shown to enhance cytotoxicity against target cells *in vitro* (10-13). Clinical

Table I. *Dose -escalation schedule* and number of patients in phase I study.*

Dose level	Dose (mg/m^2) irinotecan	cisplatin	No. of patients
1	30	30	3
2	40	30	3
3	50	30	3
4	60	30	3
5	70	30	6

*irinotecan and cisplatin on days 1 and 15.

studies of combination therapy using irinotecan and cisplatin have shown better efficacy than monotherapy in patients with gastric cancer(14, 15). Preclinical studies have also shown that the additive effects *in vitro* are highest when cells are simultaneously treated with irinotecan and cisplatin, with the cytotoxic activity depending on the area under the drug concentration-time curve and maintained when cells are intermittently exposed to treatment (10, 16). We conducted a multi-center phase I/II clinical study to identify a recommended dose of bi-weekly concomitant treatment of irinotecan and cisplatin and to determine the safety and efficacy of this combination in patients with advanced or recurrent gastric cancer.

Patients and Methods

Eligibility criteria. Patients with unresectable advanced or recurrent gastric cancer were enrolled. The patients were required to satisfy the following eligibility criteria: histologically confirmed diagnosis of gastric cancer; age of 20 to 75 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0, 1, or 2; ≤1 chemotherapy regimen completed 4 weeks before entry; leukocyte count of 4,000-12,000/mm^3 and platelet count of ≥100,000/mm^3; total bilirubin level of ≤2.0 mg/dL and aspartate aminotransferase and alanine aminotransferase levels not more than three times the upper limit of normal; creatinine level of ≤1.5 mg/dL, blood urea nitrogen level of ≤25 mg/dL, and creatinine clearance of ≥50 mL/min; and an estimated survival of at least 3 months. Before enrollment, all subjects provided a written informed consent to participate in the study. Patients with any of the following conditions were excluded: severe co-existing medical illness (intestinal paresis or ileus, interstitial pneumonia, pulmonary fibrosis, poorly controlled diabetes mellitus), active multiple cancers, severe psychiatric disturbances, or a history of hypersensitivity to either irinotecan or cisplatin. This study was conducted after being approved by the ethics committee of each participating institution.

Treatment schedule. In the phase I portion of the study, irinotecan was initially given at a dose of 30 mg/m^2 over 90 min by intravenous infusion on days 1 and 15. The irinotecan dose was then escalated in 10 mg/m^2 increments to confirm the safety of the treatment (Table I). Cisplatin, at a fixed dose of 30 mg/m^2, was given after irinotecan by intravenous infusion over 90 min with adequate hydration (a total of 1-2 L) on days 1 and 15. This treatment was repeated every 4 weeks until disease progression, refusal by the patient, or unacceptable adverse reactions.

Prior to chemotherapy, patients received antiemetics of 5-HT$_3$ receptor antagonist and steroids. Episodes of diarrhea were treated with loperamide hydrochloride as required.

The following dose adjustments were permitted: irinotecan was reduced to 50 mg/m^2 for grade 3 hematological toxicity or grade 2 non-hematological toxicity (excluding hair loss, nausea and vomiting); cisplatin was reduced to 20 mg/m^2 for renal toxicity.

Maximum-tolerated dose and recommended dose. In the phase I portion of the study, dose-limiting toxicity during the first cycle of therapy was defined as any of the following: grade 4 hematological toxicity, grade 3 non-hematological toxicity (excluding hair loss, nausea and vomiting), or withholding of treatment for 3 weeks or longer due to delayed resolution of adverse events. Three patients were assigned to each dose level. If any patient experienced dose-limiting toxicity, 3 more patients were assigned to receive the same dose. If 3 or more of the 6 patients experienced dose-limiting toxicity, the dose level was defined as the maximum-tolerated dose. The recommended dose of irinotecan for the phase II study was defined as 10 mg/m^2 lower than the maximum-tolerated dose.

Table II. *Clinical characteristics of patients in phase I and phase II studies.*

Characteristic	Phase I (n=18) No.	%	Phase II (n=40) No.	%
Age, years				
Median	63		62	
Range	(26 - 74)		(40-75)	
Sex				
Male	13	72.2	30	75.0
Female	5	27.8	10	25.0
Performance status (ECOG)				
0, 1	16	88.9	38	95.0
2	2	11.1	2	5.0
Histology				
Intestinal type	8	44.4	19	47.5
Diffuse type	10	55.5	21	52.5
Site of metastasis				
Liver	4	22.2	19	47.5
Lymph nodes	7	38.9	24	60.0
Lung	1	2.5	1	2.5
Other	2	11.1	1	2.5
Prior chemotherapy				
No			15	37.5
Yes			25	62.5
Oral fluorouracil/cisplatin			16	64.0
Oral fluorouracil alone			3	12.0
MTX/5FU/cisplatin			5	20.0
Taxanes			1	4.0

ECOG, Eastern Cooperative Oncology Group.

Table III. *Toxicity (phase I study).*

First course

Toxicity	Dose level	No. of patients	Grade				Incidence of Grade 3 & 4 (%)
			1	2	3	4	
Leukopenia	1	3	1	2	0	0	0
	2	3	3	0	0	0	0
	3	3	1	1	0	0	0
	4	3	1	1	0	0	0
	5	6	2	2	1[*1]	1[*2]	33.3
Neutropenia	1	3	0	1	1	0	33.3
	2	3	1	1	0	0	0
	3	3	0	0	1	0	33.3
	4	3	0	0	0	0	0
	5	6	0	1	2	1[*2]	50.0
Thrombocytopenia	1	3	0	0	0	0	0
	2	3	0	0	0	0	0
	3	3	0	0	0	0	0
	4	3	0	0	0	0	0
	5	6	1	0	0	0	0
Decreased hemoglobin	1	3	0	2	0	0	0
	2	3	0	1	1	0	33.3
	3	3	2	1	0	0	0
	4	3	1	0	1	0	33.3
	5	6	3	1	1	0	16.7
Diarrhea	1	3	0	0	0	0	0
	2	3	0	0	0	0	0
	3	3	1	0	0	0	0
	4	3	0	0	0	0	0
	5	6	0	1[*3]	1[*2]	0	16.7
Nausea / vomiting	1	3	0	1	0	0	0
	2	3	0	0	0	0	0
	3	3	2	1	0	0	0
	4	3	1	0	0	0	0
	5	6	1	0	0	0	0

[*1] Delayed for 28 days for leukopenia.
[*2] One patient had Grade 3 diarrhea and Grade 4 leukopenia and neutropenia.
[*3] Delayed for 28 days for diarrhea.

Evaluation. The tumor response was evaluated based on changes in the size of measurable lesions and assessment of evaluable lesions. Measurable lesions and evaluable lesions were defined and efficacy evaluated in accordance with the Japanese Criteria for Evaluating the Efficacy of Chemotherapy and Radiation Therapy in the Treatment of Gastric Cancer (17). In brief, complete remission was defined as the disappearance of all evidence of the tumor for at least 4 weeks. Partial remission was defined as 50% or greater reduction in the sum of the products of the perpendicular diameters of all measurable lesions for at least 4 weeks without any evidence of new lesions or the progression of any existing lesions. Stable disease was defined as less than 50% reduction or less than 25% increase in the sum of the products of the perpendicular diameters of all lesions for at least 4 weeks, without any evidence of new lesions or the progression of any existing lesions. Progressive disease was defined as a ≥25% increase of one or more lesions or the appearance of new lesions. Tumor measurements were performed every 4 weeks using computed tomography, plain chest X-ray films, upper gastrointestinal endoscopy and ultrasonography. Primary tumors were classified into the following 3 categories based on X-ray and endoscopic findings: measurable, not measurable but evaluable, and diffuse infiltration.

World Health Organization criteria were applied to evaluate adverse events. The eligibility and suitability for assessment of the subjects and response to treatment were reviewed by an independent review committee.

Results

Phase I Study

Patient characteristics. A total of 18 patients (13 men, 5 women) were enrolled in the phase I study between November 1997 and September 1999. The clinical characteristics of the patients

Table IV. *Response (phase II study).*

		CR		PR		NC		PD		NE		RR	
	No.	No.	%	No.	%	No.	%	No.	%	No.	%	%	95% CI
Overall	40	0	0.0	13	32.5	14	35.0	11	27.5	2	5.0	32.5	18.6-49.1
Prior chemotherapy													
Yes	25	0	0.0	5	20.0	10	40.0	9	36.0	1	4.0	20.0	6.8-40.7
Oral fluorouracil/cisplatin	16	0	0.0	4	25.0	6	37.5	6	37.5	0	0.0	25.0	
Oral fluorouracil alone	3	0	0.0	1	33.3	2	66.6	0	0.0	0	0.0	33.3	
MTX/5FU/cisplatin	5	0	0.0	0	0.0	2	40.0	3	60.0	0	0.0	0.0	
Taxanes	1	0	0.0	0	0.0	1	100.0	0	0.0	0	0.0	0.0	
No	15	0	0.0	7	53.3	4	26.7	2	13.3	1	6.7	53.3	21.3-73.4
Primary	30	0	0.0	10	33.3	14	46.7	4	13.3	0	0.0	33.3	17.3-52.8
Liver	20	0	0.0	7	35.0	9	45.0	4	20.0	0	0.0	35.0	15.4-59.2
Lymph nodes	23	1	4.3	7	30.4	13	56.5	2	8.7	0	0.0	34.8	16.4-57.3
Lung	1	0	0.0	1	100.0	0	0.0	0	0.0	0	0.0	100.0	

CR, complete response; PR, partial response; NC, no change; PD, progressive disease; NE, not evaluable; RR, response ratio.

are shown in Table II. The median age was 63 years (range, 26-74). The performance status was 0 or 1 in 16 patients and 2 in 2 patients. Histologically, 8 patients had intestinal type adenocarcinoma and 10 had diffuse type adenocarcinoma. Safety was evaluable in all 18 patients. Three patients were initially assigned to receive dose level 1. Cohorts of 3 patients each were likewise assigned to dose levels 2, 3, 4 and 5. Dose-limiting toxicity occurred at dose level 5, and 3 patients were added to this dose cohort. Table I summarizes the number of patients in each dose cohort.

Dose-limiting toxicity and recommended dose for phase II study. Three out of 6 patients at dose level 5 (irinotecan 70 mg/m^2 + cisplatin 30 mg/m^2 on days 1 and 15) experienced dose-limiting toxicity. One patient developed grade 4 leukopenia/neutropenia and grade 3 diarrhea. Two patients exhibited delayed resolution of adverse events, persisting beyond day 28, of whom one patient had leukopenia that persisted for longer than 28 days (Table III). The other patient had persistent diarrhea that required postponement of treatment. Thus, 70 mg/m^2 was established as the maximum-tolerated irinotecan dose, and a combination of irinotecan 60 mg/m^2 and cisplatin 30 mg/m^2, given on days 1 and 15 in a 28-day cycle, was recommended for use in the phase II study.

Phase II Study

Patient characteristics. Forty patients were enrolled in the phase II study between October 1999 and December 2000. The clinical characteristics of the patients are shown in Table II. All patients met the entry criteria and were included in the analysis. The subjects consisted of 30 men (75.0%) and 10 women (25.0%). Fifteen patients (37.5%) had not received prior chemotherapy, while 25 patients (62.5%) had . The median age of the patients was 62 years (range, 40-75). Histologically, 19 patients had intestinal type adenocarcinoma and 21 had diffuse type adenocarcinoma. Performance status was 0 or 1 in 38 patients (95.0%).

Tumor response and survival. Among the 40 patients with evaluable lesions, 13 (32.5%) exhibited a partial response. The response rate in the patients who had not received prior chemotherapy was 53.3% (8/15), compared to 20.0% (5/25) in those who had received prior chemotherapy (p=0.041, Fisher's exact test). The response rate according to site was 33.3% (10/30) at primary sites, 35.0% (7/20) for liver metastases, 34.8% (8/23) for abdominal lymph node metastases, including 1 complete response, and 100% (1/1) for lung metastases (Table IV). The response rate according to histological type was 21.1% (4/19) in the intestinal type and 42.9% (9/21) in the diffuse type. The median time to progression was 162 days (range, 14-395 days). By Kaplan-Meier analysis, the median survival was 288 days in the 40 subjects (Figure 1), 302 days in the patients who had received no prior chemotherapy, and 274 days in the patients who had received prior chemotherapy. The median number of treatment cycles given was 3.5 (range, 1-7.5; 143 courses, 275 administrations in total).

Patients were taken off the study because of the emergence of a new lesion (11 cases), worsening of the primary disease (19 cases), ineffectiveness (2 cases), deterioration in the patient's general condition (2 cases), request by the patient (5 cases), an adverse reaction (1 case), or a severe complication (2 cases).

Safety. Hematological toxicities of grade 3 or higher observed were leukopenia in 27.5% of the patients, neutropenia in 40.0%, thrombocytopenia in 5.0%, and

decreased hemoglobin level in 30.0% (Table V).The non-hematological toxicities of grade 3 or higher were elevated aspartate aminotransferase (in 5.0% of the patients), elevated alanine aminotransferase (5.0%), elevated total bilirubin (2.5%), elevated alkaline phosphatase (2.5%) and diarrhea (2.5%). There were no treatment-related deaths.

Dose intensity. The actual administered dose in the first two courses was 27.1 mg/m^2/week for irinotecan and 13.6 mg/m^2/week for cisplatin, which correspond to 90.3% and 90.6% of the planned doses.

The drug administration was postponed or skipped in 4 patients on day 15 in the first cycle, and 2 patients postponed the second drug administration in the first cycle. Ten patients delayed the start of the next cycle.

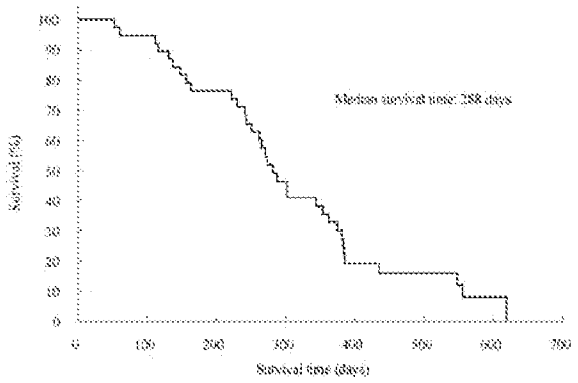

Figure 1. *Survival Curve Derived by Kaplan-Meier Analysis.*

Table V. *Toxicity (phase II study).*

Toxicity (n = 40)	Grade 1	2	3	4	Incidence of Grade 3 & 4 (%)
Leukopenia	6	16	11	0	27.5
Neutropenia	3	11	12	4	40.0
Thrombocytopenia	6	4	2	0	5.0
Decreased hemoglobin	7	17	9	3	30.0
AST	3	4	2	0	5.0
ALT	5	1	2	0	5.0
T-Bilirubin	0	0	1	0	2.5
Al-p	2	3	1	0	2.5
Diarrhea	5	5	1	0	2.5
Nausea/vomiting	14	8	0	0	0.0

AST, L-Aspartate aminotransferase; ALT, L-Alanine aminotransferase; Al-p, Alkaline phosphatase.

Discussion

The phase I study established the maximum-tolerated dose of irinotecan to be 70 mg/m^2 when given with 30 mg/m^2 of cisplatin. Thus, the recommended dose for the phase II study was 60 mg/m^2 of irinotecan and 30 mg/m^2 of cisplatin given intravenously on days 1 and 15 in a 4-week, repeated cycle. This dosage was effective and caused no dose-limiting toxicity, such as severe diarrhea, leukopenia, or neutropenia.

In the phase II study, the tumor response rate with acceptable toxicity was 32.5% (13/40, 95% CI, 18.6%-49.1%) overall and 53.3% (8/15, 95% CI, 27.9%-78.7%) in patients who had not received prior chemotherapy. The median survival was 288 days overall, and 302 days in patients who had not received prior chemotherapy.

These findings show that the recommended dose of irinotecan is 60 mg/m^2. Regarding the recommended dose of irinotecan in colorectal cancer, Cerea *et al.* suggested that the dose reduction of CPT-11 does not influence its efficacy, because they found no significant difference in the disease control (PR + SD) between patients treated with a weekly dose of 125 mg/m^2 and those who received a half-dose (18).

The combination of irinotecan and cisplatin has previously been studied in various tumor types. Kobayashi *et al.* conducted a phase I clinical study of a weekly regimen of irinotecan and cisplatin in patients with non-small cell lung cancer (irinotecan 60 mg/m^2 + cisplatin 27-40 mg/m^2, days 1, 8, 15, one week rest) and reported a high efficacy (19), although the dose-limiting toxicity of irinotecan, such as diarrhea and leukopenia, occurred frequently 7 days after administration. Ajani *et al.* conducted a phase II clinical study of a weekly regimen of irinotecan and cisplatin in advanced, untreated gastric cancer or cancer of the gastroesophageal junction (irinotecan 65 mg/m^2 + cisplatin 30 mg/m^2, weekly x 4, two weeks rest) and reported a high efficacy, although modifying the dose and schedule is necessary, because 66% of the patients experienced adverse effects, requiring a delay or cancellation of drug administration (20). These findings indicate that administering irinotecan and cisplatin weekly on schedule is difficult due to toxicity.

Boku *et al.* (14) conducted a phase II clinical study of irinotecan and cisplatin in metastatic gastric cancer (irinotecan 70 mg/m^2 on days 1 and 15 + cisplatin 80 mg/m^2 on day 1 in a four-week cycle) and reported a high efficacy with overall response rate of 48% (21/44), and 27% in previously-treated patients (4/15). However, leukopenia (59.1%), neutropenia (88.6%) and grade 3 or worse diarrhea (20.5%) were observed. They reported that the second dose of irinotecan was postponed in 82 (56%) cycles and was not given in 34 (23%) cycles out of 146 cycles overall. It was indicated that combining cisplatin once every four weeks with irinotecan bi-weekly did not reduce the

number of cycles delayed or skipped. In contrast, the adverse events of grade 3 and higher in our study were leukopenia (27.5%), neutropenia (40%) and diarrhea (2.5%). Among all the cycles administered, the second dose in the cycle was postponed in 19 cycles (13%) and skipped in 11 cycles (7.7%), so that the two agents could be administered essentially according to schedule.

Regarding the actual dose in the two doses of the initial cycle, Boku *et al.* (14) reported that the actual dose administered for irinotecan was 28.5 mg/m^2/week and cisplatin was 18 mg/m^2/week, so that the actual dose/planned dose was 81.4% for irinotecan and 89.9% for cisplatin. Although this schedule differs from our schedule, we nearly replicated the equivalent dose intensity. The response rate as second-line treatment of 20% and the median survival of 274 days indicate good efficacy. The study of second-line treatment of gastric or gastroesophageal junction carcinoma by Ajani *et al.* (15) reported a response rate of 31% and median survival of 5 months. We observed a response rate of 25% (4/16), even in patients previously treated with cisplatin plus oral 5FU. We conclude that our regimen may also be effective as a second-line treatment.

Phase III studies are necessary to establish the clinical utility of irinotecan/cisplatin.

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References

1 Cancer Statistics in Japan Editorial Board: Cancer Statistics in Japan 2003. Tokyo, Foundation for Promotion of Cancer Research, 2003.

2 Glimelius B, Hoffman K, Haglund U *et al*: Initial or delayed chemotherapy with best supportive care in advanced gastric cancer. Ann Oncol 5: 189-190, 1994.

3 Pyrhonen S, Kuitunen T, Nyandoto P *et al*: Randomised comparison of fluorouracil, epidoxorubicin and methotrexate (FEMTX) plus supportive care with supportive care alone in patients with non-resectable gastric cancer. Br J Cancer *71*: 587-591, 1995.

4 Vanhoefer U, Hansjochen P, Michel H *et al*: Final results of a randomized phase III trial of sequential high-dose MTX/5FU/LV *vs* infusional 5FU/CDDP in advanced gastric cancer. J Clin Oncol *18*: 2648-2657, 2000.

5 Wall ME, Wani MC, Cook CE *et al*: Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. J Am Chem Soc *88*: 3888-3890, 1966.

6 Kunimoto T, Nitta K, Tanaka T *et al*: Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin, against murine tumors. Cancer Res *47*: 5944-5947, 1987.

7 Andoh T, Ishii K, Suzuki Y *et al*: Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase. I. Proc Natl Acad Sci USA *84*: 5565-5569,1987.

8 Hsiang YH, Liu LF, Wall ME *et al*: DNA topoisomerase I mediated DNA cleavage and cytotoxicity of camptothecin analogues. Cancer Res *49*: 4385-4389, 1989.

9 Futatsuki K, Wakui A, Nakao I *et al*: Late phase II study of irinotecan hydrochloride (CPT-11) in advanced gastric cancer. Jpn J Cancer Chemother *21*: 1033-1038, 1994.

10 Itoh K, Takada M, Kudo S *et al*: Synergistic effects of CPT-11 and cisplatin or etoposide on human lung cancer cell lines demonstrated by computer analysis. Lung Cancer *7*: 124, 1991.

11 Kudoh S, Takada M, Masuda N *et al*: Enhanced antitumor efficacy of a combination of CPT-11, a new derivative of camptothecin, and cisplatin against human lung tumor xenografts. Jpn J Cancer Res *84*: 203-207, 1993.

12 Minagawa Y, Kigawa J, Ishihara H *et al*: Synergistic enhancement of cisplatin cytotoxicity by SN-38, an active metabolite of CPT-11, for cisplatin-resistant HeLa cells. Jpn J Cancer Res *85*: 966-971, 1994.

13 Kondo H, Shimada Y, Shirao K *et al*: Combination effects of CPT-11 and CDDP against human cell line: *in vitro* and *in vivo* examinations. Jpn J Cancer Res *85*: 616, 1994.

14 Boku N, Ohtsu A, Shimada Y *et al*: Phase II study of a combination of irinotecan and cisplatin against metastatic gastric cancer. J Clin Oncol *17*: 319-323, 1999.

15 Ajani JA, Baker J, Pisters PW *et al*: Irinotecan/cisplatin in advanced, treated gastric or gastroesophageal junction carcinoma. Oncology *16*: 16-18, 2002.

16 Masumoto N, Nakano S, Esaki T *et al*: Inhibition of cis-diamminedichloroplatinum (II) - induced DNA interstrand cross-link removal by 7-ethyl-10-hydroxy-camptothecin in HST-1 human squamous-carcinoma cells. Int J Cancer *62*: 70-75, 1995.

17 Japanese Research Society for Gastric Cancer: Japanese Classification of Gastric Carcinoma, 1st English ed. Tokyo, Kanehara, 1995.

18 Cerea G, Vaghi M, Ardizzoia A *et al*: Biomodulation of cancer chemotherapy for metastatic colorectal cancer: Randomized study of weekly low-dose irinotecan alone *versus* irinotecan plus the oncostatic pineal hormone melatonin in metastatic cololectal cancer patients progressing on 5-fluorouracil-containing combinations. Anticancer Res *23*: 1951-1954, 2003.

19 Kobayashi K, Shinbara A, Kamimura M *et al*: Irinotecan (CPT-11) in combination with weekly administration of cisplatin (CDDP) for non-small-cell lung cancer. Cancer Chemother Pharmacol *42*: 53-58, 1998.

20 Ajani JA, Baker J, Pisters PW *et al*: CPT-11 plus cisplatin in patients with advanced, untreated gastric or gastroesophageal junction carcinoma: results of a phase II study. Cancer *94*: 641-646, 2002.

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DEVELOPMENTAL THERAPEUTICS - EXPERIMENTAL THERAPEUTICS

Phase I study of the safety, pharmacokinetics (PK), and pharmacodynamics (PD) of the poly(ADP-ribose) polymerase (PARP) inhibitor veliparib (ABT-888; V) in combination with irinotecan (CPT-11; Ir) in patients (pts) with advanced solid tumors.

P. LoRusso, J. J. Ji, J. Li, L. K. Neilburn, G. Shapiro, E. A. Sausville, S. A. Boerner, D. W. Smith, M. J. Pilat, J. Zhang, A. P. Chen, N. Nechiporchik, R. E. Parchment

Karmanos Cancer Institute, Detroit, MI; SAIC-Frederick, Bethesda, MD; Karmanos Cancer Institute, Wayne State University, Detroit, MI; Dana-Farber Cancer Institute, Boston, MA; University of Maryland School of Medicine and University of Maryland Greenebaum Cancer Center, Baltimore, MD; National Cancer Institute, Bethesda, MD

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Abstract

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Background: The nuclear enzyme PARP is essential in recognition and repair of DNA damage. In preclinical models, PARP inhibitors work as sensitizing agents for DNA-damaging agents such as Ir. V is an orally bioavailable PARP 1 and 2 inhibitor. This phase I study determined the maximum tolerated dose (MTD), dose-limiting toxicities (DLTs), PK and PD of V together with Ir. Methods: Eligibility included patients with performance status 0-2; ≥ age 18; adequate bone

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marrow, hepatic and renal function. Cycles were 21 days. Ir was given i.v. 100 mg/m^2 over 90 min on Days 1 and 8. Twice daily (BID) oral dosing of V (10-50mg) occurred Days 3-14 (Cycle 1) and Days -1-14 (subsequent cycles) followed by a 6-day rest. PK studies were conducted with both agents alone and in combination. Tumor biopsies and matched peripheral blood mononuclear cells (PBMCs) were collected for PD evaluation of PAR, γ-H2AX, PARP1, TOPO1, and ERCC1 after Ir alone and after the combination. Results: 32 pts were treated (2 lung, 14 breast, 4 esophageal, 7 ovarian, 4 colon, 1 anal). Median age was 53 (range 31-73). Most frequent drug-related toxicities included: diarrhea (59%), nausea (56%), leucopenia (50%), fatigue (47%), neutropenia (47%), anemia (34%), and vomiting (31%). DLTs included fatigue, diarrhea, febrile neutropenia (gr 3), leukopenia and, neutropenia (gr 4). Response has been determined for 28 pts. A clinical benefit (CB) rate of 61% (5 PR, 2 MR, 10 SD) was observed. V exhibited linear PK over the dose range of 10 to 50 mg BID; co-administration of Ir did not affect PK of V. Exploratory analysis of pre/post-V treatment changes indicated median ERCC1 levels in tumor tissue and PBMCs differed somewhat by CB status. PAR levels in paired tumor biopsies showed > 50% reduction in 17 of 19 pts and > 90% reduction in 10 of 19 pts. Conclusions: The MTD and recommended phase II dose was established as 100 mg/m^2 of Ir given i.v. Days 1 and 8 combined with 40 mg of V given BID 15 days on/6 days off (21 day cycle). Exploratory analysis demonstrated that V reduced PAR levels in tumor. Support: NCI U01-CA062487 and NCI R21-CA135572.

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Low-dose Irinotecan as a Second-line Chemotherapy for Recurrent Small Cell Lung Cancer

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Objective: Irinotecan is a potent inhibitor of deoxyribonucleic acid topoisomerase 1 and the weekly schedule of 100–125 or 350 mg/m^2 administration on Day 1 every 3 weeks is recommended for recurrent small cell lung cancer. However, severe gastrointestinal toxic effects and myelosuppression are often observed in this dose setting. We conducted a retrospective study to evaluate the efficacy and safety of low-dose irinotecan monotherapy (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) as second-line chemotherapy for small cell lung cancer.

Methods: The medical charts of small cell lung cancer patients who had received second-line chemotherapy at the National Cancer Center Hospital East between April 2003 and June 2012 were reviewed. Consecutive 57 patients who were treated with low dose of irinotecan (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) were analyzed in this study.

Results: Median age was 70 years (range, 51–83). Fifty-two (91%) were male, 36 (63%) had an Eastern Cooperative Oncology Group performance status 0–1 and 26 (46%) were sensitive relapse. The median number of chemotherapy cycles was 2. The objective response rate was 32% (95% confidence interval: 20–45%).The median progression-free survival and the median overall survival were 2.9 months (95% confidence interval: 1.9–3.4 months) and 5.3 months (95% confidence interval: 3.6–7.6 months), respectively. The incidence of Grade 3/4 neutropenia, diarrhea and nausea/vomiting was 21, 4 and 5%, respectively.

Conclusions: Low-dose irinotecan monotherapy for recurrent small cell lung cancer might be effective with favorable toxicity. Randomized trial of 60 mg/m^2 versus standard dose of irinotecan is warranted.

Key words: small cell lung cancer – second-line – irinotecan – sensitive relapse – refractory relapse

INTRODUCTION

Lung cancer remains the leading cause of cancer-related deaths worldwide (1). Small cell lung cancer (SCLC) accounts for ~10–15% of all types of lung cancer. Despite a high sensitivity to the initial therapy, the majority of patients develop disease recurrence. The treatment options for patients with recurrent SCLC remain limited (2–4).

Irinotecan is a potent inhibitor of DNA topoisomerase 1 (5) and has been reported to be active in recurrent SCLC patients in several Phase II trials. Patients were treated with irinotecan at a dosage of 100–125 mg/m^2 weekly or 350 mg/m^2 on Day 1 every 3 weeks in these studies (6–9). However, severe gastrointestinal toxicities and myelosuppression were often observed at these dose settings.

Actually, the dose setting of 100 mg/m^2 of irinotecan monotherapy weekly is based on Phase I trials for non-SCLC patients (10), and this dose setting was also adopted in Phase II trials for recurrent SCLC patients in Japan (6). Because

SCLC is one of the most chemosensitive solid tumors, lower-dose irinotecan regimens might have the benefit of being less toxic, compared with the recommended dose in previous reports. In terms of dosage of irinotecan with weekly schedule, another Phase I study in France was reported (11). In the study, no Grade 2 or more serious diarrhea was observed at the dosage of 50 and 66 mg/m^2. On the other hand, prolonged diarrhea (defined as >4 days in the study) was observed two (one with Grade 2 and the other with Grade 3) of four patients at the dosage of 75 mg/m^2. In addition, irinotecan combination therapy is effective in a first-line setting and the recommended dose of irinotecan when used in combination with a platinum agent was 60 mg/m^2 on Days 1, 8 and 15 every 4 weeks (12).

Based on these findings, we have adopted a low-dose irinotecan monotherapy regimen (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) as a treatment option for recurrent SCLC in our clinical practice at the National Cancer Center Hospital East. In this retrospective study, we evaluated the efficacy and safety of irinotecan monotherapy at this dosage.

PATIENTS AND METHODS

Patient Selection

This retrospective cohort study was approved by the ethical review committee of the National Cancer Center, Tokyo, Japan. We retrospectively reviewed the medical records of patients with SCLC who were treated at the National Cancer Center Hospital East between April 2003 and June 2012. Patients were selected for this study according to the following criteria: (i) patients with a histological or cytological diagnosis of SCLC, (ii) patients with refractory or relapsed SCLC after initial chemotherapy or chemoradiotherapy and (iii) patients who were treated with a low dose of irinotecan monotherapy (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) as a second-line chemotherapy. For sensitive relapse SCLC, topotecan monotherapy is the standard care for sensitive relapse SCLC in second-line setting (2,3) and intravenous topotecan for five consecutive days at a dose of 1.0 mg/m^2 is approved in Japan (13). However, high frequency of serious myelotoxicity was reported in the previous studies with this treatment (3,13,14). In our clinical practice, we determined the optimal second-line regimen in each patient taking its toxicity profile and patient's feasibility into consideration. Although the application criteria of low-dose irinotecan regimen were not defined strictly due to the retrospective study from clinical practice, we generally applied the low-dose irinotecan monotherapy when we were concerned about the myelotoxicities in each patient.

Data Collection

We collected clinical data from the medical records, including the patient age, sex, initial chemotherapy regimen, sensitivity to initial therapy, Eastern Cooperative Oncology Group performance status (PS) at recurrence, clinical stage (limited disease or extensive disease according to the International Association for the Study of Lung Cancer's consensus report) at disease progression and type of relapse (sensitive relapse defined as relapse at an interval of 90 days after the completion of initial chemotherapy and refractory relapse defined as no response to initial chemotherapy or relapse within 90 days after the completion of initial chemotherapy).

Treatment

Irinotecan was administered on Days 1, 8 and 15 via a 90-min intravenous infusion at a dose of 60 mg/m^2. The treatment cycles were repeated every 4 weeks. When the leukocyte or platelet count was inappropriate or diarrhea occurred on Day 8 or 15, the treatment was skipped.

Evaluation of Response and Toxicity

The objective tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors guideline, version 1.1 (15). Efficacy evaluation schedules were not defined strictly due to the retrospective study from clinical practice. However, we performed chest computed tomography (CT) evaluation every 2–3 months in principle with our clinical practice. The investigator (M.M.) reviewed all radiological imaging and re-evaluated tumor responses in each patient. In the study, confirmation of complete response (CR) and partial response (PR) were not performed. Toxicity was assessed according to the Common Terminology Criteria for Adverse Events (v4.0). The data of toxicity were collected minutely from medical records, and we could obtain each toxicity profile weekly due to the weekly schedule regimen.

Statistical Analysis

The overall survival (OS) and progression-free survival (PFS) times were estimated using the Kaplan–Meier method. The PFS was measured from the date of the start of irinotecan chemotherapy to the documented date of disease progression, death or the last follow-up. The OS was measured from the date of the start of irinotecan chemotherapy to the date of death or the last follow-up. The statistical analyses were performed using JMP software, version 10.0.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patient Characteristics

Between April 2003 and June 2012, a total of 321 patients with recurrent SCLC received second-line chemotherapy. Among them, 57 consecutive patients with recurrent SCLC were treated with a low dose of irinotecan as a second-line therapy. The patient characteristics are listed in Table 1. The median age was 70 years (range, 51–83 years). The majority

Table 1. Patient characteristics ($n = 57$)

	Number of patients (%)
Age, median (range)	70 (51–83)
Sex	
Male	52 (91)
Female	5 (9)
ECOG PS	
0	5 (9)
1	31 (54)
2	17 (30)
3	4 (7)
Type of relapse	
Refractory relapse	31 (54)
Sensitive relapse	26 (46)
Stage at recurrence	
Limited disease	13 (23)
Extensive disease	44 (77)
First-line chemotherapy	
ETP + CDDP	27 (47)
ETP + CBDCA	26 (46)
CPT + CDDP	2 (3)
CPT + ETP + CDDP	1 (2)
AMR + CDDP	1 (2)
Thoracic irradiation	
−	33 (58)
+	24 (42)
PCI	
−	44 (77)
+	13 (23)

ECOG PS, Eastern Cooperative Oncology Group performance Status; ETP, etoposide; CDDP, cisplatin; CBDCA, carboplatin; CPT, irinotecan; AMR, amrubicin; PCI, prophylactic cranial irradiation.

Table 2. Tumor response

	n (%)
CR	0 (0)
PR	18 (32)
SD	15 (26)
PD	19 (33)
NE	5 (9)
Response rate (95% CI)	32% (20–45%)
Disease control rate (95% CI)	58% (44–71%)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

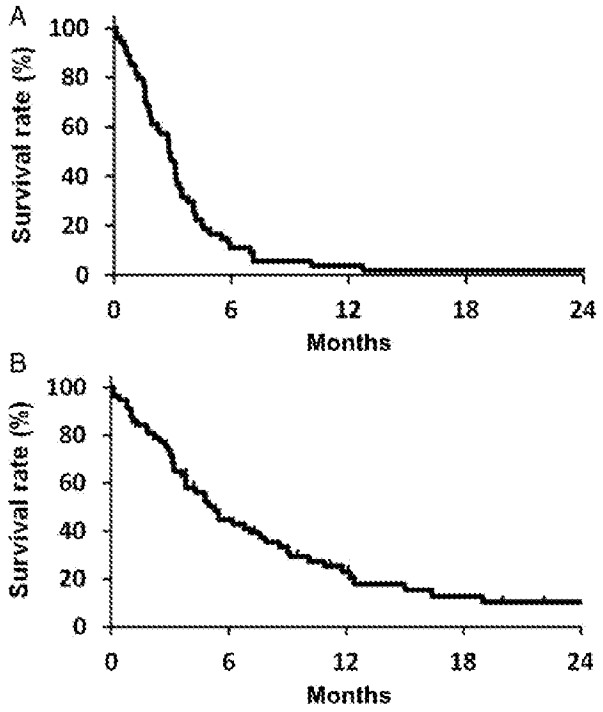

Figure 1. (A) Progression-free survival (PFS) and (B) overall survival (OS) ($n = 57$).

of patients were male (91%). In total, 158 cycles were administered. The median number of treatment cycles was 2 (range, 1–8). Of the total 158 cycles, 85 cycles (54%) were performed without skipping an administration. Sixty-four cycles (41%) were performed with the skipping of one administration on Day 8 or 15. Only nine cycles (5%) were performed with the skipping of both administrations on Days 8 and 15. The major reasons for the skipping of administration on Day 8 or 15 were leukopenia, diarrhea and fatigue.

Efficacy

The objective response rate (ORR) is shown in Table 2. Eighteen PRs were obtained, and the ORR was 32% (95% confidence interval (CI): 20–45%). The disease control rate was 58% (95% CI, 44–71%). We performed a subgroup analysis of the tumor response according to the type of relapse (sensitive relapse versus refractory relapse). The ORRs in patients with sensitive relapse and refractory relapse were 38% (95% CI, 20–59%) and 26% (95% CI, 12–45%), respectively. At the time of analysis, 56 patients (98%) had experienced disease progression and 47 (82%) patients had died. The median PFS and the median OS were 2.9 months (95% CI, 1.9–3.4 months) and 5.3 months (95% CI: 3.6–7.6 months), respectively (Fig. 1). The median PFS in patients with sensitive relapse and refractory relapse was 3.7 months (95% CI: 2.8–4.2 months) and 2.2 months (95% CI, 1.5–3.1 months), respectively. The median OS in patients with sensitive relapse and refractory relapse was 5.9 months (95% CI,

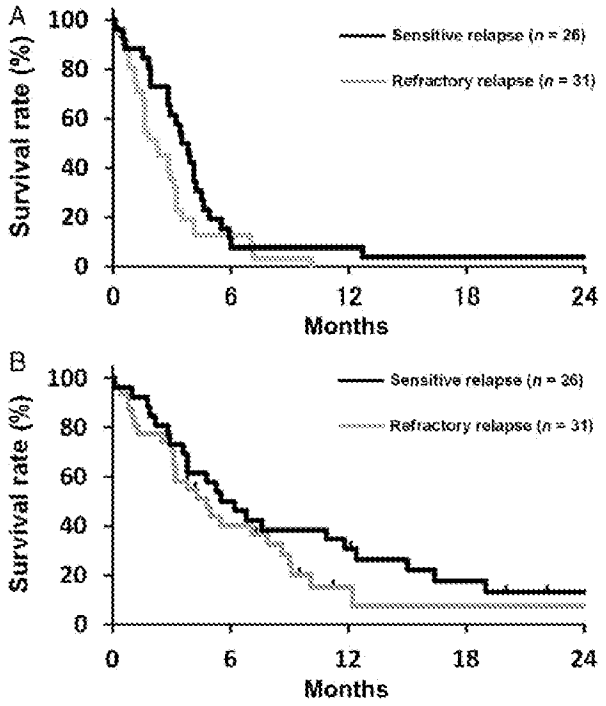

Figure 2. (A) PFS in patients with sensitive relapse and refractory relapse. (B) OS in patients with sensitive relapse and refractory relapse.

3.6–11.8 months) and 4.8 months (95% CI, 3.1–7.9 months), respectively (Fig. 2). In addition, the median OS according to PS was 7.1 months (95% CI: 3.8–10.1 months) with PS0-1 and 3.2 months (95% CI, 1.8–4.8 months) with PS2-3.

Safety

The worst grades of adverse events are listed in Table 3. The most common adverse events were neutropenia (all grades, 61%), nausea/vomiting (all grades, 61%) and diarrhea (all grades, 53%). The incidence of Grade 3/4 neutropenia, diarrhea and nausea/vomiting was 21, 4 and 5%, respectively. Treatment-related death was observed in two patients. One of them developed febrile neutropenia, resulting in septic shock. The other patient died of respiratory failure despite receiving methylprednisolone pulse treatment. We judged this adverse event as Grade 5 interstitial lung disease possibly related to irinotecan because congestive heart failure and bacterial pneumonia were clinically excluded by a chest X-ray and chest CT scan findings, in addition to the absence of symptoms such as systematic edema, fever and/or yellow sputum.

DISCUSSION

In this study, low-dose irinotecan monotherapy (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) achieved an ORR of 32% and a median PFS of 2.9 months, with a favorable toxicity profile. The incidence of Grade 3/4 neutropenia was 21%, and the

Table 3. Safety

	Grade				Grades 3–4
	1	2	3	4	% of patients
Leukopenia	20	15	10	1	19
Neutropenia	8	15	8	4	21
Thrombocytopenia	7	2	3	0	5
Diarrhea	14	14	1	1	4
Fatigue	12	4	2	–	4
Nausea/vomiting	28	4	3	0	5
Anorexia	12	11	4	0	7
Febrile neutropenia	–	–	1	1[a](Gr5)	4 (Gr3–5)
ILD	0	0	0	1[b](Gr5)	2 (Gr3–5)
Edema	0	1	0	0	0
Fever	1	0	0	0	0
Neuropathy	0	0	0	0	0
Rash	0	0	0	0	0
AST increased	2	0	0	0	0
ALT increased	2	0	0	0	0
Cre increased	0	1	0	0	0

Gr, grade; ILD, interstitial lung disease; ALT, alanine transaminase; AST, aspartate transaminase; Cre, creatinine.
[a]Grade 5 febrile neutropenia was observed in one patient.
[b]Grade 5 ILD was observed in one patient.

incidences of Grade 3/4 diarrhea and nausea/vomiting were 4 and 5%, respectively. Several Phase II trials showed that irinotecan monotherapy (100–125 mg/m^2 weekly or 350 mg/m^2 on Day 1 every 3 weeks) was promising in a second-line setting for recurrent SCLC (6–9). An ORR of 16–47% was achieved in these studies, but these dose settings of irinotecan are associated with a high incidence of Grade 3/4 neutropenia (25–58%), diarrhea (7–37%) and nausea/vomiting (13–22%). The review of efficacy and safety data of previous studies and our study is summarized in Table 4. Ando et al. (16) reported that polymorphisms of UDP-glucuronosyltransferase (UGT) 1A1 were associated with severe neutropenia and/or diarrhea caused by irinotecan. The significance of UGT1A1 polymorphisms for predicting severe neutropenia was confirmed in an irinotecan dose setting of weekly 100 mg/m^2, biweekly 150 mg/m^2 and 350 mg/m^2 on Day 1 every 3 weeks (17,18). On the other hand, no difference in the toxicity profile was observed according to UGT1A1 polymorphisms in a dose setting of weekly 70 mg/m^2 irinotecan combined with 25 mg/m^2 of docetaxel (19). In the present study, we could not evaluate the UGT1A1 polymorphism status because this study analyzed a retrospective cohort. However, our low-dose irinotecan setting (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) might reduce the risk of severe toxicity caused by genetic polymorphisms of UGT1A1.

Table 4. Efficacy and safety data of previous studies and our study

Authors	*n*	% of SR Pts	Dose (mg/m^2)	Schedule	RR (%)	Grade 3/4 (%)		
						Neutropenia	Diarrhea	Nausea/vomiting
Masuda et al. (6)	15	93	100	Weekly	47	33[a]	7	13
Negoro et al. (7)[b]	27	–	100	Weekly	33	25[a]	18	18
DeVore et al. (8)	44	39	125	d1.8.15.22 q6weeks	16	27	27	–/9[c]
Le Chevalier (9)	32	–	350	d1, q3weeks	16	58	37	22
Current study	57	46	60	d1. 8. 15, q4weeks	32	21	4	5

SR, sensitive relapse; Pts, patients; RR, response rate.
[a]The rate of leukopenia.
[b]Evaluation for incidence of toxicity was performed in a total of 146 lung cancer patients, including 27 previously treated small cell lung cancer patients.
[c]The rate of vomiting.

A higher dose does not always result in a higher efficacy for highly chemosensitive malignant tumors. In patients with recurrent SCLC patients, amrubicin, a topoisomerase II inhibitor, exhibited favorable activity with lower dose than standard dose decided by initial Phase I studies (20). In addition, in patients with ovarian cancer that is highly chemosensitive as well as SCLC, lower-dose topotecan (topoisomerase I inhibitor as well as irinotecan) might have equal activity compared with the standard dose of topotecan (21,22). Our results suggest that a lower dose of irinotecan might have an equal efficacy with a less toxic profile for patients with recurrent SCLC.

The median OS of 5.3 months in our study seems shorter compared with recently reported Japanese studies for recurrent SCLC. In these studies, the median OS were ranged 7.0–11.2 months (14,23–26). In our retrospective study, consecutive patients treated with low-dose irinotecan were enrolled to minimize selection bias. Therefore, 37% of PS2-3 included in our study, while only 0–24% of PS2 and no PS3 patients were included in these studies. Actually, the median OS in patients with PS0-1 was 7.1 months in our study. The difference of patient characteristics between our study and previous studies might influence the results of OS although our results are needed to validate by prospective studies.

This study has several limitations. First, this is a retrospective study in a single institution. Efficacy evaluation schedules were not defined strictly due to the retrospective study from clinical practice. In addition, confirmation of CR/PR and external review of radiological imaging were not performed in this study. In regard to toxicities, the data were collected minutely from medical records and we could obtain each toxicity profile weekly due to the weekly schedule regimen; however, prospective comparative studies are needed to verify the less toxicity of low-dose irinotecan monotherapy.

In conclusion, low-dose irinotecan monotherapy (60 mg/m^2 on Days 1, 8 and 15 every 4 weeks) for recurrent SCLC was potentially active with a favorable toxicity profile. Further prospective trials are warranted to compare standard-dose irinotecan monotherapy versus low-dose irinotecan monotherapy for patients with recurrent SCLC.

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Conflict of interest statement

None declared.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013;63:11–30.
2. O'Brien ME, Ciuleanu TE, Tsekov H, et al. Phase III trial comparing supportive care alone with supportive care with oral topotecan in patients with relapsed small-cell lung cancer. *J Clin Oncol* 2006;24:5441–47.
3. Eckardt JR, von Pawel J, Pujol JL, et al. Phase III study of oral compared with intravenous topotecan as second-line therapy in small-cell lung cancer. *J Clin Oncol* 2007;25:2086–92.
4. Kim YH, Mishima M. Second-line chemotherapy for small-cell lung cancer (SCLC). *Cancer Treat Rev* 2011;37:143–50.
5. Saijo N. Progress in treatment of small-cell lung cancer: role of CPT-11. *Br J Cancer* 2003;89:2178–83.
6. Masuda N, Fukuoka M, Kusunoki Y, et al. CPT-11: a new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol* 1992;10:1225–29.
7. Negoro S, Fukuoka M, Niitani H, et al. A phase II study of CPT-11, a camptothecin derivative, in patients with primary lung cancer. CPT-11 Cooperative Study Group. *Gan To Kagaku Ryoho* 1991;18:1013–9.
8. DeVore RF, Blanke CD, Denham CA, et al. Phase II study of irinotecan in patients with previously treated small cell lung cancer. *Proc Amer Soc Clin Oncol* 1998;17:451a.
9. Le Chevalier T, Ibrahim N, Chomy P, et al. A phase II study of irinotecan in patients with samll cell lung cancer progressing after initial response to first line chemotherapy. *Proc Amer Soc Clin Oncol* 1997;16:450a.
10. Negoro S, Fukuoka M, Masuda N, et al. Phase I study of weekly intravenous infusions of CPT-11, a new derivative of camptothecin, in the treatment of advanced non-small-cell lung cancer. *J Natl Cancer Inst* 1991;83:1164–68.
11. de Forni M, Bugat R, Chabot GG, et al. Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients. *Cancer Res* 1994;54:4347–54.
12. Noda K, Nishiwaki Y, Kawahara M, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* 2002;346:85–91.

13. Takeda K, Negoro S, Sawa T, et al. A phase II study of topotecan in patients with relapsed small-cell lung cancer. *Clin Lung Cancer* 2003;4:224–28.
14. Inoue A, Sugawara S, Yamazaki K, et al. Randomized phase II trial comparing amrubicin with topotecan in patients with previously treated small-cell lung cancer: North Japan Lung Cancer Study Group Trial 0402. *J Clin Oncol* 2008;26:5401–06.
15. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009;45:228–47.
16. Ando Y, Saka H, Ando M, et al. Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. *Cancer Res* 2000;60:6921–26.
17. Minami H, Sai K, Saeki M, et al. Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. *Pharmacogenet Genomics* 2007;17:497–504.
18. Innocenti F, Undevia SD, Iyer L, et al. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. *J Clin Oncol* 2004;22:1382–88.
19. Font A, Sanchez JM, Taron M, et al. Weekly regimen of irinotecan/docetaxel in previously treated non-small cell lung cancer patients and correlation with uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) polymorphism. *Invest New Drugs* 2003;21:435–43.
20. Igawa S, Yamamoto N, Ueda S, et al. Evaluation of the recommended dose and efficacy of amrubicin as second- and third-line chemotherapy for small cell lung cancer. *J Thorac Oncol* 2007;2:741–44.
21. Rodriguez M, Rose PG. Improved therapeutic index of lower dose topotecan chemotherapy in recurrent ovarian cancer. *Gynecol Oncol* 2001;83:257–62.
22. Mitchell SK, Carson LF, Judson P, et al. Efficacy and tolerability of lower-dose topotecan in recurrent ovarian cancer: a retrospective case review. *Int J Gynecol Cancer* 2005;15:793–98.
23. Onoda S, Masuda N, Seto T, et al. Phase II trial of amrubicin for treatment of refractory or relapsed small-cell lung cancer: Thoracic Oncology Research Group Study 0301. *J Clin Oncol* 2006;24:5448–53.
24. Hirose T, Nakashima M, Shirai T, et al. Phase II trial of amrubicin and carboplatin in patients with sensitive or refractory relapsed small-cell lung cancer. *Lung Cancer* 2011;73:345–50.
25. Nogami N, Hotta K, Kuyama S, et al. A phase II study of amrubicin and topotecan combination therapy in patients with relapsed or extensive-disease small-cell lung cancer: Okayama Lung Cancer Study Group Trial 0401. *Lung Cancer* 2011;74:80–4.
26. Murakami H, Yamamoto N, Shibata T, et al. A single-arm confirmatory study of amrubicin therapy in patients with refractory small-cell lung cancer: Japan Clinical Oncology Group Study (JCOG0901). *Lung Cancer* 2014;84:67–72.

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Original Research

First-line liposomal irinotecan with oxaliplatin, 5-fluorouracil and leucovorin (NALIRIFOX) in pancreatic ductal adenocarcinoma: A phase I/II study☆,☆☆

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KEYWORDS
Liposomal irinotecan;
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Abstract ***Background:*** This open-label, phase I/II study evaluated safety and efficacy for first-line liposomal irinotecan + oxaliplatin + 5-fluorouracil + leucovorin (NALIRIFOX). ***Methods:*** Patients (aged ≥18 years) had locally advanced/metastatic pancreatic ductal adenocarcinoma (mPDAC), with an Eastern Cooperative Oncology Group performance status score

☆ Prior presentation: The contents of this article satisfy the criteria for originality. Results from this final data cutoff have been presented at: the European Society for Medical Oncology World Congress on Gastrointestinal Cancer 2020 – Virtual, 1–4 July, 2020; and the European Society for Medical Oncology Virtual Congress 2020, 19–21 September 2020.

☆☆ Results from earlier data cutoff dates were presented at: the American Association for Cancer Research Special Conference on Pancreatic Cancer: Advances in Science and Clinical Care, 2019, Boston, MA, 6–9 September, 2019; the European Society for Medical Oncology World Congress on Gastrointestinal Cancer 2019, Barcelona, Spain, 3–6 July, 2019; and the American Society of Clinical Oncology Annual Conference, Chicago, IL, 1–5 June, 2018.

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[1] At the time the study was conducted.

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'Irinotecan');
Locally advanced pancreatic adenocarcinoma;
Metastatic pancreatic adenocarcinoma (MeSH: 'pancreatic neoplasms', 'carcinoma, pancreatic ductal', 'neoplasm metastasis');
Clinical trial (MeSH: 'clinical trials as topic')

of 0/1 and adequate organ function. Primary objectives were to determine the maximum tolerated dose (MTD) and to evaluate safety and tolerability. Treatment-emergent adverse events (TEAEs) were graded using National Cancer Institute Common Terminology Criteria for Adverse Events v4.03. Efficacy end-points included progression-free survival (PFS) and overall survival (OS); disease assessments used Response Evaluation Criteria in Solid Tumors 1.1.
Results: The MTD (liposomal irinotecan 50 mg/m^2 [free-base equivalent], oxaliplatin 60 mg/m^2, 5-fluorouracil 2400 mg/m^2, leucovorin 400 mg/m^2 every 2 weeks) was based on dose-limiting toxicities and cumulative safety data in four dose-exploration cohorts. The MTD was received by 32 of 56 patients, seven during dose exploration and 25 during dose expansion (median age 58.0 years [range, 39–76], 28 [87.5%] with metastatic disease at diagnosis [29 at study entry], and one receiving study treatment at data cutoff [26 February 2020]). Of these patients, 22 of 32 had grade ≥3 treatment-related TEAEs, most commonly neutropenia (31.3%), febrile neutropenia (12.5%) and hypokalaemia (12.5%); ten had serious treatment-related TEAEs; and three died from TEAEs considered unrelated to treatment. Median PFS and OS were 9.2 (95% CI: 7.69–11.96) and 12.6 (8.74–18.69) months, respectively.
Conclusion: First-line NALIRIFOX for patients with locally advanced/mPDAC was generally manageable and tolerable. A randomised, controlled phase III study is underway.

1. Introduction

Advanced pancreatic cancer is associated with poor clinical outcomes [1]. Preferred first-line treatment options for patients with metastatic pancreatic ductal adenocarcinoma (mPDAC) include gemcitabine + albumin-bound paclitaxel (gemcitabine/nab-paclitaxel) and non-liposomal irinotecan + oxaliplatin + 5-fluorouracil/leucovorin (5-FU/LV) (FOLFIRINOX) [2,3]. Although both regimens provided significant improvements in survival outcomes compared with gemcitabine monotherapy in clinical trials [4,5], survival rates for pancreatic cancer have remained low [6–8]. The research imperative for the treatment of patients with mPDAC therefore remains developing and testing new agents and new combinations in the first-line setting.

The non-liposomal formulation of the topoisomerase I inhibitor irinotecan is a well-established component of various combination therapies [9], including FOLFIRINOX in mPDAC [2,10,11]. However, preclinical and clinical data suggest there may be additional benefits if liposomal irinotecan (ONIVYDE®; historically nal-IRI; Ipsen Biopharmaceuticals, Inc., Cambridge, MA, USA) is substituted for the non-liposomal formulation. Liposomal irinotecan (70 mg/m^2 free-base equivalent), in combination with 5-FU (2400 mg/m^2) and LV (400 mg/m^2), is already a recommended treatment option for patients with mPDAC following progression with gemcitabine-based therapy, based on the results of the NAPOLI-1 phase III trial [2,3,12]. Preclinically, the active metabolite, SN-38, persists longer in tumours after administration of liposomal irinotecan (up to 168 h) than after administration of non-liposomal irinotecan (<48 h) [13]. Furthermore, in patients with mPDAC receiving liposomal irinotecan + 5-FU/LV during NAPOLI-1 [12], longer exposures to unencapsulated SN-38 above a key threshold and higher average plasma concentrations of total irinotecan, total SN-38 and unencapsulated SN-38 were all associated with better overall survival (OS) and progression-free survival (PFS) [14]. Improved anti-tumour activity has also been observed with liposomal versus non-liposomal irinotecan, when administered with oxaliplatin + 5-FU, in a patient-derived xenograft model [15].

This open-label, phase I/II study used the NALIRIFOX regimen, in which liposomal irinotecan replaced the non-liposomal irinotecan component of FOLFIRINOX. It was designed to establish a recommended dose for further study, and to investigate safety/tolerability, efficacy and pharmacokinetics (PK) in patients with locally advanced or mPDAC who had not been treated previously in the advanced/metastatic setting.

2. Methods

2.1. Patients

The study comprised two parts: dose exploration followed by dose expansion. Eligible patients were ≥18 years of age, had histologically or cytologically confirmed pancreatic adenocarcinoma that was locally advanced or metastatic, and had not been treated previously in the advanced/metastatic setting. Patients also had measurable disease using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 [16]; adequate haematological, hepatic and renal function; and an Eastern Cooperative Oncology Group (ECOG) performance status score of 0 or 1 [17] (dose-exploration part) or a Karnofsky Performance Status score of ≥70 [18] (dose-expansion part). Exclusion criteria included

any second malignancy in the previous 3 years and use of strong CYP3A4 inhibitors or inducers, or strong UGT1A1 inhibitors.

2.2. Study design and treatment

This open-label, two-part, phase I/II study enrolled patients between 26 October 2015 and 29 October 2018. The study was conducted at 21 sites in Australia, Spain and the USA. The data cutoff for the long-term follow-up results presented here was 26 February 2020.

Patients received study treatment every 2 weeks (days 1 and 15 of each 28-day cycle). Intravenous treatment was administered sequentially beginning with liposomal irinotecan, then oxaliplatin, LV 400 mg/m^2 and 5-FU 2400 mg/m^2 (no bolus; continuous infusion over 46 h); see Appendix for further details. Patients were intended to receive study treatment until radiologically determined progressive disease (PD) or unacceptable toxicity related to study treatment. Patients could discontinue oxaliplatin alone at the investigator's discretion; otherwise, discontinuation was of all four study drugs. Granulocyte colony stimulating factors (G-CSF) were permitted at investigator discretion, to manage neutropenia or as prophylaxis if patients were considered high risk (see Appendix). Oxaliplatin dose reductions were permitted for sensory neuropathy (see protocol). Survival data and information about subsequent mPDAC therapies were obtained every 8 weeks after discontinuation until death or study completion.

Dose exploration used a traditional 3 + 3 design (see Appendix); with dosing based on that administered in the NAPOLI-1 (liposomal irinotecan 70 mg/m^2 free-base equivalent) and PRODIGE 4 (FOLFIRINOX; oxaliplatin 85 mg/m^2) pivotal studies [5,12]. Doses (in order of testing) were cohort A: liposomal irinotecan 70 mg/m^2 free-base equivalent + oxaliplatin 60 mg/m^2 (70/60); cohort B: 50/60; cohort C: 50/85 (all predetermined); and cohort D: 55/70 (introduced in a protocol amendment, see Appendix). Dose-limiting toxicities (DLTs, defined in Appendix) were measured during cycle 1 (28-day DLT period). Progression to the next cohort occurred after safety evaluation was complete for the last patient enrolled in a cohort.

During dose expansion, patients received the maximum tolerated dose (MTD); those withdrawing were not replaced.

2.3. Assessments and end-points

For dose exploration, the primary objectives were to characterise DLTs and determine the recommended dose. Overall, the primary study objectives were safety and tolerability, with secondary objectives of efficacy and PK.

Treatment-emergent adverse events (TEAEs) were coded using Medical Dictionary for Regulatory Activities (MedDRA) version 20.1, and toxicity was graded using National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.03. Key TEAEs were defined in the clinical study report (CSR) using MedDRA terms, based on monitoring and the known safety profiles of liposomal irinotecan and oxaliplatin: diarrhoea (grade $\geq$3), febrile neutropenia (any grade), neutropenic sepsis (any grade), neutropenia (grade $\geq$3), thrombo-embolic events (any grade), peripheral neuropathy (grade $\geq$3).

Computerised tomography or magnetic resonance imaging was performed at screening (baseline), every 8 weeks thereafter until radiologically determined PD, and at end of treatment. Disease was evaluated by investigators using RECIST version 1.1. Efficacy end-points included PFS, OS, overall response rate (ORR), the disease control rate at 16 weeks (DCR_{16}) and the duration of response (DoR).

PK analyses and exploratory analyses of survival in *post hoc* subgroups are described in the Appendix.

2.4. Statistical analyses

The sample size for dose exploration was dependent on the number of patients enrolled into cohorts and the toxicity rate. The recommended dose was to be received by at least 30 patients; there was no efficacy hypothesis.

The median PFS and OS were calculated using the Kaplan–Meier method (with hazard ratios [HRs] determined using Cox regression for biomarker subgroups); 95% confidence intervals (CIs) were calculated using Brookmeyer–Crowley methods. For measures of clinical response, patients without a postbaseline tumour assessment were classified as not evaluable. DoR was analysed using the Kaplan–Meier method and 95% CIs were calculated using the Clopper–Pearson and Brookmeyer–Crowley methods for ORR and DCR_{16}, respectively.

Analyses were conducted for the safety and PK populations. Statistical analyses were performed with SAS® software version 9.3 or higher (SAS Institute, Inc., Cary, NC, USA). Censoring rules (Table A1) and population definitions are provided in the Appendix.

2.5. Study oversight

The study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonisation Consolidated Guideline on Good Clinical Practice. Study documentation was approved by an independent ethics committee and institutional review board. Patients provided written informed consent at screening. Protocol amendments made after the study started are described in the protocol.

3. Results

3.1. Dose exploration

Of the 31 patients enrolled for dose exploration, five experienced ≥1 DLT. The doses used in cohorts A and C were not considered tolerable because two patients in each cohort experienced ≥1 DLT. In cohort A (70/60, seven patients), neutropenic infection (grade 4) was reported in one patient and neutropenic sepsis (grade 4) in another patient. In cohort C (50/85, 10 patients), diarrhoea and vomiting were reported in one patient (both grade 4 and > 3 days in duration); and diarrhoea (grade 3, >3 days in duration), anal fissure, anal inflammation and proctalgia (all grade 2 and delayed the next scheduled dose by >14 days) were reported in another patient. Although no patients had DLTs in cohort D (55/70, seven patients), the dose was not considered tolerable following review of grade ≥3 TEAEs. Finally, one patient had a DLT of febrile neutropenia (grade 3) in cohort B (50/60, seven patients). Following review of cumulative safety in this cohort, 50/60 was the MTD recommended for expansion (Fig. 1).

3.2. Population receiving the recommended dose

3.2.1. Patient disposition and baseline characteristics

In total, 32 of the 56 patients enrolled in the study received the recommended dose, seven during dose exploration and 25 during dose expansion (Fig. 1). These patients had a median age of 58.0 years; 87.5% had metastatic disease at diagnosis, 43.8% had liver metastases and 56.3% had an ECOG performance status score of 1 (Table 1).

3.2.2. Treatment

Treatment durations and cumulative doses are reported in Table 2. In total, 31 of 32 patients receiving the recommended dose discontinued study treatment, most commonly because of PD (14 patients) (Fig. 1, Table A2). Of those who discontinued treatment, 25 subsequently received second-line therapy, most commonly gemcitabine/nab-paclitaxel (15 patients) (Appendix).

3.2.3. Safety and tolerability

All 32 patients receiving the recommended dose experienced ≥1 TEAE considered related to treatment (Table

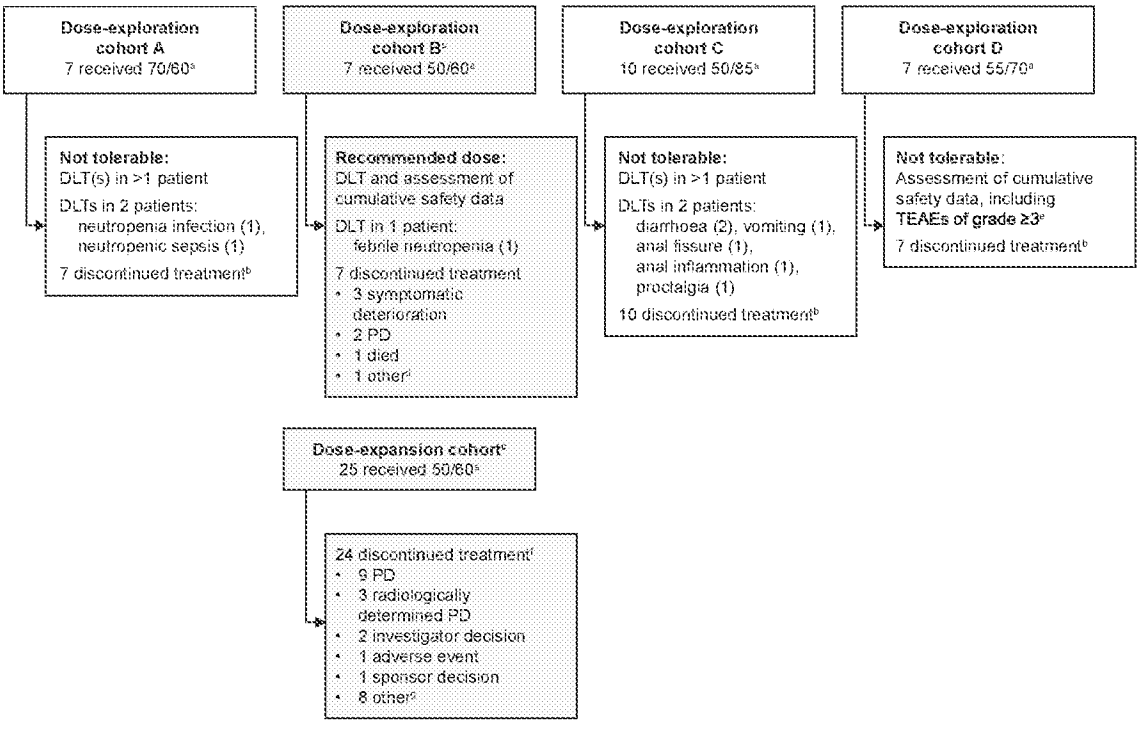

Fig. 1. Flow of patients through the study. [a] Dose of liposomal irinotecan (free-base equivalent)/dose of oxaliplatin expressed in mg/m^2 to be administered in combination with 5-fluorouracil 2400 mg/m^2 and leucovorin 400 mg/m^2 on days 1 and 15 of each 28-day cycle. [b] Reasons for discontinuation are provided in Table A2. [c] Cohorts receiving the recommended dose were included in the pooled population 50/60 for the analysis of efficacy and safety. [d] Owing to patient decision. [e] All TEAEs of grade ≥3 are provided in Table A3. [f] One patient was still receiving treatment at the data cutoff. [g] Owing to patient decision (four patients), clinical progression (two patients), consent withdrawn (one patient) and lack of clinical benefit/adverse event (one patient). [h] All patients in the enrolled population (completed screening successfully with documented enrolment date) were included in the safety and PK populations. Abbreviations: DLT, dose-limiting toxicity; PD, progressive disease; PK, pharmacokinetic.

Table 1
Demographic and disease characteristics at baseline.

Characteristic	Dose-exploration cohorts				Dose-expansion cohort	Pooled population
	A (70/60[a]) (n = 7)	B (50/60[a]) (n = 7)	C (50/85[a]) (n = 10)	D (55/70[a]) (n = 7)	(50/60[a]) (n = 25)	(50/60[a,b]) (n = 32)
Age, years						
Mean (SD)	66.7 (7.87)	60.4 (10.66)	65.5 (5.21)	63.1 (7.17)	56.8 (9.95)	57.6 (10.05)
Median (range)	64.0 (58–78)	57.0 (44–74)	66.5 (57–73)	61.0 (54–73)	58.0 (39–76)	58.0 (39–76)
Women, No. (%)	6 (85.7)	4 (57.1)	2 (20.0)	2 (28.6)	14 (56.0)	18 (56.3)
Race, No. (%)						
White	6 (85.7)	7 (100)	9 (90.0)	7 (100)	21 (84.0)	28 (87.5)
Black or African American	0	0	0	0	2 (8.0)	2 (6.3)
Asian	1 (14.3)	0	1 (10.0)	0	1 (4.0)	1 (3.1)
Missing	0	0	0	0	1 (4.0)	1 (3.1)
ECOG performance status score, No. (%)						
0	1 (14.3)	6 (85.7)	6 (60.0)	5 (71.4)	8 (32.0)	14 (43.8)
1	6 (85.7)	1 (14.3)	4 (40.0)	2 (28.6)	17 (68.0)	18 (56.3)
*UGT1A1*28* allele status, No. (%)						
Negative	4 (57.1)	3 (42.9)	5 (50.0)	3 (42.9)	11 (44.0)[c]	14 (43.8)[c]
Homozygous (7/7)	1 (14.3)	1 (14.3)	1 (10.0)	0	1 (4.0)	2 (6.3)
Heterozygous (7/6)	2 (28.6)	2 (28.6)	3 (30.0)	4 (57.1)	11 (44.0)	13 (40.6)
Missing	0	1 (14.3)	1 (10.0)	0	1 (4.0)	2 (6.3)
Tumour stage at diagnosis, No. (%)[d]						
IIA	0	0	0	0	1 (4.0)	1 (3.1)
III	3 (42.9)	1 (14.3)	2 (20.0)	2 (28.6)	2 (8.0)	3 (9.4)
IV	4 (57.1)	6 (85.7)	8 (80.0)	5 (71.4)	22 (88.0)	28 (87.5)
Tumour location, No. (%)						
Head	5 (71.4)	4 (57.1)	3 (30.0)	2 (28.6)	6 (24.0)	10 (31.3)
Body	0	2 (28.6)	6 (60.0)	3 (42.9)	2 (8.0)	4 (12.5)
Tail	1 (14.3)	1 (14.3)	0	1 (14.3)	8 (32.0)	9 (28.1)
Head and body	0	0	0	0	1 (4.0)	1 (3.1)
Body and tail	0	0	1 (10.0)	1 (14.3)	4 (16.0)	4 (12.5)
Missing	1 (14.3)	0	0	0	4 (16.0)	4 (12.5)
Metastatic lesion locations, No. (%)						
Liver	3 (42.9)	2 (28.6)	4 (40.0)	3 (42.9)	12 (48.0)	14 (43.8)
Lung	0	1 (14.3)	2 (20.0)	4 (57.1)	3 (12.0)	4 (12.5)
Lymph nodes	0	0	0	0	1 (4.0)	1 (3.1)
Other	2 (28.6)	4 (57.1)	4 (40.0)	1 (14.3)	16 (64.0)	20 (62.5)
Missing	4 (57.1)	1 (14.3)	4 (40.0)	3 (42.9)	4 (16.0)	5 (15.6)

Abbreviations: ECOG, Eastern Cooperative Oncology Group; SD, standard deviation.
[a] Dose of liposomal irinotecan (free-base equivalent)/dose of oxaliplatin expressed in mg/m^2 to be administered in combination with 5-fluorouracil 2400 mg/m^2 and leucovorin 400 mg/m^2 every on days 1 and 15 of each 28-day cycle.
[b] Comprises cohorts assigned to receive liposomal irinotecan 50 mg/m^2 and oxaliplatin 60 mg/m^2 during the dose-exploration or dose-expansion parts of the study.
[c] Excludes one patient with compound heterozygosity for the TA5 and TA7 polymorphisms.
[d] One patient in the dose-expansion cohort received a diagnosis of stage IIA disease but entered the study with stage IV disease.

2). Key TEAEs defined in the CSR (using MedDRA v20.1) were experienced by 19 patients: grade ≥3 neutropenia (10 patients, all considered treatment-related); febrile neutropenia (four patients, all grade ≥3 and considered treatment-related); grade ≥3 diarrhoea (four patients, considered treatment-related in three); thrombo-embolic events (five patients); no patients experienced neutropenic sepsis or grade ≥3 peripheral neuropathy (which was present only in cohort C [50/85, one patient]; Table A3).

The most common grade ≥3 TEAEs apart from neutropenia, febrile neutropenia and diarrhoea (see above) were hypokalaemia (six patients), neutrophil count decreased and alanine aminotransferase increased (four patients each) (Table 2, Table A3).

Grade ≥3 treatment-related TEAEs occurred in 22 of 32 patients; the most common apart from neutropenia, febrile neutropenia and diarrhoea (see above) were hypokalaemia (four patients), nausea (three patients) and neutrophil count decreased (3 patients) (Table A4). The following grade ≥3 treatment-related liver function abnormalities were reported: increases in alanine aminotransferase (two patients), gamma-glutamyltransferase (two patients), aspartate aminotransferase (one patient) and blood alkaline phosphatase (one patient); and hepatotoxicity (one patient).

Table 2
Duration of treatment, cumulative doses and overview of TEAEs.

	Dose-exploration cohorts				Dose-expansion cohort	Pooled population
	A (70/60[a]) (n = 7)	B (50/60[a]) (n = 7)	C (50/85[a]) (n = 10)	D (55/70[a]) (n = 7)	(50/60[a]) (n = 25)	(50/60[a,b]) (n = 32)
Duration of treatment in weeks,[c] mean (SD)						
Liposomal irinotecan	3.8 (5.02)	44.6 (49.26)	23.2 (31.62)	14.0 (16.20)	28.4 (20.36)	31.9 (28.93)
Oxaliplatin	3.8 (5.02)	44.6 (49.26)	15.1 (17.67)	14.0 (16.20)	25.8 (18.61)	29.9 (28.28)
5-Fluorouracil	4.1 (5.02)	44.9 (49.30)	23.5 (31.62)	14.3 (16.20)	28.7 (20.36)	32.2 (28.94)
Leucovorin	3.8 (5.02)	44.6 (49.26)	23.2 (31.62)	14.0 (16.20)	28.4 (20.36)	31.9 (28.94)
Cumulative doses in mg, median (range)						
Liposomal irinotecan	160.5 (79.1 −398.1)	620.5 (59.7 −3574.1)	185.8 (59.8 −2748.2)	326.5 (64.7 −794.3)	632.0 (58.8 −1683.2)	626.2 (58.8 −3574.1)
Oxaliplatin	120.3 (59.4 −359.6)	705.8 (59.7 −3087.7)	269.8 (84.8 −1636.5)	353.3 (69.7 −1221.1)	596.3 (58.8 −1440.4)	598.8 (58.8 −3087.7)
5-Fluorouracil	4813.7 (2373.9 −14444.4)	22844.1 (2400.0 −143350.5)	7867.6 (2400.0 −108238.0)	12081.1 (2388.1 −41865.3)	25347.4 (2352.9 −67326.2)	24862.7 (2352.9 −143350.5)
Leucovorin	802.3 (395.7 −2407.4)	4805.8 (400.0 −23926.0)	1406.8 (400.0 −17966.3)	2012.5 (394.2 −9170.1)	4953.8 (411.8 −12411.1)	4879.8 (400.0 −23926.0)
Any TEAE	7 (100)	7 (100)	10 (100)	7 (100)	25 (100)	32 (100)
Any treatment-related[d] TEAE	6 (85.7)	7 (100)	9 (90.0)	7 (100)	25 (100)	32 (100)
Grade ≥3	6 (85.7)	4 (57.1)	8 (80.0)	5 (71.4)	18 (72.0)	22 (68.8)
Any TEAE leading to dose discontinuation[e]	5 (71.4)	1 (14.3)	3 (30.0)	3 (42.9)	7 (28.0)	8 (25.0)
Any TEAE leading to dose adjustment[f]	2 (28.6)	4 (57.1)	7 (70.0)	4 (57.1)	22 (88.0)	26 (81.3)
Any serious TEAE	6 (85.7)	2 (28.6)	7 (70.0)	4 (57.1)	15 (60.0)	17 (53.1)
Leading to death[g]	0	1 (14.3)	1 (10.0)	1 (14.3)	2 (8.0)	3 (9.4)
Treatment-related[g]	4 (57.1)	1 (14.3)	5 (50.0)	4 (57.1)	9 (36.0)	10 (31.3)
TEAE of grade ≥3 occurring in ≥5% of the pooled population						
Neutropenia	1 (14.3)	2 (28.6)	3 (30.0)	1 (14.3)	8 (32.0)	10 (31.3)
Hypokalaemia	3 (42.9)	2 (28.6)	2 (20.0)	3 (42.9)	4 (16.0)	6 (18.8)
Diarrhoea	3 (42.9)	1 (14.3)	4 (40.0)	1 (14.3)	3 (12.0)	4 (12.5)
Neutrophil count decreased	1 (14.3)	0	1 (10.0)	0	4 (16.0)	4 (12.5)
Febrile neutropenia	0	1 (14.3)	0	0	3 (12.0)	4 (12.5)
Alanine aminotransferase increased	0	0	0	0	4 (16.0)	4 (12.5)
Vomiting	1 (14.3)	0	3 (30.0)	0	3 (12.0)	3 (9.4)
Anaemia	0	1 (14.3)	0	0	2 (8.0)	3 (9.4)
Nausea	0	0	3 (30.0)	0	3 (12.0)	3 (9.4)
Abdominal pain	0	0	0	1 (14.3)	3 (12.0)	3 (9.4)
Lymphocyte count decreased	0	0	0	0	3 (12.0)	3 (9.4)
Hypoalbuminemia	1 (14.3)	0	0	0	2 (8.0)	2 (6.3)
Back pain	0	1 (14.3)	0	0	1 (4.0)	2 (6.3)
Dyspnoea	0	0	0	0	2 (8.0)	2 (6.3)
Gamma-glutamyltransferase increased	0	0	0	0	2 (8.0)	2 (6.3)
Hyperglycaemia	0	0	0	0	2 (8.0)	2 (6.3)
Hyponatraemia	0	0	0	0	2 (8.0)	2 (6.3)
White blood cell count decreased	0	0	0	0	2 (8.0)	2 (6.3)

Data are no. (%) of patients from the safety population unless stated otherwise. Events were coded in accordance with the preferred terms in the Medical Dictionary for Regulatory Activities version 20.1 and toxicity was graded using the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03.

Abbreviations: SD, standard deviation; TEAE, treatment-emergent adverse event.

[a] Dose of liposomal irinotecan (free-base equivalent)/dose of oxaliplatin expressed in mg/m^2 to be administered in combination with 5-fluorouracil 2400 mg/m^2 and leucovorin 400 mg/m^2 on days 1 and 15 of each 28-day cycle.

[b] Comprises cohorts assigned to receive liposomal irinotecan 50 mg/m^2 and oxaliplatin 60 mg/m^2 during the dose-exploration or dose-expansion parts of the study.

[c] Duration of treatment (in days) was calculated as (date of last exposure − date of first exposure) + 1, before being converted to weeks.

[d] Comprises TEAEs considered by the investigator to be related to any of the four treatments administered or for which the relationship was missing.

[e] Refers to discontinuation of oxaliplatin alone or all four treatments administered, as described in the protocol. In the PP 50/50, TEAEs leading to discontinuation were peripheral neuropathy (two patients); abdominal pain, biliary dilatation, enterocolitis, malignant gastrointestinal obstruction, neurotoxicity, decreased platelet count, thrombocytopenia, upper gastrointestinal haemorrhage and decreased white blood cell count (one patient in each case); in some patients more than one TEAE contributed to discontinuation.

[f] Refers to an adjustment in the dose of any of the four treatments administered.

[g] TEAEs leading to death, considered unrelated to treatment: cohort B, upper gastrointestinal haemorrhage (n = 1); cohort C, subdural haematoma (n = 1), dose-expansion cohort, malignant gastrointestinal obstruction (n = 1, considered unrelated to treatment), disease progression (n = 1, considered unrelated to treatment); considered related to treatment: cohort D, colitis (n = 1).

Serious TEAEs were reported for 17 patients (Table 2, Table A5) and were considered treatment-related in 10 patients (Table A6). Three patients died from TEAEs considered unrelated to treatment (Table 2).

TEAEs led to discontinuation (of oxaliplatin alone or all four study treatments) in eight patients and dose adjustments of any study treatment in 26 (Table 2). Sixteen patients received G-CSF (Table A8).

Clinically significant laboratory test abnormalities were reported as TEAEs. Laboratory and other safety assessment results were in line with the expected safety profile of the study regimen.

3.2.4. Efficacy

The median PFS was 9.2 months (95% CI: 7.69–11.96; Fig. 2A) in patients receiving the recommended dose. Fifteen patients had censored data, of whom one was still receiving treatment. The median OS was 12.6 months (95% CI: 8.74–18.69; Fig. 2B), with 20 deaths reported. Best overall response, ORR, DCR_{16} and DoR are reported in Table 3.

3.2.5. Other end-points

Results of PK and exploratory analyses are reported in the Appendix.

4. Discussion

Improvements in survival rates remain elusive for patients with pancreatic cancer [6,19], underscoring the need for improved treatment options [1]. To date, only one phase III trial of targeted therapy added to chemotherapy has shown improvement in survival for patients newly diagnosed with mPDAC [1,11,20–22], highlighting the need for more durable combination chemotherapy regimens as the backbone for future first-line treatment. In this phase I/II study, patients with locally advanced or mPDAC received a new combination first line: liposomal irinotecan 50 mg/m^2 + oxaliplatin 60 mg/m^2 + 5-FU 2400 mg/m^2 + LV 400 mg/m^2 every 2 weeks (NALIRIFOX).

The safety of NALIRIFOX cannot be reliably compared with that of established therapies without head-to-head studies. However, no unexpected safety outcomes were apparent based on the known safety profiles of the drugs. Of the key TEAEs, grade ≥3 neutropenia was the most common among patients receiving the recommended dose (31.3%), followed by any grade of thrombo-embolic events (15.6%), then any grade of febrile neutropenia and grade ≥3 diarrhoea (12.5% for each). In addition, grade ≥3 neutrophil count decreased was reported in 12.5% of patients. G-CSF was administered to 50% of patients, to manage neutropenia or as prophylaxis in those considered high risk. G-CSF is permitted at the investigator's discretion in the ongoing NAPOLI-3 phase III study of NALIRIFOX (ClinicalTrials.gov NCT04083235; EudraCT 2018-003585-14). In the final long-term analysis of the NAPOLI-1 study, the most common grade ≥3 TEAEs (using MedDRA v14.1) [23] in patients receiving liposomal irinotecan + 5-FU/LV were neutropenia (32%; comprising neutropenia, neutrophil count

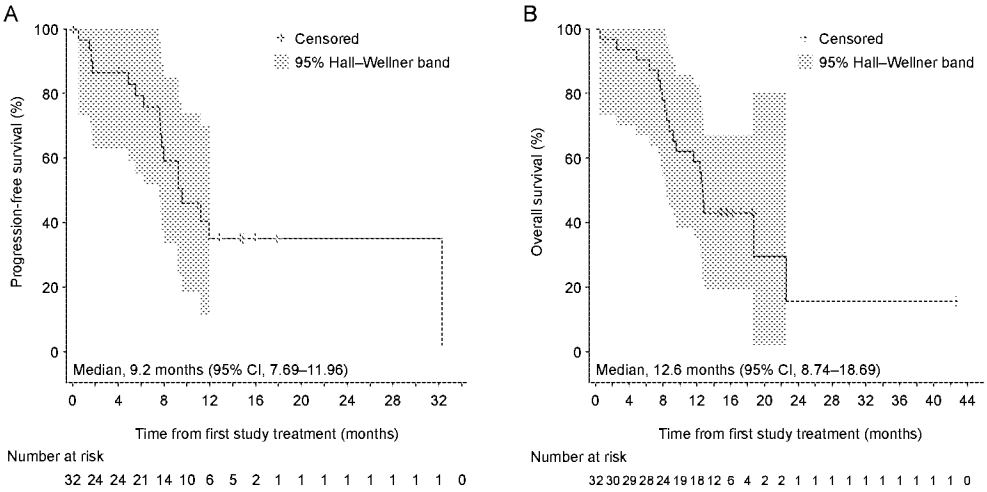

Fig. 2. (A) PFS and (B) OS in the pooled population of patients receiving the recommended dose (50/60[a]). Data are from the safety population (n = 32). Median PFS and OS were calculated using the Kaplan–Meier method, with 95% CIs calculated using Brookmeyer–Crowley methods. Confidence bands are 95% Hall–Wellner bands. One patient with minimal progressive disease per RECIST version 1.1 was approved for treatment continuation as the investigator believed there was a benefit from treatment. PFS for this patient ended at the date of minimal progressive disease. [a]Comprises cohorts assigned to receive liposomal irinotecan 50 mg/m^2 (free-base equivalent) and oxaliplatin 60 mg/m^2, in combination with 5-fluorouracil 2400 mg/m^2 and leucovorin 400 mg/m^2, on days 1 and 15 of each 28-day cycle during either the dose-exploration or dose-expansion parts of the study. Abbreviations: CI, confidence interval; OS, overall survival; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors.

Table 3
Clinical response.

	Dose-exploration cohorts				Dose-expansion cohort	Pooled population
	A (70/60[a]) (n = 7)	B (50/60[a]) (n = 7)	C (50/85[a]) (n = 10)	D (55/70[a]) (n = 7)	(50/60[a]) (n = 25)	(50/60[a,b]) (n = 32)
Best overall response[c], No. (%)						
CR	0	0	0	0	1 (4.0)	1 (3.1)[d]
PR	0	3 (42.9)	3 (30.0)	1 (14.3)	7 (28.0)	10 (31.3)
SD	2 (28.6)	3 (42.9)	1 (10.0)	3 (42.9)	12 (48.0)	15 (46.9)
PD	1 (14.3)	0	2 (20.0)	1 (14.3)	3 (12.0)	3 (9.4)
Non-PD/non-CR[e]	1 (14.3)	0	0	0	0	0
Not evaluable	3 (42.9)	1 (14.3)	4 (40.0)	2 (28.6)	2 (8.0)	3 (9.4)
Overall response (CR + PR), rate [95% CI][f]	0 [0, 41.0]	42.9 [9.9, 81.6]	30.0 [6.7, 65.2]	14.3 [0.4, 57.9]	32.0 [14.9, 53.5]	34.4 [18.6, 53.2]
DCR at 16 weeks (CR + PR + SD), rate [95% CI][g]	42.9 [9.9, 81.6]	71.4 [29.0, 96.3]	40.0 [12.2, 73.8]	28.6 [3.7, 71.0]	72.0 [50.6, 87.9]	71.9 [53.3, 86.3]
Duration of response[h]	(n = 0)	(n = 3)	(n = 3)	(n = 1)	(n = 8)	(n = 11)
Median, months [95% CI]	NE [NE, NE]	28.4 [3.52, NE]	NE [NE, 16.39]	NE [NE, NE]	9.4 [2.20, NE]	9.4 [3.52, NE]

Data are from the safety population and responses were determined using RECIST version 1.1.
Abbreviations: CI, confidence interval; CR, complete response; DCR, disease control rate; NE, not estimable; PD, progressive disease; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease.
[a] Dose of liposomal irinotecan (free-base equivalent)/dose of oxaliplatin expressed in mg/m^2 to be administered in combination with 5-fluorouracil 2400 mg/m^2 and leucovorin 400 mg/m^2 every on days 1 and 15 of each 28-day cycle.
[b] Comprises cohorts assigned to receive liposomal irinotecan 50 mg/m^2 and oxaliplatin 60 mg/m^2 during the dose-exploration or dose-expansion parts of the study.
[c] Best response recorded from the start of study treatment until disease progression or the start of new anti-cancer therapy.
[d] Patient received a diagnosis of locally advanced stage III disease.
[e] As per the protocol (version 1.0) at the time of their screening, one patient had a measurable lesion in a lymph node at screening that was too small to be considered a target lesion in accordance with RECIST version 1.1. Consequently, this patient was followed only for non-target lesions (included in the table above as 'non-PD/non-CR') but was included in the summary of overall response. The protocol was later amended to require the presence of target lesion(s).
[f] Proportion of patients with a CR or PR as the best overall response; 95% CIs were calculated using the Clopper–Pearson method.
[g] Proportion of patients with CR, PR or SD at the week-16 assessment; patients who died, whose tumours were no longer assessed, or who started new anti-cancer treatment before the week-16 assessment were not considered to have achieved disease control at week 16.
[h] Time from the first date of response (CR or PR) to the date of the first documented radiologically determined PD; duration of response was not calculated for patients who started a new anti-cancer treatment before the first response.

decreased, neutropenic sepsis, febrile neutropenia and several other terms), fatigue (14%), diarrhoea (13%) and vomiting (12%) [24]. Similarly, in the PRODIGE 4 study, the most common grade 3–4 TEAEs (using CTCAE v3.0) in patients receiving FOLFIRINOX were neutropenia (45.7%), fatigue (23.6%), vomiting (14.5%) and diarrhoea (12.7%) [8].

Grade ≥3 sensory neuropathy is a particular concern with oxaliplatin-containing regimens [25]. For the recommended NALIRIFOX regimen, none was reported. By contrast, in PRODIGE 4, grade 3–4 sensory neuropathy was experienced by 9.0% of patients receiving FOLFIRINOX (for persistent grade 2 sensory neuropathy, an oxaliplatin dose reduction from 85 to 65 mg/m^2 was permitted) [8].

The efficacy of first-line NALIRIFOX warrants further investigation, given a median PFS of 9.2 months (95% CI: 7.69–11.96) and median OS of 12.6 months (8.74–18.69), although direct comparisons with other studies cannot be made. The outcomes of the PRODIGE 4 study are of interest, as these underpin the recommendations for the FOLFIRINOX regimen as first-line therapy in mPDAC [2,10,11,26]. FOLFIRINOX was associated with a median PFS of 6.4 months (95% CI: 5.5–7.2) and median OS of 11.1 months (9.0–13.1), using RECIST v1.0 [8]. However, important differences between the study populations include the proportions of patients with metastatic disease at study entry (recommended NALIRIFOX regimen: 90.6%; FOLFIRINOX in PRODIGE 4: 100%), the proportions with liver metastases (43.8% and 87.6%, respectively) and the median ages (58 and 61 years, respectively) [8].

Limitations inherent in the present study design include the small number of patients, which limits the precision of efficacy parameter estimates; the lack of an efficacy hypothesis; the non-randomised design; and the absence of a control group. Although only patients with adequate performance status were included, similar restrictions were used in PRODIGE 4 [8].

5. Conclusions

The present phase I/II study demonstrated that first-line NALIRIFOX had tolerability that was generally manageable for patients with locally advanced or

mPDAC, with no unexpected safety outcomes. Ultimately, an important, as-yet-unanswered question is the preferred treatment for patients newly diagnosed with mPDAC. NAPOLI-3, an ongoing, large randomised, controlled, phase III study, will compare the efficacy (primary endpoint, OS) and safety of first-line NALIRIFOX with gemcitabine/nab-paclitaxel in this population, using the doses established here.

Clinical trial information

ClinicalTrials.gov number, NCT02551991 (https://www.clinicaltrials.gov/); EudraCT 2015-003086-28 (https://www.clinicaltrialsregister.eu/).

Data sharing statement

If patient data can be anonymised, Ipsen will share all individual patient data that underlie the results reported in this article with qualified researchers who provide a valid research question. Study documents, such as the clinical study report, are not always available. Proposals should be submitted to DataSharing@Ipsen.com and will be assessed by a scientific review board. Data are available beginning 6 months, and ending 5 years, after publication; after this time, only raw data may be available.

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Author contributions

Conception and design: A. Wainberg, Bruce Belanger, Fiona Maxwell, Tiffany Wang, Bin Zhang and Andrew Dean.

All authors: acquisition, analysis or interpretation of data for the work.

All authors: drafting the work or revising it critically for important intellectual content.

All authors: final approval of the manuscript.

All authors are accountable for all aspects of the work.

Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: **Note**: relationships are self-held unless noted. I = Immediate Family Member, Inst = My Institution.

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Arunthathi Thiagalingam: Former employee: Ipsen, **Stock and Other Ownership Interests:** Ipsen.

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Appendix A. Supplementary data

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References

[1] Hall BR, Cannon A, Atri P, Wichman CS, Smith LM, Ganti AK, et al. Advanced pancreatic cancer: a meta-analysis of clinical trials over thirty years. Oncotarget 2018;9:19396–405. https://doi.org/10.18632/oncotarget.25036.

[2] National Comprehensive Cancer Network. Pancreatic adenocarcinoma, version 2. February 25 2021. Available from: https://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf. [Accessed 8 March 2021].

[3] ESMO Guidelines Committee. eUpdate – cancer of the pancreas treatment recommendations. Available from: https://www.esmo.org/guidelines/gastrointestinal-cancers/pancreatic-cancer/eupdate-cancer-of-the-pancreas-treatment-recommendations2. [Accessed 8 March 2021].

[4] Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 2013;369:1691–703. https://doi.org/10.1056/NEJMoa1304369.

[5] Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 2011;364:1817–25. https://doi.org/10.1056/NEJMoa1011923.

[6] American Cancer Society. Cancer facts & figures 2020. Available from: https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2020.html. [Accessed 8 March 2021].

[7] Foundation for Promotion of Cancer Research. Cancer statistics in Japan – 2019. Available from: https://ganjoho.jp/data/reg_stat/statistics/brochure/2019/cancer_statistics_2019.pdf. [Accessed 8 March 2021].

[8] Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries and 25 major cancers in 2018. Eur J Canc 2018;103:356–87. https://doi.org/10.1016/j.ejca.2018.07.005.

[9] Bailly C. Irinotecan: 25 years of cancer treatment. Pharmacol Res 2019;148:104398. https://doi.org/10.1016/j.phrs.2019.104398.

[10] Ducreux M, Cuhna AS, Caramella C, Hollebecque A, Burtin P, Goere D, et al. Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2015;26(S5):v56–68. https://doi.org/10.1093/annonc/mdv295.

[11] Sohal DPS, Kennedy EB, Cinar P, Conroy T, Copur MS, Crane CH, et al. Metastatic pancreatic cancer: ASCO guideline update. J Clin Oncol 2020;38:3217–30. https://doi.org/10.1200/JCO.20.01364.

[12] Wang-Gillam A, Li CP, Bodoky G, Dean A, Shan YS, Jameson G, et al. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global, randomised, open-label, phase 3 trial. Lancet 2016;387:545–57. https://doi.org/10.1016/s0140-6736(15)00986-1.

[13] Kalra AV, Kim J, Klinz SG, Paz N, Cain J, Drummond DC, et al. Preclinical activity of nanoliposomal irinotecan is governed by tumor deposition and intratumor prodrug conversion. Canc Res 2014;74:7003–13. https://doi.org/10.1158/0008-5472.can-14-0572.

[14] Adiwijaya BS, Kim J, Lang I, Csoszi T, Cubillo A, Chen JS, et al. Population pharmacokinetics of liposomal irinotecan in patients with cancer. Clin Pharmacol Ther 2017;102:997–1005. https://doi.org/10.1002/cpt.730.

[15] Gaddy DF, Lee H, Paz N, Leonard SC, Kalra A, Straubinger NL, et al. Preclinical anti-tumor activity of nanoliposomal irinotecan (Nal-IRI, MM-398) + 5-FU + oxaliplatin in pancreatic cancer. Canc Res 2016;76(14 Supplement):4830 [abstract].

[16] Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Canc 2009;45:228–47. https://doi.org/10.1016/j.ejca.2008.10.026.

[17] Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the eastern cooperative Oncology Group. Am J Clin Oncol 1982;5:649–55.

[18] Karnofsky DA, Abelmann WH, Craver LF, Burchena JH. The use of the nitrogen mustards in the palliative treatment of carcinoma – with particular reference to bronchogenic carcinoma. Cancer 1948;1:634–56.

[19] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA A Cancer J Clin 2018;68:394–424. https://doi.org/10.3322/caac.21492.

[20] Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 2007;25:1960–6. https://doi.org/10.1200/JCO.2006.07.9525.

[21] Tempero M, Oh DY, Tabernero J, Reni M, Van Cutsem E, Hendifar A, et al. Ibrutinib in combination with nab-paclitaxel and gemcitabine for first-line treatment of patients with metastatic pancreatic adenocarcinoma: phase 3 RESOLVE study. Ann Oncol 2021. https://doi.org/10.1016/j.annonc.2021.01.070.

[22] Van Cutsem E, Tempero MA, Sigal D, Oh DY, Fazio N, Macarulla T, et al. Randomized phase III trial of pegvorhyaluronidase alfa with nab-paclitaxel plus gemcitabine for patients with hyaluronan-high metastatic pancreatic adenocarcinoma. J Clin Oncol 2020;38:3185–94. https://doi.org/10.1200/JCO.20.00590.

[23] National Library of Medicine (US). Study of MM-398 with or without 5-FU/LV, versus 5-FU/LV in patients with metastatic pancreatic cancer (NAPOLI-1). Identifier: NCT01494506. 17. Jun 2016. Available from: https://clinicaltrials.gov/ct2/show/results/NCT01494506?view=results. [Accessed 8 March 2021].

[24] Wang-Gillam A, Hubner RA, Siveke JT, Von Hoff DD, Belanger B, de Jong FA, et al. NAPOLI-1 phase 3 study of liposomal irinotecan in metastatic pancreatic cancer: final overall

survival analysis and characteristics of long-term survivors. Eur J Canc 2019;108:78–87. https://doi.org/10.1016/j.ejca.2018.12.007.

[25] Park SB, Goldstein D, Krishnan AV, Lin CS, Friedlander ML, Cassidy J, et al. Chemotherapy-induced peripheral neurotoxicity: a critical analysis. CA A Cancer J Clin 2013;63:419–37. https://doi.org/10.3322/caac.21204.

[26] Sohal DP, Mangu PB, Khorana AA, Shah MA, Philip PA, O'Reilly EM, et al. Metastatic pancreatic cancer: American Society of Clinical Oncology clinical practice guideline. J Clin Oncol 2016;34:2784–96. https://doi.org/10.1200/jco.2016.67.1412.