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(71) Applicant: **MERRIMACK PHARMACEUTICALS, INC.** [US/US]; One Kendall Square, Suite B7201, Cambridge, MA 02139 (US).

(72) Inventors: **FITZGERALD, Jonathan, Basil**; 32 Magnolia Street, Arlington, MA 02474 (US). **KEARNS, Jeffrey, D.**; 42 Everett Street, Apt. 2, Arlington, MA 02474 (US). **LEE, Helen**; 120 Rindge Avenue, Apt. 100, Cambridge, MA 02140 (US). **NERING, Rachel, C.**; 12 Myrtle Road, Stoneham, MA 02180 (US).

(74) Agent: **DAY, Noel, E.**; Honigman Miller Schwartz and Cohn LLP, 350 East Michigan Avenue, Suite 300, Kalamazoo, MI 49007 (US).

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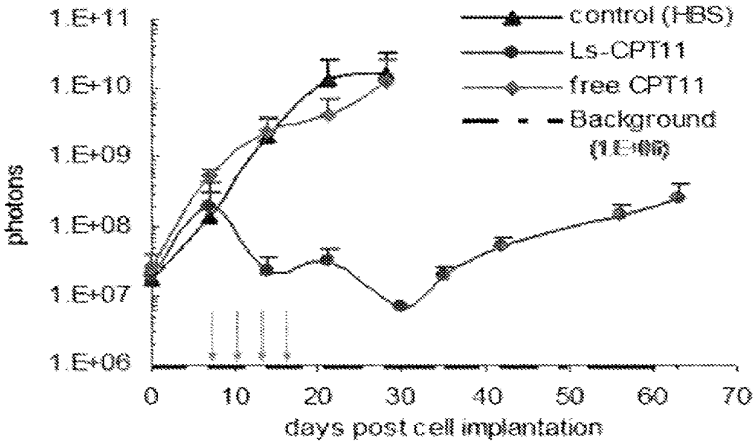
(54) Title: METHODS FOR TREATING CANCER USING COMBINATION THERAPIES COMPRISING AN OLIGOCLONAL ANTI-EGFR ANTIBODY PREPARATION AND LIPSOMAL IRINOTECAN

FIG. 1



(57) Abstract: Provided are methods for treating colorectal cancer (including RAS wild type colorectal cancer) in a patient by administering liposomal irinotecan (MM-398) in combination with anti- EGFR antibodies such as MM-151. The liposomal irinotecan (MM-398) can be co-administered with 5-fluorouracil and leucovorin.

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METHODS FOR TREATING CANCER USING COMBINATION THERAPIES COMPRISING AN OLIGOCLONAL ANTI-EGFR ANTIBODY PREPARATION AND LIPOSOMAL IRINOTECAN

CROSS-REFERENCE

This patent application claims priority to each of the following pending U.S. provisional patent applications, each incorporated herein by reference in their entirety: 62/315,129 (filed March 30, 2016), 62/324,389 (filed April 19, 2016), 62/324,986 (filed April 20, 2016), 62/338,080 (filed May 18, 2016), 62/345,506 (filed June 3, 2016) and 62/370,886 (filed August 4, 2016).

SEQUENCE LISTING

Incorporated by reference in its entirety is a computer-readable sequence listing submitted concurrently herewith and identified as follows: One 24.0 KB ASCII (Text) file named "1119sequencelisting_ST25.txt."

TECHNICAL FIELD

The specification relates to the treatment of cancer with a combination of an EGFR inhibitor, liposomal irinotecan, leucovorin and 5-fluorouracil, including the treatment of colorectal cancer.

BACKGROUND

Despite improvements in cancer treatments, there remains a critical need to further improve therapies so as to prolong patients' lives while maintaining quality of life, particularly in the case of advanced cancers resistant to current therapeutic modalities. Colorectal Cancer (CRC) remains a leading cause of cancer death worldwide and is the third most common cancer in men and women. The global incidence of CRC was 1.4 million in 2012 with an expected increase to 2.4 million by 2035. In 2014, an estimated 136,830 new CRC cases arose and 50,310 CRC-related deaths occurred in the US alone. Approximately 20% of patients present with advanced, metastatic disease and their 5 year survival remains poor (<10%) with current therapies, highlighting the need for improved treatments.

Treatment for mCRC is rapidly evolving. Approved agents include 5-FU, irinotecan, oxaliplatin, capecitabine, bevacizumab, aflibercept, cetuximab, panitumumab, regorafenib, and trifluridine / tipiracil hydrochloride. Optimum sequencing and/or combinations of these therapies has not been clearly defined, but aggressive therapy improves overall survival. FOLFOX based chemotherapy is a standard first line option in these patients within the

United States.

Combination therapies including folinic acid (leucovorin or levoleucovorin), 5-fluorouracil, and irinotecan (FOLFIRI), folinic acid, 5-fluorouracil, irinotecan and oxaliplatin (FOLFIRINOX), or a combination of folinic acid, 5-fluorouracil, and oxaliplatin (FOLFOX) are also used to treat some cancers. Bevacizumab, cetuximab and panitumumab are frequently combined with these regimens depending on patient presentation, biomarker status and physician preference. Irinotecan is 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycampothecin, IUPAC name (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. Irinotecan is a member of the topoisomerase I inhibitor class of drugs and is a semi-synthetic and water soluble analog of the naturally-occurring alkaloid, camptothecin. Also known as CPT-11, irinotecan is currently marketed formulated as an aqueous solution as Camptosar® (irinotecan hydrochloride injection). Topoisomerase I inhibitors such as irinotecan work to arrest uncontrolled cell growth by inhibiting the unwinding of DNA and thereby preventing DNA replication.

The pharmacology of irinotecan is complex, with extensive metabolic conversions involved in the activation, inactivation, and elimination of the drug. Irinotecan is a prodrug that is converted by nonspecific carboxylesterases into a 100-1000 fold more active metabolite, SN-38. SN-38 is not recognized by P-glycoprotein, a drug transporter that plays an important role in acquired drug resistance by pumping certain drugs out of cells, so irinotecan is likely to be active in tumors resistant to other standard chemotherapies. In the body, SN-38 is cleared via glucuronidation, for which major pharmacogenetic variability has been described, and biliary excretion. These drug properties contribute to the marked heterogeneities in efficacy and toxicity observed clinically with irinotecan. Irinotecan hydrochloride injection is approved in the United States for treatment of metastatic colon or renal cancer and is also used to treat colorectal, gastric, lung, uterine cervical and ovarian cancers.

EGFR signaling is important for the proliferation and survival of many epithelial malignancies affecting humans. Although EGFR-targeting antibodies have been approved for use in human malignancies that appear to be driven by EGFR, there still remains a high unmet need in these tumors, as limited efficacy is attributable to de novo or acquired resistance mechanisms to these targeted therapies. One reason for this state of affairs might stem from observations that EGFR signaling is very robust due to amplification of cell signaling downstream of the receptor, thus making the need for complete inhibition of the

pathway imperative for potential success in the clinic. EGFR signaling is further complicated by the presence in tumors of multiple ligands that bind to EGFR with either high or low affinity and result in activation of the pathway downstream of EGFR. Preclinical evidence obtained at Merrimack suggests that EGFR signaling driven by high affinity ligands is not always inhibited by currently approved EGFR antagonists. Additional mutations and resistance mechanisms both upstream (extracellular) and downstream of the receptor have been described by Merrimack and others.

Certain anti-EGFR monoclonal antibodies have demonstrated a lack of efficacy in patients with mCRC containing KRAS mutations, based on retrospective analyses. In these trials, patients received standard of care (i.e., BSC or chemotherapy) and were randomized to receive either an anti-EGFR antibody (cetuximab or panitumumab) or no additional therapy. In all studies, investigational tests were used to detect KRAS mutations in codon 12 or 13. The percentage of study populations for which KRAS status was assessed ranged from 23% to 92%. In CRC tumor cells with activating KRAS somatic mutations, the mutant KRAS protein is continuously active and appears independent of EGFR regulation. Retrospective subset analyses of metastatic or advanced colorectal cancer trials have not shown a treatment benefit for cetuximab in patients whose tumors had KRAS mutations in codon 12 or 13. Use of cetuximab is not recommended for the treatment of colorectal cancer with these mutations. Similarly, panitumumab is not indicated for the treatment of patients with colorectal cancer that harbor somatic mutations in exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of either KRAS or NRAS. Retrospective subset analyses across several randomized clinical trials were conducted to investigate the role of RAS mutations on the clinical effects of anti-EGFR-directed monoclonal antibodies (panitumumab or cetuximab). Anti-EGFR antibodies in patients with tumors containing RAS mutations resulted in exposing those patients to anti-EGFR related adverse reactions without clinical benefit from these agents. Additionally, in Study 3, 272 patients with RAS-mutant mCRC tumors received panitumumab in combination with FOLFOX and 276 patients received FOLFOX alone. In an exploratory subgroup analysis, OS was shorter (HR = 1.21, 95% CI 1.01-1.45) in patients with RAS- Panitumumab is not indicated for the treatment of patients with colorectal cancer that harbor somatic mutations in exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of either KRAS or NRAS.

SUMMARY

Provided are methods for treating cancer in a patient (*i.e.*, a human patient)

comprising administering to the patient liposomal irinotecan (e.g., irinotecan sucrose octasulfate salt liposome injection, also referred to as MM-398, nal-IRI, or ONIVYDE) in combination with 5-fluorouracil (5-FU) and leucovorin (together, 5-FU/LV) and an EGFR inhibitor, according to a particular clinical dosage regimen. Compositions adapted for use in such methods are also provided. In particular, a combination of the EGFR inhibitor MM-151, MM-398 liposomal irinotecan, 5-fluorouracil and leucovorin can be used to treat metastatic colorectal cancer (mCRC).

As an active drug in mCRC, irinotecan can be combined with EGFR inhibitors to treat RAS wild type tumors. Nal-IRI (also disclosed as MM-398 or ONIVYDE®) is a nanoliposomal formulation of irinotecan. The nanoliposomal encapsulation improves the pharmacokinetics of irinotecan and results in a lower C_{max}, longer half-life, and higher levels of irinotecan and SN-38 in tumor tissue compared with standard irinotecan. Nal-IRI is approved by the FDA under the brand name ONIVYDE, in combination with 5-FU and leucovorin in gemcitabine refractory pancreatic adenocarcinoma. In a non-comparative study evaluating the use of nal-IRI + 5-FU + LV as a 2nd line therapy in mCRC (PEPCOL study), the nal-IRI containing arm met the threshold of early responses and had lower incidence of diarrhea and neutropenia than the free irinotecan arm.

As both cetuximab and irinotecan represent established treatments, in a combination regimen or in combination with 5-FU/leucovorin (comprising the FOLFIRI regimen), the present disclosure provides methods of treating colorectal cancer by administering both MM-151 (a next-generation EGFR antibody) and ONIVYDE (also called MM-398 or nal-IRI) in combination with 5-FU and leucovorin.

1. In some embodiments, a therapeutically effective combination of the anti-EGFR therapy MM-151 can be administered in combination with liposomal irinotecan, 5-fluorouracil and leucovorin for the treatment of patients diagnosed as having colorectal cancer (CRC), including patients diagnosed as having metastatic CRC (mCRC), containing KRAS mutations. The CRC can be mCRC with tumor cells containing RAS mutations. For example, the patient can be diagnosed with mCRC that harbors somatic mutations in exon 2 (codons 12 or 13), exon 3 (codons 59 and 61) and/or exon 4 (codons 117 and 146) of either KRAS or NRAS. In another embodiment, the patient can be diagnosed with mCRC that harbors a BRAF mutation. In one example, the BRAF mutation could be the BRAF V600E mutation. In another example, the BRAF mutation is a somatic mutation in one or more of codons 464, 466, 469, 595, 596, and 601. In another embodiment, the patient can be diagnosed with mCRC that harbors an EGFR mutation. For example, the EGFR mutation

can be a somatic mutation in one or more codons of exon 12. In another embodiment, the patient can be diagnosed with mCRC that harbors a somatic mutation in the region coding for the catalytic subunit of PI3K, PIK3CA. For example, the PIK3CA mutation is mutation in one or more of codons 542, 545, and 1047. a m

MM-151 is a combination of three fully human IgG1 monoclonal antibodies. MM-151 antagonizes high-affinity EGFR ligands more effectively than approved inhibitors, including cetuximab and panitumumab, and has been observed to elicit a greater decrease in signal amplification. MM-151 has been evaluated in a phase I study of solid tumors and demonstrated a safety profile that was comparable to other EGFR inhibitors. The recommended phase II dose (RP2D) identified in the MM-151 monotherapy phase I study was 10.5 mg/kg QW and this is the starting dose for MM-151 in this study.

MM-151 was combined with nal-IRI in a xenograft experiment with the LoVo colorectal cancer cell line. The data showed that this combination enhances anti-tumor activity as compared to either therapy alone. MM-151 was combined with nal-IRI and 5-FU in a xenograft experiment with the LIM1215 colorectal cancer cell line. The data showed that this combination enhances anti-tumor activity as compared to the combination of cetuximab, irinotecan (at equivalent SN-38 exposure), and 5-FU. These results, combined with the preliminary efficacy data seen in the PEPCOL study, suggest that the combination of MM-151 + nal-IRI + 5-FU + LV may be a viable therapeutic option in mCRC.

In one aspect, a method for treatment (e.g. effective treatment) of colorectal cancer in a patient is provided, the method comprising intravenously administering to a human patient in need thereof a single administration of 6.0, 7.5, 9.0 or 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2; and a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours, to treat the colorectal cancer in the patient.

In another aspect, a method for treatment of colorectal cancer in a patient is provided, the method comprising co-administering to the patient an effective amount each of liposomal irinotecan, 5-fluorouracil (5-FU), and leucovorin, wherein the method comprises at least one cycle of administration, wherein the cycle is a period of 2 weeks, and wherein for each cycle (in any order of administration, unless otherwise indicated):

(a) once-weekly administration of 10.5 mg/kg MM-151 in combination with

(b) a single administration of liposomal irinotecan once every two weeks administered to patients not homozygous for the UGT1A1*28 allele on day 1 of each cycle at a dose of 70 mg/m^2 irinotecan (free base) in an MM-398 irinotecan liposome, and to patients homozygous for the UGT1A1*28 allele on day 1 of cycle 1 at a dose of 50 mg/m^2 irinotecan (free base) in an MM-398 irinotecan liposome, and on day 1 of each subsequent cycle at a dose of ranging from 50 mg/m^2 to 70 mg/m^2 (*e.g.*, 60 mg/m^2 or 70 mg/m^2 or 80 mg/m^2) irinotecan (free base) in an MM-398 irinotecan liposome;

(c) a single administration of 5-FU administered at a dose of 2400 mg/m^2 once every two weeks in combination with the MM-398 irinotecan liposome and

(d) leucovorin administered once every two weeks at a dose of 200 mg/m^2 (*l* form, or levo-leucovorin) or 400 mg/m^2 (*l* + *d* racemic form) in combination with the MM-398 irinotecan liposome and the 5-FU.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the anti-tumor activity of MM-398 in an orthotopic pancreatic tumor model expressing luciferase (L3.6pl).

Figure 2 is a graph showing accumulation of SN-38 in tumors following treatment with free irinotecan or liposomal irinotecan (MM-398).

Figure 3 is a graph showing the effect of MM-398 on Carbonic Anhydrase IX Staining in a HT29 Xenograft Model.

Figure 4 shows the effect of MM-398 on perfusion of small molecule Hoechst stain.

Figure 5 summarizes the pharmacokinetics of MM-398 in q3w (irinotecan, liposome + free drug).

Figure 6 summarizes the pharmacokinetics of MM-398 in q3w (SN-38).

Figure 7 summarizes the pharmacokinetic parameters of individual MM-151 monoclonal antibodies in patients treated with MM-151 monotherapy.

Figure 8A shows the simulated serum concentrations of MM-151 in patients administered with two priming doses of MM-151 followed by 10.5 mg/kg q1w dosing schedule.

Figure 8B is a graph showing the effect of weekly MM-151 dose intensity on the probability to experience rash event in patients treated with MM-151. Top: any grade rash; Bottom: grade 3 or higher. Error bars indicate standard errors of the mean.

Figure 9 shows the mean plasma concentrations of total irinotecan and SN-38 following the administration of either nal-IRI (100 mg/mg^2) based on the amount of irinotecan trihydrate hydrochloride) or irinotecan HCl (300 mg/m^2) in study PEP0206.

Figure 10 is a graph showing model predictions of tumor and plasma drug metabolites (CPT-11 and SN-38) in patients treated with nal-IRI.

Figures 11A and **11B** show levels of CPT-11 and SN-38, respectively, measured in patient tumor biopsies and plasma samples.

Figure 12 is a schematic of a clinical treatment program.

Figure 13 is a schematic of a clinical treatment program.

Figure 14 is a sequence listing for the P1X antibody component of MM-151.

Figure 15 is a sequence listing for the P2X antibody component of MM-151.

Figure 16 is a sequence listing for the P3X antibody component of MM-151.

Figure 17 is a cartoon schematic that shows the MM-151 oligoclonal design with the three component IgG1 antibodies—P1X, P2X, and P3X—bound to the EGFR extracellular domains (marked with Roman numerals I, II, III, and IV). P1X and P3X have binding epitopes on EGFR extracellular domain III while P2X has a binding epitope on EGFR extracellular domain I.

Figure 18 is a graph showing the results of a ligand antagonism cell binding assay, demonstrating the EGF ligand blocking ability of P1X (dark gray triangle), P2X (black square) or P3X (light gray circle) alone at low doses as compared to EGF ligand alone (black diamond). The concentrations for the antibodies (P1X = 0.97 nM; P2X = 2.00 nM; P3X = 4.68 nM) represent a sub-saturating concentration (approx. EC90 concentration) of cell binding. The EGFR-expressing A431 cell line was incubated with one dose of single antibody for 1 hr. followed by a dilution series of biotin-labeled EGF ligand and the amount of cell bound biotin-EGF ligand measured by quantitative flow cytometry (MFI = mean fluorescence intensity).

Figure 19 is a graph showing the results of a phospho-EGFR (pEGFR) inhibition experiment, demonstrating pEGFR inhibition by single-agent treatment with P1X (dark gray triangle), P2X (black square) or P3X (light gray circle) antibody at the indicated doses. A431 cells were incubated with antibody for 1 hr. followed by 10 min treatment with 8 nM EGF ligand and the amount of pEGFR measured by ELISA (MFI = mean fluorescence intensity).

Figure 20 demonstrates improved activity of nal-IRI + MM-151 TC combination compared to the two agents alone in LoVo subcutaneous CRC xenograft model.

Figure 21 shows the anti-tumor activity of nal-IRI (5 mg/kg) compared to irinotecan (25

mg/kg) at similar SN-38 tumor exposure in a LIM1215 subcutaneous CRC xenograft model.

Figure 22 shows the effects of irinotecan+5-FU+cetuximab and nal-IRI+5-FU+MM-151 TC combinations in LIM1215 subcutaneous CRC xenografts. Doses of 5-FU, cetuximab, and MM-151 TC were kept constant, (Figure 22A) low doses of nal-IRI (1.25 mg/kg) and irinotecan (6.25 mg/kg) and (Figure 22B) high doses of nal-IRI (5 mg/kg) and irinotecan (25 mg/kg) were utilized for comparison at which tumor SN-38 were comparable at these dose levels.

Figure 23 provides the amino acid sequences of the Complementarity Determining Regions (CDRs) of an antibody used in Example 8.

Figure 24 is a diagram showing detectable pretreatment somatic mutations within the CRC patient subset.

Figure 25 is a summary of the 24 colorectal cancer patients discussed in Example 17 and discloses the treatment regimens each patient received.

Figure 26 is a table detailing the patient treatment parameters, the duration of treatment, the best overall response, duration of treatment and mutation status (wild type, "WT" or mutant, "MT") for 24 of 29 patients within the CRC efficacy cohort who were evaluated for RECIST response in the clinical trial described in Example 16

DETAILED DESCRIPTION

I. Definitions

As used herein, the term "subject" or "patient" is a human cancer patient.

As used herein, "effective treatment" refers to treatment producing a beneficial effect, e.g., amelioration of at least one symptom of a disease or disorder. A beneficial effect can take the form of an improvement over baseline, i.e., an improvement over a measurement or observation made prior to initiation of therapy according to the method. A beneficial effect can also take the form of arresting, slowing, retarding, or stabilizing of a deleterious progression of a marker of a cancer. Effective treatment may refer to alleviation of at least one symptom of a cancer. Such effective treatment may, e.g., reduce patient pain, reduce the size and/or number of lesions, may reduce or prevent metastasis of a cancer tumor, and/or may slow growth of a cancer tumor.

The term "effective amount" refers to an amount of an agent that provides the desired biological, therapeutic, and/or prophylactic result. That result can be reduction, amelioration, palliation, lessening, delaying, and/or alleviation of one or more of the signs, symptoms, or

causes of a disease, or any other desired alteration of a biological system. In reference to cancers, an effective amount comprises an amount sufficient to cause a tumor to shrink and/or to decrease the growth rate of the tumor (such as to suppress tumor growth) or to prevent or delay other unwanted cell proliferation. In some embodiments, an effective amount is an amount sufficient to delay tumor development. In some embodiments, an effective amount is an amount sufficient to prevent or delay tumor recurrence. An effective amount can be administered in one or more administrations. The effective amount of the drug or composition may: (i) reduce the number of cancer cells; (ii) reduce tumor size; (iii) inhibit, retard, slow to some extent and may stop cancer cell infiltration into peripheral organs; (iv) inhibit (i.e., slow to some extent and may stop) tumor metastasis; (v) inhibit tumor growth; (vi) prevent or delay occurrence and/or recurrence of tumor; and/or (vii) relieve to some extent one or more of the symptoms associated with the cancer.

The terms "combination therapy," "co-administration," "co-administered" or "concurrent administration" (or minor variations of these terms) include simultaneous administration of at least two therapeutic agents to a patient or their sequential administration within a time period during which the first administered therapeutic agent is still present in the patient when the second administered therapeutic agent is administered.

The term "monotherapy" refers to administering a single drug to treat a disease or disorder in the absence of co-administration of any other therapeutic agent that is being administered to treat the same disease or disorder.

"Dosage" refers to parameters for administering a drug in defined quantities per unit time (*e.g.*, per hour, per day, per week, per month, etc.) to a patient. Such parameters include, *e.g.*, the size of each dose. Such parameters also include the configuration of each dose, which may be administered as one or more units, *e.g.*, taken at a single administration, *e.g.*, orally (*e.g.*, as one, two, three or more pills, capsules, etc.) or injected (*e.g.*, as a bolus). Dosage sizes may also relate to doses that are administered continuously (*e.g.*, as an intravenous infusion over a period of minutes or hours). Such parameters further include frequency of administration of separate doses, which frequency may change over time.

"Dose" refers to an amount of a drug given in a single administration.

As used herein, "cancer" refers to a condition characterized by abnormal, unregulated, malignant cell growth. In one embodiment, the cancer is colorectal cancer. The terms "resistant" and "refractory" refer to tumor cells that survive treatment with a therapeutic agent. Such cells may have responded to a therapeutic agent initially, but subsequently exhibited a reduction of responsiveness during treatment, or did not exhibit an adequate

response to the therapeutic agent in that the cells continued to proliferate in the course of treatment with the agent.

II. Irinotecan sucrose sulfate liposome injection (ONIVYDE, MM-398, PEP02, nal-IRI)

Nal-IRI can be administered by IV infusion over 90 minutes (±10 minutes) every two weeks at a dose of 70 mg/m^2 (free base), on Days 1 and 15 of each 28-day cycle. The first cycle Day 1 is a fixed day; subsequent doses should be administered on the first day of each cycle +/- 2 days. Prior to administration, the appropriate dose of nal-IRI must be diluted in 5% Dextrose Injection solution (D5W) or normal saline to a final volume of 500 mL. Care should be taken not to use in-line filters or any diluents other than D5W or normal saline. Nal-IRI can be administered at a rate of up to 1 mL/sec (30 mg/sec).

The actual dose of nal-IRI to be administered will be determined by calculating the patient's body surface area at the beginning of each cycle. A +/- 5% variance in the calculated total dose will be allowed for ease of dose administration. Since nal-IRI vials are single use vials, site staff must not store any unused portion of a vial for future use and they must discard unused portions of the product.

All patients can be pre-medicated prior to nal-IRI infusion and 5-FU/LV infusion with standard doses of dexamethasone and a 5-HT3 antagonist, or equivalent other anti-emetics according to standard institutional practices for irinotecan and 5-FU administration. Atropine may be prescribed prophylactically, according to standard institutional practices, for patients who experienced acute cholinergic symptoms in the previous cycles.

Patients will be tested for UGT1A1*28 status during screening, however the result of the test is not required prior to the initial dose of nal-IRI. All patients will begin dosing at 70 mg/m^2 (free base), however future doses may be reduced for patients who are positive (i.e. homozygous) for UGT1A1*28 7/7 genotype. Depending on the overall safety profile seen after the first dose, the dose may be reduced to 50 mg/m^2 (free base) after discussion between the PI, Sponsor and Medical Monitor.

As provided herein, irinotecan is administered in a stable liposomal formulation as irinotecan sucrose sulfate liposome injection (otherwise termed "irinotecan sucrose octasulfate salt liposome injection" or "irinotecan sucrosofate liposome injection"), the formulation referred to herein as "MM-398" (also known as PEP02, see US 8,147,867). MM-398 may be provided as a sterile, injectable parenteral liquid for intravenous injection. The required amount of MM-398 may be diluted, *e.g.*, in 500mL of 5% dextrose injection USP and infused over a 90 minute period.

An MM-398 liposome is a unilamellar lipid bilayer vesicle of approximately 80-140 nm in diameter that encapsulates an aqueous space which contains irinotecan complexed in a gelated or precipitated state as a salt with sucrose octasulfate. The lipid membrane of the liposome is composed of phosphatidylcholine, cholesterol, and a polyethyleneglycol-derivatized phosphatidyl-ethanolamine in the amount of approximately one polyethyleneglycol (PEG) molecule for 200 phospholipid molecules.

This stable liposomal formulation of irinotecan has several attributes that may provide an improved therapeutic index. The controlled and sustained release improves activity of this schedule-dependent drug by increasing duration of exposure of tumor tissue to drug, an attribute that allows it to be present in a higher proportion of cells during the S-phase of the cell cycle, when DNA unwinding is required as a preliminary step in the DNA replication process. The long circulating pharmacokinetics and high intravascular drug retention in the liposomes can promote an enhanced permeability and retention (EPR) effect. EPR allows for deposition of the liposomes at sites, such as malignant tumors, where the normal integrity of the vasculature (capillaries in particular) is compromised resulting in leakage out of the capillary lumen of particulates such as liposomes. EPR may thus promote site-specific drug delivery of liposomes to solid tumors. EPR of MM-398 may result in a subsequent depot effect, where liposomes accumulate in tumor associated macrophages (TAMs), which metabolize irinotecan, converting it locally to the substantially more cytotoxic SN-38. This local bioactivation is believed to result in reduced drug exposure at potential sites of toxicity and increased exposure at cancer cells within the tumor.

Nal-IRI comprises irinotecan encapsulated in a nanoliposome drug delivery system (nanoliposomal irinotecan; nal-IRI). The active ingredient of the nal-IRI injection, irinotecan, is a member of the topoisomerase I inhibitor class of drugs and is a semi-synthetic and water soluble analog of the naturally-occurring alkaloid, camptothecin. Topoisomerase I inhibitors work to arrest uncontrolled cell growth by preventing the unwinding of DNA and therefore preventing replication. The pharmacology of irinotecan is complex, with extensive metabolic conversions involved in the activation, inactivation, and elimination of the drug. Irinotecan is a pro-drug that is converted by nonspecific carboxylesterases into a 100-1000 fold more active metabolite, SN-38. SN-38 is cleared via glucuronidation, (for which major pharmacogenetic differences have been shown), and biliary excretion. These drug properties contribute to the marked differences in efficacy and toxicity observed in clinical studies with irinotecan.

Drug carrier technologies represent a rational strategy to improve the

pharmacokinetics and biodistribution of irinotecan while protecting it from premature metabolism. Nal-IRI employs a novel intraliposomal drug stabilization technology for encapsulation of irinotecan into long-circulating liposome-based nanoparticles with high drug load and high in vivo stability. The stable nanoliposome formulation of irinotecan has several attributes that may provide an improved therapeutic index. The controlled and sustained release should improve activity of this schedule-dependent drug by increasing duration of exposure of tumor tissue to drug, an attribute that allows it to be present in a higher proportion of cells during the more sensitive S-phase of the cell cycle. The improved pharmacokinetics, high intravascular drug retention in the liposomes, and enhanced permeability and retention (EPR) effect may result in site-specific drug delivery to solid tumors. Stromal targeting results from the subsequent depot effect, where liposomes accumulating in tumor associated macrophages (TAMs) release the active drug and convert it locally to the substantially more cytotoxic SN-38. The preferentially local bioactivation should result in reduced exposure to potential sites of toxicity and increased exposure to neighboring cancer cells within the tumor.

Pharmacogenetics of Irinotecan Glucuronidation

The enzyme produced by the UGT1A1 gene, UDP-glucuronosyltransferase 1, is responsible for bilirubin metabolism and also mediates SN-38 glucuronidation, which is the initial step in the predominant metabolic clearance pathway of this active metabolite of irinotecan. Besides its anti-tumor activity, SN-38 is also responsible for the severe toxicity sometimes associated with irinotecan therapy. Therefore, the glucuronidation of SN-38 to the inactive form, SN-38 glucuronide, is an important step in the modulation of irinotecan toxicity.

Mutational polymorphisms in the promoter of the UGT1A1 gene have been described in which there is a variable number of thymine adenine (ta) repeats. Promoters containing seven thymine adenine (ta) repeats (found in the UGT1A1*28 allele) have been found to be less active than the wild-type six repeats, resulting in reduced expression of UDP-glucuronosyltransferase 1. Patients who carry two deficient alleles of UGT1A1 exhibit reduced glucuronidation of SN-38. Some case reports have suggested that individuals who are homozygous for UGT1A1*28 alleles (referred to as having the UGT1A1 7/7 genotype, because both alleles are UGT1A1*28 alleles that contain 7 ta repeats, as opposed to the wild-type UGT1A1 6/6 genotype in which both alleles contain 6 ta repeats) and who have fluctuating elevation in serum bilirubin, (*e.g.*, Gilbert's Syndrome patients), may be at greater

risk of toxicity upon receiving standard doses of irinotecan. This suggests that there is a link between homozygosity of the UGT1A1*28 allele, bilirubin levels and irinotecan toxicity.

The metabolic transformation of MM-398 to SN-38 (*e.g.*, in plasma) includes two critical steps: (1) the release of irinotecan from the liposome and (2) the conversion of free irinotecan to SN-38. While not intending to be limited by theory, it is believed that once irinotecan leaves the liposomes, it is catabolized by the same metabolic pathways as conventional (free) irinotecan. Therefore the genetic polymorphisms in humans predictive for the toxicity and efficacy of irinotecan and those of MM-398 can be considered similar. Nonetheless, due to the smaller tissue distribution, lower clearance, higher systemic exposure and longer elimination half-life of SN-38 of the MM-398 formulation compared to free irinotecan, the deficient genetic polymorphisms may show more association with severe adverse events and/or efficacy.

Patients with Reduced UGT1A1 Activity

Individuals who are homozygous for the UGT1A1*28 allele (UGT1A1 7/7 genotype) have been shown to be at increased risk for neutropenia following initiation of irinotecan treatment. According to the prescribing information for irinotecan (Camptosar®), in a study of 66 patients who received single-agent irinotecan (350 mg/m^2 once every-3-weeks), the incidence of grade 4 neutropenia in patients homozygous for the UGT1A1*28 allele was as high as 50%, and in patients heterozygous for this allele (UGT1A1 6/7 genotype) the incidence was 12.5%. Importantly, no grade 4 neutropenia was observed in patients homozygous for the wild-type allele (UGT1A1 6/6 genotype). In other studies, a lower prevalence of life threatening neutropenia is described. For this reason, patients who are enrolled in the phase III study described in the Examples herein and are homozygous for the UGT1A1*28 allele (UGT1A1 7/7 genotype) will have MM-398 treatment initiated at a lower dose than patients with one (*e.g.*, UGT1A1 6/7) or two (UGT1A1 6/6) wild-type alleles.

Additional genotypic modifiers of irinotecan metabolism

Although the UGT1A1*28 allele is relatively common in Caucasians (estimates 10%), the prevalence is varied in other ethnic groups. Furthermore, additional UGT1A1 genotypes are found with higher prevalence for example in Asian populations and these could be important for the metabolism of irinotecan in these populations. For example, the UGT1A1*6 allele is more prevalent in Asians. This allele is not associated with a ta repeat, but with a Gly71Arg mutation that reduces enzyme activity. In previous and ongoing studies

of MM-398, pharmacogenetic information has been collected on patients being enrolled. In a study referred to as the PEP0203 study, the relationship of genetic polymorphism of UGT1A family and of DPYD (dihydropyrimidine dehydrogenase, an enzyme associated with catabolism of 5-FU) with pharmacokinetic parameters of MM-398 and toxicity did not provide a clear correlation with the small sample size of subjects evaluated. However, it was observed that patients with UGT1A1*6/*28 combined polymorphism had higher dose-normalized AUCs of SN-38 and experienced DLT.

III. 5-Fluorouracil (5-FU) and Leucovorin

5-FU can be administered at a dose of 2400 mg/m^2 as an IV infusion over 46-48 hours on Days 1 and 15 of each 28-day cycle. 5-FU should be reconstituted per the instructions on the package insert, SmPC or standard institutional guidelines for reconstitution of leucovorin.

5-FU can be administered after leucovorin and last in the treatment regimen. Actual dose of 5-FU and leucovorin to be administered will be determined by calculating the patient's body surface area prior to each cycle. A +/- 5% variance in the calculated total dose will be allowed for ease of dose administration.

Stomatitis and esophagopharyngitis (which may lead to sloughing and ulceration), diarrhea, anorexia, nausea, emesis and leukopenia are commonly seen with treatment; alopecia and dermatitis, in the form of pruritic rash usually appearing on the extremities, may also be seen (see US package insert or SmPC).

5-Fluorouracil is a pyrimidine antagonist that interferes with nucleic acid biosynthesis. The deoxyribonucleotide of the drug inhibits thymidylate synthetase, thus inhibiting the formation of thymidylic acid from deoxyuridylic acid, thus interfering in the synthesis of DNA. It also interferes with RNA synthesis.

Leucovorin (also called folinic acid) acts as a biochemical cofactor for 1-carbon transfer reactions in the synthesis of purines and pyrimidines. Leucovorin does not require the enzyme dihydrofolate reductase (DHFR) for conversion to tetrahydrofolic acid. The effects of methotrexate and other DHFR-antagonists are inhibited by leucovorin. Leucovorin can potentiate the cytotoxic effects of fluorinated pyrimidines (i.e., fluorouracil and floxuridine). After 5-FU is activated within the cell, it is accompanied by a folate cofactor, and inhibits the enzyme thymidylate synthetase, thus inhibiting pyrimidine synthesis. Leucovorin increases the folate pool, thereby increasing the binding of folate cofactor and active 5-FU with thymidylate synthetase.

Leucovorin can be administered at a dose of 400 mg/m^2 of the l + d racemic form, as

an IV infusion over 30 minutes (±5 minutes), on Days 1 and 15 of each 28-day cycle. Leucovorin should be reconstituted per the instructions on the package insert, SmPC or standard institutional guidelines for reconstitution of leucovorin.

Leucovorin should be administered after MM-151 and prior to the 5-FU infusion. Actual dose of 5-FU and leucovorin to be administered will be determined by calculating the patient's body surface area prior to each cycle. A +/- 5% variance in the calculated total dose will be allowed for ease of dose administration.

Leucovorin has dextro- and levo-isomers, only the latter one being pharmacologically useful. As such, the bioactive levo-isomer ("levoleucovorin") has also been approved by the FDA for treatment of cancer. The dosage of levoleucovorin is typically half that of the racemic mixture containing both dextro (*d*) and levo (*l*) isomers.

IV. MM-151 (preferred EGFR inhibitor)

EGFR signaling is important for the proliferation and survival of many epithelial malignancies. Although EGFR targeting antibodies have been approved for use in human malignancies driven by the EGFR pathway, there remains a high unmet need in these tumors, as limited efficacy is attributable to de novo or acquired resistance mechanisms to these targeted therapies. One reason for this state of affairs might stem from observations that EGFR signaling is very robust due to amplification of cell signaling downstream of the receptor, thus making the need for complete inhibition of the pathway imperative for potential success in the clinic. EGFR signaling is further complicated by the presence in tumors of multiple ligands that bind to EGFR with either high or low affinity and result in activation of the pathway downstream of EGFR. Preclinical evidence obtained at Merrimack suggests that EGFR signaling driven by high affinity ligands is not always inhibited by currently approved EGFR antagonists.

MM-151 is a novel mixture of three fully human monoclonal antibody EGFR antagonists designed to optimally inhibit EGFR dependent signaling. MM-151 antibodies have non-overlapping epitopes and can simultaneously engage the same EGFR receptor molecule. Preclinical studies suggest that MM-151 leads to downregulation of EGFR expression via internalization and degradation of EGFR. Preclinical studies demonstrate that MM-151 is more effective in inhibiting EGFR signaling and proliferation driven by both high and low affinity ligands, compared to currently approved EGFR targeting monoclonal antibodies which inhibit only low-affinity ligand pathway activation. Preclinical studies also demonstrate that MM-151 leads to antibody-dependent cellular cytotoxicity (ADCC) and

complement-dependent cytotoxicity (CDC), with the latter being a unique property of the antibody mixture that is not observed with currently approved EGFR-targeting antibodies. *In vivo* studies also showed that treatment with MM-151 results in inhibition of tumor growth of xenograft models from multiple indications including colorectal, non-small cell lung, head and neck, and triple-negative breast cancer.

MM-151 contains three distinct antibodies: P1X, P2X and P3X. All are colorless, liquid solutions that are formulated in 20mM Histidine, 10% sucrose and 0.02% Polysorbate 80, pH 6.0. The molecular weights of the drug P1X, P2X and P3X are 145, 148 and 146 respectively. P1X, P2X and P3X drug substances are combined in a ratio of 1:1:0.5 to form the final MM-151 drug product. Similar to the three drug substances, MM-151 drug product is formulated in 20mM Histidine, 10% sucrose and 0.02% Polysorbate 80, pH 6.0. The final concentration of drug product is 25 mg/ml per 10ml vial.

P1X is a full EGF ligand antagonist and binds an epitope on domain III of EGFR with subnanomolar affinity (11pM) resulting in significant inhibition of pEGFR. MM-151 P1X is a recombinant human IgG1 monoclonal antibody. The complete tetrameric structure of the IgG1 molecule is composed of two heavy chains (451 amino acids each) and two kappa light chains (214 amino acids each) held together by intra-chain and inter-chain disulfide bonds. The MM-151 P1X amino acid sequence predicts a molecular weight of 145 k_D for the intact IgG1, which is within 0.2% of the actual molecular weight as experimentally determined by mass spectroscopy. MM-151 P1X is produced using a recombinant CHO cell system. The heavy and light chain sequences of the P1X antibody are provided in FIG. 14.

P2X is also a full EGF ligand antagonist and binds a distinct epitope different from that of P1X located on domain I of EGFR with subnanomolar affinity (70pM) resulting in significant inhibition of pEGFR. MM-151 P2X is a recombinant human IgG1 monoclonal antibody. The complete tetrameric structure of the IgG1 molecule is composed of two heavy chains (449 amino acids each) and two kappa light chains (220 amino acids each) held together by intra-chain and inter-chain disulfide bonds. The predicted molecular weight of intact glycosylated MM-151 P2X is 148 kD, which is within 0.2% of the actual molecular weight as experimentally determined by mass spectroscopy. MM-151 P2X is produced using a recombinant CHO cell system. The heavy and light chain sequences of the P2X antibody are provided in FIG. 15.

P3X is a partial EGF antagonist and binds a third epitope on domain III distinct from those of P1X and P2X. P3X binds EGFR at a lower affinity than P1X and P2X (360pM) resulting in moderate inhibition (20%) of pEGFR. MM-151 P3X is a recombinant human

IgG1 monoclonal antibody. The complete tetrameric structure of the IgG1 molecule is composed of two heavy chains (453 amino acids each) and two kappa light chains (215 amino acids each) held together by intra-chain and inter-chain disulfide bonds. The MM-151 P3X amino acid sequence predicts a molecular weight of 146 kD for the intact IgG1, which is within 0.2% of the actual molecular weight as experimentally determined by mass spectroscopy. MM-151 P3X is produced using a recombinant CHO cell system. The heavy and light chain sequences of the P3X antibody are provided in FIG. 16.

The CDR regions of the MM151 antibodies are provided in the table below, and in publication WO2013/006547.

Table 1 CDR regions for MM151 Anti-EGFR Oligoclonal Antibody Preparation

Antibody	Heavy Chain	Light Chain
P1X	(SEQ ID NO:1) CDR1 Ser Tyr Ala Ile Ser (SEQ ID NO:2) CDR2 Ile Ile Pro Ile Phe Gly Thr Val Asn Tyr (SEQ ID NO:3) CDR3 Asp Pro Ser Val Asn Leu	(SEQ ID NO:4) CDR1 Gln Ser Ile Ser Ser Trp Trp Ala (SEQ ID NO:5) CDR2 Asp Ala Ser Ser Leu (SEQ ID NO:6) CDR3 Gln Gln Tyr His Ala His Pro
P2X	(SEQ ID NO:7) CDR1 Ser Tyr Ala Ile Ser (SEQ ID NO:8) CDR2 Ile Ile Pro Ile Phe Gly Ala Ala Asn Pro (SEQ ID NO:9) CDR3 Met Gly Arg Gly Lys Val	(SEQ ID NO:10) CDR1 Gln Ser Val Leu Tyr Ser Pro Asn Asn Lys Asn Tyr Leu Ala (SEQ ID NO:11) CDR2 Trp Ala Ser Thr Arg (SEQ ID NO:12) CDR3 Gln Gln Tyr Tyr Gly Ser Pro
P3X	(SEQ ID NO:13) CDR1 Ser Tyr Gly Ile Asn (SEQ ID NO:14) CDR2 Ile Ser Ala Tyr Asn Gly Asn Thr Tyr Tyr (SEQ ID NO:15) CDR3 Asp Leu Gly Gly Tyr Gly Ser Gly Ser	(SEQ ID NO:16) CDR1 Gln Ser Val Ser Ser Asn Leu Ala (SEQ ID NO:17) CDR2 Gly Ala Ser Thr Arg (SEQ ID NO:18) CDR3 Gln Asp Tyr Arg Thr Trp Pro Arg

Together, the mixture of these three EGFR antagonists is expected to increase the relative potency of MM-151 compared to currently approved antibody based EGFR

antagonists by completely blocking EGFR pathway activation in tumor cells through acting on three distinct epitopes of the target receptor. In *in vitro* studies, the mixture of the three antibodies inhibited EGF-mediated signaling of both the target receptor, EGFR, and its downstream effector, ERK. As EGFR is one of the key growth factor receptors used by tumors cells to proliferate and migrate to distal sites, MM-151 has the potential to treat a number of patients with unmet medical needs in oncology.

The individual components of MM-151, P1X, P2X, and P3X, are fully human anti-EGFR IgG1 monoclonal antibodies that bind to three distinct epitopes on the extracellular domain of EGFR. Surface plasma resonance experiments showed that P1X, P2X and P3X can associate simultaneously; that they associate with EGFR domains III, I and II respectively (Figure 16) and that the order of association does not matter. Additionally, P1X and P2X have been shown to be potent ligand antagonists, while P3X only partially blocks EGF ligand binding. However, together the mixture acts as potent EGF ligand blockers (Figure 17). P1X and P2X are potent inhibitors of pEGFR signaling, whereas P3X only partially blocks EGF-induced receptor signaling, (Figure 19).

MM-151 is administered by IV infusion every week. The first two doses of Cycle 1 are priming doses and the dose levels are 225 mg for priming dose 1 and 450 mg for priming dose 2. Subsequent doses of MM-151 are administered according to the dose levels outlined above. MM-151 should not be administered as a bolus or a push.

MM-151 is also described in Patent Cooperation Treaty (PCT) patent application PCT/US2012/045235, filed July 2, 2012, and published as WO2013/006547, incorporated herein by reference in its entirety.

V. Administration

Liposomal irinotecan is administered intravenously, either alone or in combination with 5-fluorouracil (5-FU) and/or leucovorin. In one embodiment, liposomal irinotecan is administered prior to 5-FU and leucovorin. In another embodiment, leucovorin is administered prior to 5-FU. In another embodiment, liposomal irinotecan is administered intravenously over 90 minutes. In another embodiment, 5-FU is administered intravenously over 46 hours. In another embodiment, leucovorin is administered intravenously over 30 minutes. In various embodiments the liposomal irinotecan is MM-398.

The concentration of MM-398 will be 43 mg/10 mL irinotecan free base as a white to slightly yellow, opaque, liposomal dispersion in a single-dose vial irinotecan in the form of the sucrosofate salt, encapsulated in liposomes for intravenous infusion. Nal-IRI must be

stored refrigerated at 2 to 8°C, with protection from light.

Nal-IRI is administered by IV infusion over 90 minutes every two weeks at a dose of 70 mg/m^2 (free base).

5-FU is a commercially available product that can be supplied at multiple concentration levels and vial sizes depending on the source country. It must be stored at room temperature with protection from light. 5-FU is administered by IV infusion over 46 hours every two weeks at a dose of 2400 mg/m^2.

LV is a commercially available product that can be supplied at multiple concentration levels and vial sizes depending on the source country. It must be stored at room temperature with protection from light. Leucovorin (l + d racemic form) will be administered by IV infusion over 30 minutes every two weeks at a dose of 400 mg/m^2.

V. Patient Populations

In one embodiment, a patient treated using the methods and compositions disclosed herein exhibits evidence of recurrent or persistent colorectal cancer following primary chemotherapy.

In another embodiment, the patient has had and failed at least one prior platinum based chemotherapy regimen for management of primary or recurrent disease, *e.g.*, a chemotherapy regimen comprising carboplatin, cisplatin, or another organoplatinum compound.

In an additional embodiment, the patient has failed prior treatment with gemcitabine or become resistant to gemcitabine.

In one embodiment a resistant or refractory tumor is one where the treatment-free interval following completion of a course of therapy for a patient having the tumor is less than 6 months (*e.g.*, owing to recurrence of the cancer) or where there is tumor progression during the course of therapy.

VII. Treatment Protocols

Suitable treatment protocols include, for example, those wherein the patient is administered an effective amount of liposomal irinotecan, wherein the treatment comprises at least one cycle, wherein the cycle is a period of 3 weeks, and wherein for each cycle the liposomal irinotecan is administered on day 1 of the cycle at a dose of 120 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride), except if the patient is homozygous for the UGT1A1*28 allele, wherein liposomal irinotecan is administered on day 1 of cycle 1 at a

dose of 80 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride). In one embodiment, the dose of liposomal irinotecan administered to the patient homozygous for the UGT1A1*28 allele is increased after one cycle in increments of 20 mg/m^2, up to a maximum of 120 mg/m^2(based on the amount of irinotecan trihydrate hydrochloride).

In another embodiment, the treatment protocol includes administering to the patient an effective amount each of liposomal irinotecan, 5-fluorouracil (5-FU), and leucovorin, wherein the treatment comprises at least one cycle, wherein the cycle is a period of 2 weeks, and wherein for each cycle: (a) liposomal irinotecan is administered on day 1 of the cycle at a dose of 80 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride), except if the patient is homozygous for the UGT1A1*28 allele, wherein liposomal irinotecan is administered on day 1 of cycle 1 at a dose of 60 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride); (b) 5-FU is administered at a dose of 2400 mg/m^2; and (c) leucovorin is administered at a dose of 200 mg/m^2 (*l* form) or 400 mg/m^2 (*l* + *d* racemic form). In a particular embodiment, the dose of liposomal irinotecan administered to the patient homozygous for the UGT1A1*28 allele is increased after one cycle to 80 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride).

VII. Outcomes

Provided herein are methods for treating cancer in a patient comprising administering an anti-EGFR antibody such as MM-151 (e.g., once weekly) to the patient in combination with administration once every two weeks of liposomal irinotecan (MM-398) in combination with 5-fluorouracil (5-FU) and leucovorin, according to a particular clinical dosage regimen. Responses to therapy may include:

Pathologic complete response (pCR): absence of invasive cancer in the breast and lymph nodes following primary systemic treatment.

Complete Response (CR): Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) which has reduction in short axis to <10 mm;

Partial Response (PR): At least a 30% decrease in the sum of dimensions of target lesions, taking as reference the baseline sum diameters;

Stable Disease (SD): Neither sufficient shrinkage to qualify for partial response, nor sufficient increase to qualify for progressive disease, taking as reference the smallest sum diameters while on study; or

Meanwhile, non-CR/Non-PD denotes a persistence of one or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits.

Progressive Disease (PD) denotes at least a 20% increase in the sum of dimensions of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of 5 mm. The appearance of one or more new lesions is also considered progression.

In one embodiment the patient so treated exhibits pCR, CR, PR, or SD.
In another embodiment, the patient so treated experiences tumor shrinkage and/or decrease in growth rate, i.e., suppression of tumor growth. In another embodiment, unwanted cell proliferation is reduced or inhibited. In yet another embodiment, one or more of the following can occur: the number of cancer cells can be reduced; tumor size can be reduced; cancer cell infiltration into peripheral organs can be inhibited, retarded, slowed, or stopped; tumor metastasis can be slowed or inhibited; tumor growth can be inhibited; recurrence of tumor can be prevented or delayed; one or more of the symptoms associated with cancer can be relieved to some extent.

In other embodiments, such improvement is measured by a reduction in the quantity and/or size of measurable tumor lesions. Measurable lesions are defined as those that can be accurately measured in at least one dimension (longest diameter is to be recorded) as $\geq$10 mm by CT scan (CT scan slice thickness no greater than 5 mm), 10 mm caliper measurement by clinical exam or >20 mm by chest X-ray. The size of non-target lesions, e.g., pathological lymph nodes can also be measured for improvement. In one embodiment, lesions can be measured on chest x-rays or CT or MRI films.

In other embodiments, cytology or histology can be used to evaluate responsiveness to a therapy. The cytological confirmation of the neoplastic origin of any effusion that appears or worsens during treatment when the measurable tumor has met criteria for response or stable disease can be considered to differentiate between response or stable disease (an effusion may be a side effect of the treatment) and progressive disease.

In some embodiments, administration of effective amounts of liposomal irinotecan, 5-FU and leucovorin according to any of the methods provided herein produce at least one therapeutic effect selected from the group consisting of reduction in size of a breast tumor, reduction in number of metastatic lesions appearing over time, complete remission, partial remission, stable disease, increase in overall response rate, or a pathologic complete response. In some embodiments, the provided methods of treatment produce a comparable clinical benefit rate (CBR = CR+ PR+ SD $\geq$ 6 months) better than that achieved by the same combinations of anti-cancer agents administered without concomitant MM-398

administration. In other embodiments, the improvement of clinical benefit rate is about 20% 20%, 30%, 40%, 50%, 60%, 70%, 80% or more compared to the same combinations of anti-cancer agents administered without concomitant MM-398 administration.

The following examples are illustrative and should not be construed as limiting the scope of this disclosure in any way; many variations and equivalents will become apparent to those skilled in the art upon reading the present disclosure.

EXAMPLES

Example 1: Activity of MM-398 in an Orthotopic Pancreas Tumor Model Expressing Luciferase (L3.6pl)

The anti-tumor activity of MM-398 was assessed in an orthotopic pancreatic cancer model (L3.6pl), a highly hypoxic preclinical tumor model. Approximately 2.5 x 10^{-5} L3.6pl pancreatic tumor cells were implanted by direct injection into the pancreas. The bioluminescence images (BLI) were followed over time for tumor burden detection/quantitation. MM-398 and free irinotecan were dosed at a dose of 20 mg/kg/dose weekly for three weeks. As shown in Figure 1, MM-398 (liposomal CPT11) had significant anti-tumor activity, as compared to a control (HBS) and free CPT11.

Example 2: Accumulation of SN-38 in Tumors Following Treatment with Free Irinotecan or Liposomal Irinotecan (MM-398)

It was hypothesized that the anti-tumor activity observed in the orthotopic pancreatic cancer model is due to the effect of macrophages in converting irinotecan to the more active SN-38 locally. To test this hypothesis, human colon cancer cells (HT-29) were injected subcutaneously into SCID mice, 40 mg/kg of free irinotecan or MM-398 was injected intravenously when the tumors reached 1000 mm^3 in size. Tumor-bearing mice were sacrificed at different time points, tumors from both groups were extracted and the concentrations of SN-38 were measured.

As shown in Figure 2, there was a 20-fold increase in the tumor AUC_{SN-38} for MM-398 as compared to free irinotecan. The long duration of exposure allows for prolonged exposure of the slow proliferating cancer cells to the active metabolite as they progress through the cell cycle. In addition, this activity was also hypothesized to result from a reduction in intra-tumoral hypoxia, and the subsequent downstream effects on angiogenesis, metastasis, and the immunosuppressive environment in tumors.

Example 3: Effect of MM-398 on Carbonic Anhydrase IX Staining in a HT29 Xenograft Model

To test whether MM-398 reduces markers of hypoxia, experiments were conducted in

a human colon cancer cell (HT-29) model. Specifically, HT-29 cells were injected subcutaneously into nude mice, on day 13 either PBS control or 1.25, 2.5, 5, 10 or 20 mg/kg MM-398 was injected intravenously. MM-398 was dosed once a week for 4 weeks at the indicated doses. Tumors from both groups (n = 5) were extracted 24 hours after the last dose. Frozen tumor sections were used for immunohistochemical staining of Carbonic Anhydrase IX (CAIX). Quantification of CAIX staining was performed using Definiens® (Definiens AG, Munich) software.

As shown in Figure 3, MM-398 reduced markers of hypoxia. Specifically, the graphs in Figure 3 show the percentage of cells that stained with medium (middle third) or high (top third) intensity for CAIX. Representative samples from each group are shown as well as the group average (mean +/- stdev). MM-398 treatment modifies the tumor microenvironment by decreasing the percentage of both medium and high CAIX positive cells in a dose-dependent manner. As hypoxia is a hallmark of resistant and aggressive disease, a reduction in hypoxia is expected to make tumor cells more sensitive to chemotherapies.

Example 4: MM-398 Increases Perfusion of Hoechst Stain

In addition to changing the chemosensitivity of tumor cells through modification of the tumor microenvironment, lowering hypoxia can indicate improved tumor vascularization, which can facilitate delivery of small molecule therapies. MM-398 treatment led to increased microvessel density 6 days after treatment as measured by CD31 (platelet endothelial cell adhesion molecule) staining in an HT29 xenograft study. To further assess the effect of MM-398 on small molecule tumor vascularization, a Hoechst 33342 perfusion experiment was conducted. Specifically, a primary pancreatic tumor was grown in NOD-SCID mice and given one dose of MM-398 (20mg/kg). After 24 hours, Hoechst 33342 stain was administered 20 minutes prior to sacrificing the animal. As shown in Figure 4, the increase in stain intensity in treated mice was statistically significant, $p < 0.001$. These data indicate that MM-398 modifies the tumor microenvironment in a manner that should make tumors more susceptible to agents such as 5-FU/LV, through decreasing tumor hypoxia and increasing small molecule perfusion.

Example 5: MM-398 Pharmacokinetics in Humans (Phase I)

The pharmacokinetic profile of MM-398 single agent was investigated in a phase I clinical study (PEP0201) in patients at 60, 120 or 180mg/m^2 dose levels and in a phase II clinical trial in gastric cancer patients (PEP0206) at 120mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride). Plasma levels of total irinotecan, SN-38 and

encapsulated irinotecan were measured in these studies.

The peak serum concentrations of total irinotecan (C_{max}) ranged from 48-79 μg/ml for 120mg/m^2 of MM-398 (based on the amount of irinotecan trihydrate hydrochloride), which was approximately 50-fold higher than 125mg/m^2 free irinotecan. The total irinotecan half-life ($t_{1/2}$) for MM-398 ranged from 21 to 48 hours, which was approximately 2-3 folds higher than 125mg/m^2 of free irinotecan. Overall, total irinotecan exposure at one week (AUC 0–T) ranged from 1200- 3000 (μg*h/ml) at a dose of 120 mg/m^2 of MM-398 (based on the amount of irinotecan trihydrate hydrochloride), approximately 50-100 fold higher than 300mg/m^2 of free irinotecan. In contrast, SN38 C_{max} levels at 120mg/m^2 of MM-398 (based on the amount of irinotecan trihydrate hydrochloride) ranged from 9 to 17 ng/ml, which was approximately 50% less than free irinotecan at 125mg/m^2. Overall, exposure of SN38 at one week (AUC 0-T) ranged from 474 to 997 ng*/ml and was only 1-2 fold higher than achieved by free irinotecan at 300mg/m^2. For both SN38 and total irinotecan, AUC increased less than proportionally with dose of MM-398. The PK parameters of encapsulated irinotecan almost matched that of total irinotecan indicates that most of irinotecan remained encapsulated in the liposomes during circulation. The MM-398 PK parameters were not significantly changed when combined with 5-FU/LV. Figures 5 and 6 summarize the PK findings in previous studies of MM 398.

Example 6: MM-398 Phase 1 Dose Escalation Study

A regimen combining fluorouracil, leucovorin, and MM-398 was studied in a phase I trial of solid tumors in 16 subjects, of whom 5 were patients with pancreatic cancer. The objective tumor response rate, duration of response, and disease control rate were efficacy endpoints of the study. Among the 15 efficacy-evaluable patients, 2 (13.3%) had confirmed PR, 9 (60.0%) had SD, and 4 (26.7%) had PD. The overall disease control rate was 73.3%. Partial response was observed in one gastric cancer patient (at 80mg/m^2 dose level, based on the amount of irinotecan trihydrate hydrochloride) and one breast cancer patient (at 100 mg/m^2 dose level, based on the amount of irinotecan trihydrate hydrochloride), with the duration of response of 142 and 76 days, respectively. Among the 6 patients who received the MTD dose of 80 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride), there were 1 PR, 4 SD and 1 PD. The tumor response rate and disease control rate were 16.7% and 83.3%, respectively. The main DLTs were grade 3 diarrhea, leucopenia, neutropenia and febrile neutropenia. The MTD for MM-398 was 80mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride).

In the phase I dose-escalation study of MM-398 in combination with 5-FU/LV in advanced solid tumors (PEP0203), a total of 401 episodes of AE were reported from the 16 treated subjects (safety population), of which 74 (18.4%) were of CTC grade 3 or above. Among all AEs, 231 (57.6%) were considered by the investigators to be treatment-related. The most common treatment-related AEs, included nausea (81.3%), diarrhea (75.0%), vomiting (68.8%), fatigue (43.8%), mucositis (43.8%), leucopenia (37.5%), neutropenia (37.5%), weight loss (37.5%), anemia (31.3%), and alopecia (31.3%). Acute cholinergic diarrhea was rarely observed. Table 2 provides the incidence of treatment-emergent adverse events by maximum CTC grade and by causality (incidence ≥ 20%), as seen in the PEP0203 study. Table 3 provides the incidence of grade 3 or higher treatment-emergent adverse events seen in the 5 pancreatic cancer patients treated in the PEP0203 study.

Table 2: Incidence of treatment-emergent adverse events by maximum CTC grade and by causality (incidence ≥ 20%) in the PEP0203 Study

System organ class Preferred Term	Total (N = 16)	Severity (Grade)[1]				Causality[2]	
		I	II	III	IV	Yes	No
Blood and lymphatic system disorders							
Anemia	7 (43.8%)	3	2	2	0	5	2
Leucopenia	6 (37.5%)	0	3	2	1	6	0
Neutropenia	6 (37.5%)	0	2	3	1	6	0
Gastrointestinal disorders							
Abdominal pain	7 (43.8%)	3	2	2	0	3	4
Constipation	6 (37.5%)	3	3	0	0	0	6
Diarrhea	12 (75.0%)	3	4	5	0	12	0
Nausea	13 (81.3%)	6	6	1	0	13	0
Vomiting	12 (75.0%)	3	8	1	0	11	1
General disorders and administration site conditions							
Fatigue	8 (50.0%)	4	3	1	0	7	1
Mucosal inflammation	7 (43.8%)	4	3	0	0	7	0
Pyrexia	7 (43.8%)	3	4	0	0	2	5
Infections and infestations							
Infection	6 (37.5%)	0	3	3	0	2	4
Investigations							
ALT increased	5 (31.3%)	3	2	0	0	4	1
AST increased	4 (25.0%)	3	1	0	0	1	3
Weight decreased	8 (50.0%)	4	4	0	0	6	2
Metabolism and nutrition disorders							
Anorexia	4 (25.0%)	1	2	1	0	3	1

System organ class Preferred Term	Total (N = 16)	Severity (Grade)[1]				Causality[2]	
		I	II	III	IV	Yes	No
Hypoalbuminaemia	4 (25.0%)	0	3	1	0	0	4
Hypocalcaemia	5 (31.3%)	1	4	0	0	0	5
Hypokalaemia	8 (50.0%)	2	0	5	1	2	6
Hyponatraemia	4 (25.0%)	2	0	0	2	0	4
Nervous system disorders							
Dizziness	4 (25.0%)	4	0	0	0	1	3
Psychiatric disorders							
Insomnia	4 (25.0%)	4	0	0	0	1	3
Respiratory, thoracic and mediastinal disorders							
Cough	5 (31.3%)	3	1	1	0	0	5
Skin and subcutaneous tissue disorders							
Alopecia	5 (31.3%)	5	0	0	0	5	0

[1:] Severity grading used the highest grading ever rated for each subject if the subject had such adverse event reported

[2:] Defined as subject ever experienced AE related to the study drug in causality or not

Table 3: Incidence of Grade 3 or higher treatment-emergent adverse events in pancreatic cancer patients in the PEP0203 Study (irinotecan dose based on the amount of irinotecan trihydrate hydrochloride)

	Overall N=5	60 mg/m^2 N=1	80 mg/m^2 N=3	120 mg/m^2 N=1
Primary system organ class Preferred term	**n (%)**	**n (%)**	**n (%)**	**n (%)**
-Any primary system organ class				
-Total	3 (60.0)	0	2 (66.7)	1 (100.0)
Infections and infestations				
-Total	3 (60.0)	0	2 (66.7)	1 (100.0)
Hepatitis viral	1 (20.0)	0	1 (33.3)	0
Infection	1 (20.0)	0	0	1 (100.0)
Pneumonia	1 (20.0)	0	1 (33.3)	0
Septic shock	1 (20.0)	0	1 (33.3)	0
Blood and lymphatic system disorders				
-Total	2 (40.0)	0	1 (33.3)	1 (100.0)
Lymphopenia	1 (20.0)	0	0	1 (100.0)
Neutropenia	1 (20.0)	0	1 (33.3)	0
White blood cell disorder	1 (20.0)	0	0	1 (100.0)
Gastrointestinal disorders				
-Total	2 (40.0)	0	1 (33.3)	1 (100.0)

	Overall N=5	60 mg/m^2 N=1	80 mg/m^2 N=3	120 mg/m^2 N=1
Primary system organ class Preferred term	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Diarrhoea	2 (40.0)	0	1 (33.3)	1 (100.0)
Abdominal pain	1 (20.0)	0	0	1 (100.0)
Gastrointestinal haemorrhage	1 (20.0)	0	1 (33.3)	0
Investigations				
-Total	2 (40.0)	0	1 (33.3)	1 (100.0)
Blood bilirubin increased	1 (20.0)	0	1 (33.3)	0
Lipase increased	1 (20.0)	0	0	1 (100.0)
Neutrophil count decreased	1 (20.0)	0	0	1 (100.0)
White blood cell count decreased	1 (20.0)	0	0	1 (100.0)
Metabolism and nutrition disorders				
-Total	2 (40.0)	0	1 (33.3)	1 (100.0)
Hypoalbuminaemia	1 (20.0)	0	1 (33.3)	0
Hypokalaemia	1 (20.0)	0	1 (33.3)	0
Hyponatraemia	1 (20.0)	0	0	1 (100.0)
Hypophosphataemia	1 (20.0)	0	0	1 (100.0)
Respiratory, thoracic and mediastinal disorders				
-Total	2 (40.0)	0	1 (33.3)	1 (100.0)
Dyspnoea	1 (20.0)	0	0	1 (100.0)
Pleural effusion	1 (20.0)	0	1 (33.3)	0
General disorders and administration site conditions				
-Total	1 (20.0)	0	0	1 (100.0)
Death	1 (20.0)	0	0	1 (100.0)

Example 7: MM-151 Phase 1 study

MM-151 has been assessed in one clinical study to date, MM-151-01-01-01. This study was a phase I study that evaluated MM-151 as a monotherapy and in combination with irinotecan, at different dosing levels and at dosing frequencies of QW, Q2W and Q3W. The study has completed, and preliminary signs of meaningful clinical benefit and an acceptable safety profile were observed, warranting further evaluation in metastatic CRC.

Rationale for MM-151 starting dose

Study	PEP0203	NAPOLI-1	PEPCOL
Tumor Type	Solid Tumors	Pancreas	Colorectal
Phase	I	III	II
Study design	Open label, dose escalation	Randomized comparison of nal-IRI and nal-IRI+ 5-FU/LV *vs* a common control of 5-FU/LV	Comparison of nal-IRI + 5FU/LV + AVASTIN versus FOLFIRI + AVASTIN
Number of Patients treated with nal-IRI	16	151 (monotherapy) 117 (combination)	28
Dosing Frequency	Q3W	Q3W (monotherapy) Q2W (combination)	Q2W
Dose Level (mg/m^2) (based on the amount of irinotecan trihydrate hydrochloride)	60 (n = 3) 80 (n = 6) 100 (n = 5) 120 (n = 2)	120 (monotherapy) 80 (combination)	80
Combination	5FU/LV	5FU/LV	5FU/LV + AVASTIN
Combination dose	2000/500 mg/m^2	2000/200 mg/m^2	2400/400 mg/m^2 (5FU/LV) 5 mg/kg (AVASTIN)
Key result	MTD identified as 80 mg/m^2	Combination arm achieved median OS 6.1 months, 1.9 month improvement over control arm (HR=0.57; p-value=0.0009)	Enrollment completed

Table 4: Summary of Studies Conducted with nal-IRI + 5-FU + LV
(Irinotecan dose based on the amount of irinotecan trihydrate hydrochloride)

The starting dose of 10.5 mg/kg is the QW RP2D that was identified in the phase I study.

Nal-IRI + 5-FU + LV clinical experience

The combination of nal-IRI + 5-FU + LV has been investigated in three clinical studies to date. One of these studies (NAPOLI-1)15 provided the basis for approval of this combination in gemcitabine-refractory pancreatic patients.

Nal-IRI + 5-FU + LV PK in humans

The pharmacokinetic profile of single agent nal-IRI has been investigated in several studies, in which plasma levels of total irinotecan, SN-38 and encapsulated irinotecan were

measured. In a single phase II clinical study (Study PEP0206), direct comparison of the pharmacokinetics of irinotecan and SN-38 in patients administered with nal-IRI or conventional (i.e. non-liposomal) irinotecan HCl (Camptosar®) was evaluated. Compared to the administration of irinotecan HCl 300 mg/m^2 q3w, administration of nal-IRI 100 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride) q3w resulted in higher exposure of total irinotecan (C_{max}: 13.4 fold, $AUC_{0-\infty}$: 46.2 fold, $t_{1/2}$: 2.0 fold), and higher SN-38 $t_{1/2}$ (3 fold) and marginally higher $AUC_{0-\infty}$ (1.4 fold), however, SN-38 C_{max} was reduced by 5.3 fold (Figure 4). In other PK studies of single agent nal-IRI, similar findings were observed when compared to standard doses of irinotecan HCl. Based on population pharmacokinetic analysis, no significant association was observed between the PK parameters of total irinotecan and SN-38 following nal-IRI monotherapy and when co-administered with 5-FU/LV. This result is consistent with the lack of drug interaction noted between irinotecan HCl and 5-FU (Camptosar US label). The pharmacokinetic parameters of total irinotecan and total SN-38 following the administration of nal-IRI 70 mg/m^2 (free base) as a single agent or part of combination chemotherapy are presented in Table 5.

Figure 9 is a graph showing the mean plasma concentrations of total irinotecan and SN-38 following the administration of either nal-IRI (100mg/m^2) (based on the amount of irinotecan trihydrate hydrochloride) or irinotecan HCl (300mg/m^2) in Study PEP0206. Gastric cancer patients received either nal-IRI at a dose of 100 mg/m^2 (blue line) (based on the amount of irinotecan trihydrate hydrochloride) or nal-IRI at a dose of 300 mg/m^2 (red line) (based on the amount of irinotecan trihydrate hydrochloride) every 3 weeks. Total irinotecan (top) and its active metabolite, SN-38 (bottom) were measured during Cycle 1. Error bars indicate 95% confidence interval. Dotted lines indicate lower limit of quantification (LLOQ); total irinotecan measurements consists of two LLOQ values because of two different irinotecan assay was used to measure low and high range of concentrations. The concentrations less than LLOQ values were set to the corresponding LLOQ.

Table 5: Summary of Mean (±Standard Deviation) Total Irinotecan and Total SN-38 (dose based on amount of irinotecan free base)

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
$t_{½}$: Terminal elimination half-life
CL: Clearance
V_d: Volume of distribution

Over the dose range of 50 to 155 mg/m^2 (free base), the Cmax and AUC of total irinotecan increases with dose. Additionally, the Cmax of total SN-38 increases proportionally with dose; however, the AUC of total SN-38 increases less than proportionally with dose.

The above PK results obtained from patients treated with either nal-IRI or irinotecan HCl confirmed the pre-clinical observation that nal-IRI extended plasma PK of both CPT-11 and SN-38 compared to treatment with irinotecan HCl. Further, a phase I clinical study of nal-IRI monotherapy (protocol MM-398-01-01-02; NCT# 01770353) investigated tumor levels of both CPT-11 and SN-38 following treatment with nal-IRI using post-treatment biopsies. Based on model predictions, SN-38 levels in tumor were expected to be higher than in plasma, suggesting local conversion of CPT-11 to SN-38 in the tumor microenvironment with nal-IRI (Figure 10). Predictions were confirmed by measuring levels of CPT-11 and SN-38 in tumor biopsy samples collected from patients 72 hours post-dose, demonstrating 5-fold higher levels of SN-38 in the tumor than the plasma (Figure 11A-11B). Collectively the evidence suggests that the prolonged systemic exposure to CPT-11 and SN-38 leads to prolonged levels of SN-38 in tumor tissue, which in turn leads to prolonged DNA damage to tumor cells, suggesting an advantage of nal-IRI compared to irinotecan HCl.

Figure 10, Figures 11A and 11B are graphs showing the clinical evidence for local activation and accumulation of SN-38 in tumor tissue. Figure 10 illustrates the mechanistic tumor PK model of nal-IRI predicted higher SN-38 levels in tumor compared to plasma when $70mg/m^2$ (based on the amount of irinotecan free base) nal-IRI is administered. The range of actual data, collected from a phase I study of patients (n=12) with advanced solid tumors following the administration of $70mg/m^2$ (based on the amount of irinotecan free base) nal-IR, is indicated by in Figure 11A (CPT-11) and Figure 11B (SN-38), as measured from patient tumor and plasma samples collected 72h post-nal-IRI infusion, which coincide with the levels as predicted by the mechanistic model.

Nal-IRI + 5-FU + LV safety in humans

It has been shown in animal and human PK studies that once irinotecan is released from the nal-IRI liposomes, the conversion of irinotecan to SN-38 is similar to that of the unencapsulated irinotecan. The safety of nal-IRI, therefore, may be indirectly compared with the safety of irinotecan, primarily based on a qualitative comparison of adverse reactions, as reported in the Camptosar US label for irinotecan. The comparison is qualitative, as both irinotecan and nal-IRI have been used in different doses and schedules as monotherapy and combination therapy with other chemotherapeutic agents; therefore, quantitative comparisons are difficult. The most common adverse reactions of irinotecan and nal-IRI are similar and consist mainly of gastrointestinal events and myelosuppression.

The common adverse reactions (>30%) observed in clinical studies with irinotecan in combination with other agents are: nausea, vomiting, abdominal pain, diarrhea, constipation, anorexia, mucositis, neutropenia, leukopenia (including lymphocytopenia), anemia, thrombocytopenia, asthenia, pain, fever, infection, abnormal bilirubin, and alopecia. The common adverse reactions (>30%) observed in single agent irinotecan therapy in clinical studies are: nausea, vomiting, abdominal pain, diarrhea, constipation, anorexia, neutropenia, leukopenia (including lymphocytopenia), anemia, asthenia, fever, body weight decreasing, and alopecia (Camptosar US label).

With respect to liposomal irinotecan, nal-IRI, when used in combination with 5-FU and leucovorin, the most common adverse reactions (≥20 %) observed in clinical trials considered to be related are: diarrhea, nausea, vomiting, decreased appetite, neutropenia, fatigue, anemia, stomatitis and pyrexia. The overall safety profile of nal-IRI is presented in detail in the related Investigator Brochure. Additionally, Table 6 summarizes ≥ Grade 3 safety data from the NAPOLI-1 trial comparing nal-IRI + 5-FU/LV (at a dose of 80 mg/m^2 based on the amount of irinotecan hydrochloride trihydrate, given on an every 2 week schedule), or nal-IRI monotherapy (at a dose of 120 mg/m^2 based on the amount of irinotecan trihydrate hydrochloride given on an every 3 week schedule), with 5-FU/LV alone (given weekly for 4 weeks followed by 2 weeks of rest) in the same population of patients who had received prior gemcitabine therapy.

Table 6: Summary of Grade 3 or Higher Adverse Events in NAPOLI-1 Study

	nal-IRI + 5-FU/LV (N=117)	**nal-IRI (N=147)**	**5-FU/LV (N=134)**

GRADE ≥3 NON-HEMATOLOGIC AEs IN >5% PATIENTS, %[1]			
Fatigue	14	6	4
Diarrhea	13	21	5
Vomiting	11	14	3
Nausea	8	5	3
Asthenia	8	7	7
Abdominal pain	7	8	6
Decreased appetite	4	9	2
Hypokalemia	3	12	2
Hypernatremia	3	6	2
GRADE ≥3 HEMATOLOGIC AES BASED ON LABORATORY VALUES, %[1,2]			
Neutrophil count decreased	20	16	2
Hemoglobin decreased	6	7	5
Platelet count decreased	2	1	0

1. Per CTCAE Version 4
2. Includes only patients who had at least one post-baseline assessment

Rationale for nal-IRI + 5-FU + LV dose levels

Nal-IRI, 5-FU and LV are all approved drugs and the dose levels used in this study are the approved dose levels for each therapy as defined in the package insert. In this phase I study, the doses of nal-IRI, 5-FU and LV will remain constant and the dose of MM-151 will be adjusted until an MTD is identified.

Rationale for combining MM-151 + nal-IRI + 5-FU + LV in CRC

Irinotecan is a well-established therapy in the frontline treatment of mCRC, alone or in combination, and can be combined with EGFR inhibitors to treat RAS wild type tumors; Nal-IRI (Onivyde™) is a nanoliposomal formulation of irinotecan. The nanoliposomal encapsulation improves the pharmacokinetics of irinotecan and results in a lower Cmax, longer half-life, and higher levels of irinotecan and SN-38 in tumor tissue compared with standard irinotecan. Nal-IRI is approved by the FDA in combination with 5-FU and leucovorin in gemcitabine refractory pancreatic adenocarcinoma under the brand name Onivyde. In a non-comparative study evaluating the use of nal-IRI + 5-FU + LV as a 2nd line therapy in mCRC (PEPCOL study), the nal-IRI containing arm met the threshold of early responses and had lower incidence of diarrhea and neutropenia than the FOLFIRI containing arm. Combination treatment of FOLFIRI and an EGFR inhibitor as first line treatment in mCRC are in accordance with National Comprehensive Cancer Network (NCCN) guidelines.

MM-151 is an EGFR inhibitor that combines three fully human IgG1 monoclonal antibodies. MM-151 antagonizes high-affinity EGFR ligands more effectively than another EGFR inhibitor, cetuximab, and has been observed to elicit a ~65-fold greater decrease in signal amplification. MM-151 has been evaluated in a phase I study of solid tumors and it demonstrated a safety profile that was comparable to other EGFR inhibitors, in monotherapy and in combination with irinotecan.

MM-151 was combined with nal-IRI in a LoVo colon xenograft experiment. The data showed that this combination enhances anti-tumor activity by overcoming EGFR insensitivity. This result, combined with the preliminary efficacy data seen in the PEPCOL study, suggests that the combination of MM-151 + nal-IRI + 5-FU + LV may be a viable therapeutic option in mCRC.

Potential toxicities of MM-151 + nal-IRI + 5-FU + LV

The nal-IRI+5-FU+LV regimen was studied in the NAPOLI-1 trial and the most common adverse reactions (≥20 %) observed in clinical trials considered to be related are: diarrhea, nausea, vomiting, decreased appetite, neutropenia, fatigue, anemia, stomatitis and pyrexia.

In addition, we would expect to see the known class toxicities of an EGFR inhibitor in addition to the nal-IRI+5-FU+LV regimen such as skin reaction, acneiform rash, liver toxicity, fatigue, diarrhea, stomatitis, electrolyte imbalances, paronychia, and vomiting as were seen in the FIRE-3 trial that combined FOLFIRI + cetuximab. In the phase I MM-151-01-01-01 study the most common related treatment emergent adverse events (≥20 %) in the combination of MM-151 and irinotecan were: anemia, neutropenia, diarrhea, nausea, vomiting, stomatitis, fatigue, mucosal inflammation, asthenia, infusion related reaction, weight loss, rash, dry skin, and alopecia.

Example 8

The following example employs a bin 1 antibody, "ca" which comprises the CDR regions of the Heavy and Light Chains in Figure 23. This antibody is described in the patent entitled "Antibodies Against Epidermal Growth Factor Receptor (EGFR) and Uses Thereof", U.S. Patent No. 9,044,460 as antibody "ca 34", which is herein incorporated by reference. *In vivo* studies of MM-151 alone and in combination with irinotecan were also performed in a colorectal cancer patient-derived xenograft (ST094). This study utilizes the MM-151

preclinical tool compound ("MM-151 TC") in which the antibody ca was substituted for antibody P1X. P1X, but not ca cross reacts with (binds to) mouse EGFR as well as binding to human EGFR, and the binding of P1X to the mouse antigen depletes the amount of this antibody in the mouse circulation, altering exposure of the human tumor to the antibody. Results obtained with ca are predictive of results that would be obtained in human patients with P1X, except that the antibody trio comprising P1X would be expected to be more active than the trio comprising ca in its stead. In this model, MM-151 and to a greater extent, the MM-151/irinotecan combination decreased tumor growth relative to the PBS control.

Example 9: Phase II Trial

Figure 12 is a scheme of a human clinical trial. The study can have two parts, a dose finding phase to determine the MTD and an expansion cohort after the MTD is identified. The dose levels to be used in this study are summarized in Table 7.

Table 7: Dose levels of MM-151, nal-IRI, 5-FU, and LV (irinotecan dose based on amount of irinotecan free base):

Method[1]	MM-151 QW (mg/kg)	Nal-IRI Q2W (mg/m^2)	5-FU Q2W (mg/m^2)	LV[3] Q2W (mg/m^2)
-3[2]	6.0	70	2400	400
-2[2]	7.5	70	2400	400
-1[2]	9.0	70	2400	400
1	10.5	70	2400	400
Expansion	MTD or RP2D	70	2400	400

[1]Prior to initiating the first dose in each method, there is a two week Priming Phase. In this Priming Phase, MM-151 is given at fixed doses of 225 mg and 450 mg on Weeks 1 and 2 respectively. Nal-IRI, 5-FU and Leucovorin are all administered in Week 1 of the Priming Phase at their normal doses listed in the table 7 above.
[2]Dose levels -1, -2, and -3 will be used if there is toxicity observed in Method 1 that limits further enrollment.
[3]l + d racemic form of LV will be used

30 – 90 minutes prior to infusion, patients will be administered their MM-151 premedication with all of the following:

- acetaminophen 650 mg PO or IV, and
- diphenhydramine 25-50 mg PO or IV, and
- methylprednisolone (Solumedrol®) 125 mg IV

The first two doses of MM-151 are administered during a two week priming phase. The first priming dose is given as a fixed dose of 225 mg in week 1 and the second priming

dose is given as a fixed dose of 450 mg in week 2. Subsequent dose levels are given as weight based dose levels defined per cohort.

Table 8: MM-151 Administration Rates

Infusion*	Rate (mg/hr.)	Time (hr)
1st Infusion	25 50 100	0.5 0.5 Until completion
2nd Infusion	25 50 100 200	0.5 0.5 0.5 Until completion
3rd Infusion	50 100 200 400	0.5 0.5 0.5 Until completion
4th and Subsequent Infusions	Start at 100 mg/hr and advance as tolerated	As needed

*Regardless of whether the infusion schedule contains a priming phase

The primary objective of this study is to determine the safety and tolerability of MM-151 + nal-IRI + 5-FU + LV in patients with metastatic colorectal cancer that are RAS-wildtype.

The secondary objectives of this study are:

- To characterize the adverse event profile (including DLTs)
- To determine the pharmacokinetic parameters
  To determine the immunogenicity parameters

The primary objectives of the phase IIb portion of the study are:

- To further characterize the safety and tolerability of the MM-151 + nal-IRI + 5-FU + LV combination in patients with metastatic colorectal cancer that are RAS-wildtype
- To characterize observed biomarkers from tissue, blood and/or urine and describe the impact of treatment on these biomarkers

The exploratory objectives of this study are:

- To assess preliminary efficacy as measured by objective response rate, disease control rate and progression free survival.
- To assess the relationship between biomarkers from tissue, blood and/or urine with toxicity and efficacy parameters

To be eligible for inclusion into the study patients must have/be:

- Pathologically documented, definitively diagnosed, colorectal adenocarcinoma that is locally advanced or metastatic and surgically un-resectable
- Wild type RAS gene in tumor tissue assessed via extended RAS testing for KRAS/NRAS (documentation of previously existing mutational status from an accredited laboratory will be accepted)
- Measureable disease in accordance with RECIST v1.1
- Adequate end organ function and bone marrow reserves:
- Received no more than one line of prior treatment for metastatic disease with one oxaliplatin based therapy and documentation of progression or intolerance to this therapy
- No prior treatment with irinotecan
- No prior treatment with an EGFR inhibitor

Note, the published dose of nal-IRI 80 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride) was expressed as the irinotecan hydrochloride trihydrate until October 2015. It is now expressed as the irinotecan free base. Converting a dose based on irinotecan hydrochloride trihydrate to a dose based on irinotecan free base is accomplished by substituting the Molecular Weight of irinotecan hydrochloride trihydrate (677.19 g/mole) with the Molecular Weight of irinotecan free base (586.68 g/mole), which results in a conversion factor of 0.866. Therefore, the published expression of the 80 mg/m^2 (based on the amount of irinotecan trihydrate hydrochloride) dose based on irinotecan hydrochloride trihydrate is equivalent to a 69.3 mg/m^2 dose based on irinotecan free base. This was rounded to 70 mg/m^2 to minimize any potential dosing errors.

Example 10: ONIVYDE® (MM-398 irinotecan liposome)

[0001] One preferred example of an irinotecan liposome described herein is the product that will be marketed as ONIVYDE (irinotecan liposome injection). ONIVYDE is a topoisomerase inhibitor, formulated with irinotecan hydrochloride trihydrate into a liposomal dispersion, for intravenous use. ONIVYDE indicated for the treatment of metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy.

[0002] ONIVYDE is an irinotecan liposome having a pH of about 7.25. The ONIVYDE product contains irinotecan sucrosofate encapsulated in a liposome, obtained from an irinotecan hydrochloride trihydrate starting material. The chemical name of irinotecan 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. The dosage of ONIVYDE can be calculated based on the equivalent amount of irinotecan trihydrate hydrochloride starting material used to prepare the irinotecan liposomes, or based on the amount of irinotecan in the liposome. There are about 866 mg of irinotecan per gram of irinotecan trihydrate hydrochloride. For example, an ONIVYDE dose of 80 mg based on the amount of irinotecan hydrochloride trihydrate starting material actually contains about 0.866x(80mg) of irinotecan in the final product (i.e., a dose of 80 mg/m^2 of ONIVYDE based on the weight of irinotecan hydrochloride starting material is equivalent to about 70 mg/m^2 of irinotecan in the final product). 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. Each vial of ONIVYDE contains 43 mg/10 mL irinotecan free base as a white to slightly yellow, opaque, liposomal dispersion in a single-dose vial.

[0003] The unit dosage form can be an intravenous formulation having a total volume of about 500 mL. ONIVYDE is prepared for administering by diluting the isotonic liposomal dispersion from the vial as follows: withdraw the calculated volume of ONIVYDE from the vial. ONIVYDE is diluted in 500mL 5% Dextrose Injection, USP or 0.9% Sodium Chloride Injection, USP and mix diluted solution by gentle inversion; protect diluted solution from light and 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)].

[0004] ONIVYDE (irinotecan liposome injection) is indicated, in combination with 5-fluorouracil and leucovorin, for the treatment of patients with metastatic adenocarcinoma of the pancreas that has progressed following gemcitabine-based therapy. Administer ONIVYDE prior to leucovorin and fluorouracil. The recommended dose of ONIVYDE is 70 mg/m^2 irinotecan (free base) 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 irinotecan administered by intravenous infusion over 90 minutes. The dose of ONIVYDE can be increased 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. ONIVYDE is infused as a diluted solution intravenously over 90 minutes.

[0005] Suitable treatment regimens include ONIVYDE 70 mg/m^2 with (l+d racemic form) leucovorin 400 mg/m^2 (or 200 mg/m^2 of the active l form of leucovorin) and fluorouracil 2,400 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).

Example 11: Activity of MM-151 in combination with Nal-IRI in LoVo CRC xenograft model

The combination effect of MM-151 TC (MM-151 preclinical tool compound; refer to Example 8) and nal-IRI was studied in a subcutaneous CRC xenograft model (LoVo). MM-151 TC was utilized for cross-reactivity with murine EGFR, and was administered intraperitoneally (IP) at 1 cycle/week comprised of a loading dose (mAb2E5+P2X+P3X) on C1D1, maintenance doses (mAb2E5+P2X+P3X) on D1 of subsequent cycles, and P2X maintenance doses on D3 and D5 every cycle. Nal-IRI is administered intravenously via tail vein injection weekly. LoVo tumor-bearing mice were randomized for efficacy study at Day 16 post-inoculation. A combination of nal-IRI + MM-151 has improved activity compared to the two agents alone. Table 9 illustrates response analysis based on RECIST-like criteria (modified for preclinical tumor volume analyses) on Day 30 post-inoculation. Individual tumors with -30 to -95%, -30 to 30%, and 30% change in volume were classified as partial response (PR), stable disease (SD), and progressive disease (PD), respectively. At the doses tested, nal-IRI + MM-151 resulted in 62.5% PR and 37.5% SD with no PD, while MM-151 and nal-IRI monotherapies resulted in 100% and 75% PD, respectively.

Table 9. Response of LoVo CRC xenograft based on RECIST-like criteria.

	MM-151 TC	Nal-IRI	Nal-IRI + MM-151 TC
PR	0/8 (0%)	1/8 (12.5%)	5/8 (62.5%)
SD	0/8 (0%)	1/8 (12.5%)	3/8 (37.5%)
PD	8/8 (100%)	6/8 (75%)	8/8 (0%)

Note: Response is calculated based on tumor volume change from baseline volume. PR =

between -95% to -30%, SD = -30% to 30%, PD = tumor volume > 30%.

Example 12: Activity of Nal-IRI is superior to free irinotecan in a KRAS/NRAS/BRAF wild type LIM1215 CRC xenograft at a 5-fold lower dose

Figure 21 demonstrates the anti-tumor activity of nal-IRI at 5 mg/kg compared to free irinotecan at 25 mg/kg. The doses were chosen due to similar SN-38 (active metabolite of irinotecan) exposure in the tumors based on previously published data [Kalra (2014) Cancer Research, 74(23):7003-13]. At 5-fold lower doses administered and presumably equivalent SN-38 exposure, nal-IRI has superior anti-tumor activity compared to free irinotecan. This is believed to be attributed to the prolonged duration of SN-38 present in the tumors for nal-IRI as a result of the liposomal delivery mechanism, in contrast to the free irinotecan in which the small molecules have faster clearance than the liposomal irinotecan (nal-IRI) as shown previously in Figure 2. This differential activity between nal-IRI and IRI at the equivalent SN-38 exposure doses was also previously demonstrated in HT-29 CRC xenografts [Kalra (2014) Cancer Research, 74(23):7003-13].

Example 13: Activity of nal-IRI + 5-FU + MM-151 TC vs. irinotecan + 5-FU + cetuximab

The combination of free irinotecan + 5-FU/LV + cetuximab is one of the standard of care (SOC) regimens for treating metastatic CRC. Activity of the experimental treatment, nal-IRI + 5-FU + MM-151 TC, was compared to the SOC irinotecan + 5-FU + cetuximab in LIM1215 KRAS/NRAS/BRAF wild type CRC xenograft models. Two dose levels of nal-IRI and irinotecan were chosen for combining with constant doses of 5-FU and MM-151 TC or cetuximab. "Low dose" combinations include Nal-IRI (1.25 mg/kg, IV) + 5-FU (50 mg/kg, IP) + MM-151 TC (3 mg/kg, IP) and irinotecan (6.25 mg/kg, IV) + 5-FU (50 mg/kg, IP) + cetuximab (3 mg/kg, IP). "High dose" combinations include Nal-IRI (5 mg/kg, IV) + 5-FU (50 mg/kg, IP) + MM-151 TC (3 mg/kg, IP) and irinotecan (25 mg/kg, IV) + 5-FU (50 mg/kg, IP) + cetuximab (3 mg/kg, IP). The comparative treatment groups were chosen such that the irinotecan and nal-IRI treatments have similar SN-38 tumor exposure. The dose levels of nal-IRI and IRI were below clinical dose, in which the clinical translated dose for nal-IRI (80 mg/m^2 hydrochloride trihydrate q2w for patients) is 13 mg/kg q1w in mouse, and for irinotecan (180 mg/m^2 q2w, based on the amount of irinotecan trihydrate hydrochloride) is 30 mg/kg q1w in mouse. The dose levels of MM-151 TC and cetuximab were chosen such that the exposure levels in the blood are nearly equivalent in both the first week (with the

loading dose) and the subsequent weeks (with the maintenance doses). Exposure was calculated as the area under the concentration curve (AUC) of cetuximab versus the sum total of the AUC of the three antibodies in the MM-151 TC mixture.

Figures 22A and B illustrate the anti-tumor activities of low and high dose combinations, respectively. At the 2 dose levels tested, nal-IRI + 5-FU + MM-151 TC are superior to the SOC irinotecan + 5-FU + cetuximab. In addition, Table 10 shows that high dose nal-IRI + 5-FU + MM-151 TC treatment results in 3 CR and 6 PR, while both dose levels of irinotecan + 5-FU + cetuximab did not have any CR.

Table 10. Response of LIM1215 CRC xenograft based on RECIST-like criteria.

	Low Dose		High Dose	
	Irinotecan + 5FU + Cetuximab	**Nal-IRI + 5-FU + MM-151 TC**	**Irinotecan + 5FU + Cetuximab**	**Nal-IRI + 5-FU + MM-151 TC**
CR	0/10 (0%)	0/10 (0%)	0/10 (0%)	3/10 (30%)
PR	5/10 (50%)	9/10 (90%)	6/10 (60%)	6/10 (60%)
SD	3/10 (30%)	1/10 (10%)	3/10 (30%)	1/10 (10%)
PD	2/10 (20%)	0/10 (0%)	1/10 (10%)	0/10 (0%)

Note: Response is calculated based on tumor volume change from baseline volume. CR = tumor volume <-95%, PR = between -95% to -30%, SD = -30% to 30%, PD = tumor volume > 30%.

Example 14: Initiation of Phase 1 Study of MM-151 in Combination with the ONIVYDE® (irinotecan liposome injection) Regimen in Metastatic Colorectal Cancer

This phase I clinical study examines oligoclonal EGFR (epidermal growth factor receptor) inhibitor, MM-151, in combination with ONIVYDE® (irinotecan liposome injection) plus fluorouracil (5-FU) and leucovorin in patients with RAS wild-type metastatic colorectal cancer. Data from a prior phase I study of MM-151 supports further clinical evaluation of the investigational therapy in patients with metastatic colorectal cancer. The initiation of this study advances the development path for ONIVYDE.

This phase I study will assess the safety and tolerability of the combination of MM-151, a novel antibody mixture of three human antibodies designed to target EGFR which promotes tumor growth, and ONIVYDE, also known as MM-398 or "nal-IRI," plus 5-FU and leucovorin as first or second-line treatment in patients with RAS wild-type metastatic colorectal cancer.

Preclinically, MM-151 has shown superior inhibition of the EGFR pathway compared to FDA approved EGFR inhibitors. It is expected that superior topoisomerase-1 inhibition plus superior EGFR inhibition will lead to improved efficacy in metastatic

colorectal cancer.

The trial will determine the side effect profile of MM-151 in combination with ONIVYDE plus 5-FU and leucovorin and recommended dose for subsequent trials with this combination. Eligible patients for the study must have metastatic disease, have had no prior exposure to irinotecan or an EGFR inhibitor, and have received no more than one prior line of treatment for metastatic disease.

Example 15: Evaluation of MM-151 + Nal-IRI + 5-FU + Leucovorin in RAS/RAF Wild-type Metastatic Colorectal Cancer

An open label, non-randomized phase I/II study can evaluate the combination of MM-151 + nal-IRI + 5-FU + Leucovorin in RAS/RAF wild-type Metastatic Colorectal Cancer. In the dose finding cohorts of this phase Ib/II study, a modified toxicity probability interval approach (mTPI) will be utilized to determine the maximum tolerated dose (MTD) of MM-151 in combination with nal-IRI, 5FU and leucovorin. Phase I: Approximately 8-12 patients will be enrolled to determine the maximum tolerated dose, safety and tolerability of MM-151 + nal-IRI + 5-FU + LV in patients with mCRC that are RAS wildtype. Phase II: Approximately 20-30 patients will be enrolled at the maximum tolerated dose of MM-151 in combination with nal-IRI + 5-FU + LV to characterize initial efficacy in conjunction with levels of irinotecan and SN-38 measured in tissue.

Table 11. Experimental Arms

Arms	Assigned Interventions
Experimental: Phase Ib: Safety evaluation MM-151 dose finding (beginning at 10.5 mg/kg weekly) in combination with fixed doses of nal-IRI 70mg/m^2 + LV 400 mg/m^2 + 5-FU 2400mg/m^2 every two weeks.	**Drug: MM-151** Oligoclonal antibody **Drug: nal-IRI** Nanoliposomal irinotecan Other Names: • MM-398 • Onivyde **Drug: Leucovorin** folinic acid Other Names: • folinic acid **Drug: 5-Fluorouracil** Chemotherapy Other Names: • 5-FU

Experimental: Phase IIa MM-151 (MTD or RP2D) in combination with fixed doses of nal-IRI 70mg/m^2 + LV 400 mg/m^2 + 5-FU 2400mg/m^2.	**Drug: MM-151** Oligoclonal antibody **Drug: nal-IRI** Nanoliposomal irinotecan Other Names: • MM-398 • Onivyde **Drug: Leucovorin** folinic acid Other Names: • folinic acid **Drug: 5-Fluorouracil** Chemotherapy Other Names: • 5-FU

Primary Outcome Measure:

1. Primary Objective of phase Ib

[Time Frame: 6 months] [Safety Issue: Yes]

To determine the maximum tolerated dose, safety and tolerability of MM-151 + nal-IRI + 5-FU + LV in patients with mCRC that are RAS wild-type.

2. Primary Objective of phase IIa

[Time Frame: 2 years] [Safety Issue: No]

To characterize initial efficacy in conjunction with levels of irinotecan and SN-38 measured in tissue

Secondary Outcome Measure:

3. Secondary Objectives of phase Ib

[Time Frame: 2 years] [Safety Issue: Yes]

To characterize the adverse event profile (including DLT's); To determine the pharmacokinetic parameters; To determine the immunogenicity parameters

4. Secondary Objectives of phase IIa

[Time Frame: 2 years] [Safety Issue: Yes]

To further characterize the adverse event profile; To describe the observed objective response rate (ORR) and disease control rate; To measure pretreatment and on-treatment levels of EGFR ligands; To determine the pharmacokinetic parameters; To determine the immunogenicity parameters

Other Pre-specified Outcome Measures:

5. Exploratory Objectives

[Time Frame: 2 years] [Safety Issue: Yes]

To assess the relationship between biomarkers from tissue, blood and/or urine with toxicity and efficacy parameters, including markers of the development of resistance to treatment

Eligibility:

Minimum Age: 18 Years; Gender: Both; No Healthy Volunteers.

Inclusion Criteria:

• Pathologically documented, definitively diagnosed, colorectal adenocarcinoma that is metastatic and surgically un-resectable

• For phase Ib of the study, patients must be either treatment naïve or have had no more than one prior line of therapy with an oxaliplatin-based regimen for metastatic disease

• For phase IIa of the study, patients must have had no prior therapy for metastatic disease.

• Wild-type KRAS/NRAS genes as assessed in tumor tissue via extended RAS testing

• Histologic confirmed BRAF wild-type status of primary colorectal cancer or related metastasis

• Measurable disease in accordance with RECIST v1.1

• ECOG Performance Score (PS) of 0 or 1

• Adequate bone marrow reserves as evidenced by:

ANC > 1,500/μl (unsupported by growth factors) o Platelet count > 100,000/μl o Hemoglobin > 9 g/dL o

• Adequate hepatic function as evidenced by: Serum total bilirubin ≤ ULN o Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline Phosphatase (ALP) ≤ 2.5 x ULN (≤ 5 x ULN is acceptable if bone or liver metastases are present)

• Adequate renal function as evidenced by a serum creatinine < 1.5 x ULN

• Adequate cardiac function as determined by:

An LVEF within normal institutional limits o ECG without clinically significant abnormalities including prolonged QTc interval (> 450 msecs); abnormal findings on interpretation of ECG are acceptable if principle investigator confirms it is not clinically significant.

• Recovered from the effects of any prior surgery, radiotherapy or other antineoplastic therapy to CTCAE v4.0 grade 1, baseline or less.

• Patient must be willing to provide a pre-treatment biopsy as well as an on study biopsy to

the Sponsor. In the event that a patient has an archived tumor sample from a previous biopsy and no anti-cancer treatment was given prior to that biopsy then the archived tumor sample may be submitted in place of a fresh biopsy.

• Willing to abstain from sexual intercourse or to use an effective form of contraception during the study and for 120 days following the last dose of any study therapy. This applies to women of childbearing potential as well as fertile men and their partners.

• At least 18 years of age

• Able to provide informed consent, or have a legal representative able and willing to do so

Exclusion Criteria:

• Prior pelvic radiation treatment

• Prior treatment with irinotecan (patients in phase Ib)

• Prior treatment with an EGFR inhibitor (patients in phase Ib)

• Pregnant or lactating

• Untreated (primary) or uncontrolled CNS (primary or metastatic) malignancies; patients with CNS metastases who have undergone surgery or radiotherapy or who have been on a stable dose of corticosteroids for at least 2 weeks and whose disease is stable prior to the first scheduled day of dosing will be eligible for the trial.

• Clinically significant cardiac disease, including: NYHA Class III or IV congestive heart failure, unstable angina, acute myocardial infarction within six months of planned first dose, arrhythmia requiring therapy (including torsades de pointes, with the exception of extra systoles, minor conduction abnormalities, or controlled and well treated chronic atrial fibrillation)

• 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. Patients with a history of other malignancies are eligible if they have been continuously disease free for at least 3 years.

• Clinically significant gastrointestinal disorder including hepatic disorders, occlusion, diarrhea > grade 1, malabsorption syndrome, ulcerative colitis, inflammatory bowel disease, or partial bowel obstruction

• Known hypersensitivity to any of the components of nal-IRI, other liposomal products, or any components of 5-FU or LV

• Known hypersensitivity to any of the components of MM-151

• Received other recent antitumor therapy including any standard chemotherapy or radiation within 14 days and bevacizumab within 28 days (and having passed the time of any actual or anticipated toxicities) prior to the first scheduled dose of the study treatment

• Any other medical condition deemed by the Investigator to be likely to interfere with a patient's ability to provide informed consent, cooperate and participate in the study, or to interfere with the interpretation of the results

Example 16: Final results of a first-in-human study evaluating the safety, pharmacology and initial efficacy of MM-151, an oligoclonal anti-EGFR antibody in patients with refractory solid tumors.

This is a phase I study that evaluated MM-151 when administered as a monotherapy and in combination with (non-liposomal) irinotecan. An expansion cohort was also enrolled to evaluate clinical activity in EGFR-refractory metastatic CRC patients (pts). Subset analyses and additional biomarker evaluations were performed in EGFR-driven indications.

Results: A total of 111 patients were enrolled (87 patients on monotherapy). A summary of the patient demographics are set forth in Table 12. Specific population demographics for patients with HNSCC, NSCLC, or CRC are set forth in Tables 13, 14, and 15, respectively.

Table 12: Patient demographics

Characteristic	MM-151 Monotherapy (N=87)	MM-151+ Irinotecan (N=24)	Overall (N=111)
Gender, n (%)			
Male	39 (44.8)	14 (58.3)	53 (47.7)
Female	48 (55.2)	10 (41.7)	58 (52.3)
Age (years)			
Median	62	57.5	61
Range	30-85	39-85	30-85
Type of Cancer, n (%)			
Colorectal Cancer	38 (43.7)	7 (29.2)	45 (40.5)
Head and Neck Cancer	6 (6.9)	8 (33.3)	14 (12.6)
Non Small Cell Lung Cancer	9 (10.3)	2 (8.3)	11 (9.9)
Triple Negative Breast Cancer	2 (2.3)	1 (4.2)	3 (2.7)
Other Cancer Type	32 (36.8)	6 (25.0)	38 (34.2)
Time Since First Cytological or Histo-Pathological Diagnosis (Months)			
Mean/SD	45.11 / 35.818	46.08 / 42.558	45.32 / 37.173
Median	32.66	39.38	33.61
Min/Max	2.8 / 233.7	8.2 / 226.2	2.8 / 233.7

Time Since Metastatic Diagnosis (Months)			
Mean/SD	31.58 / 25.010	27.78 / 23.032	30.78 / 24.546
Median	23.69	21.29	22.87
Min/Max	0.8 / 95.3	0.4 / 96.0	0.4 / 96.0

Table 13: HNSCC Population Demographics

Characteristics	MM-151 Monotherapy (N=4)	MM-151 + Irinotecan (N=5)	Overall (N=9)
Gender, n (%) Male Female	 2 (50.0) 2 (50.0)	 4 (80.0) 1 (20.0)	 6 (66.7) 3 (33.3)
Age (years) Median Range	 61.0 35-67	 57.0 42-77	 58.0 35-82
Time Since First Diagnosis (Months) Median	 19.22	 34.30	 26.68
Time Since Metastatic Diagnosis (Months) Median	 20.70	 17.18	 18.94
EGFR Mutation No Unknown	 1 (25.0) 3 (75.0)	 3 (60.0) 2 (40.0)	 4 (44.4) 5 (55.6)
BRAF Mutation n No Unknown	 3 1 (25.0) 2 (50.0)	 5 2 (40.0) 3 (60.0)	 8 3 (37.5) 5 (62.5)

Table 14: NSCLC Population Demographics

Characteristics	MM-151 Monotherapy (N=6)	MM-151 + Irinotecan (N=2)	Overall (N=8)
Gender, n (%) Male Female	 1 (16.7) 6 (83.3)	 1 (50.0) 1 (50.0)	 2 (25.0) 6 (75.0)
Age (years) Median Range	 70 51-82	 57 48-66	 67 48-82
Time Since First Diagnosis (Months) Mean/SD Median Min/Max	 38.03 / 13.580 35.4 21.2 / 55.7	 21.39 / 6.505 21.39 16.8 / 26.0	 33.87 / 14.040 30.8 16.8 / 55.7
Time Since Metastatic Diagnosis (Months) Mean/SD Median Min/Max	 31.82 / 12.917 30.8 12.9 / 52.5	 7.06 / 4.832 7.06 3.6 / 10.5	 25.63 / 15.934 28.02 3.6 / 52.5

EGFR Mutation			
Yes	1 (16.7)	1 (50.0)	2 (25.0)
No	3 (50.0)	1 (50.0)	4 (50.0)
Unknown	2 (33.3)	0	2 (25.0)
KRAS Mutation			
n	4	2	6
Yes	1 (16.7)	0	1 (12.5)
No	0	1 (50.0)	1 (12.5)
Unknown	3 (50.0)	1 (50.0)	4 (50.0)

Table 15: CRC Population Demographics

Characteristic	**MM-151 Monotherapy (N=22)**	**MM-151+ Irinotecan (N=7)**	**Overall (N=29)**
Gender, n (%)			
Male	12 (54.5)	5 (71.4)	17 (58.6)
Female	10 (45.5)	2 (28.6)	12 (41.4)
Age (years)			
Median	57	56	56
Min/Max	42-81	50-76	42-81
Time Since First Diagnosis (Months)			
Mean/SD	51.72 / 32.210	47.23 / 15.178	50.64 / 28.832
Median	42.56	48.16	45.6
Min/Max	8.1 / 114.1	18.6 / 63.8	8.1 / 114.1
Time Since Metastatic Diagnosis (Months)			
Mean/SD	38.56 / 26.787	36.93 / 15.722	38.17 / 24.323
Median	36.39	39.13	37.06
Min/Max	5.2 / 90.6	19.8 / 62.9	5.2 / 90.6
EGFR Mutation			
n	22	7	29
Yes	3 (13.6)	1 (14.3)	4 (13.8)
No	5 (22.7)	3 (42.9)	8 (27.6)
Unknown	14 (63.6)	3 (42.9)	17 (58.6)
KRAS Mutation			
n	17	7	24
Yes	3 (13.6)	2 (28.6)	5 (17.2)
No	11 (50.0)	5 (71.4)	16 (55.2)
Unknown	3 (13.6)	0	3 (10.3)
BRAF Mutation			
n	17	7	24
Yes	1 (4.5)	0	1 (3.4)
No	6 (27.3)	2 (28.6)	8 (27.6)
Unknown	10 (45.5)	5 (71.4)	15 (51.7)

The most common tumor types were CRC (43 [39%]), NSCLC (11 [10%]) and SCCHN (14 [13%]). Weekly dose selection was previously reported. Reported here are final safety and biomarker data. Most adverse events (AEs) were CTCAE Grades 1 and 2. The

most common Grade 3 (G3) or higher non-infusion related reaction (IRR) AEs included EGFR-pathway toxicities, such as maculopapular rash (11 [9.9%]), hypomagnesemia (10 [9%]), general rash (8 [7.2%]) and diarrhea (8 [7.2%])

Table 16: Common related, non-IRR adverse events grade 3 or higher

Adverse Event	MM-151 Monotherapy N=87 [n (%)]	MM-151 + Irinotecan N=24 [n (%)]
Rash maculo-papular	8 (9.2)	3 (12.5)
Hypomagnesaemia	7 (8.0)	3 (12.5)
Rash	7 (8.0)	1 (4.2)
Hypophosphataemia	4 (4.6)	1 (4.2)
Dermatitis acneiform	3 (3.4)	1 (4.2)
Dry skin	2 (2.3)	--
Diarrhea	1 (1.1)	4 (16.7)
Lymphocyte count decreased	1 (1.1)	1 (4.2)
Anemia	--	3 (12.5)
Neutrophil count decreased	--	3 (12.5)
Febrile neutropenia	--	2 (8.3)
Neutropenia	--	2 (8.3)
Vomiting	--	2 (8.3)
White blood cell count decreased	--	2 (8.3)
Hypokalaemia	--	2 (8.3)

G3 infusion related reactions (IRR) occurred in 8/57 (14%) of patients enrolled at the non-optimized dosing guidelines versus 1/57 (1.7%) of patients in the optimized dosing cohorts. Frequency of infusion related reactions experienced at first MM-151 infusion are summarized in Table 17.

Table 17: Frequency of infusion related reactions

	Infusion Schedule		
	Original (N=37)[1] n (%)	Modified 1 (N=17)[2] n (%)	Modified 2 (N=57)[3] n (%)
Grade 1	4 (10.8)	2 (11.8)	12 (21.1)
Grade 2	18 (48.6)	8 (47.1)	17 (29.8)
Grade 3	8 (21.6)	2 (11.8)	4 (7.0)
Grade 4	0	0	0
Grade 5	0	0	0
Any Grade	30 (81.1)	12 (70.6)	33 (57.9)

Example 17: Biomarker Studies Reveal a Low Frequency of Acquired Mutations

Following MM-151 Treatment

In the Phase 1 clinical study described in Example 16, tissue and blood samples were collected from treated patients and used in exploratory biomarker analyses. While samples were requested from all patients identified in Table 12, samples were only collected from a subset of these patients. For those patients from whom samples were obtained, blood and tissue samples were assessed for genomic alterations using next-generation sequencing assays from GuardantHealth (Guardant360) and OmniSeq (OmniSeq Comprehensive; OmniSeq PGM), respectively. The Guardant360 and OmniSeq Comprehensive assays identify somatic mutations, copy number variations, and indels/fusions in oncology-focused gene panels. Table 18 summarizes of the collected samples for biomarker analysis, from patients having EGFR-related indications (i.e., patients for whose condition anti-EGFR antibodies are an approved therapy).

Table 18: Summary of collected samples for biomarker analysis

Diagnosis	MM-151 Monotherapy	MM-151 + Irinotecan
CRC	**N=22** Archival tissue – 19 Pre-treatment blood – 14 Post-treatment blood – 9	**N=7** Archival tissue – 5 Pre-treatment blood – 6 Post-treatment blood – 2
HNSCC	**N=4** Archival tissue – 2 Pre-treatment blood – 3 Post-treatment blood – 2	**N=5** Archival tissue – 4 Pre-treatment blood – 5 Post-treatment blood – 2
NSCLC	**N=6** Archival tissue – 2 Pre-treatment blood – 6 Post-treatment blood – 4	**N=2** Archival tissue – 1 Pre-treatment blood – 2 Post-treatment blood – 2

Subset analyses were performed for a cohort of colorectal cancer patients (N=29) who received the minimum efficacious dose of $\geq$ 6 mg/kg on QW or Q2W schedules (i.e., the "CRC efficacy cohort patients," as used herein). 28 of 29 CRC efficacy cohort patients had archival tissue and/or pre-treatment serum samples for DNA sequencing. Figure 24 summarizes the pre-treatment somatic mutations within the CRC efficacy cohort. Mutually exclusive mutations in KRAS/NRAS/BRAF were observed in 39% of these patients (11/28).

Figure 25 is a table listing the cancer type, treatment schedule, duration of treatment and mutation status (wild type, "WT" or mutant, "MT") for 26 patients from whom we collected both pre- and post-treatment serum samples from the clinical trial described in

Example 16. In addition, the table in Figure 25 indicates the measured maximum change in solid tumor size as measured by RECIST 1.1 ("Maximum Percent Change") compared to the solid tumor size measured prior to treatment. The data in Figure 25 demonstrates a low frequency of acquired KRAS/NRAS/BRAF mutations following MM-151 treatment (*i.e.*, 1 of 5 CRC patients and 2 of 19 in all indications that were assessed as WT for all measured KRAS, NRAS and BRAF prior to treatment). Notably, no CRC patients acquired an EGFR exon 12 mutation, and only one of 25 wild type patients (from all indications shown in the Table of Figure 25) acquired a variant of unknown significance at a low allelic frequency. Clinically meaningful durations were achieved in patients presenting with multiple resistance markers, including RAS/RAF mutations. This demonstrated a rate of acquired mutations measured in MM-151-treated patients that was lower than observed with treatment of other EGFR antibodies in the literature.

Figure 26 is a table detailing the patient treatment parameters, the duration of treatment, the best overall response, duration of treatment and mutation status (wild type, "WT" or mutant, "MT") for 24 of 29 patients within the CRC efficacy cohort who were evaluated for RECIST response in the clinical trial described in Example 16. The Table in Figure 26 also includes patient treatment parameters listing the treatment type (MM-151 monotherapy or combination of MM-151 and irinotecan), whether the first two weekly doses of MM-151 were priming doses (225 mg and 450 mg fixed dose, respectively), dose of MM-151 administered (after the priming doses, if applicable), MM-151 dose frequency (every week or every two weeks), and (if applicable) the irinotecan dose and dose frequency. Of these 24 patients listed in the Table in Figure 26, 13 patients (54%) had a reduction in target lesions and 7 progressed at first scan, including 2 wild type (WT in KRAS, NRAS, BRAF, PIK3CA, EGFR Exon 12), 3 NRAS mutants, 1 co-occurring PIK3CA mutation, and 1 BRAF mutation. Two patients were noted as progressive having progressive disease (PD) on first scan but had <20% increase in target lesions, one patient had a new non-target lesion noted, and one patient was a clinical progression (not RECIST). Of the 15 remaining patients that did not progress outright, 7 patients were WT, 4 KRAS mutant, 3 BRAF mutant, 2 NRAS mutant, and 2 EGFR ECD mutant (including co-occurring).

Further exploratory clinical biomarker analyses were performed on subsets of patients enrolled in the clinical trial described in Example 16. Downregulation of EGFR was measured by immunohistochemistry (IHC) in matched pre-treatment and on-treatment tissue biopsies (FFPE) collected from 4 patients treated with 10.5 mg/kg MM-151 once a week after

administration of fixed dose priming doses of 225 mg and 450 mg in weeks 1 and 2 of treatment. Clinical validation of MM-151 mechanistic foundations was observed by EGFR downregulation, high affinity ligand frequency in refractory mCRC patients, and the ability to overcome both upstream and downstream mutations (acquired and *de novo*). Additionally, a decrease in measurable lesions was observed in 54% of evaluable patients in the CRC cohort and a measureable decrease in lesions was observed in both WT and mutant patients. Preliminary indications of objective clinical activity across both the EGFR-refractory and naïve populations suggest potential for broad effect. Biomarker profiling also suggests that MM-151 may overcome mechanisms of resistance.

Endnotes

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features set forth herein. The disclosure of each and every U.S., international, or other patent or patent application or publication referred to herein is hereby incorporated herein by reference in its entirety.

APPENDIX

Abbreviation	**Term**
AE	adverse event
ALT	alanine aminotransferase
ANC	absolute neutrophil count
AST	aspartate aminotransferase
AUC	area under the curve
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BSA	body surface area
BUN	blood urea nitrogen
CBC	complete blood count
CEA	carcinoembryonic antigen
C_{max}	maximum plasma concentration of the drug
CNS	central nervous system
CR	complete response per RECIST
CRC	colorectal cancer
CRF	case report form

CT	computerized tomography
CTCAE	Common Terminology Criteria for Adverse Events
DCR	disease control rate
DHFR	dihydrofolate reductase
dL	deciliter
DLT	dose limiting toxicity
ECOG	Eastern Cooperative Oncology Group
ECG	electrocardiogram
EGFR	epidermal growth factor receptor
EPR	enhanced permeability and retention
FDA	Food and Drug Administration
G-CSF	granulocyte colony-stimulating factors
GCP	Good Clinical Practice
FFPE	formalin fixed paraffin embedded
HAHA	human anti-human antibody
HIPAA	Health Insurance Protection and Portability Act
ICH	International Conference on Harmonization
INR	international normalized ratio
IRB	institutional review board
IRR	Infusion-Related Reaction
IV	intravenous
Kg	kilogram
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
LV	leucovorin
LDH	lactate dehydrogenase
LS-GMR	least squares geometric mean ratios
mCRC	metastatic colorectal cancer
m^2	meters squared
MedDRA	Medical Dictionary for Regulatory Activities
Mg	milligram
mL	milliliter
MRI	magnetic resonance imaging
MTD	maximum tolerated dose
Nal-IRI	nanoliposomal irinotecan; MM-398; Onivyde
MUGA	multiple gated accession scan
NCCN	National Comprehensive Cancer Network
NCI	National Cancer Institute
PFS	progression free survival
PK	pharmacokinetic
PO	*per os*; by mouth
PR	partial response per RECIST
PTT	partial thromboplastin time
RBC	Red blood cell
QW	once-weekly dose administration schedule

Q2W	every-two-week dose administration schedule
Q3W	every-three-week dose administration schedule
RECIST	Response evaluation Criteria In Solid Tumors
RNA	ribonucleic acid
RP2D	Recommended phase II Dose
SAE	serious adverse event
SD	stable disease per RECIST
TAM	tumor associated macrophages
T_{max}	time after administration of drug when the maximum plasma concentration is reached
$T_{1/2}$	terminal half life
ULN	upper limit of normal
Vdss	volume of distribution at steady state
WBC	white blood count
5-FU	5-Fluorouracil, Folinic acid

Claims

What is claimed is:

1. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:
   a. a single administration of 6.0, 7.5, 9.0 or 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2; and
   b. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours, to treat the colorectal cancer in the patient.
2. The method of claim 1, wherein the leucovorin is administered as 400 mg/m^2 of the (l+d) form of leucovorin.
3. The method of claims 1 or 2, wherein the leucovorin is administered over 30 minutes.
4. The method of any one of claims 1-3, wherein the MM-398 irinotecan liposome, the leucovorin and the 5-fluorouracil is administered over a total of 48 hours.
5. The method of any one of claims 1-4, wherein all of the irinotecan administered to the patient is administered in the MM-398 irinotecan liposome.
6. The method of any one of claims 1-5, wherein the single administration of the MM-151 is administered prior to the single administration of the MM-398 irinotecan liposome, leucovorin or 5-fluorouracil.
7. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:
   a. a single administration of 6.0 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2; and then
   b. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours, to treat the colorectal cancer in the patient.
8. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:

a. a single administration of 7.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2; and then

b. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours,

to treat the colorectal cancer in the patient.

9. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:

a. a single administration of 9.0 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2; and then

b. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours,

to treat the colorectal cancer in the patient.

10. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:

a. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours; and then

b. a single administration of 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2,

to treat the colorectal cancer in the patient.

11. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:

a. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin,

and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours; and then

b. a single administration of 6.0 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2,

to treat the colorectal cancer in the patient.

12. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:

a. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours; and then

b. a single administration of 7.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2,

to treat the colorectal cancer in the patient.

13. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:

a. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours; and then

b. a single administration of 9.0 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2,

to treat the colorectal cancer in the patient.

14. A method of treating colorectal cancer, the method comprising intravenously administering to a human patient in need thereof:

a. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin,

and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours; and then

b. a single administration of 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2,

to treat the colorectal cancer in the patient.

15. The method of any one of claims 7-14, wherein the leucovorin is administered as 400 mg/m^2 of the (l+d) form of leucovorin.

16. The method of any one of claims 7-15, wherein the leucovorin is administered over 30 minutes.

17. The method of any one of claims 7-16, wherein the MM-398 irinotecan liposome, the leucovorin and the 5-fluorouracil is administered over a total of 48 hours.

18. The method of any one of claims 7-17, wherein all of the irinotecan administered to the patient is administered in the MM-398 irinotecan liposome.

19. The method of any one of claims 1-18, wherein no other anti-neoplastic agent is administered to the human patient to treat the colorectal cancer.

20. The method of any one of claims 1-19, wherein the colorectal cancer comprises a RAS wild type colorectal cancer tumor.

21. A method of treating a RAS wild type colorectal cancer, the method comprising intravenously administering in any order to a human patient in need thereof:

a. a single administration of 6.0, 7.5, 9.0 or 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2; and

b. a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours,

to treat the colorectal cancer in the patient.

22. The method of claim 21, wherein the leucovorin is administered as 400 mg/m^2 of the (l+d) form of leucovorin.

23. The method of claims 21 or 22, wherein the leucovorin is administered over 30 minutes.

24. The method of any one of claims 21-23, wherein the MM-398 irinotecan liposome, the leucovorin and the 5-fluorouracil is administered over a total of 48 hours.

25. The method of any one of claims 21-24, wherein all of the irinotecan administered to the patient is administered in the MM-398 irinotecan liposome.

26. The method of any one of claims 21-25, wherein the single administration of the MM-151 is administered prior to the single administration of the MM-398 irinotecan liposome, leucovorin or 5-fluorouracil.

27. The method of any one of claims 1-19, wherein the patient is diagnosed with colorectal cancer comprising a tumor having a RAS mutation.

28. The method of any one of claims 1-19, wherein the patient is diagnosed with colorectal cancer comprising a tumor having a KRAS mutation in codon 12 or 13.

29. The method of any one of claims 1-19, wherein the patient is diagnosed with colorectal cancer that harbor somatic mutations in exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of either KRAS or NRAS.

30. A method of treating colorectal cancer having a RAS mutation, the method comprising intravenously administering in any order to a human patient in need thereof a single administration of 6.0, 7.5, 9.0 or 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2.

31. The method of claim 30, wherein the method further comprises administering in combination with the MM-151 once every two weeks a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours, to treat the colorectal cancer in the patient.

32. The method of claim 31, wherein the leucovorin is administered as 400 mg/m^2 of the (l+d) form of leucovorin.

33. The method of claims 31 or 32, wherein the leucovorin is administered over 30 minutes.

34. The method of any one of claims 31-33, wherein the MM-398 irinotecan liposome, the leucovorin and the 5-fluorouracil is administered over a total of 48 hours.

35. The method of any one of claims 31-34, wherein all of the irinotecan administered to the patient is administered in the MM-398 irinotecan liposome.

36. The method of any one of claims 31-35, wherein the single administration of the MM-151 is administered prior to the single administration of the MM-398 irinotecan liposome, leucovorin or 5-fluorouracil.

37. The method of any one of claims 30-36, wherein the patient is diagnosed with colorectal cancer comprising a tumor having a RAS mutation.

38. The method of any one of claims 30-37, wherein the patient is diagnosed with colorectal cancer comprising a tumor having a KRAS mutation in codon 12 or 13.

39. The method of any one of claims 30-38, wherein the patient is diagnosed with colorectal cancer that harbor somatic mutations in exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of either KRAS or NRAS.

40. A method of treating colorectal cancer harboring a BRAF mutation, the method comprising intravenously administering in any order to a human patient in need thereof: a single administration of 6.0, 7.5, 9.0 or 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2.

41. The method of claim 40, further comprising administering to the patient in combination with every other dose of the MM151: a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours, to treat the colorectal cancer in the patient.

42. The method of any one of claims 40-41, wherein the BRAF mutation is V600E.

43. The method of any one of claims 40-42, wherein the BRAF mutation is a somatic mutation in one or more of codons 464, 466, 469, 595, 596, and 601.

44. A method of treating colorectal cancer harboring an EGFR mutation, the method comprising intravenously administering in any order to a human patient in need thereof: a single administration of 6.0, 7.5, 9.0 or 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2.

45. The method of claim 40, further comprising administering to the patient in combination with every other dose of the MM151: a single administration once every two weeks of: a single administration of 70 mg/m2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m2 of the (l) form of leucovorin, and a single administration of 2400 mg/m2 of 5-fluorouracil over 46 hours, to treat the colorectal cancer in the patient.

46. The method of any one of claims 44-45, wherein the EGFR mutation is a somatic mutation in one or more codons in exon 12.

47. A method of treating a colorectal cancer harboring a PIK3CA mutation, the method comprising intravenously administering in any order to a human patient in need thereof: a single administration of 6.0, 7.5, 9.0 or 10.5 mg/kg of MM-151 once per week, following an initial priming dose of 225 mg MM-151 in week 1 and a second priming dose of 450 mg of MM-151 in week 2.

48. The method of claim 47, further comprising administering to the patient in combination with every other dose of the MM151: a single administration once every two weeks of: a single administration of 70 mg/m^2 irinotecan (free base) encapsulated in a MM-398 irinotecan liposome over 90 minutes, a single administration of 200 mg/m^2 of the (l) form of leucovorin, and a single administration of 2400 mg/m^2 of 5-fluorouracil over 46 hours, to treat the colorectal cancer in the patient.

49. The method of claim 48 wherein the PIK3CA mutation is a somatic mutation in one or more of codons 542, 545, and 1047.

FIG. 1

Activity of MM-398 (Ls-CPT11) in an Orthotopic Pancreas Tumor Model Expressing Luciferase (L3.6pl).

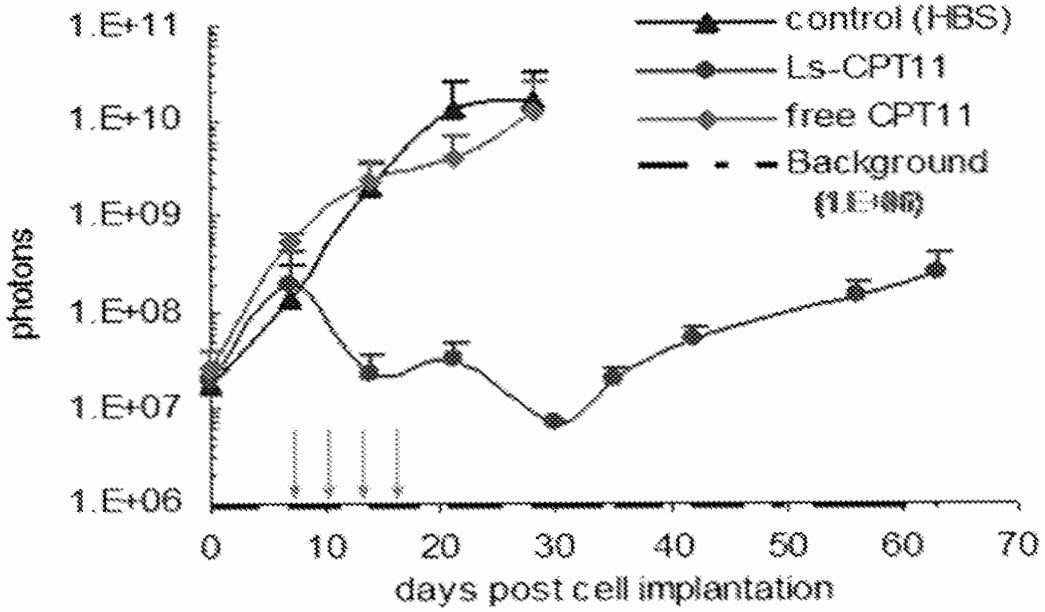


FIG. 2

Accumulation of SN-38 in Tumors Following Treatment with Free Irinotecan or Nanoliposomal Irinotecan (MM-398).

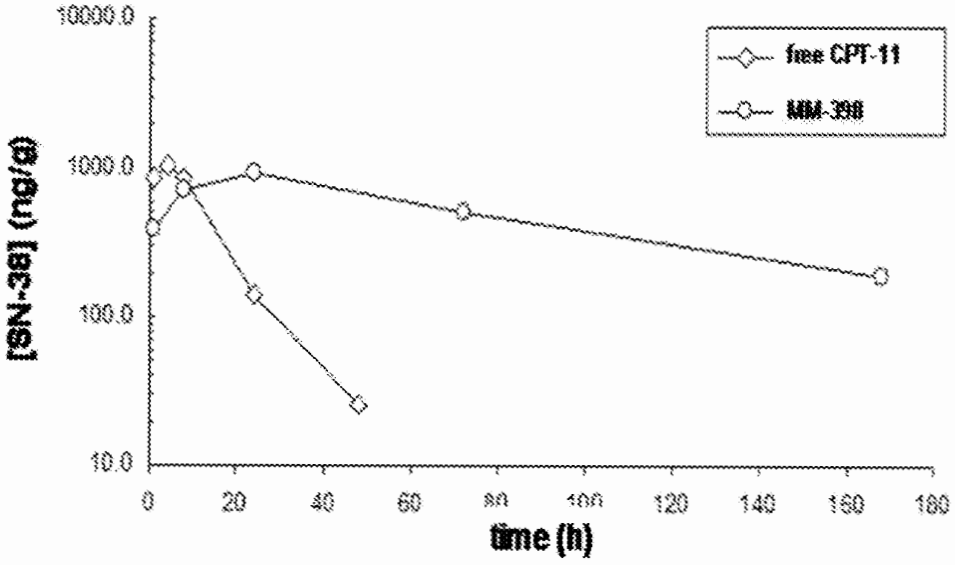


FIG. 3

Effect of MM-398 on Carbonic Anhydrase IX staining in the HT29 xenograft model.

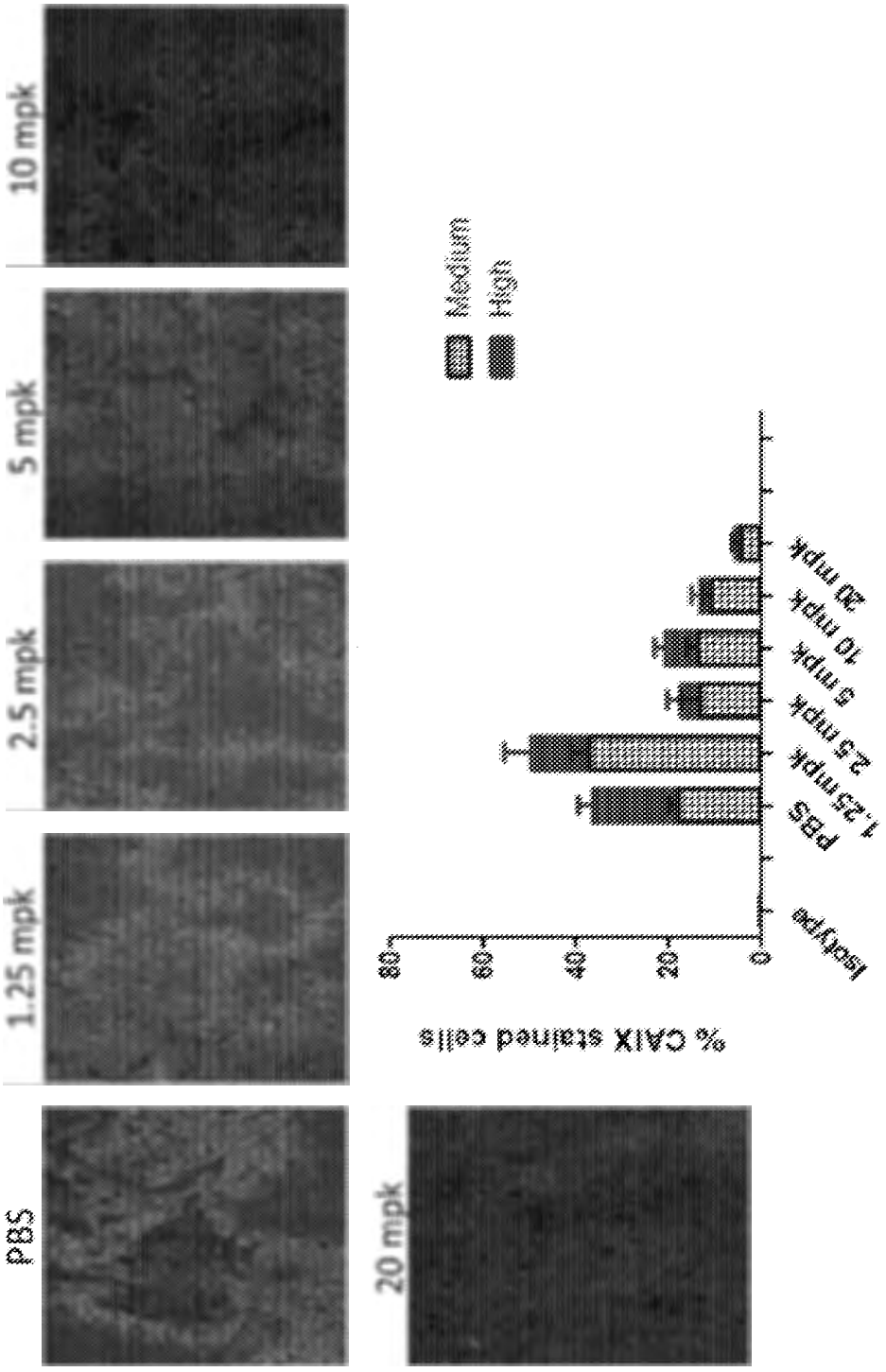


FIG. 4

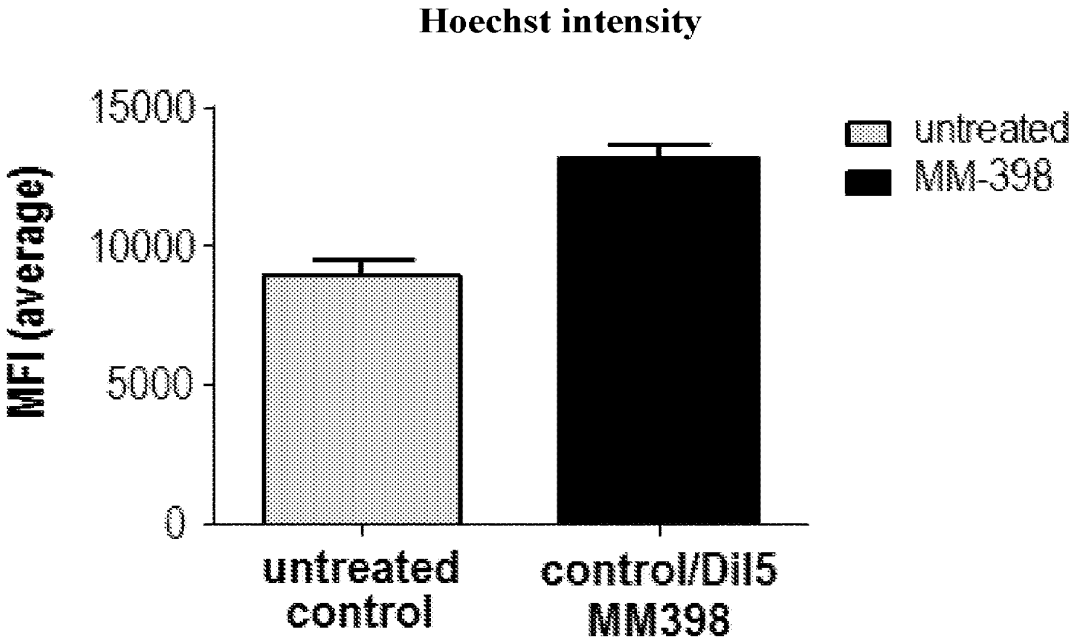

Hoechst intensity
MFI (average)
15000
10000
5000
0
untreated
MM-398
untreated control
control/Dil5 MM398

FIG. 5

MM-398 PK in q3w (irinotecan, liposomes + free drug)

Dose (mg/m^2) & Study	PEP0203				PEP0201		PEP0206		Campto® Package insert	
	60	80	100	120	120	180	PEP02	Campto®	125	340
	(n=3)	(n=6)	(n=4)	(n=2)	(n=6)	(n=4)	120	300	mg/m^2	mg/m^2
Parameters							(n=37)	(n=27)	(N=64)	(N=6)
C_{max}	28.93	29.16	44.06	47.94	79.4	102	60.8	4.3	1.66	3.392
(µg/mL)	(±15.75)	(±5.24)	(±7.65)	(±16.24)	(±13.9)	(±17.6)	(±36.6)	(±1.2)	(±0.797)	(±0.874)
$t_{1/2}$	24.02	32.09	48.11	30.65	29.5	22.2	21.2	7.7	5.8	11.7
(h)	(±16.76)	(±18.21)	(±17.41)	(±5.32)	(±17.2)	(±11.5)	(±18.3)	(±4.4)	(±0.7)	(±1.0)
AUC_{0-T}	1,047	1,116	2,193	1,117	2,835	1,945	1,651.5	24.2	10.2	20.604
(µg·h/mL)	(±1,156)	(±810)	(±1,017)	(±308)	(±1,817)	(±1,029)	(±1,412.0)	(±7.7)	(±3.27)	(±6.027)
$AUC_{0-\infty}$	1,114	1,211	2,472	1,261	2,963	1,963	1,812.2	26.2		
(µg·h/mL)	(±1,270)	(±924)	(±1261)	(±500)	(±1,947)	(±1,035)	(±1,601.9)	(±9.0)	..	..
Cl	0.1249	0.1164	0.0547	0.1033	0.0591	0.119	0.191	12.9	13.3	13.9
($L/h/m^2$)	(±0.1058)	(±0.0949)	(±0.0358)	(±0.0409)	(±0.0367)	(±0.0703)	(±0.260)	(±4.7)	(±6.01)	(±4.0)
V_{ss}	2.6	2.93	2.63	3.16	1.8	1.97	2.23	98.5	110	234
(L/m^2)	(±1.44)	(±0.60)	(±0.49)	(±0.38)	(±0.771)	(±0.342)	(±0.69)	(±29.0)	(±48.5)	(±69.6)

Note: AUC 0-T is defined as T = 24 hours for Camptosar package insert,
T= 49.5 hours for Camptosar in the PEP0206 study and
T = 169.5 hours for MM-398.

FIG. 6

Note: AUC 0-T is defined as T = 24 hours for Camptosar package insert, T= 49.5 hours for Camptosar in the PEP0206 study and T = 169.5 hours for MM-398.

Dose (mg/m^2) & Study	PEP0203				PEP0201		PEP0206		Campto® Package insert	
	60 (n=3)	80 (n=6)	100 (n=4)	120 (n=2)	120 (n=6)	180 (n=4)	PEP02 120 (n=37)	Campto® 300 (n=27)	125 mg/m^2 (N=64)	340 mg/m^2 (N=6)
Parameters										
C_{max} (ng/mL)	7.02 (±5.64)	7.98 (±4.39)	7.39 (±1.68)	16.64 (±9.36)	9.2 (±3.5)	14.3 (±6.16)	8.79 (±8.68)	44.1 (±28.2)	26.3 (±11.9)	56.0 (±28.2)
$t_{1/2}$ (h)	183.81 (±172.3)	53.75 (±15.6)	73.41 (±18.3)	26.23 (±6.53)	75.4 (±43.8)	58.0 (±32.8)	88.8 (±114.6)	22.8 (±10.9)	10.4 (±3.1)	21.0 (±4.3)
AUC_{0-T} (ng·h/mL)	367.40 (±227)	354.77 (±145)	551.40 (±381.8)	367.60 (±155.7)	710 (±395)	1,160 (±969)	467 (±310)	361 (±125)	229 (±108)	474 (±245)
$AUC_{0-\infty}$ (ng·h/mL)	1,373.3 (±1,119)	502.15 (±153)	844.28 (±444)	474.00 (±209)	997 (±680)	1,420 (±1,134)	879 (±1,426)	440 (±162)	..	..

FIG. 7

Dose proportionality of MM-151 PK parameters. Solid line represents the linear regression line with 95% confidence interval (shaded area). Cmax: maximum serum concentration, µg/ml; Cavg: average serum concentration, µg/ml; t1/2: terminal half-life, days.

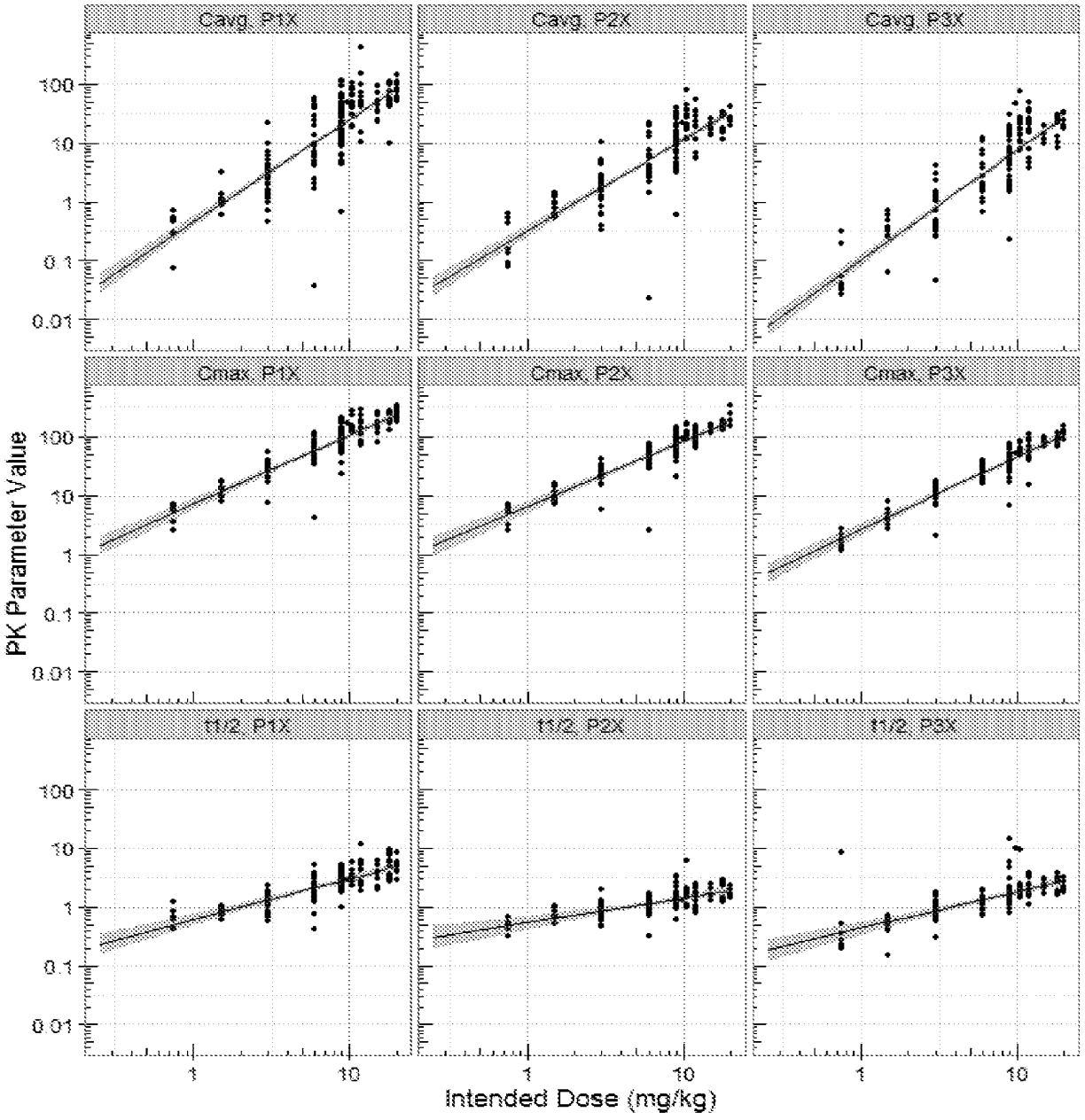


FIG. 8A

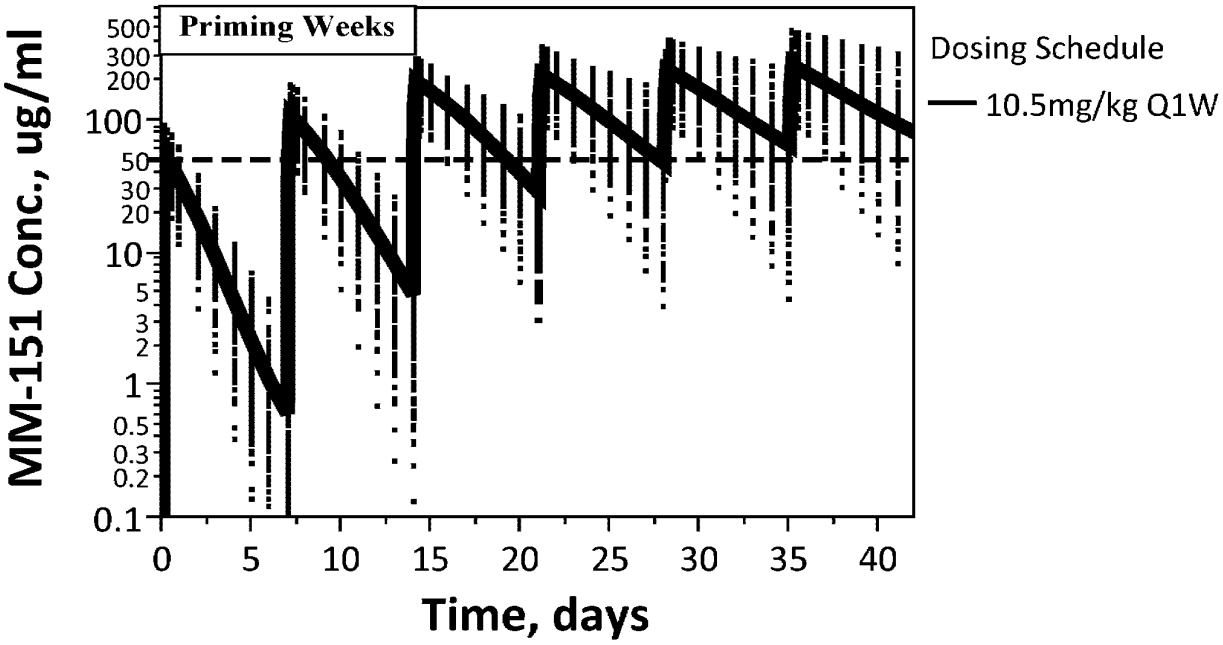

Priming Weeks
MM-151 Conc., ug/ml
500
300
200
100
50
30
20
10
5
3
2
1
0.5
0.3
0.2
0.1
0
5
10
15
20
25
30
35
40
Time, days
Dosing Schedule
10.5mg/kg Q1W

FIG. 8B

Effect of weekly MM-151 dose intensity on the probability to experience rash event in patients treated with MM-151. Top: any grade rash; Bottom: grade 3 or higher. Error bars indicate standard errors of the mean.

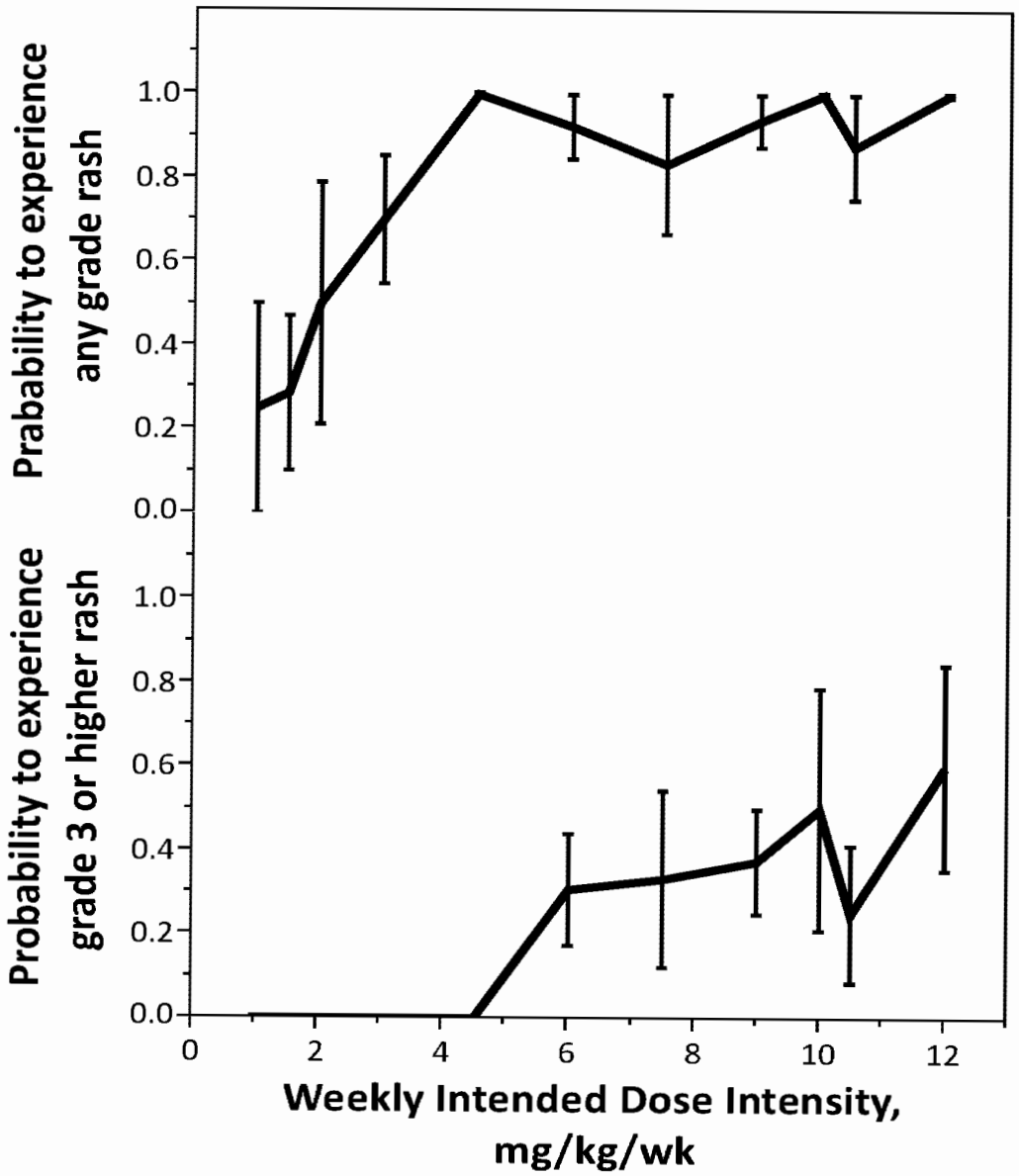


FIG. 9

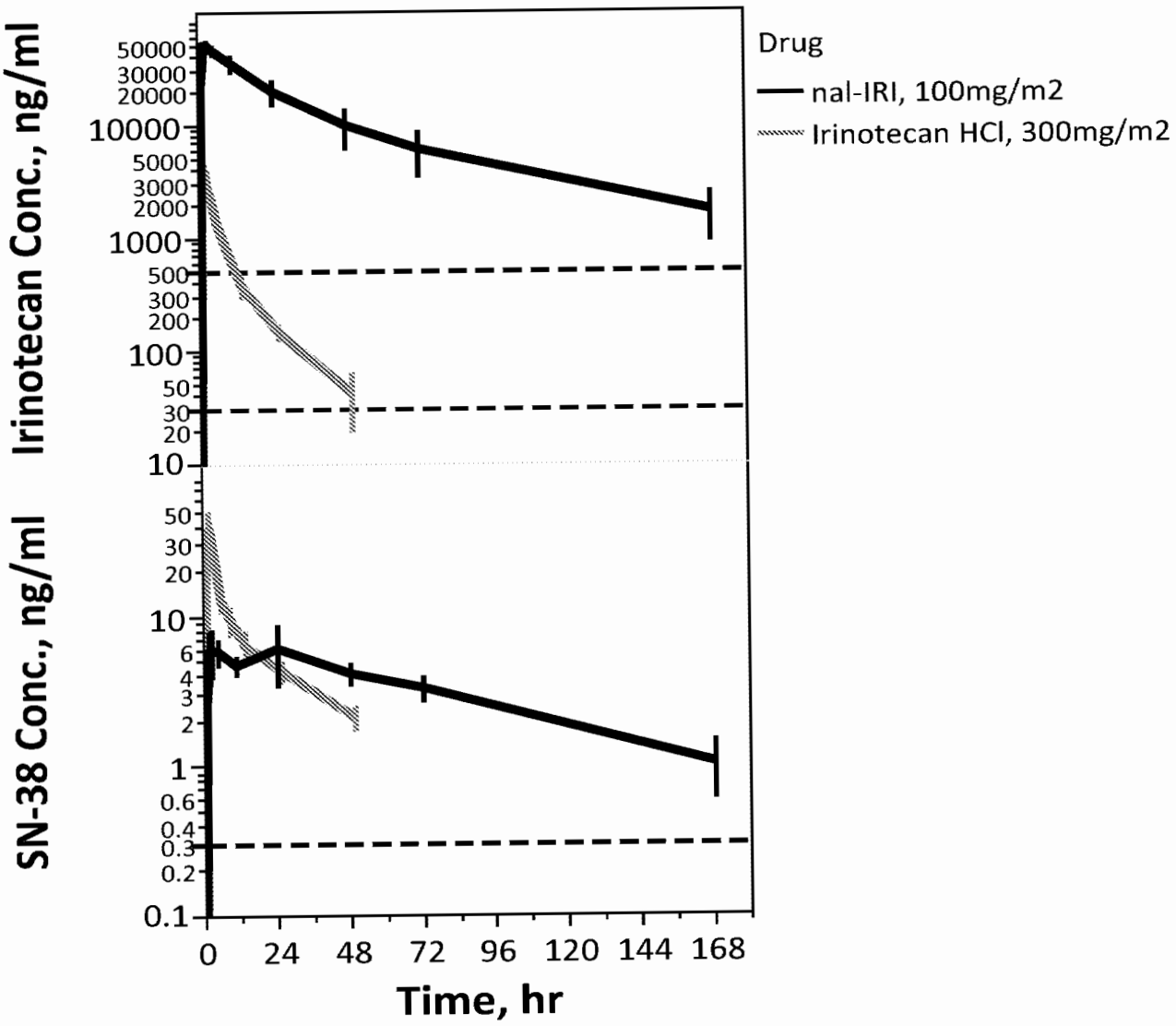

Irinotecan Conc., ng/ml
SN-38 Conc., ng/ml
50000
30000
20000
10000
5000
3000
2000
1000
500
300
200
100
50
30
20
10
50
30
20
10
6
4
3
2
1
0.6
0.4
0.3
0.2
0.1
0
24
48
72
96
120
144
168
Time, hr
Drug
nal-IRI, 100mg/m2
Irinotecan HCl, 300mg/m2

FIG. 10

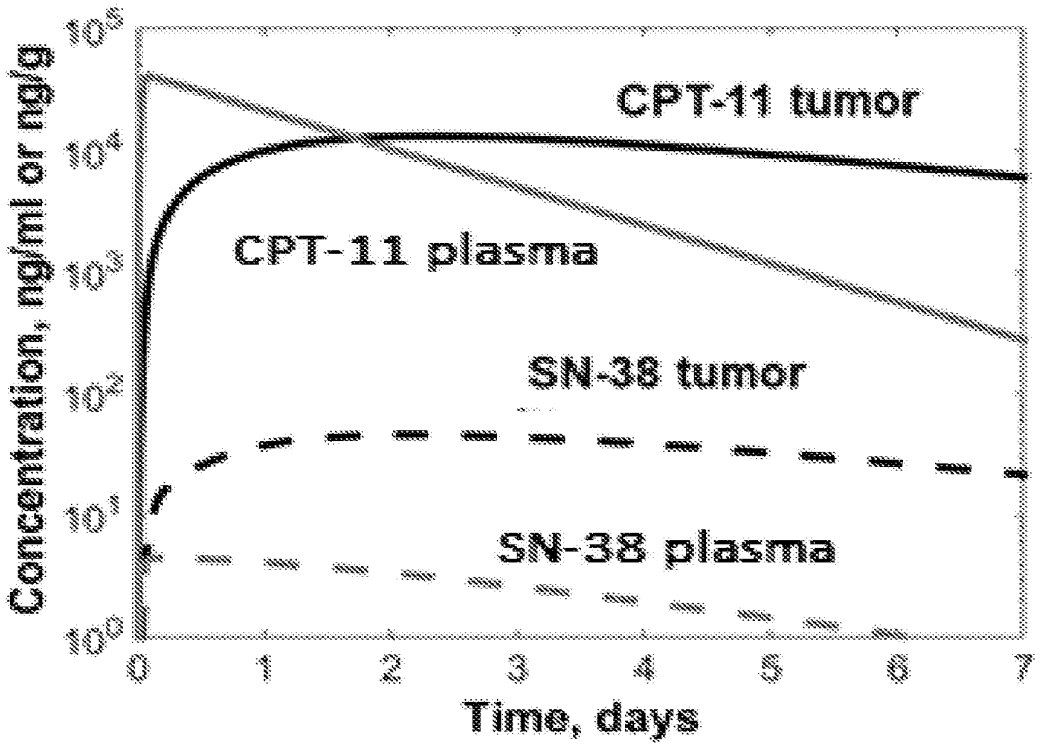

Concentration, ng/ml or ng/g
10^5
10^4
10^3
10^2
10^1
10^0
CPT-11 tumor
CPT-11 plasma
SN-38 tumor
SN-38 plasma
0
1
2
3
4
5
6
7
Time, days

FIG. 11A

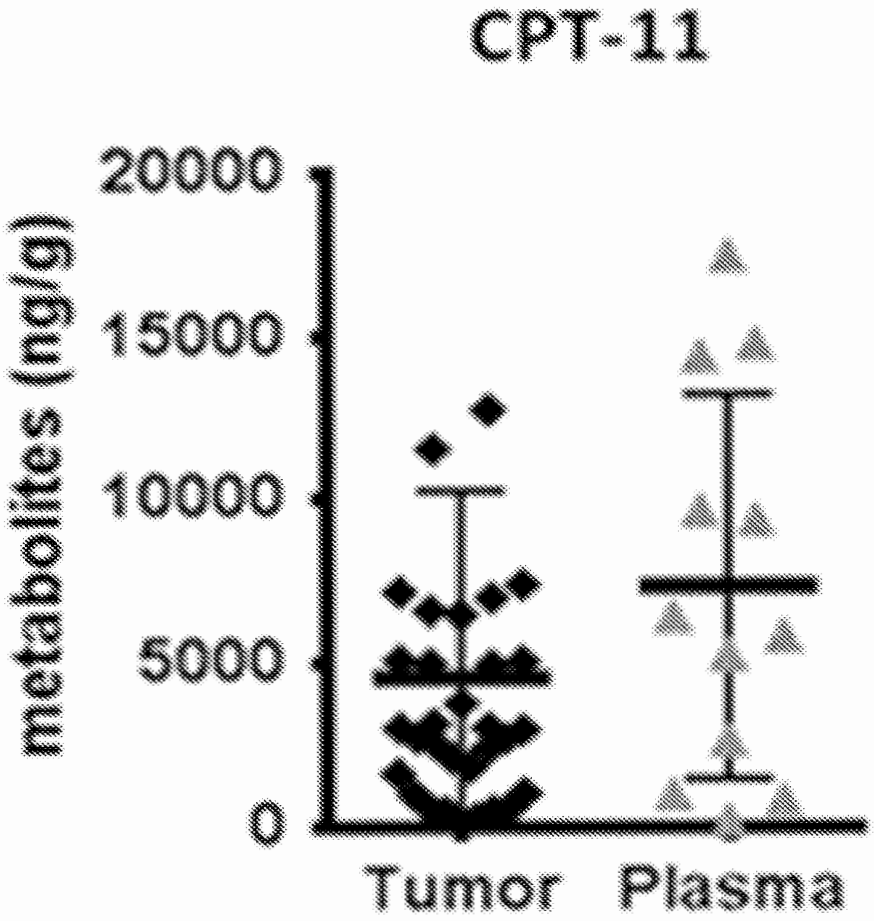

CPT-11
metabolites (ng/g)
20000
15000
10000
5000
0
Tumor
Plasma

FIG. 11B

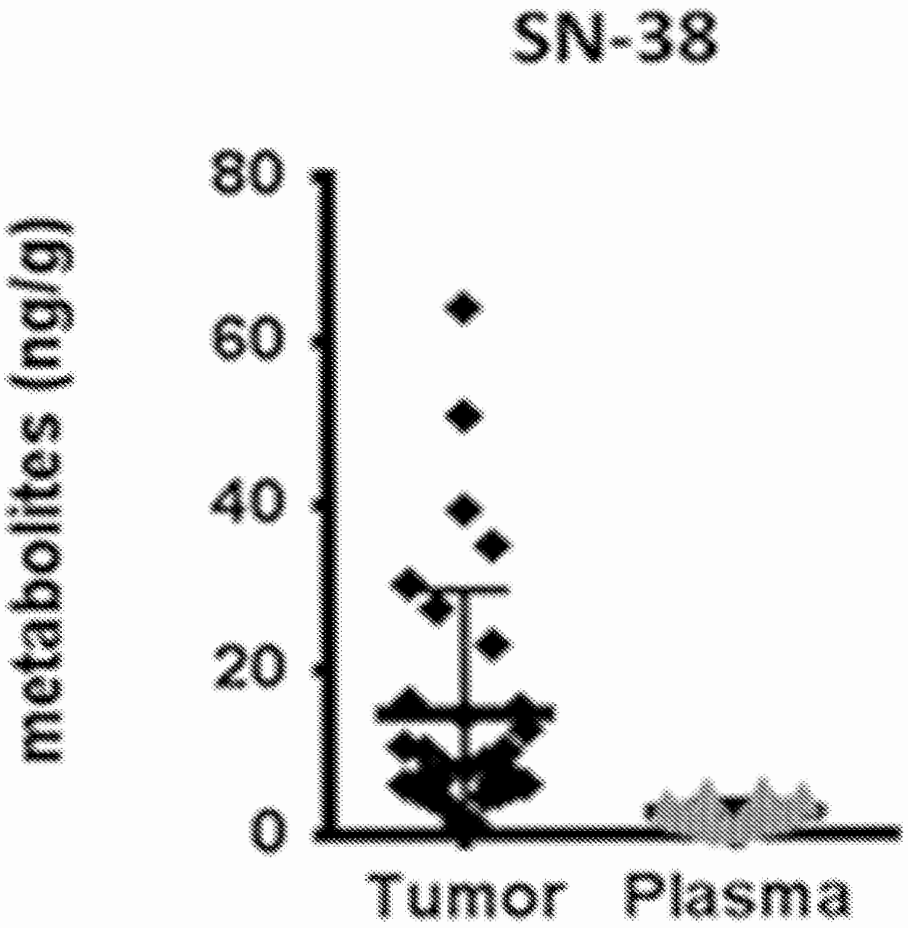


FIG. 12

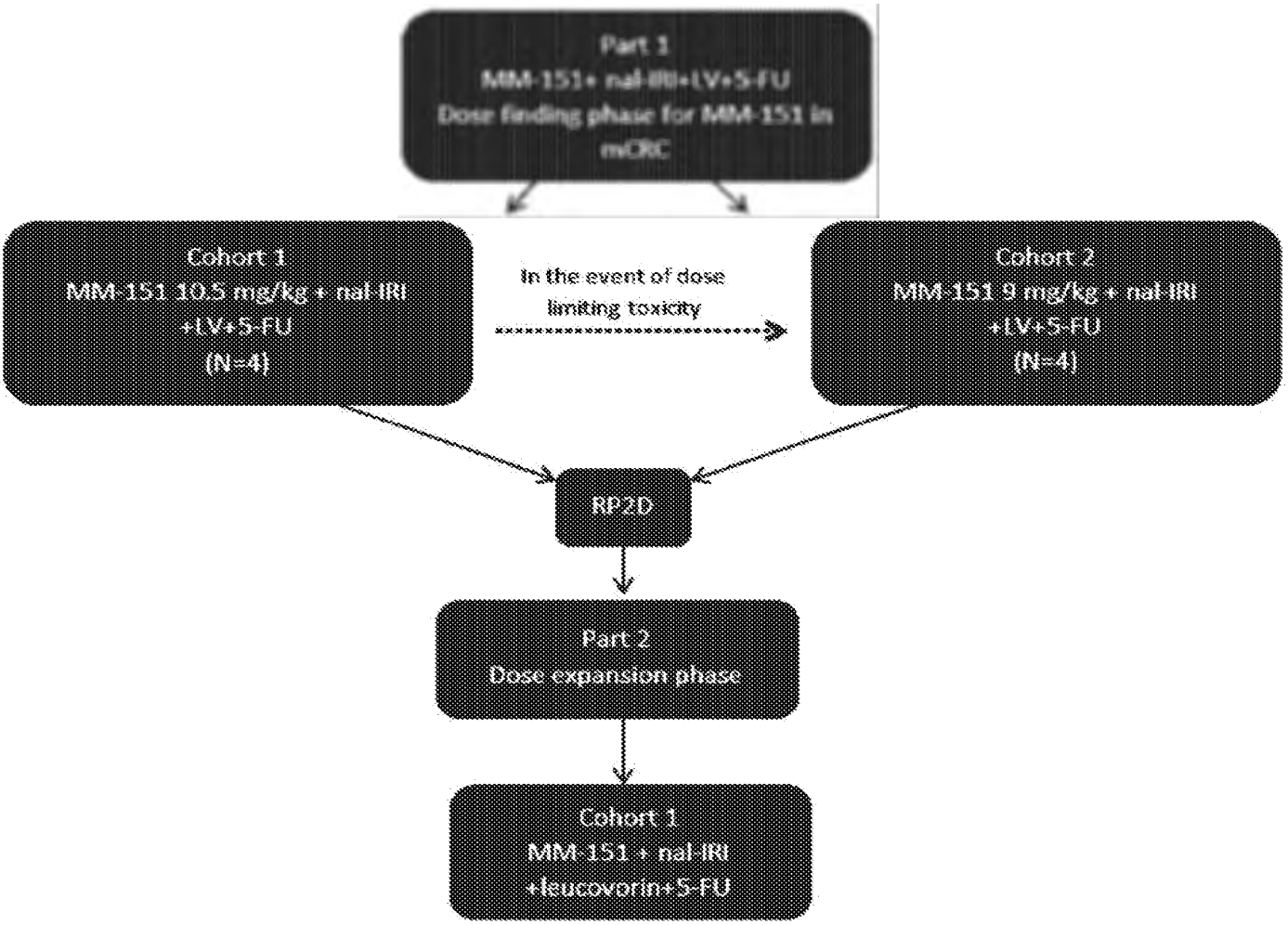

Part 1
MM-151+ nal-IRI+LV+5-FU
Dose finding phase for MM-151 in mCRC
Cohort 1
MM-151 10.5 mg/kg + nal-IRI +LV+5-FU
(N=4)
In the event of dose limiting toxicity
Cohort 2
MM-151 9 mg/kg + nal-IRI +LV+5-FU
(N=4)
RP2D
Part 2
Dose expansion phase
Cohort 1
MM-151 + nal-IRI +leucovorin+5-FU

FIG. 13

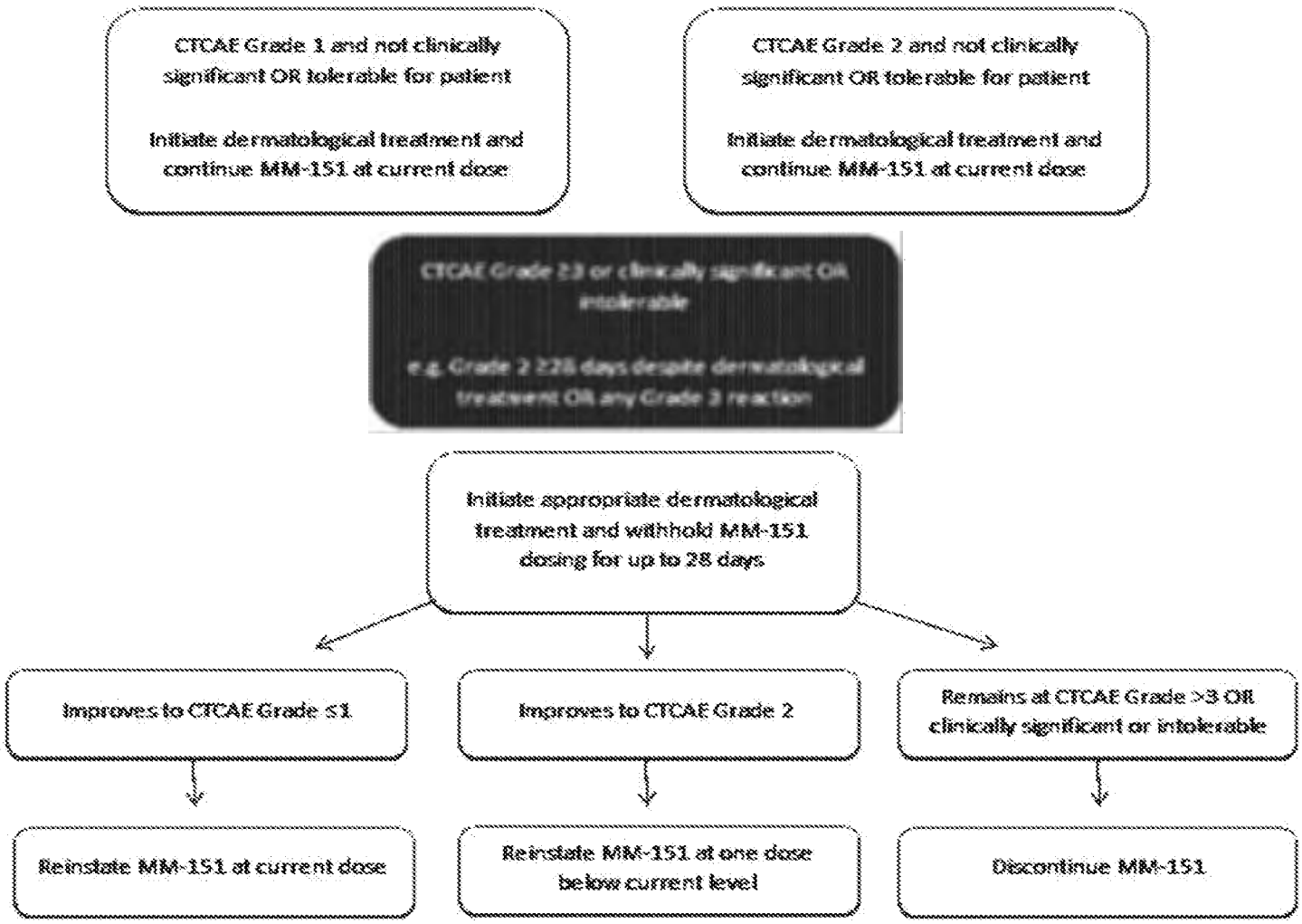

CTCAE Grade 1 and not clinically significant OR tolerable for patient
Initiate dermatological treatment and continue MM-151 at current dose
CTCAE Grade 2 and not clinically significant OR tolerable for patient
Initiate dermatological treatment and continue MM-151 at current dose
CTCAE Grade ≥3 or clinically significant OR intolerable
e.g. Grade 2 ≥28 days despite dermatological treatment OR any Grade 3 reaction
Initiate appropriate dermatological treatment and withhold MM-151 dosing for up to 28 days
Improves to CTCAE Grade ≤1
Improves to CTCAE Grade 2
Remains at CTCAE Grade >3 OR clinically significant or intolerable
Reinstate MM-151 at current dose
Reinstate MM-151 at one dose below current level
Discontinue MM-151

FIG. 14

MM-151 P1X Heavy Chain Sequence (SEQ ID NO:19)

QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGSIIPIFGT
VNYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARDPSVNLYWYFDLWGR
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

MM-151 P1X Light Chain Sequence (SEQ ID NO:20)

DIQMTQSPSTLSASVGDRVTITCRASQSISSWWAWYQQKPGKAPKLLIYDASSLESG
VPSRFSGSGSGTEFTLTISSLQPDDFATYYCQQYHAHPTTFGGGTKVEIKRTVAAPSV
FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 15

P2X Heavy Chain Sequence (SEQ ID NO:21)

QVQLVQSGAEVKKPGSSVKVSCKASGGTFGSYAISWVRQAPGQGLEWMGSIIPIFGA
ANPAQKSQGRVTITADESTSTAYMELSSLRSEDTAVYYCAKMGRGKVAFDIWGQGT
MVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC
PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

P2X Light Chain Sequence (SEQ ID NO:22)

DIVMTQSPDSLAVSLGERATINCKSSQSVLYSPNNKNYLAWYQQKPGQPPKLLIYWA
STRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYGSPITFGGGTKVEIKRT
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 16

P3X Heavy Chain Sequence (SEQ ID NO:23)

QVQLVQSGAEVKKPGASVKVSCKASGYAFTSYGINWVRQAPGQGLEWMGWISAYN
GNTYYAQKLRGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCARDLGGYGSGSVPF
DPWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK

P3X Light Chain Sequence (SEQ ID NO:24)

EIVMTQSPATLSVSPGERATLSCRASQSVSSNLAWYQQKPGQAPRLLIYGASTRATGI
PARFSGSGSGTEFTLTISSLQSEDFAVYYCQDYRTWPRRVFGGGTKVEIKRTVAAPSV
FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 17

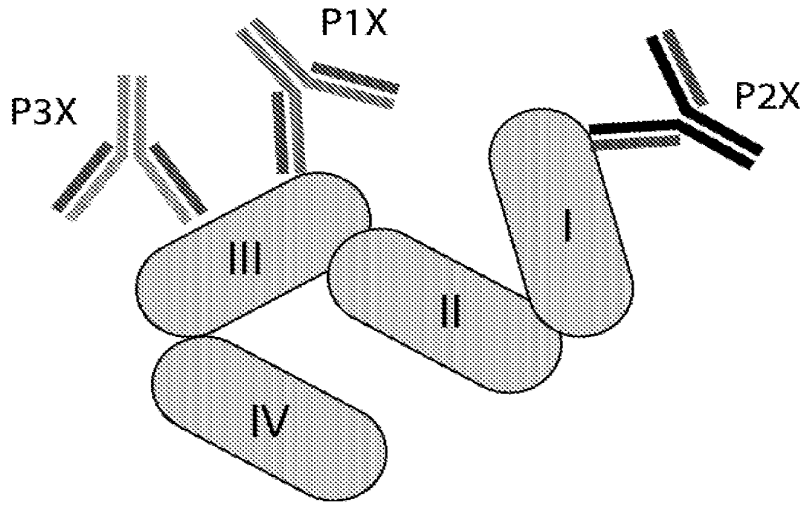


FIG. 18

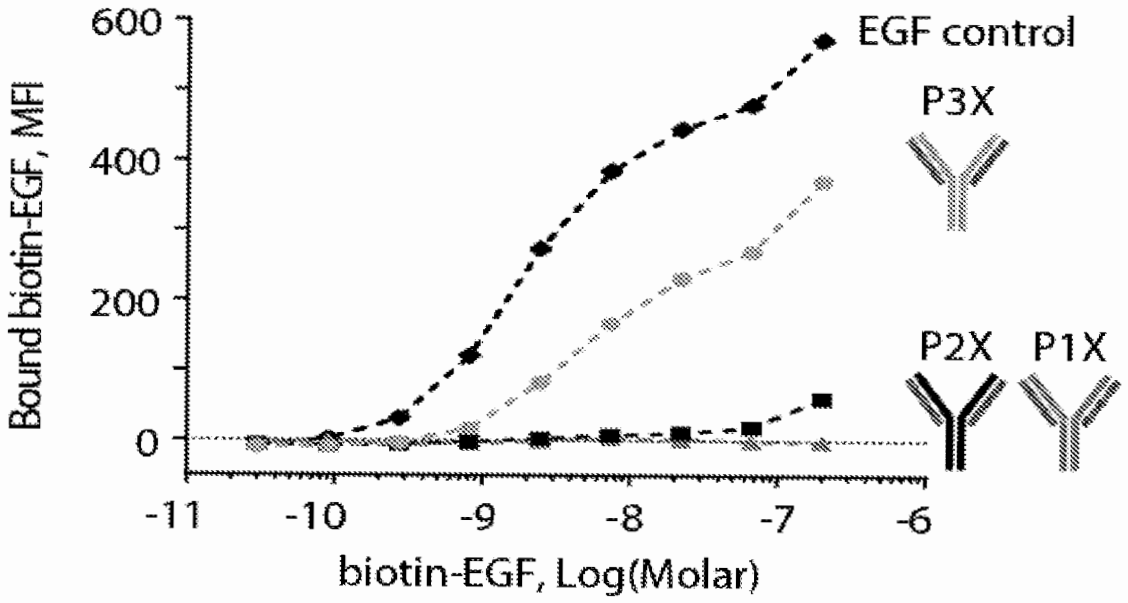

600
400
200
0
Bound biotin-EGF, MFI
-11
-10
-9
-8
-7
-6
biotin-EGF, Log(Molar)
EGF control
P3X
P2X
P1X

FIG. 19

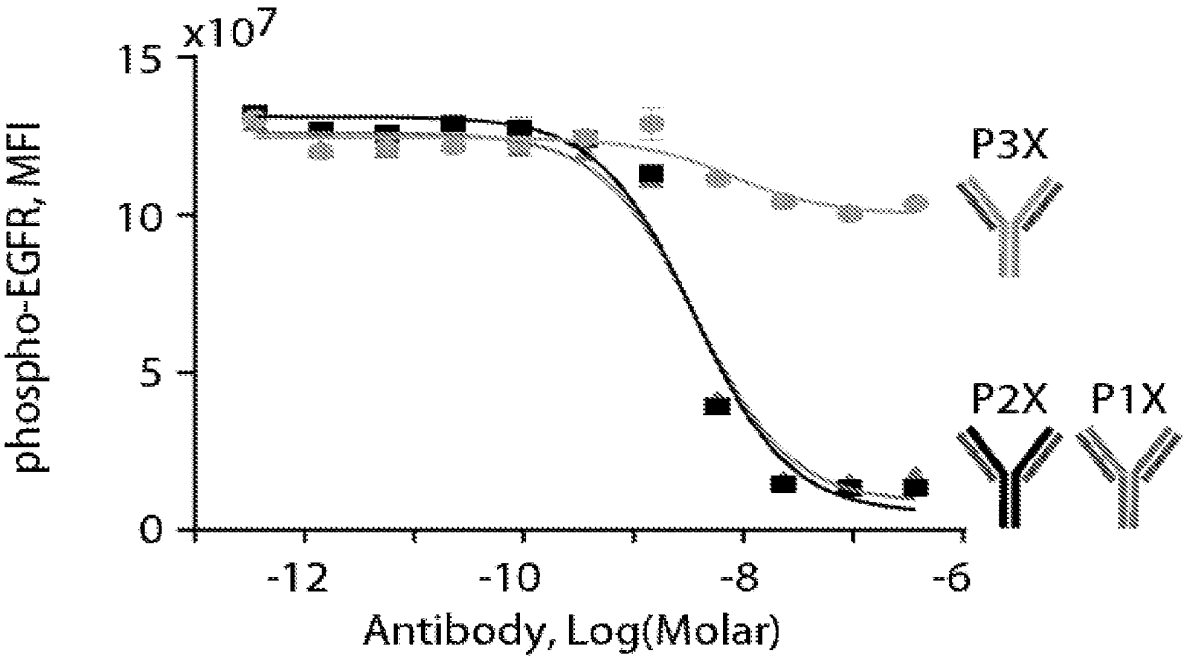

x10^7
15
10
5
0
phospho-EGFR, MFI
-12
-10
-8
-6
Antibody, Log(Molar)
P3X
P2X
P1X

FIG. 20

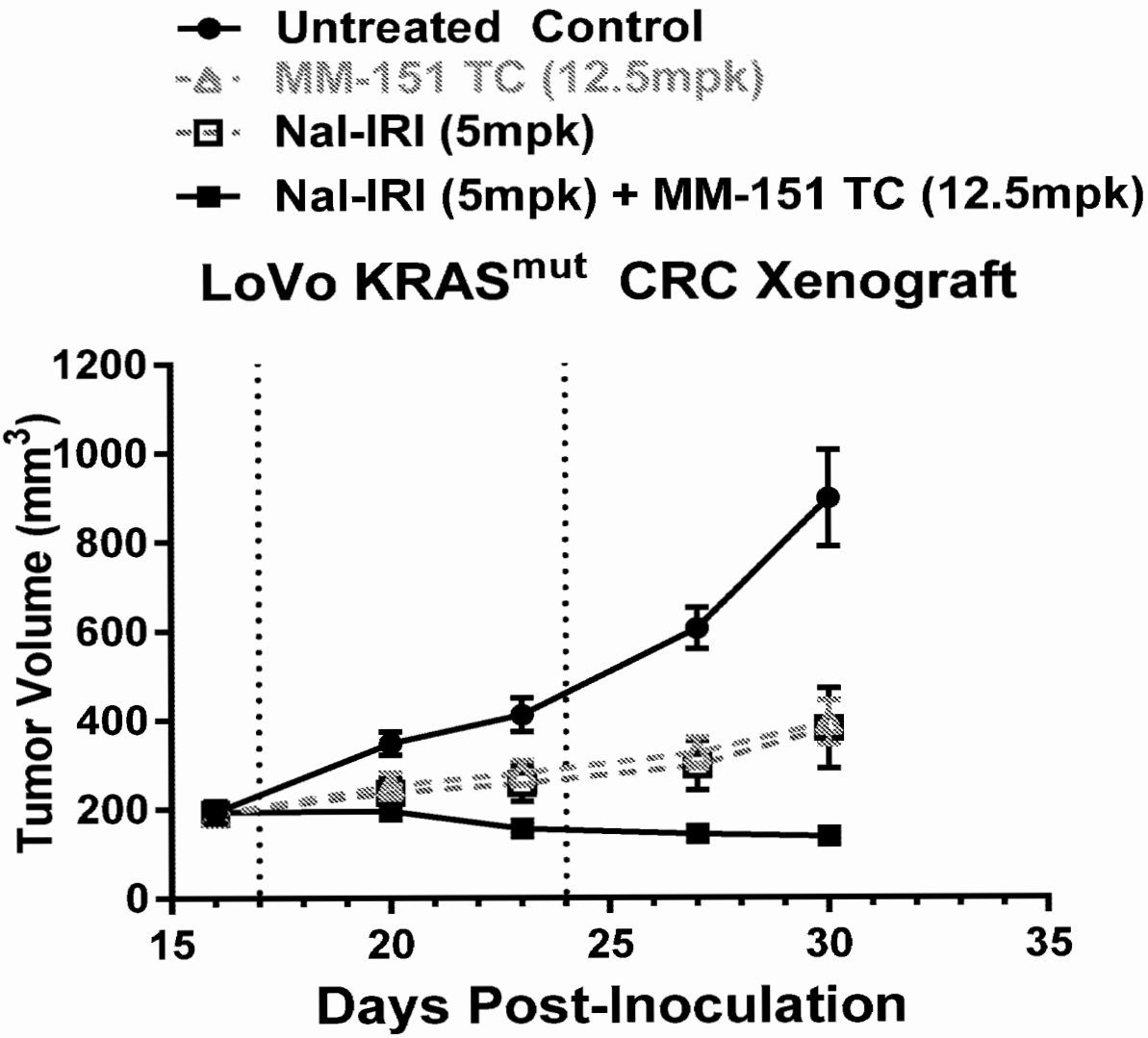

Untreated Control
MM-151 TC (12.5mpk)
Nal-IRI (5mpk)
Nal-IRI (5mpk) + MM-151 TC (12.5mpk)
LoVo KRASmut CRC Xenograft
Tumor Volume (mm3)
1200
1000
800
600
400
200
0
15
20
25
30
35
Days Post-Inoculation

FIG. 21

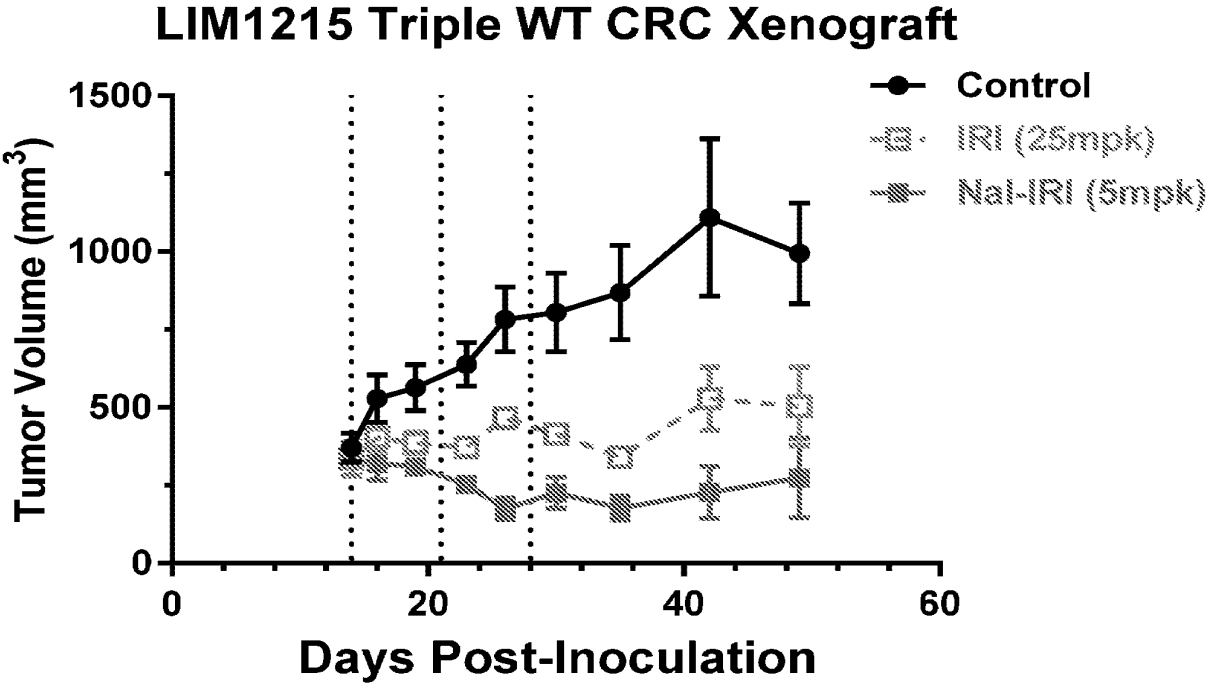

LIM1215 Triple WT CRC Xenograft
Control
IRI (25mpk)
Nal-IRI (5mpk)
Tumor Volume (mm3)
1500
1000
500
0
0
20
40
60
Days Post-Inoculation

FIG. 22A

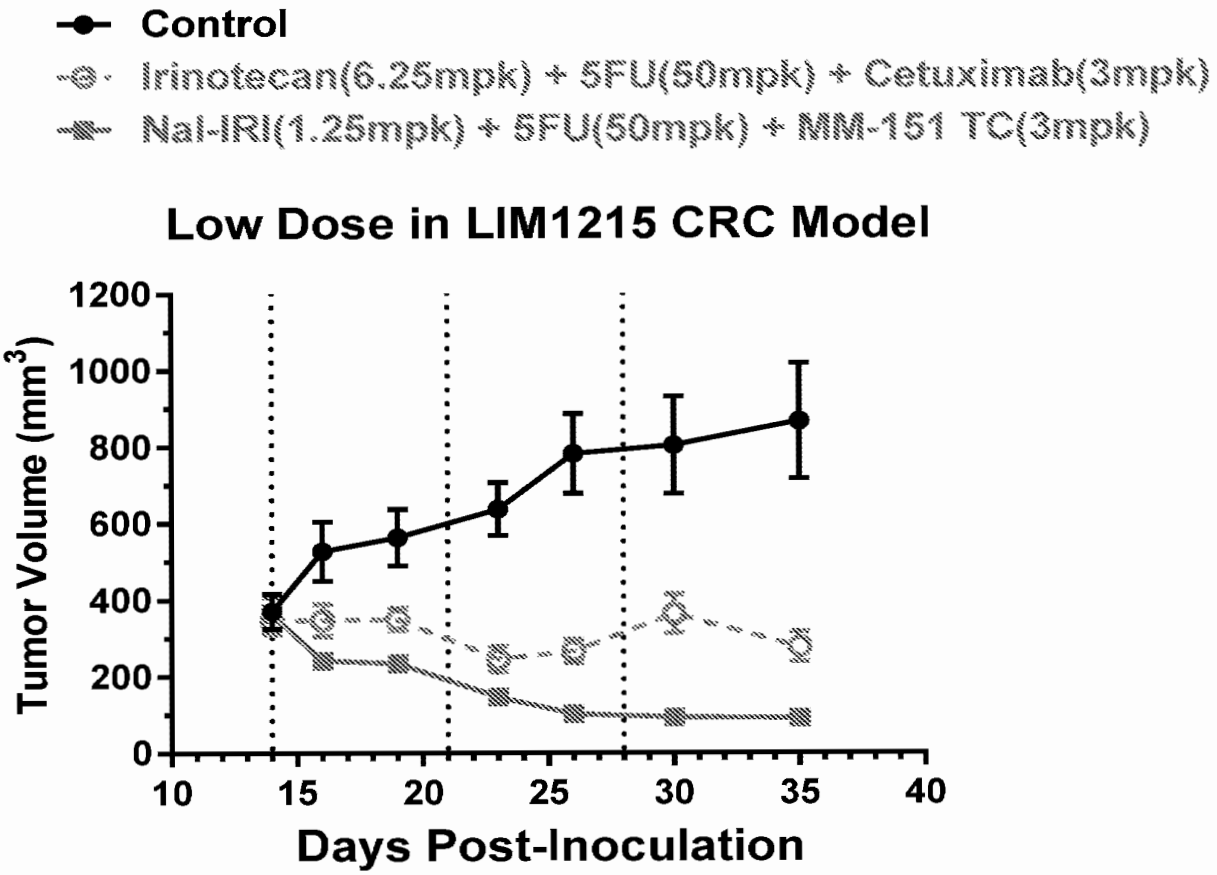

Control
Irinotecan(6.25mpk) + 5FU(50mpk) + Cetuximab(3mpk)
NaI-IRI(1.25mpk) + 5FU(50mpk) + MM-151 TC(3mpk)
Low Dose in LIM1215 CRC Model
Tumor Volume (mm3)
1200
1000
800
600
400
200
0
10
15
20
25
30
35
40
Days Post-Inoculation

FIG. 22B

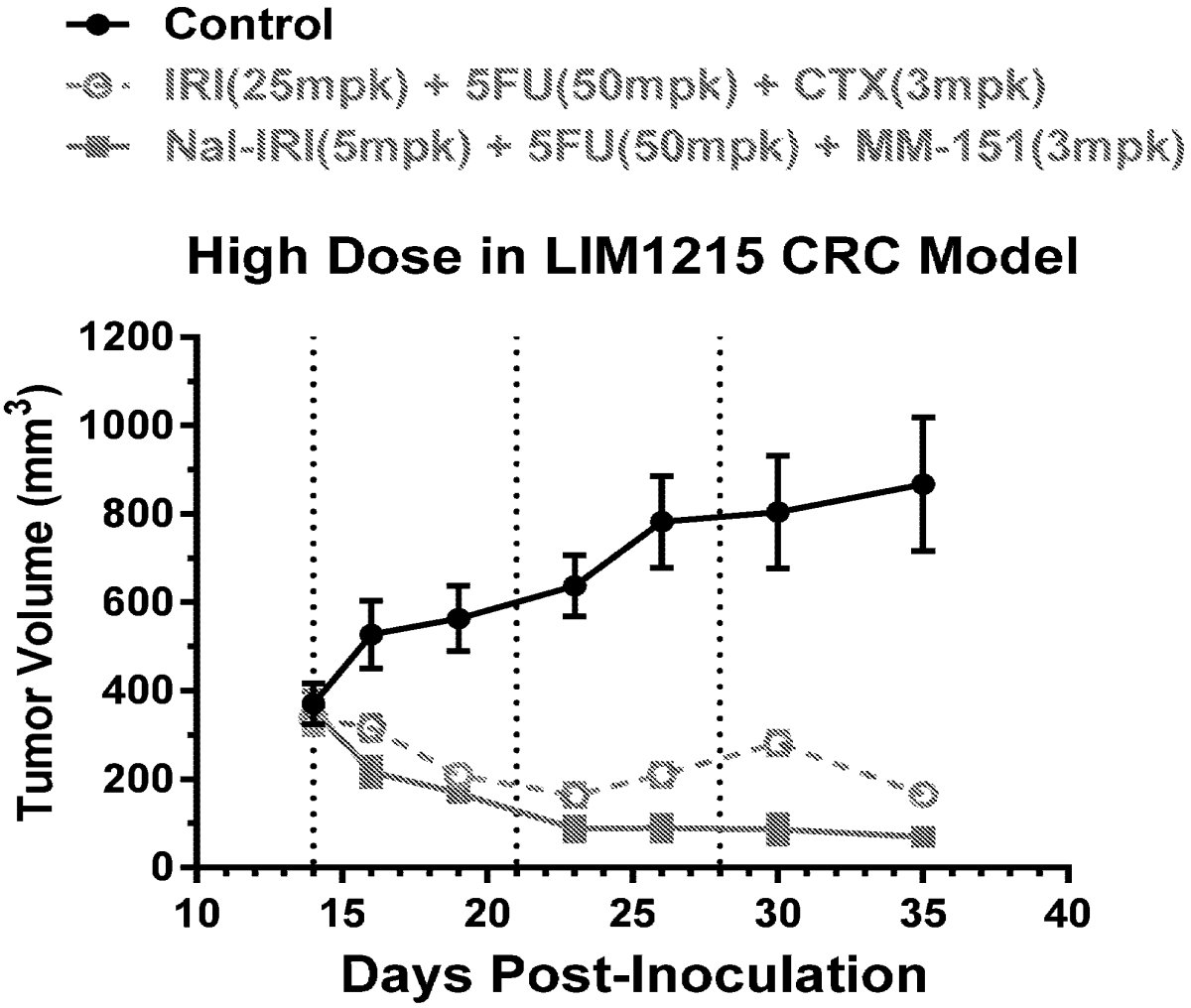

Control
IRI(25mpk) + 5FU(50mpk) + CTX(3mpk)
Nal-IRI(5mpk) + 5FU(50mpk) + MM-151(3mpk)
High Dose in LIM1215 CRC Model
Tumor Volume (mm3)
1200
1000
800
600
400
200
0
10
15
20
25
30
35
40
Days Post-Inoculation

FIG. 23

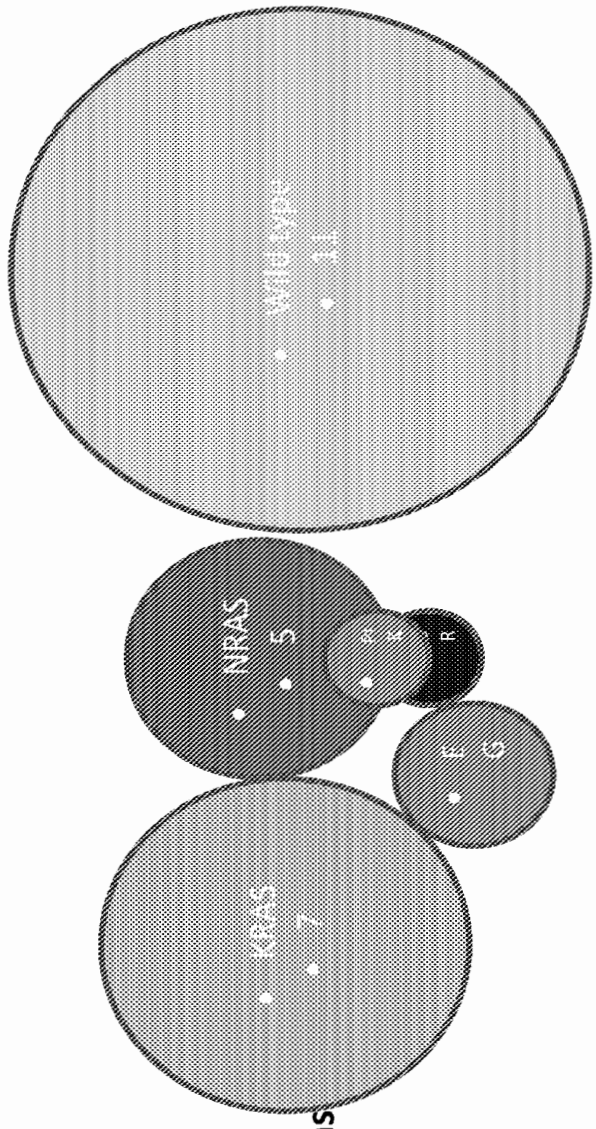
Antibody ca Heavy Chain Sequence

CDR 3: DPSVDL (SEQ ID NO:25)
CDR 2: IIPIFGTA (SEQ ID NO:26)
CDR 1: SYA (SEQ ID NO:27)

Antibody ca Light Chain Sequence

CDR 3: QQFAAHA (SEQ ID NO: 28)
CDR 2: DAS (SEQ ID NO: 29)
CDR 1: QSISSW (SEQ ID NO:30)

FIG. 24



- Pre-treatment somatic mutations
  - (N=28)

FIG. 25

Subject #	Cancer Type	Treatment and Schedule[y]	Duration of Treatment (days)	Maximum Percent Change	Timepoint	EGFR	KRAS	NRAS	BRAF codon 600	BRAF other codons	PIK3CA
4	CRC	0.75mg/kg MM-151 (QW) (dose escalated to 9mg/kg)	581	-40%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
20	CRC	3mg/kg MM-151 (Q2W) [x]	147	-10%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
22	NSCLC	3mg/kg MM-151 (Q2W) [x]	44	-1%	Pre-Treatment	WT	MT	WT	WT	WT	WT
					Post-Treatment	WT	MT	WT	WT	WT	WT
25	NSCLC	6mg/kg MM-151 (Q2W)	43	13%	Pre-Treatment	WT	MT	WT	WT	WT	WT
					Post-Treatment	WT	MT	WT	WT	WT	WT
42	CRC	9mg/kg MM-151 (Q3W) [x]	22	21%	Pre-Treatment	MT	MT	MT	WT	WT	WT
					Post-Treatment	MT	MT	MT	WT	WT	WT
54	CRC	9mg/kg MM-151 (QW)	169	-46%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
56	HEPATOCELLULAR CARCINOMA	12mg/kg MM-151 (Q2W)	57	35%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
57	NSCLC	12mg/kg MM-151 (QW)	78	15%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
60	NSCLC	12mg/kg MM-151 (Q2W)	127	10%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
63	HNSCC	12mg/kg MM-151 (Q2W)	57	17%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
68	HNSCC	6mg/kg MM-151 (QW) + 180 mg/m^2 irinotecan (Q2W)	176	-53%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
71	NSCLC	6mg/kg MM-151 (QW)	42	-2%	Pre-Treatment	WT	WT	WT	WT	WT	MT

		+ 180 mg/m^2 irinotecan (Q2W)			Post-Treatment	WT	WT	WT	WT	WT	WT
78	NSCLC	15mg/kg MM-151 (Q2W)	57	2%	Pre-Treatment	WT	**MT**	WT	WT	WT	WT
					Post-Treatment	WT	**MT**	WT	WT	WT	WT
79	MALIGNANT MELANOMA	6mg/kg MM-151 (QW) + 180 mg/m^2 irinotecan (Q2W)	85	-32%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
84	CRC	6mg/kg MM-151 (QW) + 180 mg/m^2 irinotecan (Q2W)	50	0%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
85	CRC	10.5mg/kg MM-151 (QW)	64	28%	Pre-Treatment	WT	**MT**	WT	WT	WT	WT
					Post-Treatment	WT	**MT**	WT	WT	WT	WT
88	METASTATIC CHOLANGIOCARCINOMA	18mg/kg MM-151 (Q2W)	42	47%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	**MT**	WT	WT
90	METASTATIC RENAL CELL	18mg/kg MM-151 (Q2W)	85	-5%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
98	CRC	10.5mg/kg MM-151 (QW)	155	-18%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
99	HNSCC	9mg/kg MM-151 (QW) + 180 mg/m^2 irinotecan (Q2W)	15	N.D.	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
100	NSCLC	9mg/kg MM-151 (QW) + 180 mg/m^2 irinotecan (Q2W)	57	N.D.	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
104	HNSCC	20mg/kg MM-151 (Q2W)	29	24%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	**MT** [2]	WT	WT	WT	WT	WT
106	CRC	7.5mg/kg MM-151 (QW) + 180 mg/m^2 irinotecan (Q2W)	169	-3%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	**MT**	WT	WT	WT
108	BREAST CANCER	20mg/kg MM-151 (Q2W)	55	36%	Pre-Treatment	WT	WT	WT	WT	WT	WT
					Post-Treatment	WT	WT	WT	WT	WT	WT
109	CRC	10.5mg/kg MM-151 (QW)	43	11%	Pre-Treatment	WT	**MT**	WT	WT	**MT**	WT

110	CRC	18mg/kg MM-151 (Q2W)	64	65%	Post-Treatment	Pre-Treatment	WT	**MT**	WT	WT	WT
						Post-Treatment	WT	WT	**MT**	WT	WT

[x] This patient was dosed below a target level of >= 6mg/kg MM-151 on a QW or Q2W schedule

[y] once-weekly (QW), every-other week (Q2W), every-three week (Q3W)

[z] The EGFR L448F mutation identified for this patient is a variant of unknown significance

FIG. 26

Subject #	Treatment Group	MM-151 Priming Doses (Yes/No)	MM-151 Dose (mg/kg)	MM-151 Dosing Frequency [z]	Irinotecan Dose (mg/m^2)	Irinotecan Dosing Frequency	Duration of Treatment (days)	Best Overall Response	Maximum Percent Change	Mutations
2	MM-151 Monotherapy	No	0.25 (6)[x]	QW	*N/A*	*N/A*	610	Partial Response (PR)	-36%	WT
4	MM-151 Monotherapy	No	0.75 (9)[x]	QW	*N/A*	*N/A*	581	Partial Response (PR)	-40%	WT
36	MM-151 Monotherapy	No	6	Q2W	*N/A*	*N/A*	56	Stable Disease (SD)	15%	KRAS
41	MM-151 Monotherapy	No	6	Q2W	*N/A*	*N/A*	43	Progressive Disease (PD)	32%	NRAS
48	MM-151 Monotherapy	No	9	Q2W	*N/A*	*N/A*	156	Stable Disease (SD)	-19%	WT
49	MM-151 Monotherapy	No	9	Q2W	*N/A*	*N/A*	214	Stable Disease (SD)	-6%	KRAS, PIK3CA
50	MM-151 Monotherapy	No	9	QW	*N/A*	*N/A*	8	Progressive Disease (PD)	29%	NRAS, PIK3CA
51	MM-151 Monotherapy	No	9	Q2W	*N/A*	*N/A*	155	Stable Disease (SD)	5%	EGFR-ECD, BRAF
54	MM-151 Monotherapy	No	9	QW	*N/A*	*N/A*	169	Partial Response (PR)	-46%	WT
55	MM-151 Monotherapy	No	9	QW	*N/A*	*N/A*	57	Stable Disease (SD)	-16%	KRAS
65	MM-151 Monotherapy	Yes	12	Q2W	*N/A*	*N/A*	63	Progressive Disease (PD)	44%	EGFR-ECD
80	MM-151 Monotherapy	Yes	10.5	QW	*N/A*	*N/A*	176	Partial Response (PR)	-36%	WT
84	MM-151 + Irinotecan	Yes	6	QW	180	Q2W	50	Progressive Disease (PD)	0%	WT
85	MM-151 Monotherapy	Yes	10.5	QW	*N/A*	*N/A*	64	Progressive Disease (PD)	28%	KRAS
86	MM-151 + Irinotecan	Yes	6	QW	180	Q2W	505	Partial Response (PR)	-69%	KRAS
89	MM-151 Monotherapy	Yes	18	Q2W	*N/A*	*N/A*	57	Progressive Disease (PD)	5%	WT

93	MM-151 Monotherapy	Yes	10.5	QW	*N/A*	*N/A*	52	Progressive Disease (PD)	98%	BRAF
95	MM-151 Monotherapy	Yes	18	Q2W	*N/A*	*N/A*	98	Stable Disease (SD)	-30%	EGFR-ECD
98	MM-151 Monotherapy	Yes	10.5	QW	*N/A*	*N/A*	155	Stable Disease (SD)	-18%	WT
103	MM-151 Monotherapy	Yes	20	Q2W	*N/A*	*N/A*	57	Progressive Disease (PD)	37%	WT
106	MM-151 + Irinotecan	Yes	7.5	QW	180	Q2W	169	Stable Disease (SD)	-3%	NRAS, BRAF
109	MM-151 Monotherapy	Yes	10.5	QW	*N/A*	*N/A*	43	Progressive Disease (PD)	11%	KRAS, BRAF
110	MM-151 Monotherapy	Yes	10.5	QW	*N/A*	*N/A*	64	Progressive Disease (PD)	65%	NRAS
113	MM-151 + Irinotecan	Yes	7.5	QW	180	Q2W	127	Stable Disease (SD)	-19%	NRAS, BRAF

[x] The dose level for this patient was increased during the course of treatment and the dose level is shown as [Initial Dose (Escalated Dose)]

[z] once-weekly (QW), every-other week (Q2W), every-three week (Q3W)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2017/024410

A. CLASSIFICATION OF SUBJECT MATTER

(see extra sheet)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 39/395, 31/33, 31/505, 31/7052, 33/16, 9/127, C07K 16/28, A61P 35/00

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

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

PatSearch, EMBL, NCBI, PAJ, Espacenet, DWPI, PCT Online, USPTO DB, CIPO (Canada PO), SIPO DB

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CUTSEM E.V. et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. The N.Eng.J.Med, 2009, Vol. 360, no. 14, p. 1408-1417, especially Table 1, p. 1410-1414	1-3, 7-15, 21-23, 30-33, 40-42, 44-49
Y	ARENA S. et al. MM-151 overcomes acquired resistance to cetuximab and panitumumab in colorectal cancers harboring EGFR extracellular domain mutations. Sci. Transl. Med., 3 February 2016, Vol. 8, Issue 324ra14, p. 1-10, doi.: 10/1126/scitranslmed.aad5640	1-3, 7-15, 21-23, 30-33, 40-42, 44-49
Y	HARALDSDOTTIR S. et al. Integrating anti-EGFR therapies in metastatic colorectal cancer. J Gastrointest.Oncol., 2013, Vol. 4, no. 3, p. 285-298	1-3, 7-15, 21-23, 30-33, 40-42, 44-49
Y	WO 2015/175827 A1 (MERRIMACK PHARMACEUTICALS INC.) 19.11.2015, abstract, p. 9-15, claims 17-28	1-3, 7-15, 21-23, 30-33, 40-42, 44-49

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 June 2017 (20.06.2017)	20 July 2017 (20.07.2017)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer V.Gogol Telephone No. 495 531 65 15

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A61K 33/16 *(2006.01)*
A61K 9/127 *(2006.01)*
C07K 16/28 *(2006.01)*
A61P 35/00 *(2006.01)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/074382 A1 (MERRIMACK PHARMACEUTICALS INC.) 17.03.2016, abstract, claims 1-20	1-3, 7-15, 21-23, 30-33, 40-42, 44-49
A	FUCHS Ch. et al. Irinotecan in the treatment of colorectal cancer. Can. Treat. Rev., 2006, Vol. 32, p. 491-503	1-3, 7-15, 21-23, 30-33, 40-42, 44-49

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2017/024410

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

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

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

3. ☒ Claims Nos.: 4-6, 16-12, 24-29, 34-39, 43
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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(71) Applicant: **IPSEN BIOPHARM LTD.** [GB/GB]; Ash Road, Wrexham Industrial Estate, Wrexham LL13 9UF (GB).

(72) Inventors; and
(71) Applicants: **ZHANG, Bin** [US/US]; 55 Hill Road, Apt 307, Belmont, Massachusetts 02478 (US). **BRAUN, Stephan** [DE/FR]; 37, rue Paul Valery, 75116 Paris (FR).

(72) Inventors: **FITZGERALD, Jonathan Basil**; 32 Magnolia Street, Arlington, Massachusetts 02474 (US). **KALRA, Ashish**; 19 Burnham Street, Apt. D2, Belmont, Massachusetts 02478 (US). **LEONARD, Shannon**; 28 Highland Park, Peabody, Massachusetts 01960 (US).

(74) Agent: **OATES, Edward Christopher** et al.; Carpmaels & Ransford LLP, One Southampton Row, London WC1B 5HA (GB).

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

(57) Abstract: Combination therapy regimens including liposomal irinotecan, oxaliplatin and 5-fluorouracil are useful in the treatment of gastric cancer, including treatment of patients diagnosed with previously untreated gastric cancer. The combination therapy can include the administration of liposomal irinotecan, oxaliplatin, leucovorin and 5-fluorouracil once every two weeks.

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

TECHNICAL FIELD

[001] This disclosure relates to novel therapies useful in the treatment of gastric cancer, including the use of liposomal irinotecan in combination with 5-fluorouracil and oxaliplatin for the (first line) treatment of patients diagnosed with previously untreated gastric cancer.

BACKGROUND

[002] Gastric cancer is one of the most common causes of cancer-related mortality worldwide. Approximately 18,000 metastatic gastric cancer patients were expected to be treated with pharmaceutical regimens in the United States in 2015. Of this population, approximately 7000 patients fall into the HER2-negative frontline treatment category. HER2-negative gastric cancer is a highly heterogeneous disease and targeted therapies, such as bevacizumab, rilotumumab and cetuximab, have been unsuccessful. Complicating the prognosis, many gastric cancer patients present with advanced-stage disease at diagnosis. There is currently no globally accepted standard chemotherapeutic regimen for the treatment of advanced gastric cancer, despite the fact that several treatment regimens have been investigated with limited efficacy (5 year survival rate of ~4%). The disease is commonly treated with chemotherapy, surgery and radiation therapy. Given the poor prognosis and the low median survival rate for patients with gastric cancer, new treatment options are still needed.

[003] Tolerability of multi-drug regimens is important in cancer treatment. The longer the duration of manageable treatment should translate into improved outcome due to longer drug exposure. One widely used first-line treatment of patients with gastric cancer is oxaliplatin plus infusional 5-fluorouracil (5-FU) and leucovorin (LV). Specifically, a modified FOLFOX6 regimen (mFOLFOX6) involves 5-FU/LV (400 mg/m^2 IV bolus + 2400 mg/m^2 as 46 h infusion/400 mg/m^2) + oxaliplatin (85 mg/m^2), q2w. While the treatment is generally well-tolerated, the overall survival is not increased.

[004] During the last 5 years, another combination chemotherapy regimen that has emerged as a first-line treatment of gastric cancer is the combination therapy of 5-fluorouricil (5-FU)/leucovorin (LV) + irinotecan + oxaliplatin (FOLFIRINOX). However, FOLFIRINOX is known to have significant toxicity, and use is limited to patients with better performance status (i.e. ECOG performance score of 0 or 1). With prolonged FOLFIRINOX treatment, oxaliplatin is often discontinued from the regimen due to toxicity. Therefore, if

equally effective double regimens can be identified, patients may be able to tolerate prolonged treatment better, and even poor performance status patients may receive benefit. Although the FOLFIRINOX regimen has been recommended by the National Comprehensive Cancer Network (NCCN) as a preferred option for first-line metastatic disease since 2011, there are some concerns about the toxicity associated with FOLFIRINOX. One dose regimen of FOLFIRINOX is 85 mg/m^2 oxaliplatin, 180 mg/m^2 irinotecan, and fluorouracil at a dose of 400 mg/m^2 administered by IV bolus followed by a continuous infusion of 2400 mg/m^2. 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.

[005] CPT-11 is irinotecan hydrochloride trihydrate, marketed as Camptosar® in the United States, approved for use in combination with 5-fluorouracil and leucovorin as first line therapy for patients with metastatic carcinoma of the colon or rectum, or for patients with metastatic carcinoma of the colon or rectum whose disease has recurred or progressed following initial fluorouracil-based therapy.

[006] MM-398 is a liposomal irinotecan and is marketed in the U.S. as the FDA-approved product ONIVYDE® in combination with 5-fluorouracil and leucovorin for the treatment of patients with certain forms of pancreatic cancer after disease progression following gemcitabine-based therapy.

SUMMARY

[007] Improved antineoplastic therapies for the treatment of gastric cancer provide the administration of liposomal irinotecan in combination with oxaliplatin and 5-fluorouracil to patients with previously untreated gastric cancer. The 5-fluorouracil can be administered in combination with leucovorin. The improved antineoplastic therapies can provide improved therapeutic index (e.g., improved toxicity profiles) relative to prior FOLFIRINOX regimens.

[008] A method of treating gastric cancer can comprise the administration of an antineoplastic therapy of liposomal irinotecan (e.g., MM-398), oxaliplatin, and 5-fluorouracil once every two weeks to the patient. Optionally, leucovorin can also be administered prior to each administration of the 5-fluorouracil. Each administration of the liposomal irinotecan can be administered in a total dose of 50 mg/m^2 or 55 mg/m^2 (may be referred to as 56 mg/m^2) liposomal irinotecan (dose based on free base, as defined herein) or 60 mg/m^2 or 65 mg/m^2 liposomal irinotecan (dose based on hydrochloride trihydrate as defined herein). A total of 2,400 mg/m^2 5-fluorouracil can be administered over 46 hours starting on each day when the liposomal irinotecan is administered. A total of 60, 70, 75, or 85 mg/m^2 oxaliplatin

can be administered on each day the liposomal irinotecan is administered. A total of 200 mg/m^2 (l) leucovorin can be administered prior to each administration of the 5-flurouracil (e.g., optionally administered as 400 mg/m^2 of (l+d) leucovorin). The antineoplastic therapy can be administered starting on days 1 and 15 of a 28-day treatment cycle, with the liposomal irinotecan, oxaliplatin, and optionally leucovorin administered on days 1 and 15, and initiating the 46-hour administration of the 5-fluorouracil on days 1 and 15. The total dose of each administration of liposomal irinotecan and 5-fluorouracil can be reduced by 25% for certain patients, including patients who experience a Grade 3 or 4 adverse reaction to a previous dose of the antineoplastic therapy.

[009] The invention is based in part on several pre-clinical discoveries. First, liposomal irinotecan improved anti-tumor activity of the topoisomerase 1 inhibitor SN-38 (an active metabolite of irinotecan) relative to exposure-matched doses of non-liposomal irinotecan. Second, liposomal irinotecan combined with 5-fluorouracil and oxaliplatin consistently improved tumor growth inhibition and survival in mouse xenograft models of gastric cancer relative to non-liposomal irinotecan, without exacerbating the baseline toxicities of these agents.

[010] In addition, the invention is based in part on the discovery that the administration of a dose of an initial antineoplastic therapy to humans consisting of 70 mg/m^2 liposomal irinotecan (free base) was not well tolerated in humans when administered in combination with 60 mg/m^2 oxaliplatin, 2400 mg/m^2 5-fluorouracil and 400 mg/m^2 (l+d) leucovorin. In particular, the administration of this initial antineoplastic therapy resulted in unexpected gastrointestinal adverse events. However, subsequent antineoplastic therapy consisting of a novel combination of 50 mg/m^2 liposomal irinotecan, 60 mg/m^2 oxaliplatin, 2400 mg/m^2 5-fluorouracil and 400 mg/m^2 (l+d) leucovorin did not result in any of the gastrointestinal adverse events observed with the initial antineoplastic therapy.

[011] SN-38 is a potent active metabolite of liposomal irinotecan. The average unencapsulated SN-38 (uSN38 Cavg) is associated with increased efficacy of liposomal irinotecan, while higher levels of other PK parameters are associated with reduced tolerability of liposomal irinotecan. For example, higher total irinotecan maximum plasma concentration (tIRI Cmax) is associated with diarrhea and higher unencapsulated maximum plasma concentration of SN38 (uSN38 Cmax) is associated with neutropenia. These pharmacokinetic parameters are proportional to nal dose.

[012] The administration of liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin with a dose level of 60 mg oxaliplatin and 80 mg/m^2 of liposomal irinotecan

resulted in a greater than 100% increase in the average concentration in plasma of unencapsulated uSN38 C_{max}, while administering these four agents with a dose level of 60 mg oxaliplatin and 60 mg/m^2 of liposomal irinotecan (see dose level -1 in Table 2) resulted in an increase in of about 9%. In addition, the PK parameters associated with reduced tolerability (tIRI C_{max} or uSN38 C_{max}) increased about 15% and 44% for the 60 mg/80 mg/m^2 (respectively) but decreased for dose level -1 by 3% and 27% (respectively). So unexpectedly, the administration of a lower amount of liposomal irinotecan resulted in a more tolerable dose, while at the same time retaining efficacy. In the present application the administration of oxaliplatin, liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin at dose level -1 or -3 (Table 2) increases the uSN38 C_{avg} in plasma without significantly increasing the tIRI C_{max} or uSN38 C_{max}, which allows for administration of these doses with greater tolerability

[013] Accordingly, preferred methods of treating (previously untreated) gastric cancer provide for the administration of a human-tolerated antineoplastic therapy once every two weeks, where each administration of the antineoplastic therapy is a combination of the antineoplastic agents liposomal irinotecan, oxaliplatin and 5-fluorouracil provided herein. Preferably, the antineoplastic therapy administered once every two weeks consists of: (a) a total dose of 50 mg/m^2 liposomal irinotecan (dose based on the free base, as defined herein), (b) a total dose of 60-85 mg/m^2 oxaliplatin (including, e.g., 60, 70, or 85 mg/m^2), and (c) a total of 2,400 mg/m^2 5-fluorouracil optionally administered in combination with leucovorin; or (a) a total dose of 55 mg/m^2 liposomal irinotecan (dose based on the free base, as defined herein), (b) a total dose of 60-85 mg/m^2 oxaliplatin (including, e.g., 60, 70 or 85 mg/m^2), and (c) a total of 2,400 mg/m^2 5-fluorouracil optionally administered in combination with leucovorin. Optionally, the combination can include administration of a total of 200 mg/m^2 (*l*)leucovorin (optionally administered as 400 mg/m^2 of (*l*+*d*) leucovorin), prior to initiating the administration of the 5-fluorouracil. Preferably, no other antineoplastic agent is administered during the antineoplastic therapy, other than amounts of SN-38 produced within the patient from the liposomal irinotecan, after administration of the liposomal irinotecan. For example, the antineoplastic therapy can be administered without (non-liposomal) CPT-11 irinotecan. Preferably, the liposomal irinotecan, oxaliplatin, and (optionally) leucovorin are consecutively administered as separate infusions on a single (first) day and the 5-fluorouracil is administered starting on the first day after the administration of the leucovorin (if administered) and continuing into the following day (e.g., over a total of 46 hours).

BRIEF DESCRIPTION OF THE DRAWINGS

[014] Figure 1A is a graph showing nal-IRI activity in gastric tumor model MKN-45; Figure 1B is a graph showing nal-IRI activity in gastric tumor model KATO III.

[015] Figures 2A and 2B are graphs showing anti-tumor activity of MM-398 in comparison to free irinotecan monotherapy in a xenograft model of gastric cancer. Mice bearing MKN-45 tumors were treated with saline, 12.5 mg/kg free irinotecan or 2.5 mg/kg MM-398 weekly for 3 weeks (Figure 2A) or saline, 25 mg/kg free irinotecan and 5 mg/kg MM-398 weekly for 3 weeks (Figure 2B) (days of dosing are indicated by horizontal dashed lines ; n=X per mice group).

[016] Figures 3A is a graph showing anti-tumor activity of MM-398 in comparison to free irinotecan in the context of combination therapy with 5-FU and oxaliplatin; Figure 3B is a graph showing anti-tumor activity of MM-398 in comparison to free irinotecan in the context of triplet combination therapy with 5-FU and oxaliplatin.

[017] Figures 4A-4H, are graphs showing that MM-398 displays superior anti-tumor activity in tumor models less responsive to oxaliplatin, 5-FU and free irinotecan (equal exposure) (Efficacy Study in Gastric Model (MKN-45) – Monotherapy). Figure 4A. MM-398 Tumor volume; Figure 4B. MM-398 Body weight; Figure 4C. Oxaliplatin tumor volume; Figure 4D. Oxaliplatin body weight; Figure 4E. 5-FU tumor volume; Figure 4F. 5-FU Body weight; Figure 4G. Free irinotecan Tumor volume and Figure 4H. Free irinotecan Body weight. MM-398 at 5 mg/kg has better anti-tumor activity than all other treatments tested.

[018] Figures 5A depicts FOLFIRI regimen and Figure 5B depicts FOLFOX regimen vs. MM-398 in MKN45. MM-398 monotherapy has better anti-tumor activity than FOLFIRI and FOLFOX at the doses tested.

[019] Figures 6A-6C depict body weight change in response to Figure 6A. monotherapy regimens, Figure 6B. doublet regimens and Figure 6C. triplet regimens. There was acceptable weight loss in all groups.

[020] Figures 7A and 7B are graphs showing anti-tumor activity of (A) MM-398 in comparison to (B) free irinotecan at low doses.

[021] Figures 8A and 8B are graphs showing anti-tumor activity of (A) MM-398 in comparison to (B) free irinotecan at high doses.

[022] Figure 9 is a schematic of a clinical trial in gastric cancer.

[023] Figures 10A and 10B are graphs showing duration of sustained SN-38 tumor levels: Figure 10A. Tumor SN-38 Concentration; Figure 10B. Time SN-38 concentration is predicted to be above a threshold out of a 6 week cycle.

[024] Figure 11 is a graph showing historical median OS performance of selected first line gastric cancer regimens in various trials.

[025] Figure 12 is a graph showing safety of dose level 1 and dose level -1.

[026] Figure 13 is a graph showing efficacy of Dose level 1 and dose level -1.

[027] Figure 14 is a table showing adverse events in response to treatment with Dose level -1 and dose level 1.

DETAILED DESCRIPTION

[028] Doses of nal-IRI in some studies were calculated based on the equivalent dose of irinotecan hydrochloride trihydrate (salt); in this specification, unless specified otherwise, the doses are based on irinotecan as the free base.

[029] There are about 866 mg of irinotecan per gram of irinotecan trihydrate hydrochloride. For example, a dose of 80 mg/m^2 of liposomal irinotecan based on the amount of irinotecan hydrochloride trihydrate starting material actually contains about 0.866 x (80mg/m^2) = 69.38 mg/m^2 of irinotecan free base, which may be rounded to an integer to avoid dosing errors. For example 69.38 mg/m^2 may be rounded to 70 mg/m^2 as shown in Table A.

[030] Another example is a dose of 65 mg/m^2 liposomal irinotecan based on the trihydrate salt, which refers to an amount of liposomal irinotecan free base providing the same amount of liposome encapsulated irinotecan that is present in 65 mg/m^2 of liposomal irinotecan hydrochloride trihydrate which is equivalent to a dose of 56.29 mg/m^2 liposomal irinotecan free base which may be rounded, in order to avoid dosing errors, to a dose from 55 mg/m^2 to 57 mg/m^2, for example, 55 mg/m^2, 56 mg/m^2 or 57 mg/m^2. Another example is a dose of 60 mg/m^2 liposomal irinotecan based on the trihydrate salt which refers to an amount of the liposomal irinotecan free base providing the same amount of liposome encapsulated irinotecan that is present in 60 mg/m^2 of irinotecan hydrochloride trihydrate, and is equivalent to a dose 51.96 mg/m^2 which can be rounded, in order to avoid dosing errors, to a dose of from about 50 mg/m^2 to about 52 mg/m^2, for example, to 50 mg/m^2, 51 mg/m^2, or 52 mg/m^2 of liposomal irinotecan free base. Likewise a dose of 50 mg/m^2 of liposomal irinotecan hydrochloride trihydrate can be converted to 43.30 mg/m^2 of liposomal irinotecan free base which can be rounded, in order to avoid dosing errors, to 43 mg/m^2 or 44 mg/m^2. Similarly a dose of 49 mg/m^2 of liposomal irinotecan hydrochloride trihydrate can be converted to 42.22 mg/m^2 of liposomal irinotecan free base which can be rounded, in order to avoid dosing errors, to 42 mg/m^2 or 43 mg/m^2. Another embodiment is a dose of 45 mg/m^2 of liposomal

irinotecan hydrochloride trihydrate which can be converted to 38.97 mg/m^2 of liposomal irinotecan free base which can be rounded, in order to avoid dosing errors, to 38 mg/m^2 or 39 mg/m^2. In another example, a dose of 40 mg/m^2 of liposomal irinotecan hydrochloride trihydrate can be converted to 34.64 mg/m^2 of liposomal irinotecan free base which can be rounded, in order to avoid dosing errors, to 34 mg/m^2 or 35 mg/m^2. In another example, a dose of 33 mg/m^2 of liposomal irinotecan hydrochloride trihydrate can be converted to 28.15 mg/m^2 of liposomal irinotecan free base which can be rounded, in order to avoid dosing errors, to 28 mg/m^2 or 29 mg/m^2. In another example, a dose of 30 mg/m^2 of liposomal irinotecan hydrochloride trihydrate can be converted to 32.5 mg/m^2 of liposomal irinotecan free base which can be rounded, in order to avoid dosing errors, to 32 mg/m^2 or 33 mg/m^2.

[031] Additional examples are shown in Table A.

[032] Table A

irinotecan hydrochloride trihydrate salt mg/m^2	irinotecan free base mg/m^2	Conversion from hydrochloride trihydrate salt to free base using 0.866 conversion factor
120	100	103.92
80	70	69.38
65	55	56.29
60	50	51.96
50	43	43.30
49	42	42.22
45	39	38.97
40	35	34.64
33	28	28.15
30	33	32.50

[033] As used herein, unless otherwise indicated, the term "nal-IRI" (nanoliposomal irinotecan) and "MM-398" refer to a form of liposomal irinotecan. The term "CPT-11" refers to (non-liposomal) irinotecan hydrochloride trihydrate.

[034] As used herein, "5-FU" and "5FU" and used interchangeably and refer to 5-fluorouracil.

[035] All cited documents are incorporated herein by reference.

[036] As used herein, the endpoints of an expressed range are included in the range. For example, a range from 30 mg to 70 mg, includes 30 and 70 (and all numbers between the endpoints).

[037] Testing of xenograft models of gastric cancer in Example 2 demonstrated improved anti-tumor activity of liposomal irinotecan relative to exposure-matched doses of

non-liposomal irinotecan. In the mouse animal studies in Example 2, a dose of "x" mg/kg liposomal irinotecan provides about the same exposure to the topoisomerase 1 inhibitor (irinotecan and/or SN-38) as a dose of "5x" non-liposomal irinotecan (CPT-11). The liposomal irinotecan consistently improved tumor growth inhibition and survival relative to non-liposomal irinotecan in preclinical models, both as a monotherapy and in combination with 5-FU and oxaliplatin. These findings illustrate the therapeutic potential of liposomal irinotecan in combination with 5-FU/LV and oxaliplatin and support a clinical trial of this triplet regimen in first-line gastric cancer (Example 4).

[038] An animal model of the FOLFIRINOX regimen was tested against the MM-398 + 5-FU/LV + oxaliplatin regimen in a gastric tumor xenograft mouse model. Liposomal irinotecan (MM-398) performed better than conventional (non-liposomal) irinotecan (CPT-11) at equivalent exposure doses (5 mg/kg MM-398 vs. 25 mg/kg free IRI) in the gastric xenograft cancer models (Example 2) either alone (e.g., Figure 2A), or in combination with oxaliplatin and/or 5-FU (e.g., Figure 2B).

[039] These preclinical findings support the therapeutic use of liposomal irinotecan in combination with 5-FU/LV and oxaliplatin and a clinical trial of this triplet regimen in first-line gastric cancer (Example 4). Figure 3A and 3B depicts a graphical representation of the study design employing the combination of MM-398 + 5-FU/LV + oxaliplatin as described herein.

[040] For example, use of a combination of liposomal irinotecan, oxaliplatin, and 5-fluorouracil in treating gastric cancer in a human patient who has not previously received chemotherapy to treat the gastric cancer, the use comprising administering an antineoplastic therapy to the patient a total of once every two weeks, the antineoplastic therapy consisting of: (a) 50 mg/m^2 of liposomal irinotecan, 60 mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient; (b) 50 mg/m^2 of liposomal irinotecan, 85 mg/m^2 oxaliplatin, 200 mg/m^2 of (l)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient; (c) 55 mg/m^2 of liposomal irinotecan, 70 mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient; (d) 50 mg/m^2 of liposomal irinotecan, 60 mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient wherein the liposomal irinotecan, oxaliplatin, 5-

fluorouracil and leucovorin is administered on days 1 and 15 of a 28-day treatment cycle; (e) 50 mg/m^2 of liposomal irinotecan, 85 mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient, wherein the liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin is administered on days 1 and 15 of a 28-day treatment cycle; (f) 55 mg/m^2 of liposomal irinotecan, 70 mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient wherein the liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin is administered on days 1 and 15 of a 28-day treatment cycle; (g) 50 mg/m^2 of liposomal irinotecan, 60 mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient wherein the liposomal irinotecan is administered, followed by administering the oxaliplatin, followed by administering the leucovorin, followed by administering the 5-fluorouracil; (h) 50 mg/m^2 of liposomal irinotecan, 85 mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient wherein the liposomal irinotecan is administered, followed by administering the oxaliplatin, followed by administering the leucovorin, followed by administering the 5-fluorouracil; (i) 55 mg/m^2 of liposomal irinotecan, 70 mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient wherein the liposomal irinotecan is administered, followed by administering the oxaliplatin, followed by administering the leucovorin, followed by administering the 5-fluorouracil; or (j) 50 mg/m^2 - 55 mg/m^2 of liposomal irinotecan, 60 mg/m^2-85mg/m^2 oxaliplatin, 200 mg/m^2 of (*l*)-form of leucovorin or 400 mg/m^2 of the (*l*+*d*) racemic form of leucovorin, and 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient wherein the liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin is administered on days 1 and 15 of a 28-day treatment cycle, wherein the liposomal irinotecan is administered, followed by administering the oxaliplatin, followed by administering the leucovorin, followed by administering the 5-fluorouracil, wherein the administration of the oxaliplatin begins 2 hours after completing each administration of the liposomal irinotecan. Each of these exemplary uses can be modified to replace the doses of liposomal irinotecan, oxaliplatin, leucovorin and 5-flurouracil disclosed herein in the following passages relating to these specific components. Sometimes the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in

liposomes. Sometimes, the liposomal irinotecan comprises irinotecan encapsulated in liposome vesicles consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethlyene glycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).

[041] As provided herein, irinotecan can be administered in an irinotecan liposome preparation. Preferably, the liposomal irinotecan is irinotecan sucrose sulfate liposome injection (otherwise termed "irinotecan sucrose octasulfate salt liposome injection" or "irinotecan sucrosofate liposome injection"), the formulation referred to herein as "MM-398" (also known as PEP02, see US 8,147,867) is a form of "nanoliposomal irinotecan" (also called "irinotecan liposome" or "liposomal Irinotecan"). MM-398 is irinotecan as the irinotecan sucrose octasulfate salt encapsulated in a nanoliposome drug delivery system.

[042] The liposomal irinotecan can be a pharmaceutical composition prepared for human intravenous administration. For example, the liposomal irinotecan may be provided as a sterile, injectable parenteral liquid for intravenous injection. The required amount of liposomal irinotecan may be diluted, e.g., in 500 mL of 5% dextrose injection USP, to provide a variety of concentrations, for example, 5 mg/mL, and may be infused over a 90 minute period.

[043] The active ingredient of the MM-398 injection, irinotecan, is a member of the topoisomerase I inhibitor class of drugs and is a semi-synthetic and water soluble analog of the naturally-occurring alkaloid, camptothecin. Topoisomerase I inhibitors work to arrest uncontrolled cell growth by preventing the unwinding of DNA and therefore preventing replication. The pharmacology of irinotecan is complex, with extensive metabolic conversions involved in the activation, inactivation, and elimination of the drug. Irinotecan is a pro-drug that is converted by nonspecific carboxylesterases into a 100-1000 fold more active metabolite, SN-38. SN-38 is cleared via glucuronidation, (for which major pharmacogenetic differences have been shown), and biliary excretion. These drug properties contribute to the marked differences in efficacy and toxicity observed in clinical studies with irinotecan.

[044] The liposomal irinotecan can be a unilamellar lipid bilayer vesicle of approximately 80-140 nm in diameter that encapsulates an aqueous space that contains irinotecan complexed in a gelated or precipitated state as a salt with sucrose octasulfate. The lipid membrane of the liposome is composed of phosphatidylcholine, cholesterol, and a polyethyleneglycol-derivatized phosphatidyl-ethanolamine in the amount of approximately one polyethyleneglycol (PEG) molecule for every 200 phospholipid molecules.

[045] The amount of liposomal irinotecan administered to the human patient can range from about 30 mg/m^2 to about 170 mg/m^2, preferably 50 mg/m^2 or 55 mg/m^2 or 56 mg/m^2 when administered in combination with oxaliplatin and 5-fluorouracil for treatment of gastric cancer (dose expressed in terms of free base).

[046] The plasma pharmacokinetics of total irinotecan and total SN-38 were evaluated in patients with cancer who received MM-398, as a single agent or as part of combination chemotherapy, at doses between 50 and 155 mg/m^2 (amount of irinotecan free base, equivalent to 60-180 mg/m^2 dose expressed in terms of the amount of irinotecan hydrochloride trihydrate salt) and 353 patients with cancer using population pharmacokinetic analysis. 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.

[047] The pharmacokinetic parameters of total Irinotecan and total SN-38 following administration of MM-398 50 mg/m^2 (free base) as a single agent or part of combination chemotherapy are presented in Table B.

[048] Table B: Total Irinotecan and Total SN-38

[049] Pharmacokinetic Parameters in Patients with Solid Tumors.

Dose (mg/m^2) free base	Total Irinotecan			Total SN-38	
	C_{max} [μg/mL]	$AUC_{0-\infty}$ [h·μg/mL]	$t_{1/2}$[h]	C_{max} [ng/mL]	$t_{1/2}$[h]
Max (125%)	32.5	1193.5	25.8	4.8	67.8
50	26	954.8	25.8	3.8	67.8
Min (80%)	20.8	763.8	25.8	3.0	67.8

[050] The C_{max} of SN-38 increases proportionally with liposomal irinotecan dose but the AUC of SN-38 increases less than proportionally with dose, enabling new methods of dosage adjustment. For example, the value of the parameter associated with adverse effects (C_{max}) decreases by a relatively greater extent than the value of the parameter associated with the effectiveness of treatment (AUC). Accordingly, when an adverse effect is seen, a reduction in the dosing of the liposomal irinotecan can be implemented that maximizes the difference

between the reduction in C_{max} and in AUC. The discovery means that in treatment regimens, a given SN-38 AUC can be achieved with a surprisingly low SN-38 Cmax. Likewise, a given SN-38 C_{max} can be achieved with a surprisingly high SN-38 AUC.

[051] 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.

[052] In some embodiments, the liposomal irinotecan can be characterized by the parameters in Table B. In some embodiments, the liposomal irinotecan can be MM-398 or a product that is bioequivalent to MM-398. In some embodiments, the liposomal irinotecan can be characterized by the parameters in Table C, including a C_{max} and/or AUC value that is 80-125% of the corresponding value in Table B. The pharmacokinetic parameters of total irinotecan for various alternative liposomal irinotecan formulations administering 50 mg/m^2 irinotecan free base once every two weeks is provided in Table C.

[053] Table C

[054] Total Irinotecan Pharmacokinetic Parameters in Alternative Liposomal Irinotecan Formulations

Dose (mg/m^2) Free base	Total Irinotecan	
	C_{max} [µg/mL] (n=25)	$AUC_{0-\infty}$ [h·µg/mL] (n=23)
50	20.8-32.5	763.8-1193.5

C_{max}: Maximum plasma concentration

$AUC_{0-\infty}$: Area under the plasma concentration curve extrapolated to time infinity

$t_{½}$: Terminal elimination half-life

[055] The combination treatment described herein encompasses administration of MM-398 liposomal irinotecan in combination with multiple additional active agents: oxaliplatin, leucovorin and 5-fluorouracil, in doses and schedules to human patients with gastric cancer not previously treated with a prior chemotherapeutic agent in the metastatic setting as described herein.

[056] 5-Fluorouracil is a pyrimidine antagonist that interferes with nucleic acid biosynthesis. The deoxyribonucleotide of the drug inhibits thymidylate synthetase, thus inhibiting the formation of thymidylic acid from deoxyuridylic acid, thus interfering in the synthesis of DNA. It also interferes with RNA synthesis. An exemplary effective amount of

5-fluorouracil administered to a human patient can range from about 2,000 mg/m^2 to about 3,000 mg/m^2. In some embodiments, the amount of 5-fluorouracil administered to the human patient is 2,400 mg/m^2.

[057] Leucovorin is optionally administered prior to the 5-fluorouracil. Leucovorin acts as a biochemical cofactor for 1-carbon transfer reactions in the synthesis of purines and pyrimidines. Leucovorin does not require the enzyme dihydrofolate reductase (DHFR) for conversion to tetrahydrofolic acid. The effects of methotrexate and other DHFR-antagonists are inhibited by leucovorin. Leucovorin can potentiate the cytotoxic effects of fluorinated pyrimidines (i.e., fluorouracil and floxuridine). After 5-FU is activated within the cell, it is accompanied by a folate cofactor, and inhibits the enzyme thymidylate synthetase, thus inhibiting pyrimidine synthesis. Leucovorin increases the folate pool, thereby increasing the binding of folate cofactor and active 5-FU with thymidylate synthetase. Leucovorin has dextro- and levo-isomers, only the latter one being pharmacologically useful. As such, the bioactive levo-isomer ("levo-leucovorin") has also been approved by the FDA for treatment of cancer. The dosage of leucovorin is that of the racemic mixture containing both dextro (d) and levo (*l*) isomers, or optionally the (*l*) form of leucovorin at half the dosage of the (*l*+*d*) racemic form. An exemplary effective amount of leucovorin administered to the human patient can include an amount of (*l*)-form leucovorin ranging from about 100 mg/m^2 to about 300 mg/m^2. In some embodiments, the amount of (*l*)-form leucovorin administered to the human patient is 200 mg/m^2. In other embodiments, the leucovorin administered is the (*l*+*d*)-form of leucovorin, in an amount ranging from about 200 mg/m^2 to about 600 mg/m^2. In some embodiments, the amount of (*l*+*d*)-form of leucovorin administered is 400 mg/m^2.

[058] Oxaliplatin is a platinum-based drug that acts as a DNA cross-linking agent to effectively inhibit DNA replication and transcription, resulting in cytotoxicity which is cell-cycle non-specific. Oxaliplatin is typically used in combination with infusional 5-FU/LV, and is approved for use in advanced colorectal cancer (refer to package insert for more details). The effective amount of oxaliplatin administered to the human patient can range from about 30 mg/m^2 to about 150 mg/m^2, for example, from about 40 mg/m^2 to about 100 mg/m^2, or an amount of oxaliplatin of 50 mg/m^2, 55 mg/m^2, 60 mg/m^2, 65 mg/m^2, 70 mg/m^2, 75 mg/m^2, 80 mg/m^2, 85 mg/m^2, 90 mg/m^2, or 95 mg/m^2.

[059] Dose modifications may be made to methods of administering the combination treatment described herein as a result of adverse events, include hematological and non-hematological adverse events.

[060] In some embodiments, methods of administering the combination treatment described herein to patients having one or more characteristics can include reducing or otherwise modifying the dose of MM-398 administered according to the embodiments herein. In some embodiments, the dose of MM-398 is modified according to Table 1A.

[061] Table 1A: Examples of Dose Modifications for MM-398 (salt)

Toxicity NCI CTCAE v4.0	Occurrence	MM-398 adjustment in patients receiving 60 mg/m^2‡ (salt) 50 mg/m^2 (free base)	Patients homozygous for UGT1A1*28 without previous increase to 60 mg/m^2 (salt) 50 mg/m^2 (free base)
Grade 3 or 4 adverse reactions	Withhold MM-398. 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 or baseline grade resume MM-398 at:		
	First	45 mg/m^2 (salt)	45 mg/m^2 (salt)
	Second	30 mg/m^2 (salt)	30 mg/m^2 (salt)
	Third	Discontinue MM-398	Discontinue MM-398
Interstitial Lung Disease	First	Discontinue MM-398	Discontinue MM-398
Anaphylactic Reaction	First	Discontinue MM-398	Discontinue MM-398

[062] In some embodiments, the first, second, or any subsequent dose of MM-398 can be reduced by 20-30% (including dose reductions of 20%, 25% and/or 30%) in response to patient tolerability considerations such as an adverse reaction to a first or subsequent dose of MM-398 and/or other antineoplastic agent, and/or identifying a patient as being homozygous for the UGT1A1*28 allele. In some embodiments, the second or subsequent dose of MM-398 is reduced by another about 20%, 25% or 30% (a dose reduction of about 40%, 50% or 60% of the original dose). For example, a dose of 60 mg/m^2 MM-398 reduced by 25% is 45

mg/m^2 and a second reduction of another 25% is 30 mg/m^2. In some embodiments, the dose of MM-398 is reduced by 25%. In some embodiments, the dose of MM-398 is reduced by 30%. In some embodiments, the reduced dose of MM-398 is in a range starting from 30 mg/m^2 to (and including) 55 mg/m^2. In some embodiments, the dose of MM-398 is reduced to 50 mg/m^2. In some embodiments, the dose of MM-398 is reduced to 45 mg/m^2. In some embodiments, the dose of MM-398 is reduced to 35 mg/m^2.

[063] Other dose reduction schedules are provided Tables 1B-1E below. When the starting (initial) dose of MM-398 is 50 mg/m^2, 5FU 2400mg/m^2, LV (l+d) 400mg/m^2 and oxaliplatin is either 85mg/m^2 or 60mg/m^2, then the first dose reduction in response to a grade III or IV hematotoxicity is preferably a 25% dose reduction for each of the MM-398, 5-FU and oxaliplatin doses for each administration of the antineoplastic therapy. For persistent toxicities despite the first dose reduction, an additional 25% dose reduction in each of the antineoplastic agents of MM-398, 5-fluorouracil and oxaliplatin is preferred. Further toxicity will then lead to discontinuation of treatment in some instances. For non-hematologic toxicities, the same dose reduction schema can be followed as for hematotoxicity, except for the specific toxicities associated with the drug (i.e. 5FU hand foot syndrome, and oxaliplatin neuropathy) which can be selected based on the medically appropriate dose for the patient.

[064] Table 1B Examples of Reduced Doses of MM-398 and oxaliplatin

Dose	MM-398 (mg/m^2) (salt)	Oxaliplatin (mg/m^2)	5-fluorouracil (5FU) (mg/m^2)
Initial	60	60	2400
First Reduction	45	45	1800
Second Reduction	30	30	1350

[065] Table 1C Examples of Reduced Doses of MM-398 and oxaliplatin

Dose	MM-398 (mg/m^2) (salt)	Oxaliplatin (mg/m^2)	5-fluorouracil (5FU) (mg/m^2)
Initial	60	80	2400
First Reduction	45	60	1800
Second Reduction	30	40	1350

[066] Table 1D Examples of Reduced Doses of MM-398 and oxaliplatin

Dose	MM-398 (mg/m^2) (salt)	Oxaliplatin (mg/m^2)	5-fluorouracil (5FU) (mg/m^2)
Initial	60	60	2400
First Reduction	45	45	2400
Second Reduction	30	30	1800

[067] Table 1E Examples of Reduced Doses of MM-398 and oxaliplatin

Dose	MM-398 (mg/m^2) (salt)	Oxaliplatin (mg/m^2)	5-fluorouracil (5FU) (mg/m^2)
Initial	60	80	2400
First Reduction	45	60	2400
Second Reduction	30	40	1800

[068] In some embodiments, methods of administering the combination treatment described herein to patients having one or more characteristics can include reducing or otherwise modifying the dose of oxaliplatin administered according to the embodiments herein. In some embodiments, the dose of oxaliplatin is reduced by 20-30%. In some embodiments, the, the dose of oxaliplatin is reduced by 20%. In some embodiments, the, the dose of oxaliplatin is reduced by 25%. In some embodiments, the, the dose of oxaliplatin is reduced by 30%. In some embodiments, the reduced dose of oxaliplatin is in a range from 30 mg/m^2 to 75 mg/m^2. In some embodiments, the dose of oxaliplatin is reduced to 75 mg/m^2. In some embodiments, the dose of oxaliplatin is reduced to 65 mg/m^2. In some embodiments, the dose of oxaliplatin is reduced to 60 mg/m^2. In some embodiments, the dose of oxaliplatin is reduced to 45 mg/m^2. In some embodiments, the dose of oxaliplatin is reduced to 45 mg/m^2. In some embodiments, the dose of oxaliplatin is reduced to 34 mg/m^2.

[069] In some embodiments, methods of administering the combination treatment described herein to patients having one or more characteristics can include reducing or otherwise modifying the dose of 5-fluorouracil administered according to the embodiments herein. In some embodiments, the dose of 5-fluorouracil is reduced by 20-30%. In some embodiments, the dose of 5-fluorouracil is reduced by 20%. In some embodiments, the dose of 5-fluorouracil is reduced by 25%. In some embodiments, the, dose of 5-fluorouracil is reduced by 30%. In some embodiments, the reduced dose of 5-fluorouracil is in a range from

1000 mg/m^2 to 1800 mg/m^2. In some embodiments, the dose of 5-fluorouracil is reduced to 1800 mg/m^2. In some embodiments, the dose of 5-fluorouracil is reduced to 1350 mg/m^2. In some embodiments, the dose of 5-fluorouracil is reduced to 1400 mg/m^2. In some embodiments, the dose of 5-fluorouracil is reduced to 1200 mg/m^2.

[070] In some embodiments, methods of administering the combination treatment described herein to patients having one or more characteristics can include further reducing or otherwise modifying the dose of MM-398, oxaliplatin and/or 5-fluorouracil administered according to the embodiments herein.

[071] In some embodiments, methods of administering the combination treatment described herein to patients having one or more characteristics can include reducing or otherwise modifying the dose of more than one of MM-398, oxaliplatin and 5-fluorouracil administered according to the embodiments herein.

[072] Additional dose modifications for MM-398, oxaliplatin and/or 5-fluorouracil can be found in the respective Package Inserts, which are incorporated herein by reference.

[073] In one embodiment, the method of administering the combination treatment comprises 30, 40, 50, or 55 mg/m^2 of liposomal irinotecan, 30, 36, 42, 45, 53, 60, 64, 70, or 85 mg/m^2 oxaliplatin, 200 mg/m^2 of (l)-form of leucovorin or 400 mg/m^2 of the (l+d) racemic form of leucovorin, and 1,200, 1,350, 1,800, or 2,400 mg/m^2 5-fluorouracil to treat the gastric cancer in the human patient.

[074] Thus, in some embodiments, the method of administering the combination treatment to treat the gastric cancer in the human patient comprises administration of the following dose of liposomal irinotecan, oxaliplatin and leucovorin as shown in Table 1F below. Leucovorin is generally administered at 200 mg/m^2 of (l)-form of leucovorin or 400 mg/m^2 of the (l+d) racemic form but the dose may be varied by the patient's doctor. Any of the embodiments in the table may be administered with 200 mg/m^2 of (l)-form of leucovorin or 400 mg/m^2 of the (l+d) racemic form or a different doctor prescribed dose.

[075] Table 1F Embodiments of the present invention

Dose irinotecan hydrochloride trihydrate mg/m^2 (salt)	Dose oxaliplatin mg/m^2	5-fluorouracil mg/m^2	Leucovorin
30	30	1,200	Yes
30	30	1,350	Yes
30	30	1,400	Yes
30	30	1,800	Yes
30	30	2,400	Yes

30	36	1,200	Yes
30	36	1,350	Yes
30	36	1,400	Yes
30	36	1,800	Yes
30	36	2,400	Yes
30	42	1,200	Yes
30	42	1,350	Yes
30	42	1,400	Yes
30	42	1,800	Yes
30	42	2,400	Yes
30	45	1,200	Yes
30	45	1,350	Yes
30	45	1,400	Yes
30	45	1,800	Yes
30	45	2,400	Yes
30	53	1,200	Yes
30	53	1,350	Yes
30	53	1,400	Yes
30	53	1,800	Yes
30	53	2,400	Yes
30	60	1,200	Yes
30	60	1,350	Yes
30	60	1,400	Yes
30	60	1,800	Yes
30	60	2,400	Yes
30	64	1,200	Yes
30	64	1,350	Yes
30	64	1,400	Yes
30	64	1,800	Yes
30	64	2,400	Yes
30	70	1,200	Yes
30	70	1,350	Yes
30	70	1,400	Yes
30	70	1,800	Yes
30	70	2,400	Yes
30	85	1,200	Yes
30	85	1,350	Yes
30	85	1,400	Yes
30	85	1,800	Yes
30	85	2,400	Yes
33	30	1,200	Yes
33	30	1,350	Yes
33	30	1,400	Yes
33	30	1,800	Yes
33	30	2,400	Yes
33	36	1,200	Yes
33	36	1,350	Yes
33	36	1,400	Yes

33	36	1,800	Yes
33	36	2,400	Yes
33	42	1,200	Yes
33	42	1,350	Yes
33	42	1,400	Yes
33	42	1,800	Yes
33	42	2,400	Yes
33	45	1,200	Yes
33	45	1,350	Yes
33	45	1,400	Yes
33	45	1,800	Yes
33	45	2,400	Yes
33	53	1,200	Yes
33	53	1,350	Yes
33	53	1,400	Yes
33	53	1,800	Yes
33	53	2,400	Yes
33	60	1,200	Yes
33	60	1,350	Yes
33	60	1,400	Yes
33	60	1,800	Yes
33	60	2,400	Yes
33	64	1,200	Yes
33	64	1,350	Yes
33	64	1,400	Yes
33	64	1,800	Yes
33	64	2,400	Yes
33	70	1,200	Yes
33	70	1,350	Yes
33	70	1,400	Yes
33	70	1,800	Yes
33	70	2,400	Yes
33	85	1,200	Yes
33	85	1,350	Yes
33	85	1,400	Yes
33	85	1,800	Yes
33	85	2,400	Yes
36	30	1,200	Yes
36	30	1,350	Yes
36	30	1,400	Yes
36	30	1,800	Yes
36	30	2,400	Yes
36	36	1,200	Yes
36	36	1,350	Yes
36	36	1,400	Yes
36	36	1,800	Yes
36	36	2,400	Yes
36	42	1,200	Yes

36	42	1,350	Yes
36	42	1,400	Yes
36	42	1,800	Yes
36	42	2,400	Yes
36	45	1,200	Yes
36	45	1,350	Yes
36	45	1,400	Yes
36	45	1,800	Yes
36	45	2,400	Yes
36	53	1,200	Yes
36	53	1,350	Yes
36	53	1,400	Yes
36	53	1,800	Yes
36	53	2,400	Yes
36	60	1,200	Yes
36	60	1,350	Yes
36	60	1,400	Yes
36	60	1,800	Yes
36	60	2,400	Yes
36	64	1,200	Yes
36	64	1,350	Yes
36	64	1,400	Yes
36	64	1,800	Yes
36	64	2,400	Yes
36	70	1,200	Yes
36	70	1,350	Yes
36	70	1,400	Yes
36	70	1,800	Yes
36	70	2,400	Yes
36	85	1,200	Yes
36	85	1,350	Yes
36	85	1,400	Yes
36	85	1,800	Yes
36	85	2,400	Yes
40	30	1,200	Yes
40	30	1,350	Yes
40	30	1,400	Yes
40	30	1,800	Yes
40	30	2,400	Yes
40	36	1,200	Yes
40	36	1,350	Yes
40	36	1,400	Yes
40	36	1,800	Yes
40	36	2,400	Yes
40	42	1,200	Yes
40	42	1,350	Yes
40	42	1,400	Yes
40	42	1,800	Yes

40	42	2,400	Yes
40	45	1,200	Yes
40	45	1,350	Yes
40	45	1,400	Yes
40	45	1,800	Yes
40	45	2,400	Yes
40	53	1,200	Yes
40	53	1,350	Yes
40	53	1,400	Yes
40	53	1,800	Yes
40	53	2,400	Yes
40	60	1,200	Yes
40	60	1,350	Yes
40	60	1,400	Yes
40	60	1,800	Yes
40	60	2,400	Yes
40	64	1,200	Yes
40	64	1,350	Yes
40	64	1,400	Yes
40	64	1,800	Yes
40	64	2,400	Yes
40	70	1,200	Yes
40	70	1,350	Yes
40	70	1,400	Yes
40	70	1,800	Yes
40	70	2,400	Yes
40	85	1,200	Yes
40	85	1,350	Yes
40	85	1,400	Yes
40	85	1,800	Yes
40	85	2,400	Yes
45	30	1,200	Yes
45	30	1,350	Yes
45	30	1,400	Yes
45	30	1,800	Yes
45	30	2,400	Yes
45	36	1,200	Yes
45	36	1,350	Yes
45	36	1,400	Yes
45	36	1,800	Yes
45	36	2,400	Yes
45	42	1,200	Yes
45	42	1,350	Yes
45	42	1,400	Yes
45	42	1,800	Yes
45	42	2,400	Yes
45	45	1,200	Yes
45	45	1,350	Yes

45	45	1,400	Yes
45	45	1,800	Yes
45	45	2,400	Yes
45	53	1,200	Yes
45	53	1,350	Yes
45	53	1,400	Yes
45	53	1,800	Yes
45	53	2,400	Yes
45	60	1,200	Yes
45	60	1,350	Yes
45	60	1,400	Yes
45	60	1,800	Yes
45	60	2,400	Yes
45	64	1,200	Yes
45	64	1,350	Yes
45	64	1,400	Yes
45	64	1,800	Yes
45	64	2,400	Yes
45	70	1,200	Yes
45	70	1,350	Yes
45	70	1,400	Yes
45	70	1,800	Yes
45	70	2,400	Yes
45	85	1,200	Yes
45	85	1,350	Yes
45	85	1,400	Yes
45	85	1,800	Yes
45	85	2,400	Yes
49	30	1,200	Yes
49	30	1,350	Yes
49	30	1,400	Yes
49	30	1,800	Yes
49	30	2,400	Yes
49	36	1,200	Yes
49	36	1,350	Yes
49	36	1,400	Yes
49	36	1,800	Yes
49	36	2,400	Yes
49	42	1,200	Yes
49	42	1,350	Yes
49	42	1,400	Yes
49	42	1,800	Yes
49	42	2,400	Yes
49	45	1,200	Yes
49	45	1,350	Yes
49	45	1,400	Yes
49	45	1,800	Yes
49	45	2,400	Yes

49	53	1,200	Yes
49	53	1,350	Yes
49	53	1,400	Yes
49	53	1,800	Yes
49	53	2,400	Yes
49	60	1,200	Yes
49	60	1,350	Yes
49	60	1,400	Yes
49	60	1,800	Yes
49	60	2,400	Yes
49	64	1,200	Yes
49	64	1,350	Yes
49	64	1,400	Yes
49	64	1,800	Yes
49	64	2,400	Yes
49	70	1,200	Yes
49	70	1,350	Yes
49	70	1,400	Yes
49	70	1,800	Yes
49	70	2,400	Yes
49	85	1,200	Yes
49	85	1,350	Yes
49	85	1,400	Yes
49	85	1,800	Yes
49	85	2,400	Yes
50	30	1,200	Yes
50	30	1,350	Yes
50	30	1,400	Yes
50	30	1,800	Yes
50	30	2,400	Yes
50	36	1,200	Yes
50	36	1,350	Yes
50	36	1,400	Yes
50	36	1,800	Yes
50	36	2,400	Yes
50	42	1,200	Yes
50	42	1,350	Yes
50	42	1,400	Yes
50	42	1,800	Yes
50	42	2,400	Yes
50	45	1,200	Yes
50	45	1,350	Yes
50	45	1,400	Yes
50	45	1,800	Yes
50	45	2,400	Yes
50	53	1,200	Yes
50	53	1,350	Yes
50	53	1,400	Yes

50	53	1,800	Yes
50	53	2,400	Yes
50	60	1,200	Yes
50	60	1,350	Yes
50	60	1,400	Yes
50	60	1,800	Yes
50	60	2,400	Yes
50	64	1,200	Yes
50	64	1,350	Yes
50	64	1,400	Yes
50	64	1,800	Yes
50	64	2,400	Yes
50	70	1,200	Yes
50	70	1,350	Yes
50	70	1,400	Yes
50	70	1,800	Yes
50	70	2,400	Yes
50	85	1,200	Yes
50	85	1,350	Yes
50	85	1,400	Yes
50	85	1,800	Yes
50	85	2,400	Yes
60	30	1,200	Yes
60	30	1,350	Yes
60	30	1,400	Yes
60	30	1,800	Yes
60	30	2,400	Yes
60	36	1,200	Yes
60	36	1,350	Yes
60	36	1,400	Yes
60	36	1,800	Yes
60	36	2,400	Yes
60	42	1,200	Yes
60	42	1,350	Yes
60	42	1,400	Yes
60	42	1,800	Yes
60	42	2,400	Yes
60	45	1,200	Yes
60	45	1,350	Yes
60	45	1,400	Yes
60	45	1,800	Yes
60	45	2,400	Yes
60	53	1,200	Yes
60	53	1,350	Yes
60	53	1,400	Yes
60	53	1,800	Yes
60	53	2,400	Yes
60	60	1,200	Yes

60	60	1,350	Yes
60	60	1,400	Yes
60	60	1,800	Yes
60	60	2,400	Yes
60	64	1,200	Yes
60	64	1,350	Yes
60	64	1,400	Yes
60	64	1,800	Yes
60	64	2,400	Yes
60	70	1,200	Yes
60	70	1,350	Yes
60	70	1,400	Yes
60	70	1,800	Yes
60	70	2,400	Yes
60	85	1,200	Yes
60	85	1,350	Yes
60	85	1,400	Yes
60	85	1,800	Yes
60	85	2,400	Yes
65	30	1,200	Yes
65	30	1,350	Yes
65	30	1,400	Yes
65	30	1,800	Yes
65	30	2,400	Yes
65	36	1,200	Yes
65	36	1,350	Yes
65	36	1,400	Yes
65	36	1,800	Yes
65	36	2,400	Yes
65	42	1,200	Yes
65	42	1,350	Yes
65	42	1,400	Yes
65	42	1,800	Yes
65	42	2,400	Yes
65	45	1,200	Yes
65	45	1,350	Yes
65	45	1,400	Yes
65	45	1,800	Yes
65	45	2,400	Yes
65	53	1,200	Yes
65	53	1,350	Yes
65	53	1,400	Yes
65	53	1,800	Yes
65	53	2,400	Yes
65	60	1,200	Yes
65	60	1,350	Yes
65	60	1,400	Yes
65	60	1,800	Yes

65	60	2,400	Yes
65	64	1,200	Yes
65	64	1,350	Yes
65	64	1,400	Yes
65	64	1,800	Yes
65	64	2,400	Yes
65	70	1,200	Yes
65	70	1,350	Yes
65	70	1,400	Yes
65	70	1,800	Yes
65	70	2,400	Yes
65	85	1,200	Yes
65	85	1,350	Yes
65	85	1,400	Yes
65	85	1,800	Yes
65	85	2,400	Yes

[076] Liposomal irinotecan is preferably administered intravenously, in combination with oxaliplatin, 5-fluorouracil (5-FU) and leucovorin. In one embodiment, liposomal irinotecan is administered prior to oxaliplatin, 5-FU and leucovorin. In another embodiment, leucovorin is administered prior to 5-FU. In another embodiment, the MM-398 liposomal irinotecan is administered followed by administration of the oxaliplatin, followed by administration of the leucovorin, and followed by the administration of the 5-fluorouracil. In certain embodiments, the liposomal irinotecan is administered to the patient intravenously over 90 minutes. In another embodiment, the oxaliplatin is administered to the patient intravenously over 120 minutes. In another embodiment, 5-FU is administered intravenously over 46 hours. In one embodiment, the oxaliplatin is administered from about 6 to about 72 hours after administration of the liposomal irinotecan. In another embodiment, the oxaliplatin is administered for example, 6 hours, 12 hours, 24 hours, 36 hours, 48 hours, 60 hours, or 72 hours, after administration of the liposomal irinotecan. In another embodiment, leucovorin is administered intravenously over 30 minutes. In various embodiments the liposomal irinotecan is MM-398. In various embodiments, the human patient with gastric cancer is pre-medicated with dexamethasone and a 5-HT3 antagonist or other anti-emetic prior to administering the MM-398 liposomal irinotecan, and other active agents.

[077] Further embodiments of the invention

[078] The following methods and embodiments can be considered alone, in combination other embodiments in this section, or in combination with the methods disclosed above. The invention provides methods for treating gastric cancer in a human patient, such as in a patient not

previously treated with a chemotherapeutic agent in the metastatic setting, the method comprising administering to the patient liposomal irinotecan, also referred to as MM-398 (e.g., irinotecan sucrose octasulfate salt liposome injection) in combination with oxaliplatin, leucovorin and 5-FU.

[079] 1. A method for treating gastric cancer in a human subject who has not previously received chemotherapy to treat the gastric cancer, the method comprising: administering to the subject a therapeutically effective amount of MM-398 liposomal irinotecan in combination with oxaliplatin, leucovorin, and 5-FU to treat the gastric cancer in the human subject.

[080] 2. The method of embodiment 1, wherein the amount of MM-398 liposomal irinotecan administered is administered is 50 mg/m^2 (free base) or 55 mg/m^2 (free base).

[081] 3. A method for treating gastric cancer in a human subject who has not previously received chemotherapy to treat the gastric cancer, the method comprising: administering to the subject 60 mg/m^2 (salt) or 65 mg/m^2 (salt) of MM-398 liposomal irinotecan in combination with oxaliplatin, leucovorin, and 5-FU to treat the gastric cancer in the human subject.

[082] 4. The method of any one of embodiments 1-3, wherein the amount of oxaliplatin administered is from about 50 mg/m^2 to about 100 mg/m^2, such as about 60 mg/m^2 to about 85 mg/m^2, for example 60 mg/m^2, 70 mg/m^2, 75 mg/m^2, or 85 mg/m^2.

[083] 5. The method of any one of embodiments 1-4, wherein the leucovorin administered at a dosage of 400 mg/m^2 of the (*l*+*d*) racemic form, or 200 mg/m^2 of the (*l*) form.

[084] 6. The method of any one of embodiments 1-5, wherein the amount of 5-FU administered is 2,400 mg/m^2.

[085] 7. The method of any one of embodiments 1-6, wherein the MM-398 liposomal irinotecan, oxaliplatin, leucovorin, and 5-FU are administered at least once, such as wherein the MM-398, oxaliplatin, leucovorin, and 5-FU are administered on days 1 and 15 of a 28-day cycle.

[086] 8. The method of any one of embodiments 1-7, wherein multiple cycles are administered.

[087] 9. The method of any one of embodiments 1-8, wherein the oxaliplatin is administered to the patient prior to the leucovorin, such as wherein the leucovorin is administered to the patient prior to the 5-FU, optionally wherein the MM-398 liposomal irinotecan is administered to the patient prior to the oxaliplatin, leucovorin, and 5-FU.

[088] 10. The method of embodiment 9, wherein the MM-398 is administered over 90 minutes, followed by administration of the oxaliplatin over 120 minutes, followed by administration of the leucovorin over 30 minutes, followed by the administration of the 5-FU over 46 hours.

[089] In a particular embodiment, a human patient with gastric cancer who has not previously been treated with any chemotherapeutic agent in the metastatic setting, is treated with a combination regimen of the present disclosure, the method comprising, intravenously administering to the patient, beginning on day 1 of a 2-week cycle, 50 mg/m^2 of MM-398 liposomal irinotecan over 90 minutes, followed by 60-85 mg/m^2 oxaliplatin, followed by 200 mg/m^2 of the *(l)* form of leucovorin, or 400 mg/m^2 of the *(l+d)* racemic form of leucovorin, followed by 2,400 mg/m^2 5-FU, wherein the human patient is treated with one or multiple cycles. In another particular embodiment, a human patient with gastric cancer who has not previously been treated with any chemotherapeutic agent in the metastatic setting, is treated with a combination regimen of the present disclosure, the method comprising, intravenously administering to the patient, beginning on day 1 of a 2-week cycle, 55 mg/m^2 of MM-398 liposomal irinotecan over 90 minutes, followed by 60-85 mg/m^2 oxaliplatin, followed by 200 mg/m^2 of the *(l)* form of leucovorin, or 400 mg/m^2 of the *(l+d)* racemic form of leucovorin, followed by 2,400 mg/m^2 5-FU, wherein the human patient is treated with one or multiple cycles. In the embodiments disclosed herein, the effective amount of MM-398 liposomal irinotecan administered to the human patient can range from about 30 mg/m^2 to about 60 mg/m^2, for example, from about 40 mg/m^2 to about 50 mg/m^2, or from about 50 mg/m^2 to about 55 mg/m^2. In various embodiments, the amount of MM-398 liposomal irinotecan administered to the human patient is 50 mg/m^2. In various embodiments, the amount of MM-398 liposomal irinotecan administered to the human patient is 55 mg/m^2. In the embodiments disclosed herein, the effective amount of oxaliplatin administered to the human patient can range from about 40 mg/m^2 to about 100 mg/m^2, for example, from about 60 mg/m^2 to about 85 mg/m^2, or for example, from about 60 mg/m^2 to about 70 mg/m^2. In various embodiments, the amount oxaliplatin administered to the human patient is 60 mg/m^2, 70 mg/m^2, or 85 mg/m^2. In one variant of this embodiment, oxaliplatin is administered over 120 minutes, leucovorin is administered over 30 minutes, and 5-FU is administered over 46 hours.

[090] Examples

[091] Example 1: Evaluation of *in vivo* tolerability and efficacy of nal-IRI in gastric tumor models

[092] Anti-tumor activity of MM-398 was evaluated in MKN-45 and KATO III gastric tumor models. Mice bearing xenograft tumors were treated with saline, 25 mg/kg free irinotecan, 5 mg/kg MM-398, 10 mg/kg MM-398 or 20 mg/kg MM-398 given weekly for 4

weeks (Figures 1A and 1B). All doses were well tolerated. nal-IRI displays anti-tumor activity with tumor regression at 10 and 20 mg/kg.

[093] Example 2: Evaluation of *in vivo* tolerability and efficacy of combination therapies in an animal model

[094] Anti-tumor activity of MM-398 in comparison to free irinotecan in the context of triplet combination therapy with 5-FU and oxaliplatin was evaluated. Mice bearing MKN-45 xenograft tumors were treated with saline, 100 mg/kg 5-FU + 5 mg/kg oxaliplatin, 25 mg/kg free irinotecan, 5 mg/kg MM-398, the triplet of free irinotecan + 5-FU + oxaliplatin or MM-398 + 5-FU + oxaliplatin given weekly for 3 weeks. All groups were performed in the same study and are separated into two panels for visualization purposes. 5-FU was administered intraperitoneally while all other agents were administered intravenously; days of dosing are indicated by horizontal dashed lines; n=X per mice group (Figures 2A and 2B).

[095] Example 3: Tolerability of Antineoplastic Therapies in Human Clinical Trial

[096] The tolerability of antineoplastic therapies combining liposomal irinotecan, 5-FU/leucovorin and oxaliplatin was evaluated in a human clinical trial, using two different doses: 80 mg/m^2 (salt) of liposomal irinotecan (MM-398) and 60 mg/m^2 (salt) of liposomal irinotecan (MM-398). Table 2 summarizes four dosing regimens for the treatment of previously untreated (front-line) pancreatic cancer in humans over a 28 day treatment cycle.

[097] Table 2 Dose Table (MM-398 + 5-FU/LV + oxaliplatin)

Level	Oxaliplatin		5-FU/LV		MM-398 (nal-IRI)	
	Dose (mg/m^2)[a]	Dose Day[c]	Dose (mg/m^2)[b]	Dose Day[c]	Dose (mg/m^2) (salt)	Dose Day[c]
1	60	1, 15	2400/400	1, 15	80	1, 15
2	85	1, 15	2400/400	1, 15	60	1, 15
-2A	75	1, 15	2400/400	1, 15	60	1, 15
3	70	1, 15	2400/400	1, 15	65	1, 15

[a]First dose administration in conjunction with first dose of nal-IRI; oxaliplatin to be administered 2 hours after the completion of the nal-IRI infusion.

[b]46 hour infusion, no bolus is given; leucovorin and 5-FU will be administered last, following the completion of the oxaliplatin infusion.

[c]Day indicated is part of a 28-day cycle.

Note: The dose of nal-IRI and 5-FU/LV in Dose Level 1 and 2 above is the same dose and schedule that was previously used in the NAPOLI-1 Phase 3 study.

[098] Initially, a combination of oxaliplatin, MM-398 liposomal irinotecan, leucovorin and 5-fluorouracil was evaluated at dose level 1 in Table 2 above. The results are summarized in Table 3 for dose level 1 in Table 2 above (for 80 mg/m^2 (salt) MM-398 dose), showing that the 80 mg/m^2 (salt) dose of liposomal irinotecan (MM-398) in combination with oxaliplatin and 5-fluorouracil/leucovorin at dose level 1 was not tolerated in humans.

[099] Table 3: Antineoplastic Therapy with 80 mg/m^2 liposomal irinotecan (salt) in combination with oxaliplatin/5FU/leucovorin in human clinical trials

Patient	Cycle 1 Day 1	Cycle 1 Day 15	Cycle 2 Day 1	Cycle 2 Day 15	Cycle 3 Day 1	Cycle 3 Day 15	Cycle 4 Day 1	Cycle 4 Day 15
1	✓	✓	X	X	X	X	X	X
2	✓	R*	R2*	R2*	X	X	X	X
3	✓	X	X	X	X	X	X	X
4	✓	✓	X	X	X	X	X	X
5	✓	X	X	X	X	X	X	X
6	✓	✓	R1*	R1	X	R1	R1	X
7	✓	X	X	X	X	X	X	X

*Dosing held to allow for recovery from toxicity related to the study treatment.

[0100] Table 3 summarizes the results from treating a total of seven (7) patients diagnosed with pancreatic cancer.

[0101] A "check mark" (✓) in Table 3 indicates the patient received the antineoplastic therapy of dose level 1 in Table 2 above, starting on the indicated days of 3 consecutive 28-day treatment cycles: 80 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 60 mg/m^2 oxaliplatin, 400 mg/m^2 (*l+d*) leucovorin and 2,400 mg/m^2 5-fluorouracil, as described in the protocol of Example 4.

[0102] A "R" in Table 3 indicates the patient received a reduced dose of antineoplastic therapy of dose level -1 in Table 2 on the corresponding cycle and day: 60 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride

trihydrate salt), 60 mg/m^2 oxaliplatin, 400 mg/m^2 (*l*+*d*) leucovorin and 2,400 mg/m^2 5-fluorouracil, as described in the protocol of Example 4.

[0103] An "R1" in Table 3 indicates the patient received a reduced dose of antineoplastic therapy of dose level -1 in Table 2 (Example 3 above) on the corresponding cycle and day: 60 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 60 mg/m^2 oxaliplatin, 400 mg/m^2 (*l*+*d*) leucovorin and 2400 mg/m^2 5-fluorouracil, as described in the protocol of Example 3.

[0104] An "R2" in Table 3 indicates the patient received a reduced dose of antineoplastic therapy of dose on the corresponding cycle and day: 50 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 60 mg/m^2 oxaliplatin, 400 mg/m^2 (*l*+*d*) leucovorin and 1,200 mg/m^2 5-fluorouracil (a 50% reduction compared to dose level -1 dose), as described in the protocol of Example 3.

[0105] An "X" in Table 3 indicates the patient did not receive an antineoplastic therapy combining liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin, or combining liposomal irinotecan, oxaliplatin, and 5-fluorouracil. After cycle 1, day 1 and prior to cycle 1, day 15, patient 2 was determined to be homozygous for the UGT1A1*28 allele, and subsequent reduced doses of the antineoplastic therapy were administered on days indicated in Table 3, based on the protocol of Example 4. Patients 1 and 3-7 were not homozygous for UGT1A1*28 allele.

[0106] The antineoplastic therapy of dose level 1 in Table 2 (Example 3) was only administered to 3 of these 7 patients on day 15 of (28-day) cycle 1, no patients received dose level 1 for more than 2 consecutive doses, and none of the patients received this therapy after cycle 1.

[0107] Accordingly, as noted in the Table 3, antineoplastic therapies combining a dose of 80 mg/m^2 liposomal irinotecan (salt) with 60 mg/m^2 oxaliplatin and doses of 2,400 and 400 mg/m^2 of 5-fluorouracil and (*l*+*d*) leucovorin were not well tolerated in a human clinical trial (resulting in dose limiting toxicities). Examples of antineoplastic therapies combining a dose of 80 mg/m^2 liposomal irinotecan (salt) with 60 mg/m^2 oxaliplatin and doses of 2,400 and 400 mg/m^2 of 5-fluorouracil and (*l*+*d*) leucovorin include the therapies in Table 2.

[0108] In contrast, as noted in Table 4 below, antineoplastic therapies combining a dose of 60 mg/m^2 liposomal irinotecan (salt) with 60 mg/m^2 oxaliplatin and doses of 2,400 and 400 mg/m^2 of 5-fluorouracil and (*l*+*d*) leucovorin were tolerated in a human clinical trial. In particular, dose level -1 in Table 4 (a 60 mg/m^2 (salt) M-398 dose) was administered two or more consecutive times to multiple human patients in the clinical trial described in Example

4. These antineoplastic therapies comprising the reduced 60 mg/m^2 (salt) of liposomal irinotecan (MM-398) in combination with oxaliplatin and 5-fluorouracil/leucovorin were better tolerated in humans than dose level 1 (Figures 12-14). In other embodiments, patients are administered the therapy of dose level -2B or -3 in Table 4.

[0109] Table 4 Oxaliplatin Dose Table

Level	Oxaliplatin		5-FU/LV		MM-398 (nal-IRI)	
	Dose (mg/m^2)[a]	Dose Day[c]	Dose (mg/m^2)[b]	Dose Day[c]	Dose (mg/m^2)	Dose Day[c]
-1	60	1, 15	2400/400	1, 15	60	1, 15
1	60	1, 15	2400/400	1, 15	80	1, 15
-2B	85	1, 15	2400/400	1, 15	60	1, 15
-3	70	1, 15	2400/400	1, 15	65	1, 15

[a]First dose administration in conjunction with first dose of MM-398; oxaliplatin to be administered 2 hours after the completion of the nal-IRI.

[b]46 hour infusion, no bolus is given; leucovorin and 5-FU will be administered last, following the completion of the oxaliplatin infusion.

[c]Day indicated is part of a 28-day cycle.

[0110] Table 5: Antineoplastic Therapy with 60 mg/m^2 liposomal irinotecan (salt) in combination with oxaliplatin/5FU/leucovorin in human clinical trials

Patient	Cycle 1 Day 1	Cycle 1 Day 15	Cycle 2 Day 1	Cycle 2 Day 15	Cycle 3 Day 1	Cycle 3 Day 15	Cycle 4 Day 1	Cycle 4 Day 15	Additional Cycles
1	✓	✓	X	R3	R3	R3	R3	X	4 at R3
2	✓^	X	X	X	X	X	X	X	-
3	✓	✓	✓	✓	✓	✓	✓*	✓	11 (original dose) with some dose delay
4	✓	✓	✓	✓*	✓	✓	✓	✓	1 (original dose); 11 (R4) with some dose delay
5	✓	✓	X	X	X	R5	R6	R6	2 (R6)
6	✓	✓	✓	✓	✓	✓	✓	✓	5 (original dose) with some dose delay
7	✓	✓	✓	✓	X	X	X	X	-

* Dosing held to allow for recovery from toxicity related to the study treatment.

^ Subject deceased (disease related).

[0111] Table 5 summarizes the results from treating a total of seven (7) patients diagnosed with pancreatic cancer. A "check mark" (✓) in Table 5 indicates the patient received the antineoplastic therapy of dose level -1 in Table 4 above, starting on the indicated days of 3 consecutive 28-day treatment cycles: 60 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 60 mg/m^2 oxaliplatin, 400 mg/m^2 (*l+d*) leucovorin and 2,400 mg/m^2 5-fluorouracil, as described in the protocol of Example 4.

[0112] In contrast to the antineoplastic therapy of dose level 1 in Table 2, the antineoplastic therapy of dose level -1 in Table 2 (Example 3) was administered repeatedly to patients 3, 4, and 6 for at least 3 consecutive administrations (including 14 consecutive administrations for patient 6).

[0113] The antineoplastic therapy of dose level -1 in Table 4 (Example 3) was administered to 6 of 7 patients on days 1 and 15 of (28-day) cycle 1, and days 1 and 15 of (28 day) cycle 2, and to at least 4 of 7 patients in the study, with no dose limiting toxicities.

[0114] A "check mark" (✓) in Table 5 indicates the patient received the antineoplastic therapy of dose level -1 in Table 4 above, starting on the indicated days of 3 consecutive 28-day treatment cycles: 80 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 60 mg/m^2 oxaliplatin, 400 mg/m^2 (*l+d*) leucovorin and 2,400 mg/m^2 5-fluorouracil, as described in the protocol of Example 4.

[0115] An "R3" in Table 5 indicates the patient received a reduced dose of antineoplastic therapy of dose on the corresponding cycle and day: 50 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 60 mg/m^2 oxaliplatin, 400 mg/m^2 (*l+d*) leucovorin and 1,800 mg/m^2 5-fluorouracil (a 25% reduction compared to dose level -1 dose), as described in the protocol of Example 4. One patient in Table 5 received this reduced dose in response to Grade II symptoms (non-hematologic), but without a dose limiting toxicity.

[0116] An "R4" in Table 5 indicates the patient received a reduced dose of antineoplastic therapy of dose on the corresponding cycle and day: 60 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 45 mg/m^2 oxaliplatin, 400 mg/m^2 (*l+d*) leucovorin and 2400 mg/m^2 5-fluorouracil (a 50% reduction compared to dose level -1 dose), as described in the protocol of Example 3.

[0117] An "R5" in Table 5 indicates the patient received a reduced dose of antineoplastic therapy of dose on the corresponding cycle and day: 30 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt) (a 50% reduction compared to dose level -1 dose), 30 mg/m^2 oxaliplatin (a 50% reduction compared to dose level -1 dose), 197 mg/m^2 (*l*+*d*) leucovorin and 1200 mg/m^2 5-fluorouracil (a 50% reduction compared to dose level -1 dose), as described in the protocol of Example 3.

[0118] An "R6" in Table 5 indicates the patient received a reduced dose of antineoplastic therapy of dose on the corresponding cycle and day: 36 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 36 mg/m^2 oxaliplatin, 240 mg/m^2 (*l*+*d*) leucovorin and 1440 mg/m^2 5-fluorouracil, as described in the protocol of Example 4.

[0119] Accordingly, as noted in the Table 5, antineoplastic therapies combining a dose of 60 mg/m^2 liposomal irinotecan (salt) with 60 mg/m^2 oxaliplatin and doses of 2,400 and 400 mg/m^2 of 5-fluorouracil and (*l*+*d*) leucovorin were well tolerated in a human clinical trial.

[0120] An example of an antineoplastic therapies combining a dose of 60 mg/m^2 liposomal irinotecan with 85 mg/m^2 oxaliplatin and doses of 2,400 and 400 mg/m^2 of 5-fluorouracil and (*l*+*d*) leucovorin include the therapies in Table 2.

[0121] Table 6: Antineoplastic Therapy with 60 mg/m^2 liposomal irinotecan in combination with oxaliplatin/5FU/leucovorin in human clinical trials.

Patient	Cycle 1 Day 1	Cycle 1 Day 15	Cycle 2 Day 1	Cycle 2 Day 15	Cycle 3 Day 1	Cycle 3 Day 15	Cycle 4 Day 1	Cycle 4 Day 15	Additional Cycles (dose)
1	✓^	X	X	X	X	X	X	X	-
2	✓	✓	✓	✓	✓	✓	✓	✓	5 (original dose) with some delay; 2 additional with reduced oxaliplatin
3	✓	✓	✓	✓	✓	✓	✓	X	
4	✓^	X	X	X	X	X	X	X	-
5	✓	X	X	X	X	X	X	X	-
6	✓	✓	R7*	R7	X	R7	R7	R7	3 (original dose) with some delay
7	✓	X	X	X	X	X	X	X	-
8	✓	R7*	R7	R7	X	X	X	X	-
9	✓	✓	✓	✓	✓	✓	✓	✓	2 (original

									dose) with some delay
10	✓	X	R8	R8	X	X	X	X	-

* Dosing held to allow for recovery from toxicity related to the study treatment.

^ Subject deceased (disease related).

[0122] Table 6 summarizes the preliminary clinical results from treating a total of ten (10) patients diagnosed with pancreatic cancer.

[0123] A "check mark" (✓) in Table 6 indicates the patient received the antineoplastic therapy of dose level -2B in Table 4 above, starting on the indicated days of 3 consecutive 28-day treatment cycles: 60 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 85 mg/m^2 oxaliplatin, 400 mg/m^2 (*l*+*d*) leucovorin and 2,400 mg/m^2 5-fluorouracil, as described in the protocol of Example 3.

[0124] An "R7" in Table 6 indicates the patient received a reduced dose of antineoplastic therapy of dose on the corresponding cycle and day: 50 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 85 mg/m^2 oxaliplatin, 400 mg/m^2 (*l*+*d*) leucovorin and 1,800 mg/m^2 5-fluorouracil (a 25% reduction compared to dose level -2B dose), as described in the protocol of Example 3.

[0125] An "R8" in Table 6 indicates the patient received a reduced dose of antineoplastic therapy of dose on the corresponding cycle and day: 50 mg/m^2 liposomal irinotecan (MM-398, dose based on the corresponding amount of irinotecan hydrochloride trihydrate salt), 60 mg/m^2 oxaliplatin, 400 mg/m^2 (*l*+*d*) leucovorin and 1,800 mg/m^2 5-fluorouracil (a 25% reduction compared to dose level -2B dose), as described in the protocol of Example 3.

[0126] An "X" in Table 6 indicates the patient did not receive an antineoplastic therapy combining liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin, or combining liposomal irinotecan, oxaliplatin, and 5-fluorouracil.

[0127] Example 4: Treatment of Gastric Cancer

[0128] As schematically shown in Figure 9, the present study is an open-label, phase 3 comparative study to assess the safety, tolerability, and efficacy of MM-398 in combination with other anticancer therapies, compared to mFOLFOX6, in patients with gastric cancer who have not received prior chemotherapy. This study assesses the following regimens: (1) MM-398 + 5-FU/LV + oxaliplatin (Arm 1) and (2) 5-FU/LV + oxaliplatin (Arm 2).

[0129] This phase 3 study evaluates the efficacy of oxaliplatin + 5-FU/LV with or without MM-398 in patients with previously untreated gastric cancer. The study may also

provide important information on the impact of MM-398 combination treatment on patient HRQL and identify potential biomarkers of response.

[0130] In the study, MM-398 is administered instead of conventional irinotecan to improve the safety, tolerability, and ultimately efficacy of a FOLFIRINOX regimen. The addition of oxaliplatin to the NAPOLI-1 regimen is included to increase DNA damage and potentiate efficacy. Further, due to the MM-398 prolonged PK properties and sustained tumor exposure, using MM-398 instead of conventional irinotecan is designed to further improve upon the efficacy of FOLFIRINOX.

[0131] A modified triplet combination regimen of liposomal irinotecan, oxaliplatin, 5-fluorouracil (5-FU)/leucovorin is provided herein, whereby no bolus of 5-FU will be administered. The target dose of oxaliplatin, 60, 70, or 85 mg/m^2, is evaluated in the Arm 1 combination regimen with the continuous infusion dose of 5-FU (excluding the bolus), and the every 2 week dose of MM-398 previously shown to be tolerable and efficacious in combination with 5-FU. Note that with MM-398 dosing, the C_{max} of SN-38 is expected to be lower than would be expected for standard dosing with free irinotecan.

[0132] Based on previous experience with irinotecan, individuals who are homozygous for the UGT1A1*28 allele (UGT1A1 7/7 genotype) are at increased risk for neutropenia following initiation of irinotecan treatment. According to the prescribing information for irinotecan, in a study of 66 patients who received single-agent irinotecan (350 mg/m^2 once every-3-weeks), the incidence of grade 4 neutropenia in patients homozygous for the UGT1A1*28 allele was as high as 50%, and in patients heterozygous for this allele (UGT1A1 6/7 genotype) the incidence was 12.5%. Importantly, no grade 4 neutropenia was observed in patients homozygous for the wild-type (WT) allele (UGT1A1 6/6 genotype). In other studies, a lower prevalence of accompanying life threatening neutropenia is described (for details refer to the prescribing information for irinotecan). Population PK studies of MM-398 have not identified a relationship between UGT1A1*28 homozygosity and increased SN-38 exposure (see Investigator Brochure). In a Phase I study, no differences in toxicity were seen in cohorts of heterozygous or WT patients, and DLTs of diarrhea with or without accompanying dehydration or fatigue, were seen in both cohorts. For these reasons, and because the prevalence of UGT1A1*28 homozygosity is relatively low, testing results are not required prior to the first dose of MM-398 on this study and the starting dose for all patients will be 60 mg/m^2 (salt) which is equivalent to 50 mg/m^2 (free base). However, if patients are known to be homozygous for UGT1A1*28, the dose of MM-398 may be reduced as described herein.

[0133] Patients will be randomized to treatment (1:1:1) to either MM-398 + 5-FU/LV + oxaliplatin or 5-FU/LV + oxaliplatin. The randomization is stratified based on region (East Asia vs. rest of the world) and performance status (ECOG 0 vs. 1).

[0134] The following adverse events are common (≥ 40%) with past oxaliplatin treatment in combination with 5-FU/LV and are to be expected with the MM-398-containing combination regimen: peripheral sensory neuropathy, neutropenia, thrombocytopenia, anemia, nausea, increases in transaminases and alkaline phosphatase, diarrhea, fatigue, emesis, and stomatitis. Additional adverse events may be anticipated, as described in the package insert for oxaliplatin, including allergic and anaphylactic reactions. In a Phase 3 study of the FOLFIRINOX combination, the most common (> 5%) Grade 3-4 adverse events were: neutropenia, fatigue, vomiting, diarrhea, thrombocytopenia, sensory neuropathy, anemia, elevated alanine aminotransferase (ALT) level, thromboembolism, and febrile neutropenia. Considering these expected toxicities, Arm 1 is evaluated for safety and tolerability.

[0135] A dose of oxaliplatin of 70 mg/m^2 or 85 mg/m^2 is the target dose for this study. The study will confirm whether these doses are compatible when MM-398 is used instead of conventional irinotecan. In case there are any unexpected toxicities, patients may be treated at a lower dose of oxaliplatin (60 mg/m^2).

[0136] Table 7 Arm 1 Dosing of (MM-398 + 5-FU/LV + oxaliplatin)

Oxaliplatin		5-FU/LV		MM-398 (nal-IRI)	
Dose (mg/m^2)[a]	Dose Day[c]	Dose (mg/m^2)[b]	Dose Day[c]	Dose (mg/m^2)	Dose Day[c]
60	1, 15	2400/400	1, 15	50	1, 15
85	1, 15	2400/400	1, 15	50	1, 15
70	1, 15	2400/400	1, 15	55	1, 15

[a]First dose administration in conjunction with first dose of MM-398; oxaliplatin to be administered 2 hours after the completion of the nal-IRI infusion.

[b]46 hour infusion, no bolus is given; leucovorin and 5-FU will be administered last, following the completion of the oxaliplatin infusion

[c]Day indicated is part of a 28-day cycle

[0137] Arm 1: MM-398 + 5-FU/LV + oxaliplatin

[0138] The order of the infusions to be administered in the clinic is as follows: MM-398 administered first, followed by oxaliplatin, then LV, followed by 5-FU.

[0139] Patients receive the oxaliplatin infusion 2 hours after the completion of the MM-398 infusion. If no infusion reactions are seen, patients can receive oxaliplatin directly after completion of the MM-398 infusion. If any grade 3 or higher infusion reactions are seen in patients, the DSMB may elect to revert back to administration of oxaliplatin two hours after the completion of the MM-398 infusion.

[0140] Arm 2: 5-FU/LV + oxaliplatin

[0141] Patients receive the oxaliplatin (85 mg/m^2) infusion, followed by leucovorin and 5-FU (400 mg/m^2 IV bolus + 2400 mg/m^2 as 46 h infusion/400 mg/m^2).

[0142] Premedication

[0143] All patients must be premedicated prior to MM-398 infusion, 5-FU/LV infusion, and oxaliplatin infusion with standard doses of dexamethasone and a 5-HT3 antagonist, or equivalent other anti-emetics according to standard institutional practices for irinotecan, 5-FU, and oxaliplatin administration, or the Summary of Product Characteristics (SmPC) for sites located in the European Union (EU). Atropine may be prescribed prophylactically for patients who experienced acute cholinergic symptoms in the previous cycles.

[0144] Doses and Administration of MM-398 (Arm 1)

[0145] MM-398 is administered by intravenous (IV) infusion over 90 minutes (±10 minutes) every two weeks. The first cycle Day 1 is a fixed day; subsequent doses should be administered on the first day of each cycle +/- 2 days.

[0146] Prior to administration, the appropriate dose of MM-398 must be diluted in 5% Dextrose Injection solution (D5W) or normal saline to a final volume of 500 mL. Care should be taken not to use in-line filters or any diluents other than D5W or normal saline. MM-398 can be administered at a rate of up to 1 mL/sec (30 mg/sec).

[0147] The actual dose of MM-398 to be administered will be determined by calculating the patient's body surface area at the beginning of each cycle. A +/- 5% variance in the calculated total dose will be allowed for ease of dose administration. Since MM-398 vials are single-use vials, site staff must not store any unused portion of a vial for future use and they must discard unused portions of the product.

[0148] Doses and Administration of 5-FU and Leucovorin (Arms 1 and 2)

[0149] Leucovorin is administered at a dose of 400 mg/m^2 of the (*l* + *d*)- racemic form, or (*l*) form 200 mg/m^2, as an IV infusion over 30 minutes (±5 minutes), on Days 1 and 15 of each 28-day cycle

[0150] 5-FU is administered at a dose of 2400 mg/m^2 as an IV infusion over 46-hours (±60 minutes), on Days 1 and 15 of each 28-day cycle. In Arm 2, a 400 mg/m^2 IV bolus of 5-FU is administered in addition to the 46 hour infusion.

[0151] Leucovorin should be reconstituted per the instructions on the package insert, SmPC or standard institutional guidelines for reconstitution of leucovorin.

[0152] Leucovorin should be administered prior to the 5-FU infusion and may be given concurrently with oxaliplatin. Actual dose of 5-FU and leucovorin to be administered is determined by calculating the patient's body surface area prior to each cycle. A +/- 5% variance in the calculated total dose will be allowed for ease of dose administration.

[0153] Doses and Administration of Oxaliplatin (Arms 1 and 2)

[0154] Oxaliplatin is administered at a dose of 70 mg/m^2 or 85 mg/m^2, IV over 120 minutes (±10 minutes), on Days 1 and 15 of each 28-day cycle (if target dose is confirmed in accordance with methods described herein). If the target dose is not tolerated, the dosage of oxaliplatin can be adjusted to 60 mg/m^2.

[0155] Oxaliplatin should be prepared according to the instructions on the package insert, SmPC or per standard institutional guidelines for preparation and administration of oxaliplatin.

[0156] Oxaliplatin should be administered following MM-398 infusion in Arm 1. Actual dose of oxaliplatin to be administered is determined by calculating the patient's body surface area prior to each cycle. A +/- 5% variance in the calculated total dose is allowed for ease of dose administration.

[0157] Dose Limiting Toxicities (DLTs)

[0158] For MM-398 administered in combination with 5-FU/LV and oxaliplatin, the following adverse events are considered as dose limiting toxicities (DLTs) if they occur during the first cycle of treatment and are deemed related to the study treatment regimen:

[0159] Grade 4 neutropenia or thrombocytopenia that does not resolve within 7 days despite optimal therapy (withholding study drug and administering concomitant medication, e.g. G-CSF administration for neutropenia);

[0160] Grade 4 neutropenia complicated by fever ≥ 38.5 °C (i.e. febrile neutropenia) and/or Grade 3 neutropenia with infection;

[0161] Inability to begin subsequent treatment course within 14 days of the scheduled date, due to drug-related toxicity; and

[0162] Any grade 4 non-hematologic toxicity with the specific exclusion of: Fatigue/asthenia < 2 weeks in duration, increases in alkaline phosphatase level, nausea and vomiting ≤3 days duration (only considered dose limiting if they last > 72 hours after treatment with an optimal anti-emetic regimen), and diarrhea ≤3 days duration (only considered dose limiting if diarrhea lasts > 72 hours after treatment with an optimal anti-diarrheal regimen)

[0163] Any toxicity that is related to disease progression will not be considered a DLT.

[0164] The safety assessment period for purposes of DLT evaluation and dose escalation decisions is one cycle of treatment (i.e. 28 days; or 14 days after the 2nd dose of study treatment if there is a treatment delay according as described herein). The dose can escalate to the next level only after the safety data have been evaluated at the current dose level (once the last patient enrolled in the cohort completes the first cycle of treatment) and the criteria for safety and tolerability of the optimal dose have not been exceeded. In addition, any drug-related toxicities of Grade 3 or higher that arise after Cycle 1 (if applicable) are assessed for their potential relationship to cumulative MM-398 or combination therapy doses and considered in the decision to escalate the dose. PK data may be available, but is not be required for decisions on dose escalation.

[0165] Table 8

Inclusion Criteria	Exclusion Criteria
In order for inclusion into the study, patients must have/be: Pathologically confirmed gastric cancer that has not been previously treated in the metastatic setting Measurable or non-measurable disease as defined by RECIST v1.1 ECOG performance status of 0 or 1 Adequate biological parameters as evidenced by the following blood counts: ANC > 1,500 cells/μl without the use of hematopoietic growth factors,	Patients must meet all the inclusion criteria and none of the following exclusion criteria: Prior treatment of gastric cancer in the metastatic setting with surgery, radiotherapy, chemotherapy or investigational therapy Prior treatment of gastric cancer with cytotoxic doses of chemotherapy (patients receiving prior treatment with chemotherapy as a radiation sensitizer are eligible if ≥ 6 months has elapsed from completion of therapy) Known metastasis to the central nervous system Clinically significant gastrointestinal disorder including hepatic disorders, bleeding, inflammation, occlusion, diarrhea > grade 1, malabsorption syndrome, ulcerative

Inclusion Criteria	Exclusion Criteria
Platelet count > 100,000 cells/µl, and Hemoglobin > 9 g/dL Adequate hepatic function as evidenced by: Serum total bilirubin ≤ ULN (biliary drainage is allowed for biliary obstruction), and AST and ALT ≤ 2.5 x ULN (≤ 5 x ULN is acceptable if liver metastases are present) Adequate renal function as evidenced by serum creatinine ≤ 1.5 x ULN, and calculated clearance ≥60 mL/min/1.72 m^2 for patients with serum creatinine levels above or below the institutional normal value. Actual body weight should be used for calculating creatinine clearance using the Cockcroft-Gault Equation (CreatClear = Sex * ((140 - Age) / (SerumCreat)) * (Weight / 72); for patients with body mass index (BMI) >30 kg/m^2, lean body weight should be used instead. Normal ECG or ECG without any clinically significant findings Recovered from the effects of any prior surgery or radiotherapy ≥ 18 years of age Agreeable to submit unstained archived tumor tissue for analysis, if available Able to understand and sign an informed consent (or have a legal representative who is able to do so)	colitis, inflammatory bowel disease, or partial bowel obstruction 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. Patients with a history of other malignancies are eligible if they have been continuously disease free for at least 3 years. Known hypersensitivity to any of the components of MM-398, other liposomal products, or any components of 5-FU, leucovorin or oxaliplatin Known hypersensitivity to any of the components of nab-paclitaxel or gemcitabine (Part 2 only) Concurrent illnesses that would be a relative contraindication to trial participation such as active cardiac or liver disease, including: Severe arterial thromboembolic events (myocardial infarction, unstable angina pectoris, stroke) less than 6 months before inclusion NYHA Class III or IV congestive heart failure, ventricular arrhythmias or uncontrolled blood pressure Known historical or active infection with HIV, hepatitis B, or hepatitis C Active infection or an unexplained fever > 38.5°C during screening visits or on the first scheduled day of dosing (at the discretion of the investigator, patients with tumor fever may be enrolled), which in the investigator's opinion might compromise the patient's participation in the trial or affect the study outcome Use of strong CYP3A4 inhibitors or inducers, or presence of any other contraindications for irinotecan Presence of any contraindications for 5-FU, leucovorin, or oxaliplatin Use of strong CYP2C8 inhibitors or inducers, or presence of any other contraindications for nab-paclitaxel or gemcitabine (Part 2 only)

Inclusion Criteria	Exclusion Criteria
	Any other medical or social condition deemed by the Investigator to be likely to interfere with a patient's ability to sign informed consent, cooperate and participate in the study, or interfere with the interpretation of the results Pregnant or breast feeding; females of child-bearing potential must test negative for pregnancy at the time of enrollment based on a urine or serum pregnancy test. Both male and female patients of reproductive potential must agree to use a highly effective method of birth control, during the study and for 3 months following the last dose of study drug.

[0166] Dose Modifications

[0167] The toxicity of each cycle must be recorded prior to the administration of a subsequent cycle and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) (Version 4.03). All dose reductions for all arms should be based on the worst preceding toxicity.

[0168] Dosing may be held for up to 2 weeks from when it was due to allow for recovery from toxicity related to the study treatment. If the time required for recovery from toxicity is more than 2 weeks, the patient should be discontinued from the study, unless the patient is benefiting from the study treatment, in which case the patient's continuation on study should be discussed between Investigator and Sponsor regarding risks and benefits of continuation. If oxaliplatin is not well tolerated in patients enrolled in Arm 1, oxaliplatin may be discontinued and patients may continue to receive MM-398 + 5-FU/LV at the discretion of the Investigator.

[0169] If a patient's dose is reduced during the study due to toxicity, it should remain reduced for the duration of the study; dose re-escalation to an earlier dose is not permitted. Any patient who has 2 dose reductions and experiences an adverse event that would require a third dose reduction must be discontinued from study treatment.

[0170] Dose Modifications

[0171] Prior to each dosing, patients must have: ANC $\geq$ 1500/mm^3, WBC $\geq$ 3500/ mm^3, Platelet count $\geq$ 100,000/mm^3 and Diarrhea $\leq$ Grade 1.

[0172] Treatment should be delayed to allow sufficient time for recovery to levels noted above, and upon recovery, treatment should be administered according to the guidelines in the tables below. If the patient had febrile neutropenia, the ANC must have resolved to ≥ 1500/mm^3 and the patient must have recovered from infection. For Grade 3 or 4 non-hematological toxicities, treatment should be delayed until they resolve to Grade 1 or baseline. Guidelines for dose adjustments of each individual treatment within the regimen are found in the tables below. In case a patient experiences an infusion reaction, either institutional guidelines or the guidelines provided for infusion reaction management should be followed.

[0173] For all tables below, patient should be withdrawn from study treatment if more than 2 dose reductions are required or if MM-398 reductions lower than 30 mg/m^2 are required. No dose adjustments for toxicity are required for leucovorin. Leucovorin must be given immediately prior to each 5-FU dose; hence, if 5-FU dose is held, leucovorin dose should be held as well.

[0174] Treatment discontinuation that is required due to MM-398 or 5-FU toxicity will result in discontinuation from the study. However, for Arm 1, toxicity that requires discontinuation from oxaliplatin only (e.g. neuropathy) will result in the option to continue on study treatment with MM-398 + 5-FU/LV only for all future dosing.

[0175] The starting dose of ONIVYDE will be either 50 mg/m^2 or 55 mg/m^2, 5FU 2400 mg/m^2, LV 400 mg/m^2 and oxaliplatin will be 85 mg/m^2, 70 mg/m^2 or 60 mg/m^2. Dose reduction will be 25% reduction in all agents for any grade III-IV Hematotoxicity. For persistent toxicities despite the first dose reduction, and additional 25% dose reduction in all agents will occur. Further toxicity will then lead to discontinuation from trial.

[0176] For non-hematologic toxicities, the dose reduction will be the same dose reduction schema as for hematotoxicity, except for the specific toxicities associated with the drug (i.e. 5-FU hand foot syndrome, and oxaliplatin neuropathy) which will be as shown in Table 3.

[0177] Table 9: Arm 1 Dose Modifications

Worst Toxicity by CTCAE Grade	Nal-IRI (MM-398)	5-FU	Oxaliplatin
Hematological Toxicities			
Grade 2 hematotoxicity	100 % of previous dose		
Grade 3 or 4 neutropenia (ANC ≤ 1000/mm^3) or febrile	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)		

Worst Toxicity by CTCAE Grade	Nal-IRI (MM-398)	5-FU	Oxaliplatin
neutropenia and/or thrombocytopenia[a]			
Other Grade 3 or 4 hematologic toxicities not specifically listed above	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)		
Non-Hematological Toxicities Other than Asthenia and Grade 3 Anorexia			
Grade 1 or 2, including diarrhea	100 % of previous dose	100% of previous dose, except for Grade 2 hand foot syndrome, Grade 2 cardiac toxicity, or any grade neurocerebellar toxicity	100 % of previous dose, except for Grade 2 sensory neuropathy
Grade 3 or 4, including diarrhea (except nausea and vomiting)[d]	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose) *except for Grade 3 or 4 hand foot syndrome	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)
Grade 3 or 4 nausea and/or vomiting despite anti-emetic therapy[d]	Optimize anti-emetic therapy AND 1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)		
Grade 2 hand foot syndrome	100 % of previous dose	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)	100 % of previous dose
Grade 3 or 4 hand foot syndrome	100 % of previous dose	Discontinue therapy	100 % of previous dose
Any grade neurocerebellar or ≥ Grade 2 cardiac toxicity	100 % of previous dose	Discontinue therapy	100 % of previous dose

Worst Toxicity by CTCAE Grade	Nal-IRI (MM-398)	5-FU	Oxaliplatin
Sensory neuropathy	100 % of previous dose No dose modifications required	100 % of previous dose No dose modifications required	Grade 2, persistent: Reduce dose from 85 mg/m^2 to 60 mg/m^2 or from 60 mg/m^2 to 45 mg/m^{2}by 25% Grade 3, recovers prior to next cycle: Reduce dose from 85 mg/m^2 to 60 mg/m^2 or from 60 mg/m^2 to 45 mg/m^{2}by 25% Grade 3, persistent: Discontinue therapy Grade 4: Discontinue therapy

[a]Consider the use of G-CSF for patients who experience ≥ Grade 3 neutropenia or febrile neutropenia.

[b]Asthenia and Grade 3 Anorexia do not require dose modification

[c]Grade 1 diarrhea: 2-3 stools/day > pretreatment; Grade 2 diarrhea: 4-6 stools/day > pretreatment

[d]Grade 3 diarrhea: 7-9 stools/day > pretreatment; Grade 4 diarrhea: > 10 stools/day > pretreatment

[0178] Infusion reactions will be monitored. Infusion reactions will be defined according to the National Cancer Institute CTCAE (Version 4.0) definition of an allergic reaction/infusion reaction and anaphylaxis, as defined below:

[0179] Table 10

Grade 1: Transient flushing or rash, drug fever <38° C (<100.4° F); intervention not indicated
Grade 2: Intervention or infusion interruption indicated; responds promptly to symptomatic treatment (e.g., antihistamines, NSAIDS, narcotics); prophylactic medications indicated for <24 hours
Grade 3: Symptomatic bronchospasm, with or without urticaria; parenteral intervention indicated; allergy-related edema/angioedema; hypotension
Grade 4: Life-threatening consequences; urgent intervention indicated

[0180] Study site policies or the following treatment guidelines shall be used for the management of infusion reactions.

[0181] Table 11

Grade 1 Slow infusion rate by 50% Monitor patient every 15 minutes for worsening of condition
Grade 2 Stop infusion Administer diphenhydramine hydrochloride 50 mg IV, acetaminophen 650 mg orally, and oxygen Resume infusion at 50% of the prior rate once infusion reaction has resolved Monitor patient every 15 minutes for worsening of condition For all subsequent infusions, premedicate with diphenhydramine hydrochloride 25-50 mg IV
Grade 3 Stop infusion and disconnect infusion tubing from patient Administer diphenhydramine hydrochloride 50 mg IV, dexamethasone 10 mg IV, bronchodilators for bronchospasm, and other medications or oxygen as medically necessary No further treatment with MM-398 will be permitted
Grade 4 Stop the infusion and disconnect infusion tubing from patient Administer epinephrine, bronchodilators or oxygen as indicated for bronchospasm Administer diphenhydramine hydrochloride 50 mg IV, dexamethasone 10 mg IV Consider hospital admission for observation No further treatment with MM-398 will be permitted

[0182] For patients who experience a Grade 1 or Grade 2 infusion reaction, future infusions may be administered at a reduced rate (over 120 minutes), with discretion.

[0183] For patients who experience a second grade 1 or 2 infusion reaction, administer dexamethasone 10 mg IV. All subsequent infusions should be premedicated with diphenhydramine hydrochloride 50 mg IV, dexamethasone 10 mg IV, and acetaminophen 650 mg orally.

[0184] MM-398 Dose Modifications for Hematological Toxicities

[0185] Prior to initiating a new cycle of therapy, the patients must have:

[0186] ANC $\geq$ 1500/mm^3

[0187] Platelet count $\geq$ 100,000/mm^3

[0188] Treatment should be delayed to allow sufficient time for recovery and upon recovery, treatment should be administered according to the guidelines in the tables below. If the patient had febrile neutropenia, the ANC must have resolved to $\geq$ 1500/mm^3 and the patient must have recovered from infection.

[0189] Table 12: MM-398 Dose Modifications for Neutrophil Count

ANC: cells/mm^3 (Worst CTCAE grade)	MM-398 Dose for Next Cycle		
	Arm A: Patients Not Homozygous for UGT1A1*28	Arm A: Patients Homozygous for UGT1A1*28 Arm C: Patients Not Homozygous for UGT1A1*28	Arm C: Patients Homozygous for UGT1A1*28
$\geq$ 1000 to 1999 (Grade 1 or 2)	100% of previous dose	100% of previous dose	100% of previous dose
< 1000 (Grade 3/4) or febrile neutropenia	Reduce dose by 20 mg/m^2 to a minimum dose of 30 mg/m^2	Reduce dose to 40 mg/m^2 for the first occurrence and to 30 mg/m^2 for the second occurrence	Reduce dose to 40 mg/m^2 for the first occurrence and to 30 mg/m^2 for the second occurrence

[0190] Table 13: MM-398 Dose Modifications for Other Hematologic Toxicity

Worst Toxicity CTCAE Grade	MM-398 Dose for Next Cycle		
	Arm A: Patients Not Homozygous for UGT1A1*28	Arm A: Patients Homozygous for UGT1A1*28 Arm C: Patients Not Homozygous for UGT1A1*28	Arm C: Patients Homozygous for UGT1A1*28
$\leq$ Grade 2	100% of previous dose	100% of previous dose	100% of previous dose
Grade 3/4	Reduce dose by 20 mg/m^2 to a minimum dose of 30 mg/m^2	Reduce dose to 40 mg/m^2 for the first occurrence and to 30 mg/m^2 for the second occurrence	Reduce dose to 40 mg/m^2 for the first occurrence and to 30 mg/m^2 for the second occurrence

[0191] MM-398 Dose Modifications for Non-Hematological Toxicities

[0192] Treatment should be delayed until diarrhea resolves to ≤ Grade 1, and for other Grade 3 or 4 non-hematological toxicities, until they resolve to Grade 1 or baseline. Guidelines for dose adjustment of MM-398 for drug related diarrhea and other Grade 3 or 4 non-hematological toxicities are provided below. Infusion reactions should be handled as described above.

[0193] Table 14: MM-398 Dose Modifications for Diarrhea

Worst Toxicity CTCAE Grade	MM-398 Dose for Next Cycle[a]		
	Arm A: Patients Not Homozygous for UGT1A1*28	Arm A: Patients Homozygous for UGT1A1*28 Arm C: Patients Not Homozygous for UGT1A1*28	Arm C: Patients Homozygous for UGT1A1*28
Grade 1 or 2 (2-3 stools/day > pretreatment or 4-6 stools/day > pretreatment)	100% of previous dose	100% of previous dose	100% of previous dose
Grade 3 (7-9 stools/day > pretreatment) or Grade 4 (>10 stools/day > pretreatment)	Reduce dose by 20 mg/m^2 to a minimum dose of 30 mg/m^2	Reduce dose to 40 mg/m^2 for the first occurrence and to 30 mg/m^2 for the second occurrence	Reduce dose to 40 mg/m^2 for the first occurrence and to 30 mg/m^2 for the second occurrence

[0194] Table 15: MM-398 Dose Modifications for Non-Hematological Toxicities Other than Diarrhea, Asthenia and Grade 3 Anorexia

Worst Toxicity CTCAE Grade	MM-398 Dose for Next Cycle		
	Arm A: Patients Not Homozygous for UGT1A1*28	Arm A: Patients Homozygous for UGT1A1*28 Arm C: Patients Not Homozygous for UGT1A1*28	Arm C: Patients Homozygous for UGT1A1*28
Grade 1 or 2	100% of previous	100% of previous dose	100% of previous dose

	dose		
Grade 3 or 4 (except nausea and vomiting)	Reduce dose by 20 mg/m^2 to a minimum dose of 30 mg/m^2	Reduce dose to 40 mg/m^2 for the first occurrence and to 30 mg/m^2 for the second occurrence	Reduce dose to 40 mg/m^2 for the first occurrence and to 30 mg/m^2 for the second occurrence
Grade 3 or 4 nausea and or vomiting despite anti emetic therapy	Optimize anti-emetic therapy AND reduce dose by 20 mg/m^2 to a minimum dose of 30 mg/m^2	Optimize anti-emetic therapy AND reduce dose to 30 mg/m^2	Optimize anti-emetic therapy AND reduce dose to 30 mg/m^2

[0195] 5-FU and Leucovorin Dose Modifications:

[0196] Guidelines for 5-FU dose modifications are provided below. No dose adjustments for toxicity are required for leucovorin. Leucovorin must be given immediately prior to each 5-FU dose; hence, if 5-FU dose is held, leucovorin dose should be held as well. In case a patient experiences an infusion reaction, either institutional guidelines or the guidelines provided for MM-398 infusion reaction management should be used.

[0197] 5-FU Dose Modifications for Hematological Toxicities

[0198] Prior to the next dose in a cycle or prior to initiating a new cycle of therapy, the patients must have:

[0199] ANC $\geq 1500/mm^3$

[0200] WBC $\geq 3500/mm^3$

[0201] Platelet count $\geq 75,000/mm^3$ (according to the European summary of product characteristics for 5-FU, the platelets should have recovered to $\geq 100,000/mm^3$ prior to initiating therapy)

[0202] Treatment should be delayed to allow sufficient time for recovery and upon recovery, treatment should be administered according to the guidelines provided in the table below.

[0203] Table 16 5-FU Dose Modifications for Hematological Toxicities (Arm B & C)

ANC ($cells/mm^3$)		Platelets ($cells/mm^3$)	5-FU Dose for D8, D15, D22[a]	5-FU Dose for Next Cycle[a]
≥ 1000	and	$\geq 50,000$	100% of previous dose	100% of previous dose

500 - 999	or	<50,000 – 25,000	Hold; when resolved, reduce dose by 25% [b]	Reduce dose by 25%[b]
< 500 or febrile neutropenia	or	< 25,000 or thrombocytopenia with bleeding	Hold dose; when resolved, reduce dose by 25%[b]	Reduce dose by 25% [b]

[a] All dose modifications should be based on the worst preceding toxicity

[b] Patients who require more than 2 dose reductions must be withdrawn from the study

[0204] 5-FU Dose Modifications for Non-Hematological Toxicities

[0205] Treatment should be delayed until all Grade 3 or 4 non-hematological toxicities resolve to Grade 1 or baseline. Guidelines for dose adjustment of 5-FU related toxicities are provided below.

[0206] Table 17: 5-FU Dose Modifications for Non-Hematological Toxicities Other than Asthenia and Grade 3 Anorexia[c]

Worst Toxicity CTCAE Grade	5-FU Dose for D8, D15, D22[a]	5-FU Dose for Next Cycle[a]
Grade 1 or 2	100% of previous dose, except for Grade 2 hand foot syndrome, Grade 2 cardiac toxicity, or any grade neurocerebellar toxicity	100% of previous dose, except for Grade 2 hand and foot syndrome, Grade 2 cardiac toxicity, or any grade neurocerebellar toxicity
Grade 2 hand foot syndrome	Reduce dose by 25% [b]	Reduce dose by 25% [b]
Any grade neurocerebellar or ≥ Grade 2 cardiac toxicity	Discontinue therapy	Discontinue therapy
Grade 3 or 4	Hold; when resolved, reduce dose by 25% [b], except for Grade 3 or 4 hand foot syndrome	Reduce dose by 25%[b],except for Grade 3 or 4 hand foot syndrome

Grade 3 or 4 hand foot syndrome	Discontinue therapy	Discontinue therapy

[a] All dose modifications should be based on the worst preceding toxicity

[b] Patients who require more than 2 dose reductions must be withdrawn from the study

[c] Asthenia and Grade 3 Anorexia do not require dose modification

[0207] MM-398 Dose Modifications for UGT1A1*28 Positive Patients (Arm 1)

[0208] Patients are tested for UGT1A1*28 status during screening, however the result of the test is not required prior to the initial dose of MM-398. All patients will begin dosing at 50 mg/m^2 (free base), however future doses may be reduced for patients who are positive (i.e. homozygous) for UGT1A1*28 7/7 genotype. Any patients who receive a reduced dose during Cycle 1 due to UGT1A1*28 homozygosity will not be evaluable for the cohort and are replaced.

[0209] Table 18: Part 2 Arm 2 (nal-IRI + 5-FU/LV) Dose Modifications for Hematologic Toxicities

Worst Toxicity by CTCAE Grade	Nal-IRI	5-FU
Grade 2 neutropenia (ANC<1500-1000 cells/mm^3)	100% of previous dose	
Grade 3 or 4 neutropenia (ANC $\leq$ 1000/mm^3) or febrile neutropenia[a]	1st occurrence: Reduce dose to 60 mg/m^2 2nd occurrence: Reduce dose to 50mg/m^2	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)

≥Grade 2 thrombocytopenia (Grade 2: platelets ≤ 75,000/mm^3 – 50,000/mm^3 or Grade 3-4: platelets < 50,000/mm^3)	If Grade 2: 100% of previous dose If ≥ Grade 3: 1st occurrence: Reduce dose to 60 mg/m^2 2nd occurrence: : Reduce dose to 50mg/m^2	If Grade 2: 100% of previous dose If ≥ Grade 3: 1st occurrence: Reduce dose by 25% 2nd occurrence: : Reduce dose another 25% (50% of original dose)
Other hematologic toxicities not specifically listed above	If Grade 2: 100% of previous dose If ≥ Grade 3: 1st occurrence: Reduce dose to 60 mg/m^2 2nd occurrence: : Reduce dose to 50mg/m^2	If Grade 2: 100% of previous dose If ≥ Grade 3: 1st occurrence: Reduce dose by 25% 2nd occurrence: : Reduce dose another 25% (50% of original dose)

[a] Consider the use of G-CSF for patients who experience ≥ Grade 3 neutropenia or febrile neutropenia.

[0210] Table 19: Part 2 Arm 2 (nal-IRI + 5-FU/LV) Dose Modifications for non-Hematological Toxicities other than Asthenia and Grade 3 Anorexia[a, d, e]

Worst Toxicity by CTCAE Grade	Nal-IRI	5-FU
Grade 1 or 2, Including diarrhea[b]	100% of previous dose	100% of previous dose, except for Grade 2 hand foot syndrome, Grade 2 cardiac toxicity , or any grade neurocerebellar toxicity
Grade 3 or 4, Including diarrhea[c] (except nausea and vomiting)	1st occurrence: Reduce dose to 60 mg/m^2 2nd occurrence: Reduce dose to 50mg/m^2	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)

		Note: except for Grade 3 or 4 hand foot syndrome
Grade 3 or 4 nausea and/or vomiting despite anti-emetic therapy	Optimize anti-emetic therapy AND reduce dose to 60 mg/m^2; if the patient is already receiving 60 mg/m^2, reduce dose to 50 mg/m^2	Optimize anti-emetic therapy AND reduce dose by 25%; if the patient is already receiving a reduced dose, reduce dose an additional 25%
Grade 2 hand foot syndrome	100% of previous dose	1st occurrence: Reduce dose by 25% 2nd occurrence: Reduce dose another 25% (50% of original dose)
Grade 3 or 4 hand foot syndrome	100% of previous dose	Discontinue therapy
Any grade neurocerebellar or ≥Grade 2 cardiac toxicity	100% of previous dose	Discontinue therapy

[a] Aesthenia and Grade 3 Anorexia do not require dose modification.

[b] Grade 1 diarrhea: 2-3 stools/day > pretreatment; Grade 2 diarrhea: 4-6 stools/day > pretreatment.

[c] Grade 3 diarrhea: 7-9 stools/day > pretreatment; Grade 4 diarrhea: > 10 stools/day >

[d] Any toxicity ≥ Grade 2, except anemia and alopecia, can justify a dose reduction if medically indicated.

[e] Patients who require more than 2 dose reductions must be withdrawn from the study.

[0211] Disease Evaluation

[0212] Tumor responses are evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, to establish disease progression by CT or MRI. In addition, other imaging procedures, as deemed appropriate by the Investigator, are performed to assess sites of neoplastic involvement. The same method of assessment must be used throughout the study. Investigators should select target and non-target lesions in accordance with RECIST v1.1 guidelines. Follow up measurements and overall response should also be in accordance with these guidelines.

[0213] Tumor assessments should be completed until it has been determined that the patient has progressive disease (in accordance with RECIST v1.1). For patients who do not have documented disease progression per RECIST v. 1.1 at the time of treatment termination, imaging studies should be continually performed into the follow-up period every 8 weeks until disease progression is documented. Continued imaging follow-up on schedule is recommended to reduce potential bias in the evaluations of the impacts of the experimental treatments on disease.

[0214] EORTC-QLQ-C30 and EQ-5D-5L

[0215] Health-related quality of life (HRQL) is assessed by the EORTC-QLQ-C30 and EQ-5D-5L instruments. The EORTC-QLQ-C30 is a reliable and valid measure of the quality of life of cancer patients in multicultural clinical research settings. It incorporates nine multi-item scales: five functional scales (physical, role, cognitive, emotional, and social); three symptom scales (fatigue, pain, and nausea and vomiting); and a global health and quality-of-life scale. Several single-item symptom measures are also included. EQ-5D is a generic, preference-based measurement of HRQL. The EQ-5D-5L descriptive system comprises the following 5 dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each dimension has 5 levels: no problems, slight problems, moderate problems, severe problems, and unable to do.

[0216] Patients are required to complete both questionnaires at time points outlined in the Schedule of Assessments. On days that the patient is to receive study drug, assessments should be completed prior to study drug administration. Only those patients for whom validated translations of the questionnaires are available will be required to complete the questionnaire.

[0217] Efficacy Analysis

[0218] In the assessments of efficacy, the MM-398-containing arm is compared to the control arm. Efficacy comparisons use stratified analyses, incorporating randomization strata. Each comparison uses 0.10 level one-sided testing to evaluate whether the MM-398 - containing arm improves the efficacy parameter. Confidence intervals are presented at two-sided 95% level for descriptive purposes. Hypothesis tests and confidence intervals are not adjusted for multiple comparisons. The primary efficacy comparisons are based on the ITT population, which includes all randomized patients.

[0219] Tumor evaluation is measured according to RECIST v1.1. For each patient, progression free survival time is determined as the time from randomization to the first documented radiographical Progression of Disease (PD), per investigator using RECIST 1.1,

or death from any cause, whichever comes first. If the progression or death occurs at a time point that is greater than 12 weeks after the non-PD last tumor assessment, then progression-free survival time is censored at the time of the last non-PD tumor assessment.

[0220] A primary analysis is conducted when the Week 24 progression-free status for all randomized patients can be determined, anticipated at approximately 24 weeks after the last patient is randomized. A subsequent analysis for PFS and other endpoints is performed when PFS events have occurred in at least 120 (i.e. 80% of randomized patients) patients.

[0221] Primary Efficacy Analysis

[0222] In the intention-to-treat (ITT) analysis, a patient is considered to have achieved progression-free survival at 24 weeks if the patient has data to indicate the patient has not progressed at 24 weeks. That is, a patient is considered a responder if there is at least one non-PD assessment, prior to progression or new anticancer therapy, at Week 24 or later.

[0223] Patients who do not meet the 24-week progression-free achievement criteria (e.g. patients progressed/died up to Week 24, patients censored prior to Week 24), if progression or death occurs at a time point that is greater than 12 weeks after the non-PD last tumor assessment.

[0224] For each arm, the progression-free survival achievement rate at 24 weeks is estimated by the number of patients meeting the 24 week achievement criteria divided by the number of ITT patients in the arm. The rate estimates are presented with corresponding 95% confidence intervals. The MM-398 containing arm is assessed for increase in rate relative to the control arm using a one-sided Cochran-Mantel-Haenszel test, incorporating randomization stratification factors, at 0.10 level of significance.

[0225] Secondary Efficacy Analyses

[0226] Progression-Free Survival (PFS) is descriptively summarized for each arm using Kaplan-Meier methodology. Median PFS time and corresponding 95% confidence limits are presented. For the MM-398-containing arm, PFS is compared to the control arm. Hypothesis tests are conducted for differences in PFS using a one-sided stratified log-rank test. Hazard ratios (with 95% confidence interval) for PFS are estimated using stratified Cox models.

[0227] Best Overall Response (BOR) is defined as the best response as recorded from the start of study drug until disease progression. Patients without a post-baseline tumor assessment are considered to be non-evaluable for BOR. To classify BOR as stable disease (SD), there should be a qualifying SD assessment at least 6 weeks from randomization. Objective Response Rate (ORR) is defined as the proportion of patients with a BOR characterized as either a Complete Response (CR) or Partial Response (PR) relative to the

total number of evaluable patients. Only patients with measurable disease at baseline will be included in the analysis of the objective response. Estimates of objective response rate and its corresponding 95% CI are calculated for each treatment arm. For each MM-398-containing arm, ORR is compared to the control arm. Differences in objective response rate between the MM-398-containing arm and control arm are provided with 95% CIs. Cochran-Mantel-Haenszel tests, adjusting by randomization strata, are used to compare objective response rates.

[0228] Overall Survival (OS) is the time from randomization to the date of death from any cause. Patients who are alive or lost to follow-up at the time of the analysis will be censored at the last known alive date. OS is descriptively summarized for each arm using Kaplan-Meier methodology. For the MM-398-containing arm, OS is compared to the control arm. Hypothesis tests are conducted for differences in OS using a one-sided stratified log-rank test. Hazard ratios (with 95% confidence interval) for PFS are estimated using stratified Cox models.

[0229] Quality of Life Analyses

[0230] Quality of life analyses are performed using patients in the analysis populations for each quality of life instrument (EORTC-QLC-C30, EQ-5D-5L). EORTC-QLQ-30 and EQ-5D-5L results will be summarized at each visit by treatment group.

[0231] For each EORTC QLQ-C30 administered, scores are computed for the following scales: Global Health Status, Physical Functioning, Role Functioning, Emotional Functioning, Cognitive Functioning, Social Functioning, Fatigue, Nausea and vomiting, Pain, Dyspnea, Insomnia, Appetite Loss, Constipation, Diarrhea, Financial difficulties.

[0232] Scoring is carried out as described in the EORTC QLQ-C30 Scoring Manual (Fayers, Aaronson, Bjordal, Curran, & Groenvald, 2001). Linear transformations are applied to the raw scores so that the reported score will have range 0-100 for all scales. Summary statistics are presented for each subscale. A summary health state index value is computed for each EQ-5D-5L assessment. Summary statistics are presented for summary health state index. For each EQ-5D-5L attribute (mobility, self-care, usual activities, pain/discomfort, and anxiety/depression), responses are tabulated.

[0233] Safety Analysis

[0234] Safety analyses (adverse events and laboratory analyses) will be performed using the safety population. Adverse events are reported by the MedDRA version 17.1 or higher. Toxicity is graded according to the NCI CTCAE version 4.03.

[0235] The period for treatment-emergent adverse events and safety findings is from the time of first study drug administration to 30 days after the date of last study drug administration. If an adverse event begins on the date of first study drug administration with no time recorded, the event is then considered as treatment-emergent.

[0236] Tabular summaries are to be presented for all adverse events, pre-treatment adverse events, treatment-emergent adverse events (TEAE), serious adverse events, adverse events leading to study drug discontinuation, TEAE-related to study drug and TEAE Grade 3/4. Adverse events are to be summarized by System Organ Class and preferred term. All adverse event data is to be listed by patient.

[0237] Laboratory data is presented by cycle. Abnormal laboratory values are assessed using all available data and toxicity grading will be assigned according to NCI CTCAE toxicity scale, where criteria are available to do so. Maximum and minimum decrease/increase in continuous laboratory data are reported. Frequency and percent of abnormal laboratory values (L/ULN, 2*L/ULN) are assessed. Shift to most severe toxicity grade are summarized.

[0238] Vital signs and ECG are tabulated for the change from baseline by time point. Additional analyses may be performed as described in detail within the SAP.

[0239] Vital signs are tabulated for the change from baseline by time point. Additional analyses may be performed as described in detail within the SAP.

[0240] Biomarker Subgroup Analysis

[0241] Analyses are performed to assess the associations between potential biomarkers (from plasma and archived tissue) and efficacy parameters (ORR, percent change in target lesion size, and PFS or as appropriate). Graphical displays are performed when appropriate.

[0242] Pharmacokinetics Analysis

[0243] Plasma concentrations of MM-398 and oxaliplatin can be used to characterize PK parameters. Due to the sparse PK sampling schedule, PK parameters for individual patients can be estimated based on the Empirical Bayesian Estimation method with priors from the previously estimated (MM-398) or published (oxaliplatin) population PK model parameters. The model simulated exposures, e.g., C_{max}, AUC (area under the curve), are used to examine any possible interactions between MM-398 and oxaliplatin by comparing the least squares geometric mean ratios (LS-GMR) of drug exposures. NONMEM®, Version 7.3, is used to estimate individual PK parameters and simulate plasma exposures.

[0244] Example 5: ONIVYDE® (irinotecan liposome injection) Liposomal Irinotecan

[0245] One preferred example of an irinotecan liposome described herein is the product marketed as ONIVYDE® (irinotecan liposome injection). ONIVYDE® is a topoisomerase inhibitor, formulated with irinotecan in a liposomal dispersion, for intravenous use.

[0246] The finished ONIVYDE® product is a white to slightly yellow opaque sterile concentrate for infusion. It consists of an isotonic dispersion of liposomes containing irinotecan hydrochloride trihydrate. The liposomes are small unilamellar lipid bilayer vesicles, approximately 110 nm in diameter, enclosing an aqueous compartment that contains irinotecan in a gelated or precipitated state, as sucrosofate 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. The liposomes are dispersed in an aqueous buffered solution.

[0247] The ONIVYDE® product contains irinotecan sucrosofate encapsulated in a liposome, obtained from an irinotecan hydrochloride trihydrate starting material. The chemical name of irinotecan 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. The dosage of ONIVYDE® can be calculated based on the equivalent amount of irinotecan trihydrate hydrochloride starting material used to prepare the irinotecan liposomes, or based on the amount of irinotecan in the liposome. There are about 866 mg of irinotecan per gram of irinotecan trihydrate hydrochloride. For example, an ONIVYDE® dose of 80 mg based on the amount of irinotecan hydrochloride trihydrate starting material actually contains about 0.866x (80mg) of irinotecan in the final product (i.e., a dose of 80 mg/m^2 of ONIVYDE® based on the weight of irinotecan hydrochloride starting material is clinically equivalent to about 70 mg/m^2 of irinotecan in the final product). Each 10 mL single-dose vial contains 43 mg irinotecan free base at a concentration of 4.3 mg/mL.

Claims

1. A method of treating gastric cancer in a human patient who has not previously received an antineoplastic agent to treat the gastric cancer, the method comprising administering an antineoplastic therapy to the patient a total of once every two weeks, the antineoplastic therapy consisting of administering to the patient a total of:
    a. 50 or 55 mg/m^2 of liposomal irinotecan,
    b. 60, 70 or 85 mg/m^2 oxaliplatin,
    c. 200 mg/m^2 of (*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 to treat the gastric cancer in the human patient.
2. The method of claim 1, wherein a total of 50 mg/m^2 of liposomal irinotecan is administered to the patient during the antineoplastic therapy once every two weeks.
3. The method of claim 1, wherein a total of 55 mg/m^2 oxaliplatin is administered to the patient during the antineoplastic therapy once every two weeks.
4. The method of any one of claims 1-3, wherein a total of 60 mg/m^2 oxaliplatin is administered to the patient during the antineoplastic therapy once every two weeks.
5. The method of any one of claims 1-3, wherein a total of 70 mg/m^2 oxaliplatin is administered to the patient during the antineoplastic therapy once every two weeks.
6. The method of any one of claims 1-3, wherein a total of 85 mg/m^2 oxaliplatin is administered to the patient during the antineoplastic therapy once every two weeks.
7. The method of claim 1, wherein a total of 50 mg/m^2 of liposomal irinotecan and a total of 60 mg/m^2 oxaliplatin is administered to the patient during the antineoplastic therapy once every two weeks.
8. The method of claim 1, wherein a total of 55 mg/m^2 of liposomal irinotecan and a total of 70 mg/m^2 oxaliplatin is administered to the patient during the antineoplastic therapy once every two weeks.
9. The method of claim 1, wherein a total of 50 mg/m^2 of liposomal irinotecan and a total of 85 mg/m^2 oxaliplatin is administered to the patient during the antineoplastic therapy once every two weeks.
10. The method of any one of claims 1-9, wherein each administration of the oxaliplatin begins 2 hours after completing each administration of the liposomal irinotecan.
11. The method of any one of claims 1-10, wherein the 5-fluorouracil is administered as an infusion over 46 hours.

12. The method of any one of claims 1-11, wherein the leucovorin is administered immediately prior to the 5-fluorouracil.
13. The method of any one of claims 1-12, wherein the liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin are administered on days 1 and 15 of a 28-day treatment cycle.
14. The method of any one of claims 1-13, wherein the liposomal irinotecan is administered as an infusion over a total of about 90 minutes.
15. The method of any one of claims 1-14, wherein the liposomal irinotecan is administered, followed by administering the oxaliplatin, followed by administering the leucovorin, followed by administering the 5-fluorouracil.
16. The method of any one of claims 1-15, wherein the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposomes.
17. The method of any one of claims 1-16, wherein the liposomal irinotecan comprises irinotecan encapsulated in liposome vesicles consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethlyene glycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).
18. The method of claim 2, wherein the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposomes, and the liposomal irinotecan comprises irinotecan encapsulated in liposome vesicles consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethlyene glycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).
19. The method of claim 18, wherein the liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin are administered on days 1 and 15 of a 28-day treatment cycle; each administration of the liposomal irinotecan is administered prior to the leucovorin; the leucovorin is administered immediately prior to each administration of the 5-fluorouracil and each administration of 5-fluorouracil is administered as an infusion over 46 hours.
20. The method of claim 10, wherein the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated in liposomes, and the liposomal irinotecan comprises irinotecan encapsulated in liposome vesicles consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethlyene glycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE).
21. The method of claim 20, wherein the liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin are administered on days 1 and 15 of a 28-day treatment cycle; each

administration of the liposomal irinotecan is administered prior to the leucovorin; the leucovorin is administered immediately prior to each administration of the 5-fluorouracil and each administration of 5-fluorouracil is administered as an infusion over 46 hours.

22. A method of treating gastric cancer in a human patient who has not previously received gemcitabine to treat the gastric cancer, the method comprising administering an antineoplastic therapy to the patient a total of once every two weeks, the antineoplastic therapy consisting of administering to the patient a total of:
    a. 50 mg/m^2 of liposomal irinotecan,
    b. 85 mg/m^2 oxaliplatin,
    c. 200 mg/m^2 of (*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 to treat the gastric cancer in the human patient.
23. A method of treating gastric cancer in a human patient who has not previously received gemcitabine to treat the gastric cancer, the method comprising administering an antineoplastic therapy to the patient a total of once every two weeks, the antineoplastic therapy consisting of administering to the patient a total of:
    a. 55 mg/m^2 of liposomal irinotecan,
    b. 70 mg/m^2 oxaliplatin,
    c. 200 mg/m^2 of (*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 to treat the gastric cancer in the human patient.
24. A method of treating gastric cancer in a human patient who has not previously received gemcitabine to treat the gastric cancer, the method comprising administering an antineoplastic therapy to the patient a total of once every two weeks, the antineoplastic therapy consisting of administering to the patient a total of:
    a. 50 mg/m^2 of liposomal irinotecan,
    b. 60 mg/m^2 oxaliplatin,
    c. 200 mg/m^2 of (*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 to treat the gastric cancer in the human patient.
25. A method of treating gastric cancer in a human patient who has not previously received gemcitabine to treat the gastric cancer, the method comprising administering

an antineoplastic therapy to the patient a total of once every two weeks, the antineoplastic therapy consisting of administering to the patient a total of:

a. 55 mg/m^2 of liposomal irinotecan,
b. 85 mg/m^2 oxaliplatin,
c. 200 mg/m^2 of (*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 to treat the gastric cancer in the human patient.

26. The method of any one of claims 22-25, wherein
    a. the liposomal irinotecan comprises irinotecan sucrose octasulfate encapsulated liposome vesicles comprising 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and a N-(carbonylmethoxypolyethlyene glycol-2000)-1,2-distearoly-sn-glycero-3-phosphoethanolamine (MPEG-2000-DSPE);
    b. the liposomal irinotecan, oxaliplatin, 5-fluorouracil and leucovorin are administered on days 1 and 15 of a 28-day treatment cycle;
    c. each administration of the liposomal irinotecan is administered prior to the leucovorin;
    d. the leucovorin is administered immediately prior to each administration of the 5-fluorouracil; and
    e. each administration of 5-fluorouracil is administered as an infusion over 46 hours.
27. The method of claim 26, wherein each administration of the oxaliplatin begins after completing each administration of the liposomal irinotecan, and the method further comprises administering a corticosteroid and anti-emetic to the patient prior to the antineoplastic therapy.

FIG. 1A

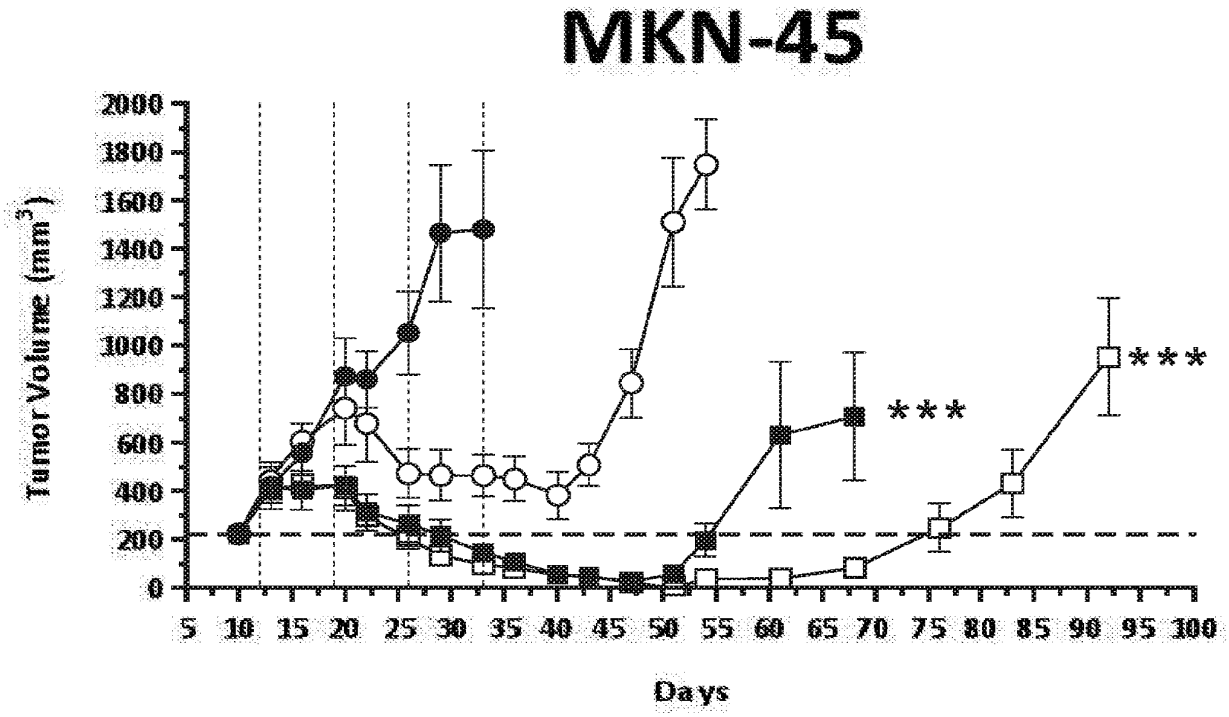

MKN-45
Tumor Volume (mm3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Days
***
***

FIG. 1B

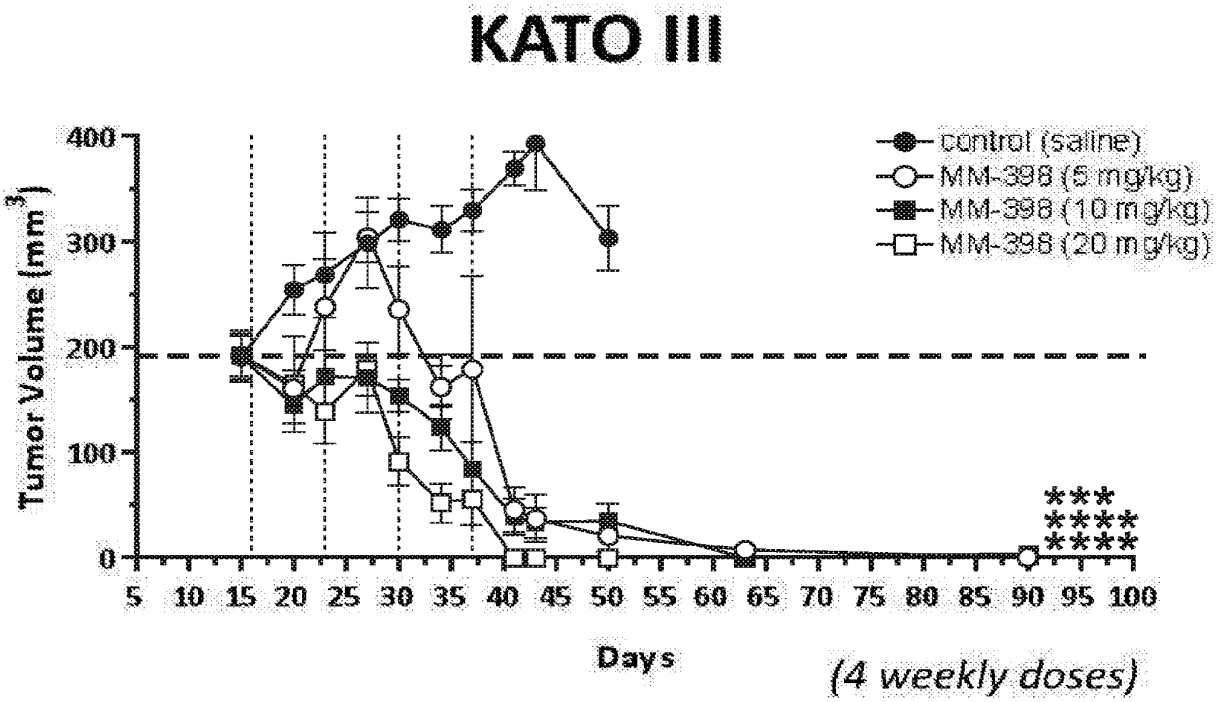

KATO III
Tumor Volume (mm3)
400
300
200
100
0
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Days
control (saline)
MM-398 (5 mg/kg)
MM-398 (10 mg/kg)
MM-398 (20 mg/kg)
***
****
****
(4 weekly doses)

FIG. 2A

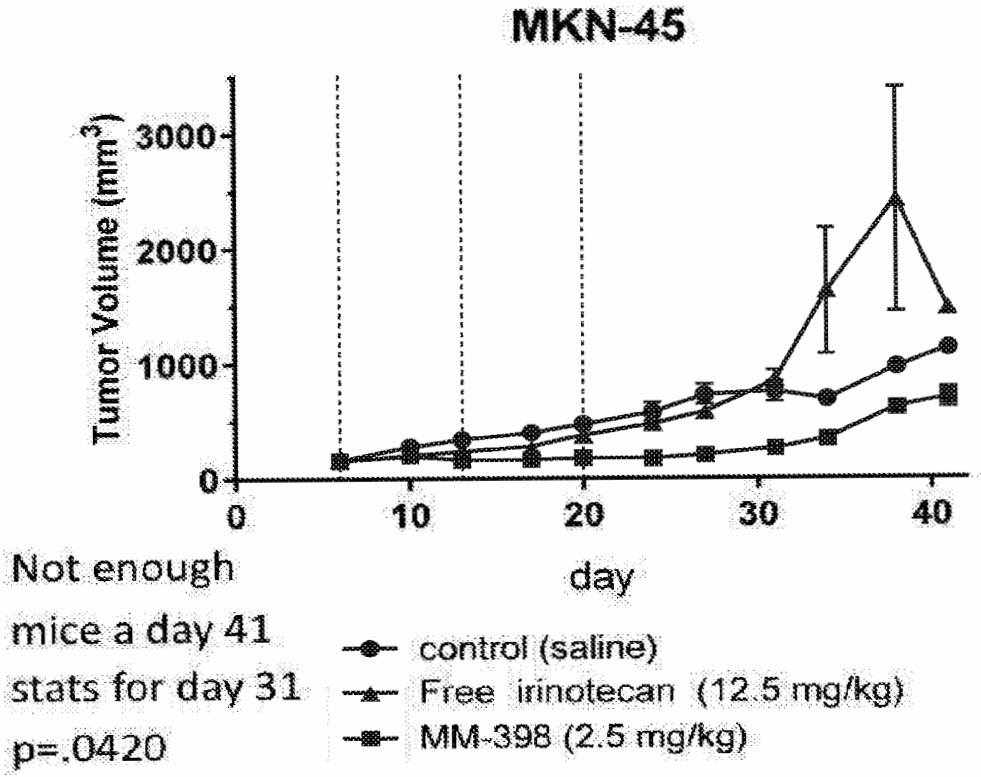

MKN-45
Tumor Volume (mm3)
3000
2000
1000
0
0
10
20
30
40
day
Not enough mice a day 41
stats for day 31
p=.0420
control (saline)
Free irinotecan (12.5 mg/kg)
MM-398 (2.5 mg/kg)

FIG. 2B

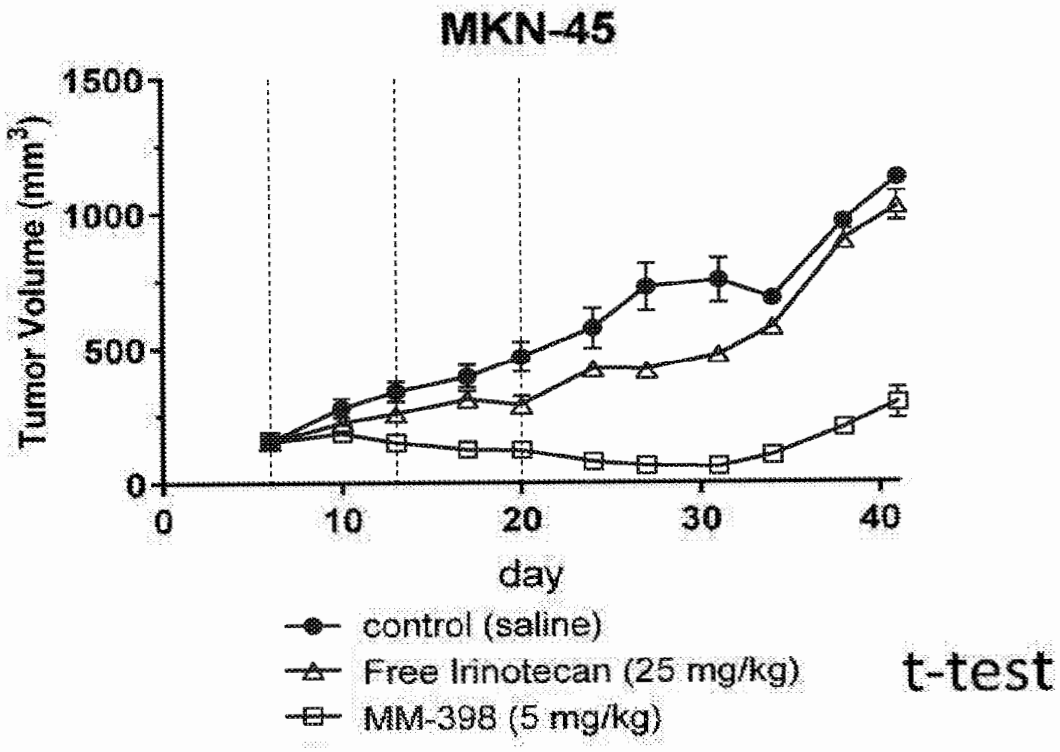

MKN-45
Tumor Volume (mm3)
1500
1000
500
0
0
10
20
30
40
day
control (saline)
Free Irinotecan (25 mg/kg)
MM-398 (5 mg/kg)
t-test

FIG. 3A

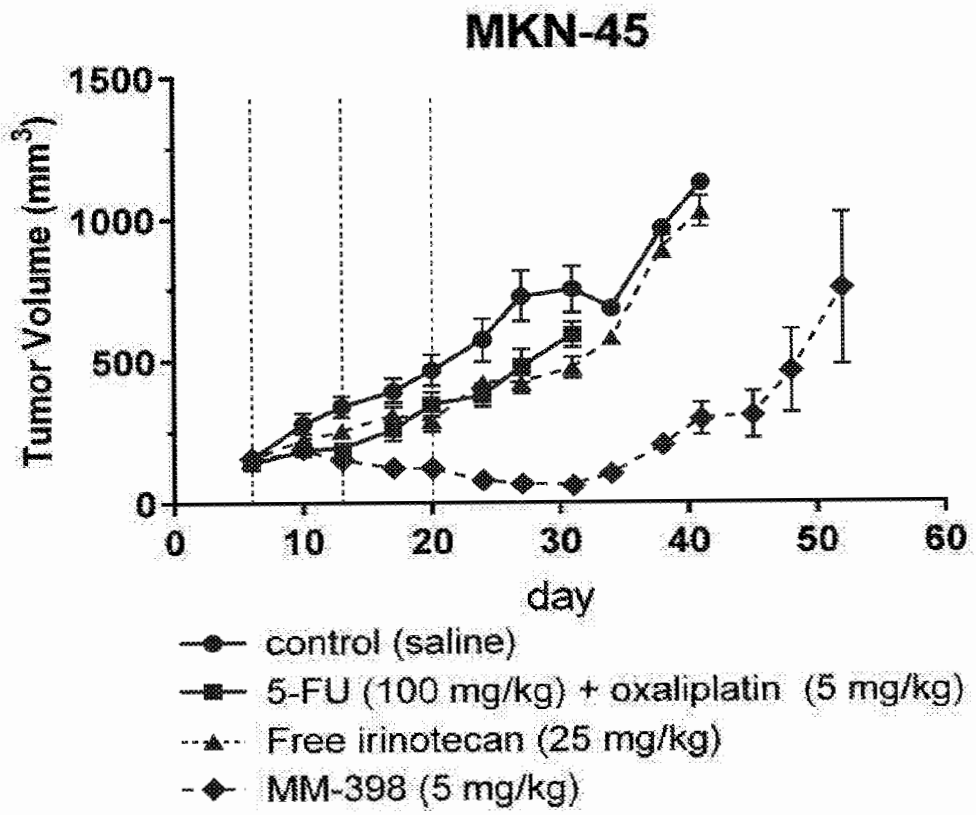

MKN-45
Tumor Volume (mm3)
1500
1000
500
0
0
10
20
30
40
50
60
day
control (saline)
5-FU (100 mg/kg) + oxaliplatin (5 mg/kg)
Free irinotecan (25 mg/kg)
MM-398 (5 mg/kg)

FIG. 3B

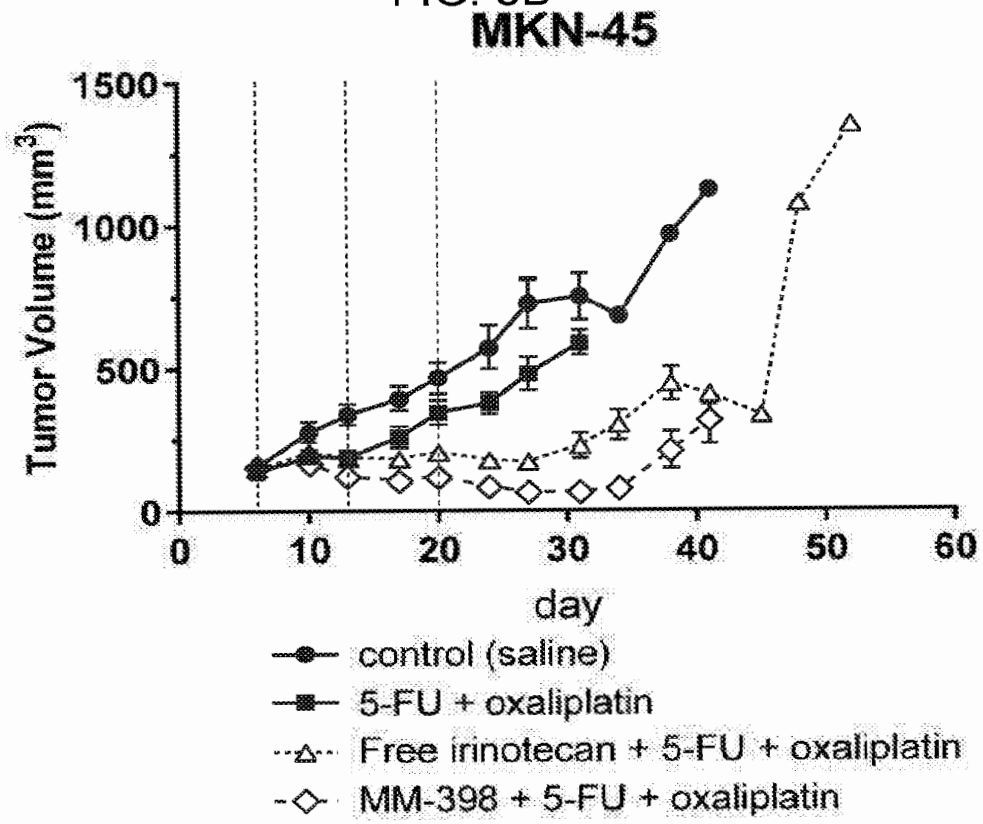

MKN-45
Tumor Volume (mm3)
1500
1000
500
0
0
10
20
30
40
50
60
day
control (saline)
5-FU + oxaliplatin
Free irinotecan + 5-FU + oxaliplatin
MM-398 + 5-FU + oxaliplatin

FIG. 4A

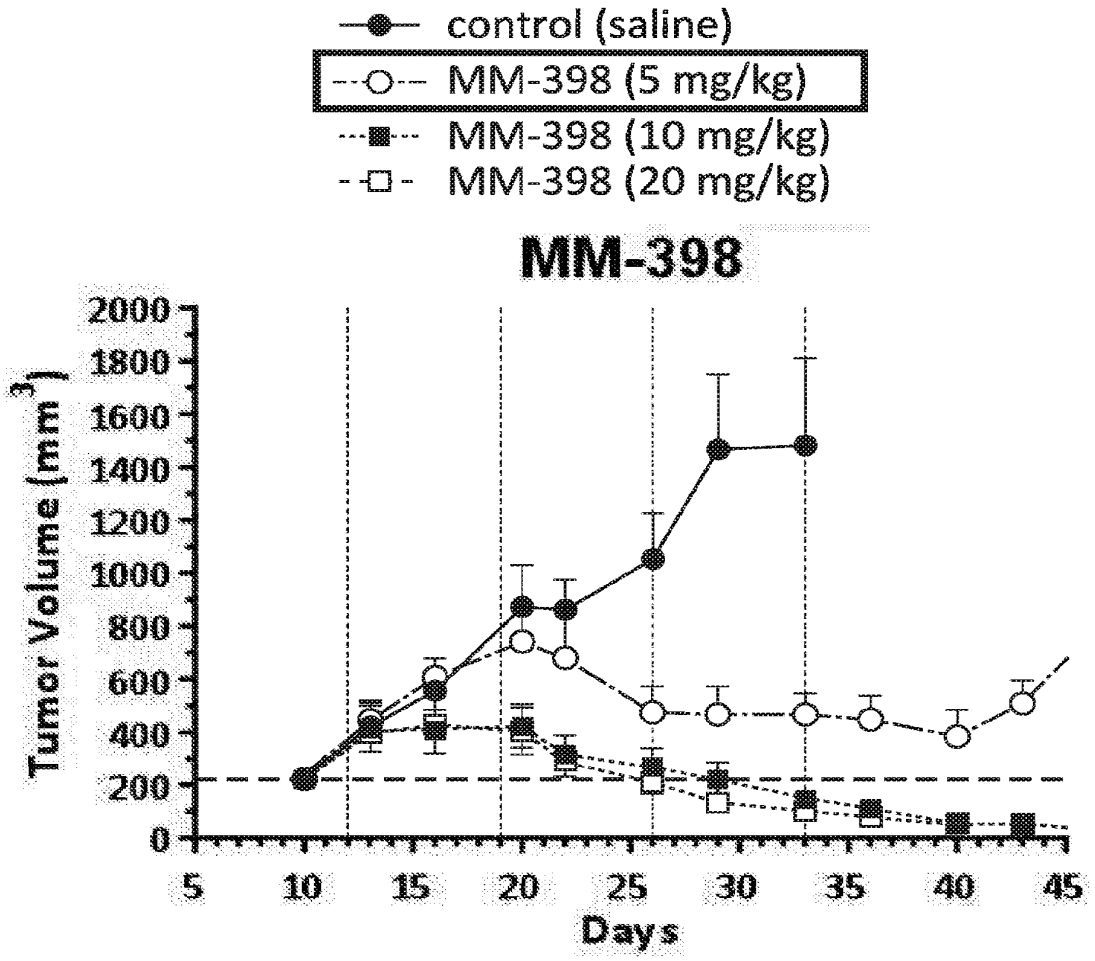

control (saline)
MM-398 (5 mg/kg)
MM-398 (10 mg/kg)
MM-398 (20 mg/kg)
MM-398
Tumor Volume (mm3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
5
10
15
20
25
30
35
40
45
Days

FIG. 4B

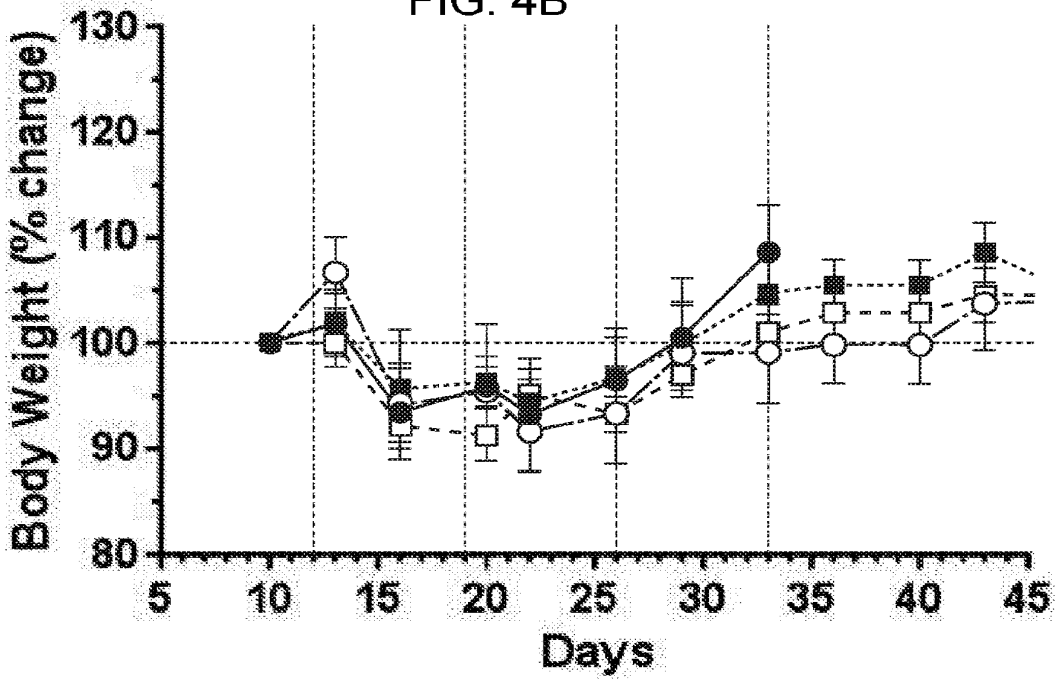

Body Weight (% change)
130
120
110
100
90
80
5
10
15
20
25
30
35
40
45
Days

FIG. 4C

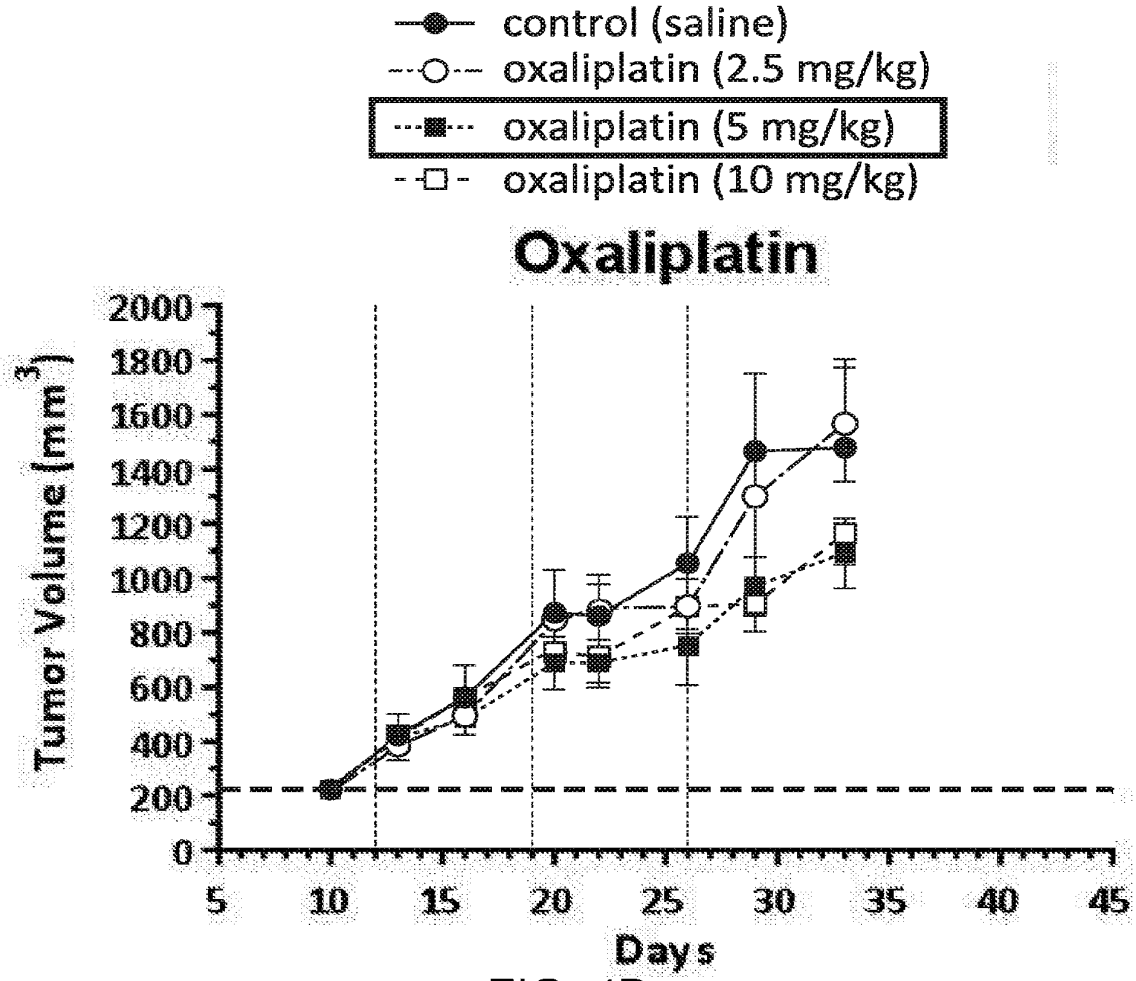

control (saline)
oxaliplatin (2.5 mg/kg)
oxaliplatin (5 mg/kg)
oxaliplatin (10 mg/kg)
Oxaliplatin
Tumor Volume (mm3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
5
10
15
20
25
30
35
40
45
Days

FIG. 4D

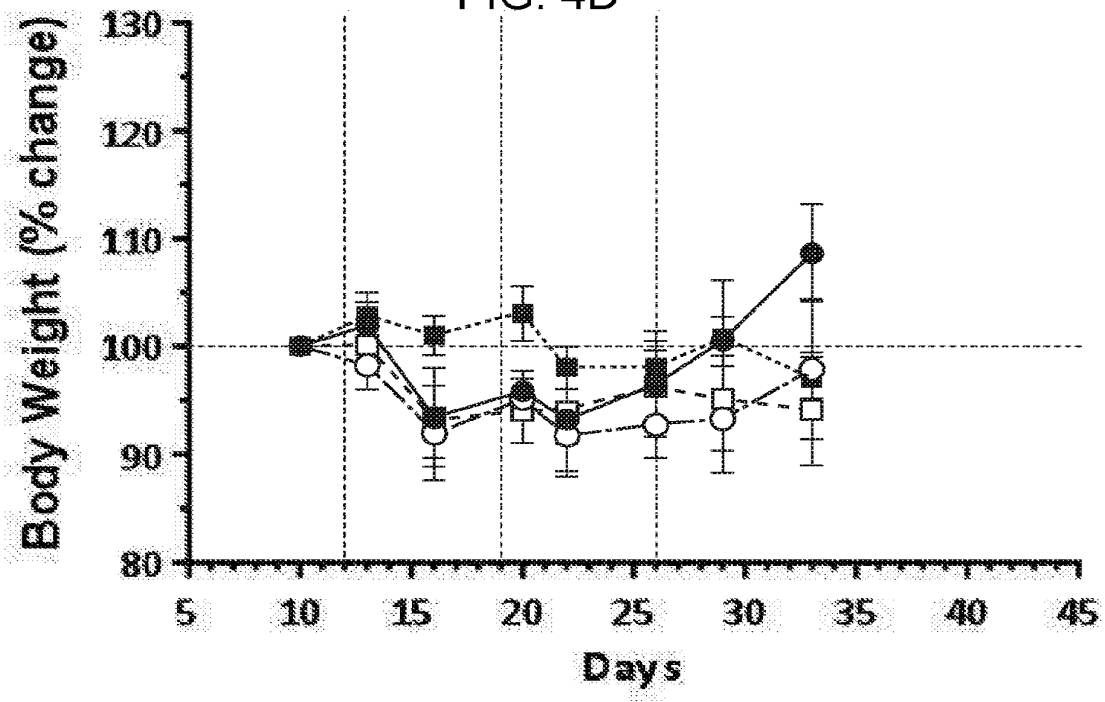

Body Weight (% change)
130
120
110
100
90
80
5
10
15
20
25
30
35
40
45
Days

FIG. 4E

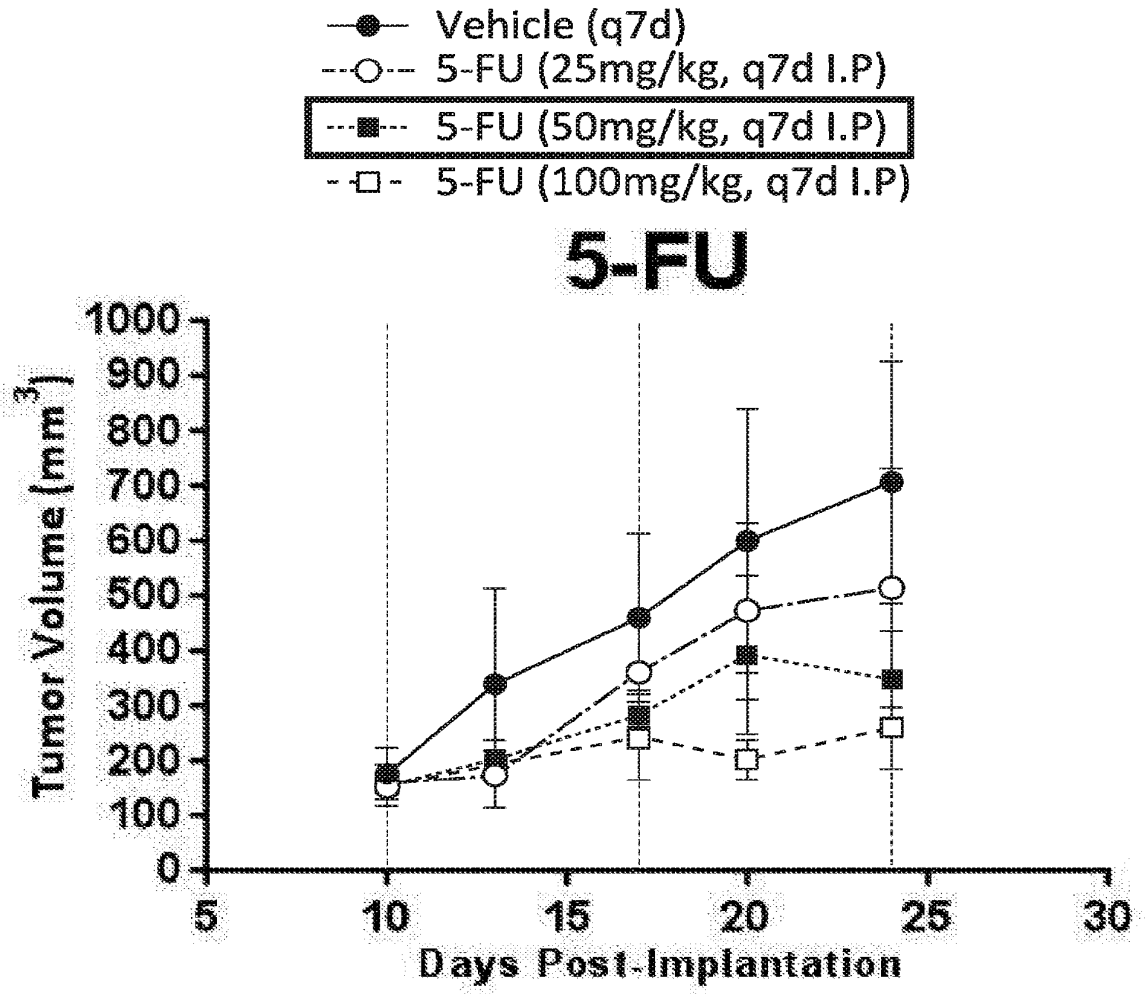

Vehicle (q7d)
5-FU (25mg/kg, q7d I.P)
5-FU (50mg/kg, q7d I.P)
5-FU (100mg/kg, q7d I.P)
5-FU
Tumor Volume (mm3)
1000
900
800
700
600
500
400
300
200
100
0
5
10
15
20
25
30
Days Post-Implantation

FIG. 4F

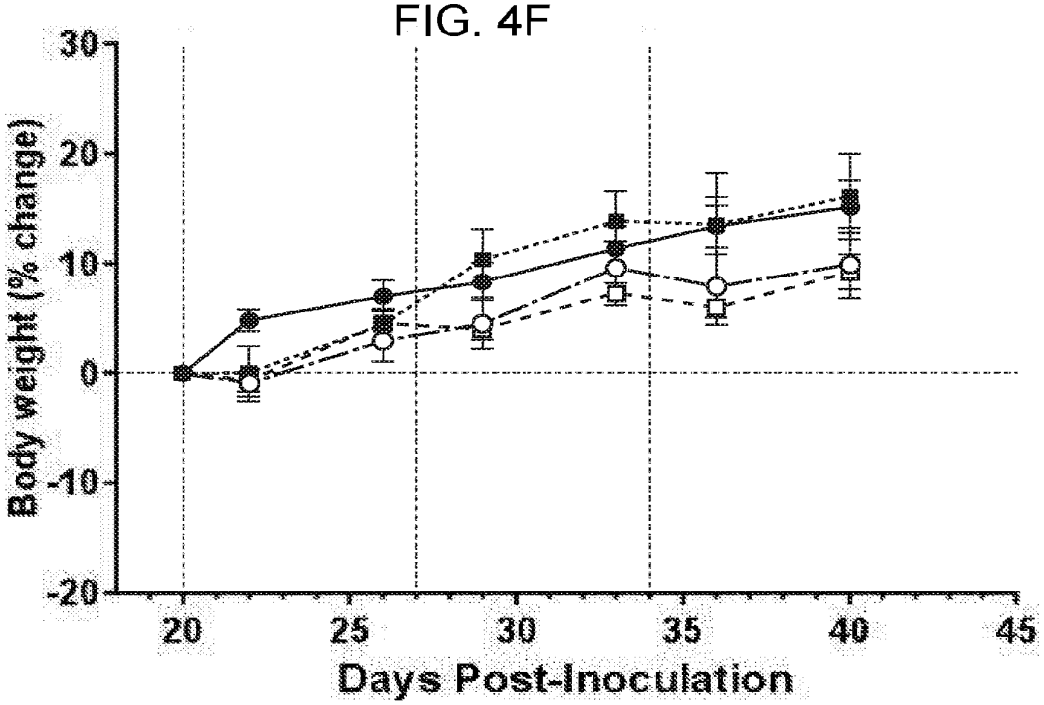

Body weight (% change)
30
20
10
0
-10
-20
20
25
30
35
40
45
Days Post-Inoculation

FIG. 4G

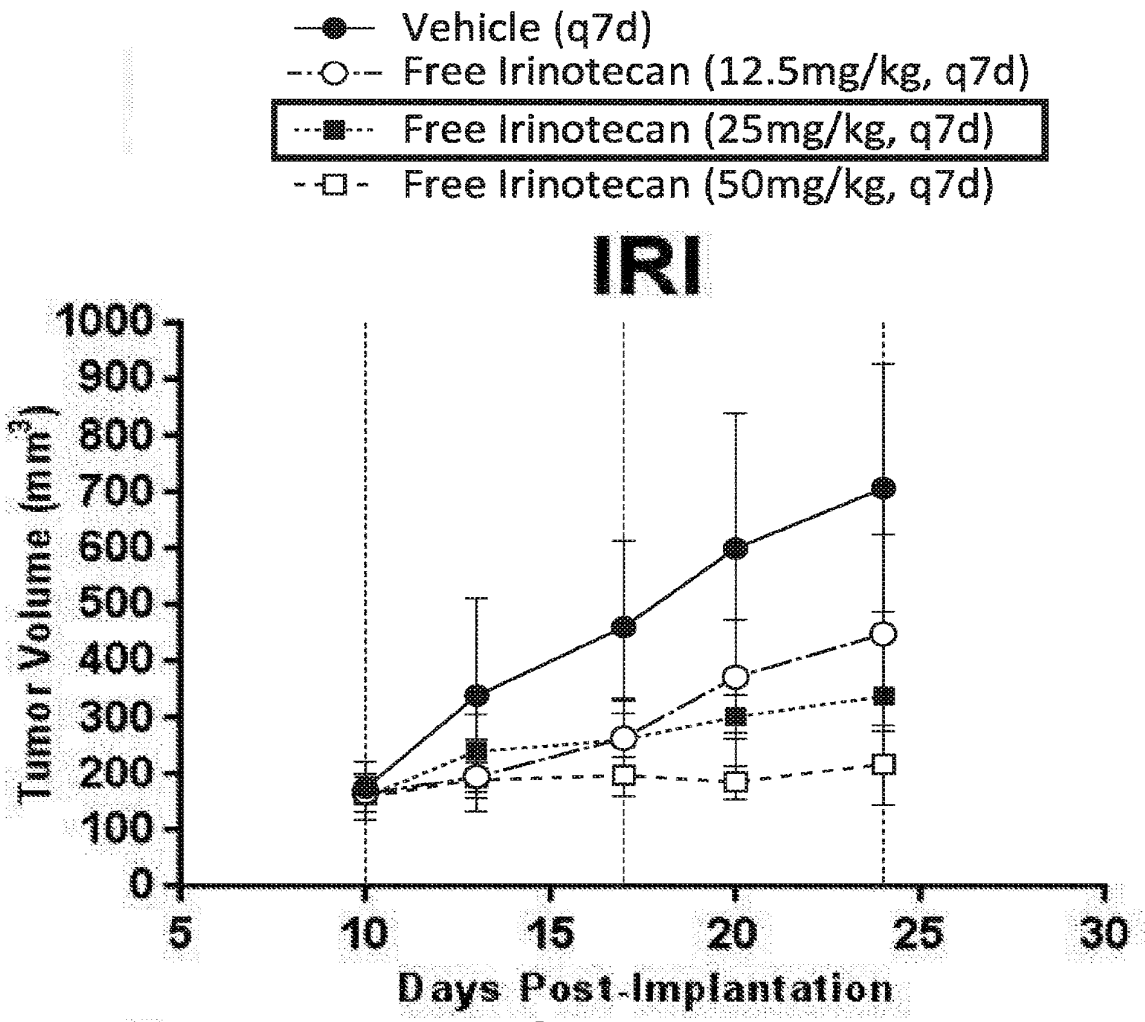

Vehicle (q7d)
Free Irinotecan (12.5mg/kg, q7d)
Free Irinotecan (25mg/kg, q7d)
Free Irinotecan (50mg/kg, q7d)
IRI
Tumor Volume (mm3)
1000
900
800
700
600
500
400
300
200
100
0
5
10
15
20
25
30
Days Post-Implantation

FIG. 4H

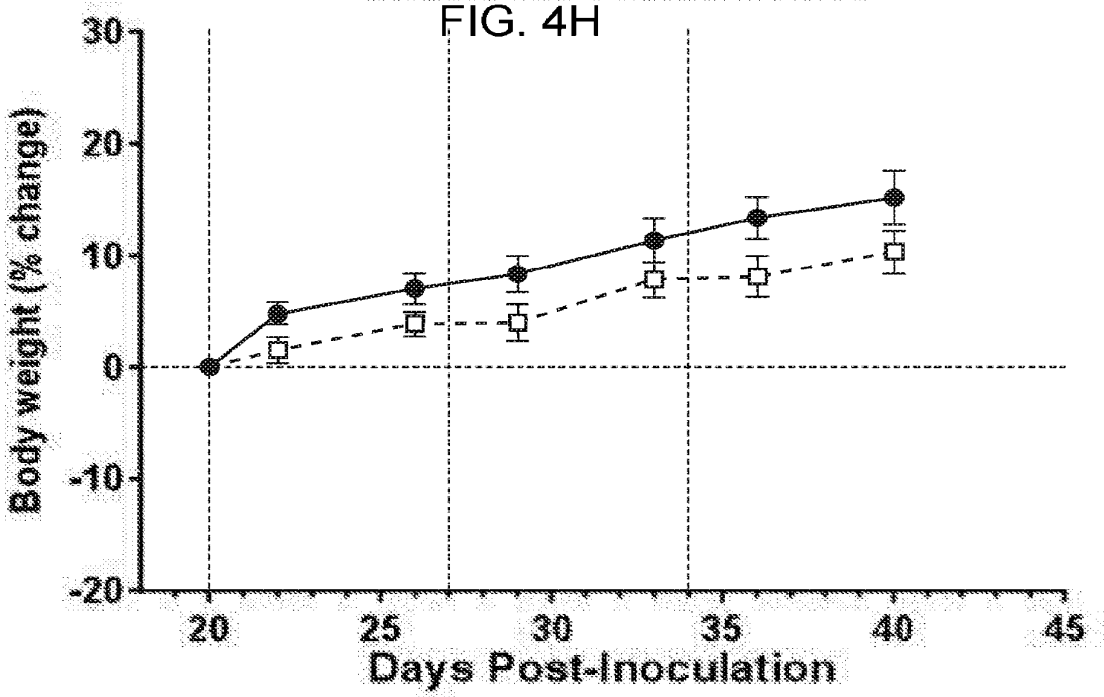

Body weight (% change)
30
20
10
0
-10
-20
20
25
30
35
40
45
Days Post-Inoculation

FIG. 5A

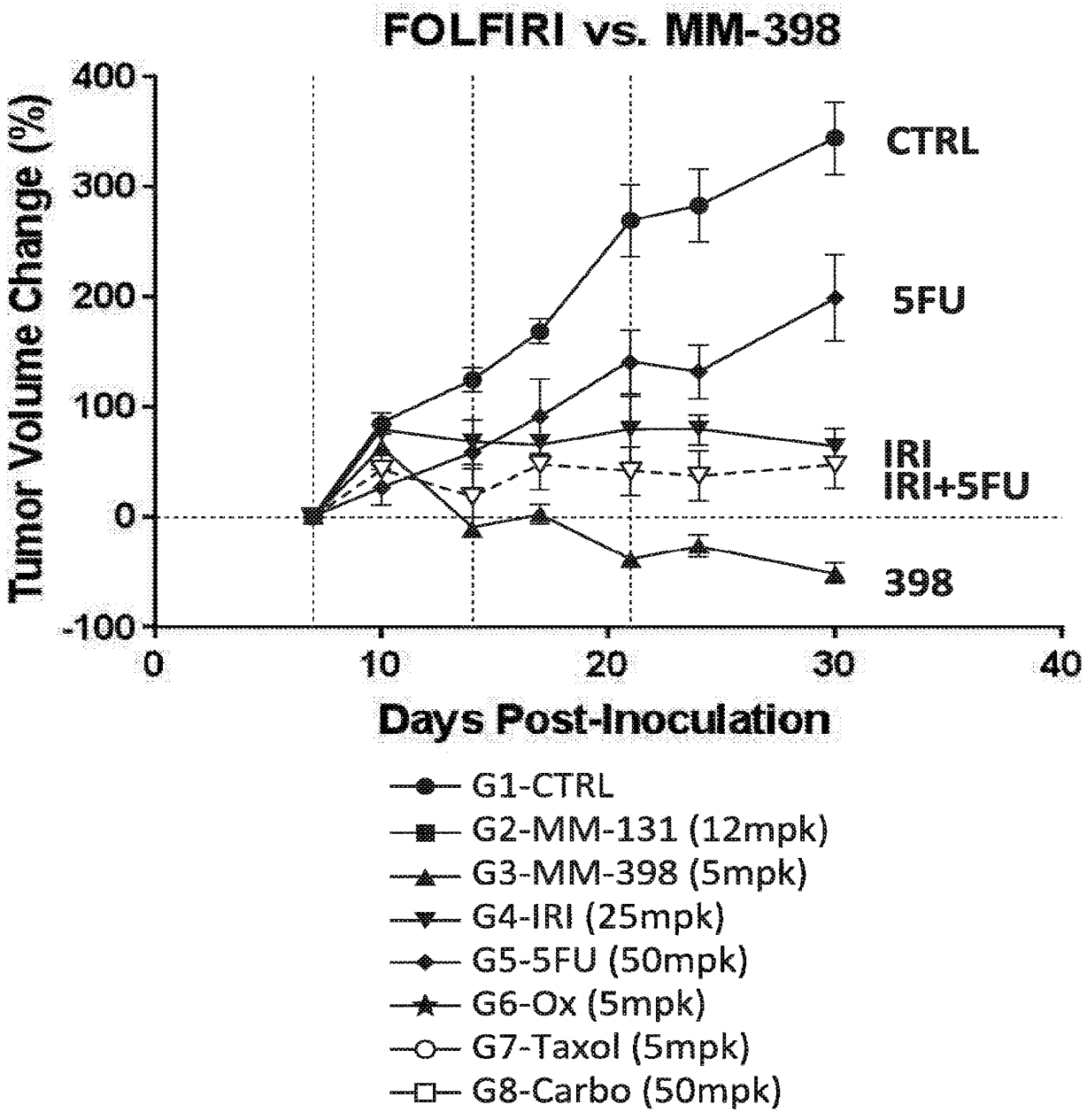

FOLFIRI vs. MM-398
Tumor Volume Change (%)
400
300
200
100
0
-100
0
10
20
30
40
Days Post-Inoculation
CTRL
5FU
IRI
IRI+5FU
398
G1-CTRL
G2-MM-131 (12mpk)
G3-MM-398 (5mpk)
G4-IRI (25mpk)
G5-5FU (50mpk)
G6-Ox (5mpk)
G7-Taxol (5mpk)
G8-Carbo (50mpk)

FIG. 5B

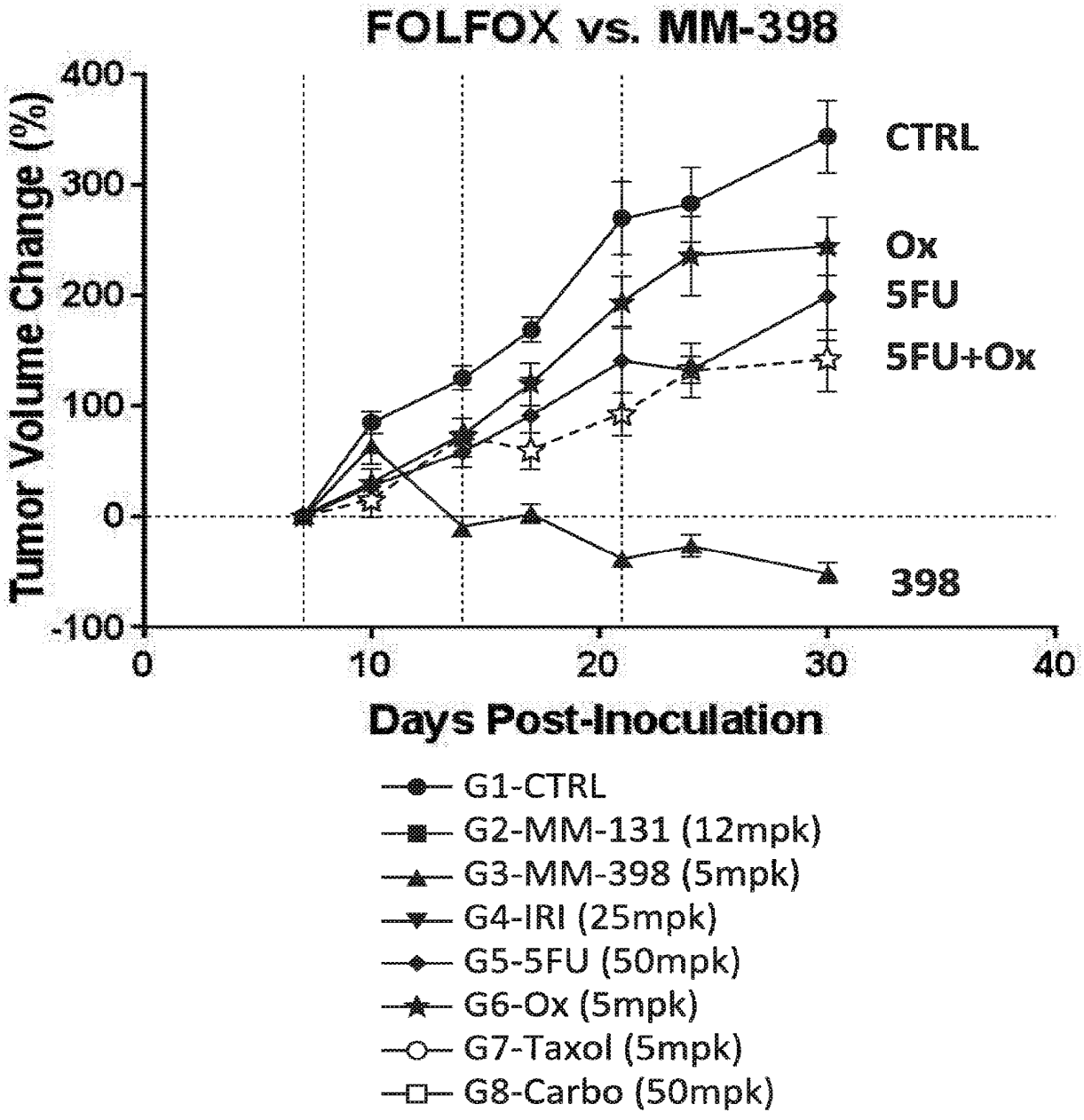

FOLFOX vs. MM-398
Tumor Volume Change (%)
400
300
200
100
0
-100
0
10
20
30
40
Days Post-Inoculation
CTRL
Ox
5FU
5FU+Ox
398
G1-CTRL
G2-MM-131 (12mpk)
G3-MM-398 (5mpk)
G4-IRI (25mpk)
G5-5FU (50mpk)
G6-Ox (5mpk)
G7-Taxol (5mpk)
G8-Carbo (50mpk)

FIG. 6A

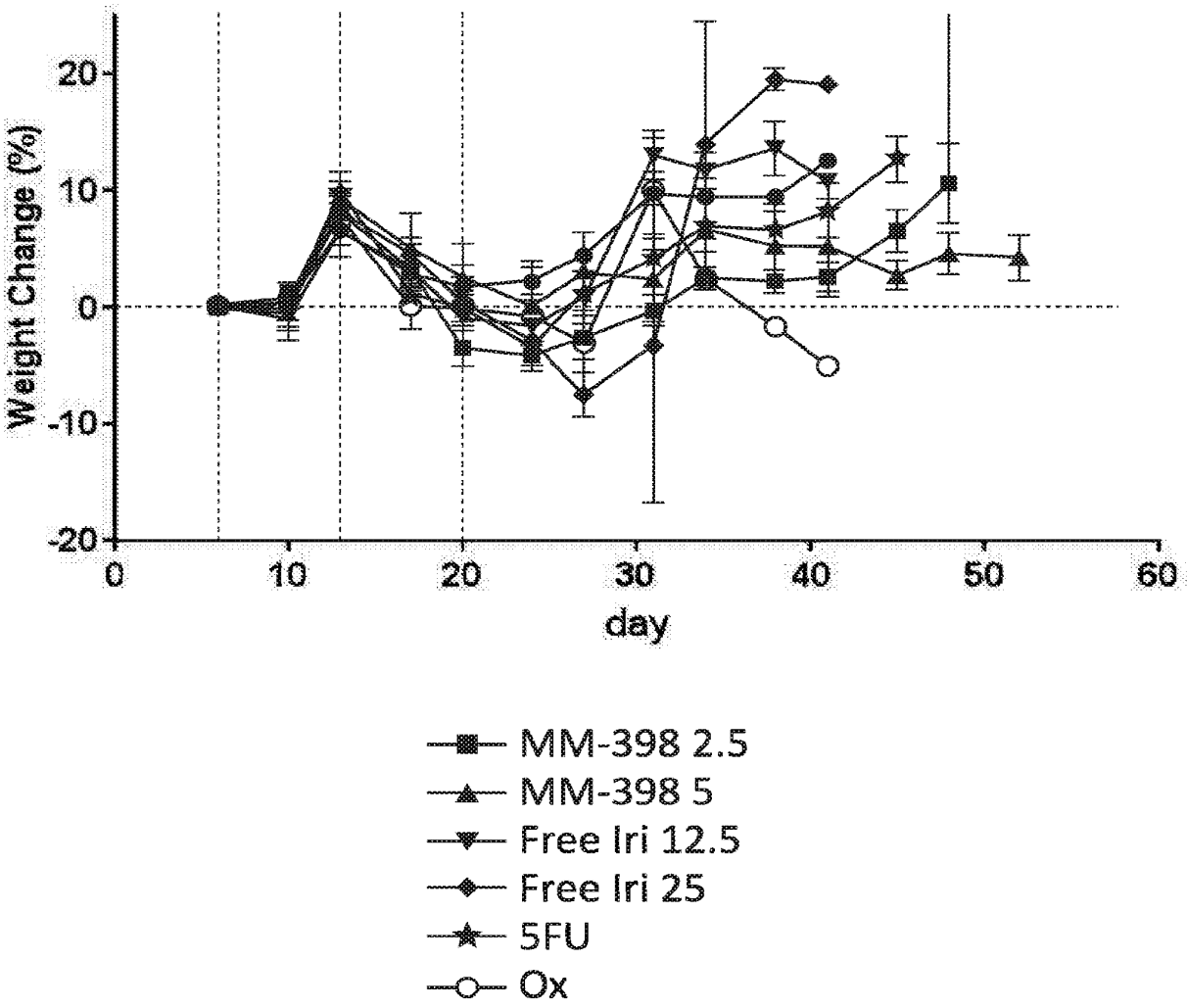

Weight Change (%)
20
10
0
-10
-20
0
10
20
30
40
50
60
day
MM-398 2.5
MM-398 5
Free Iri 12.5
Free Iri 25
5FU
Ox

FIG. 6B

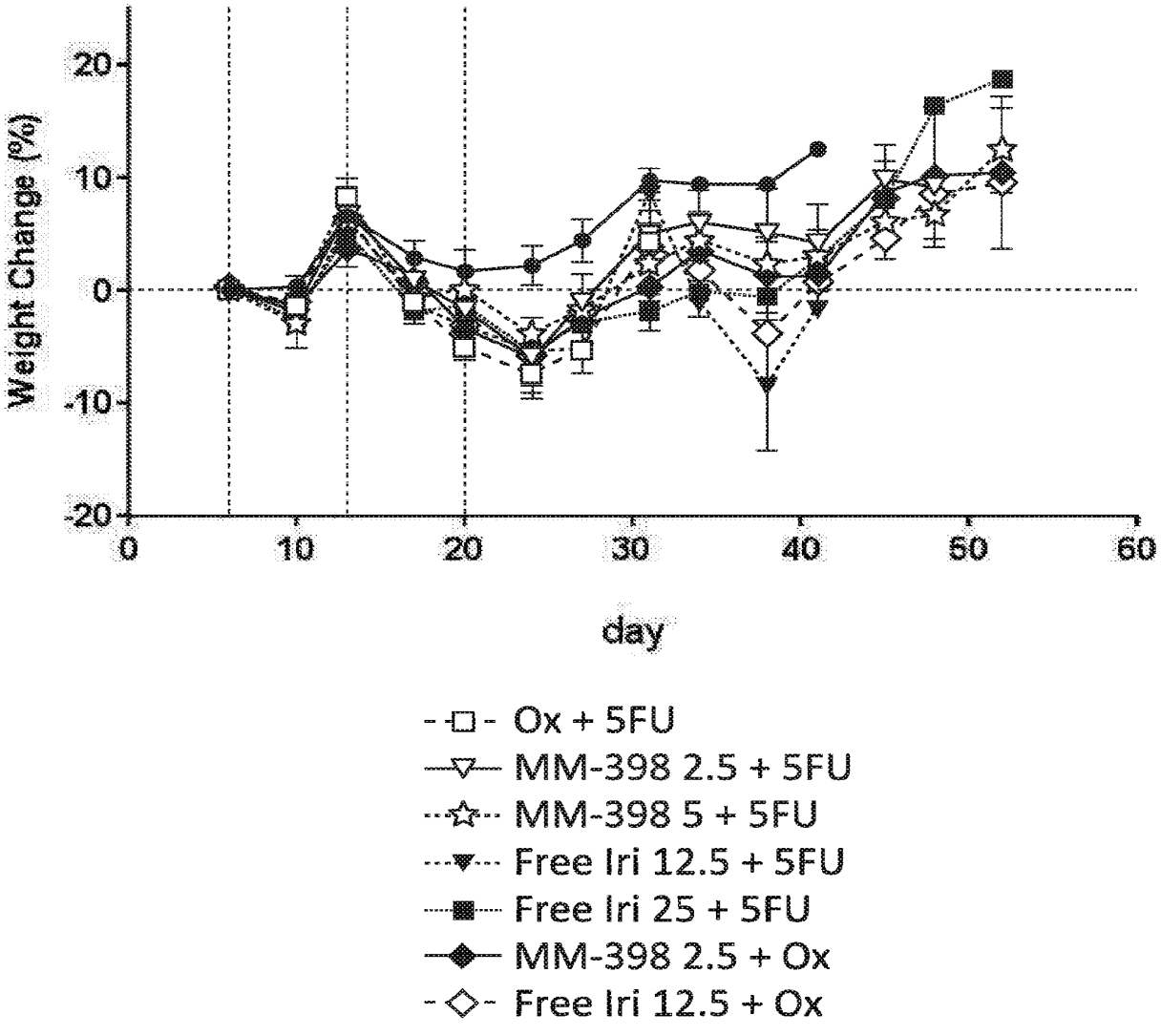

Weight Change (%)
20
10
0
-10
-20
0
10
20
30
40
50
60
day
Ox + 5FU
MM-398 2.5 + 5FU
MM-398 5 + 5FU
Free Iri 12.5 + 5FU
Free Iri 25 + 5FU
MM-398 2.5 + Ox
Free Iri 12.5 + Ox

FIG. 6C

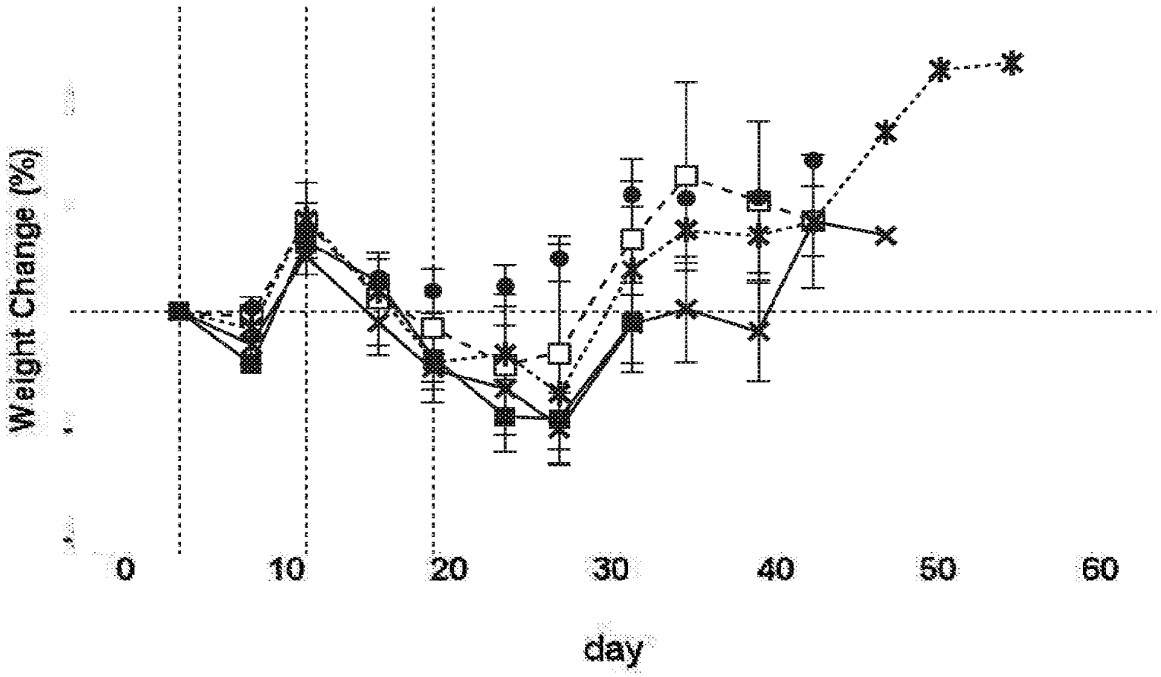

Weight Change (%)
0
10
20
30
40
50
60
day
MM-398 2.5 + 5FU + Ox
MM-398 5 + 5FU + Ox
Free Iri 12.5 + 5FU + Ox
Free Iri 25 + 5FU + Ox

FIG. 7

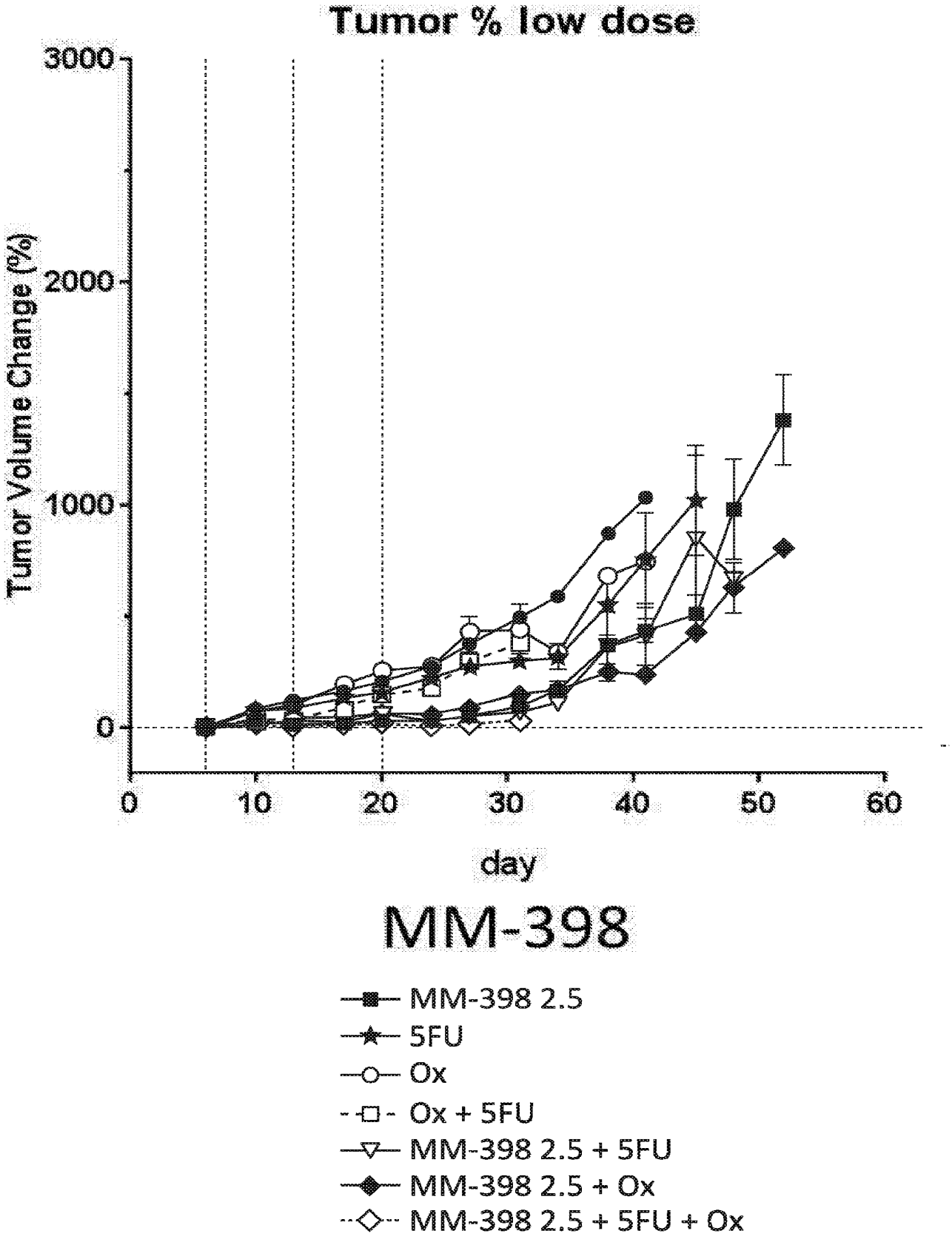

Tumor % low dose
Tumor Volume Change (%)
3000
2000
1000
0
0
10
20
30
40
50
60
day
MM-398
MM-398 2.5
5FU
Ox
Ox + 5FU
MM-398 2.5 + 5FU
MM-398 2.5 + Ox
MM-398 2.5 + 5FU + Ox

FIG. 7B

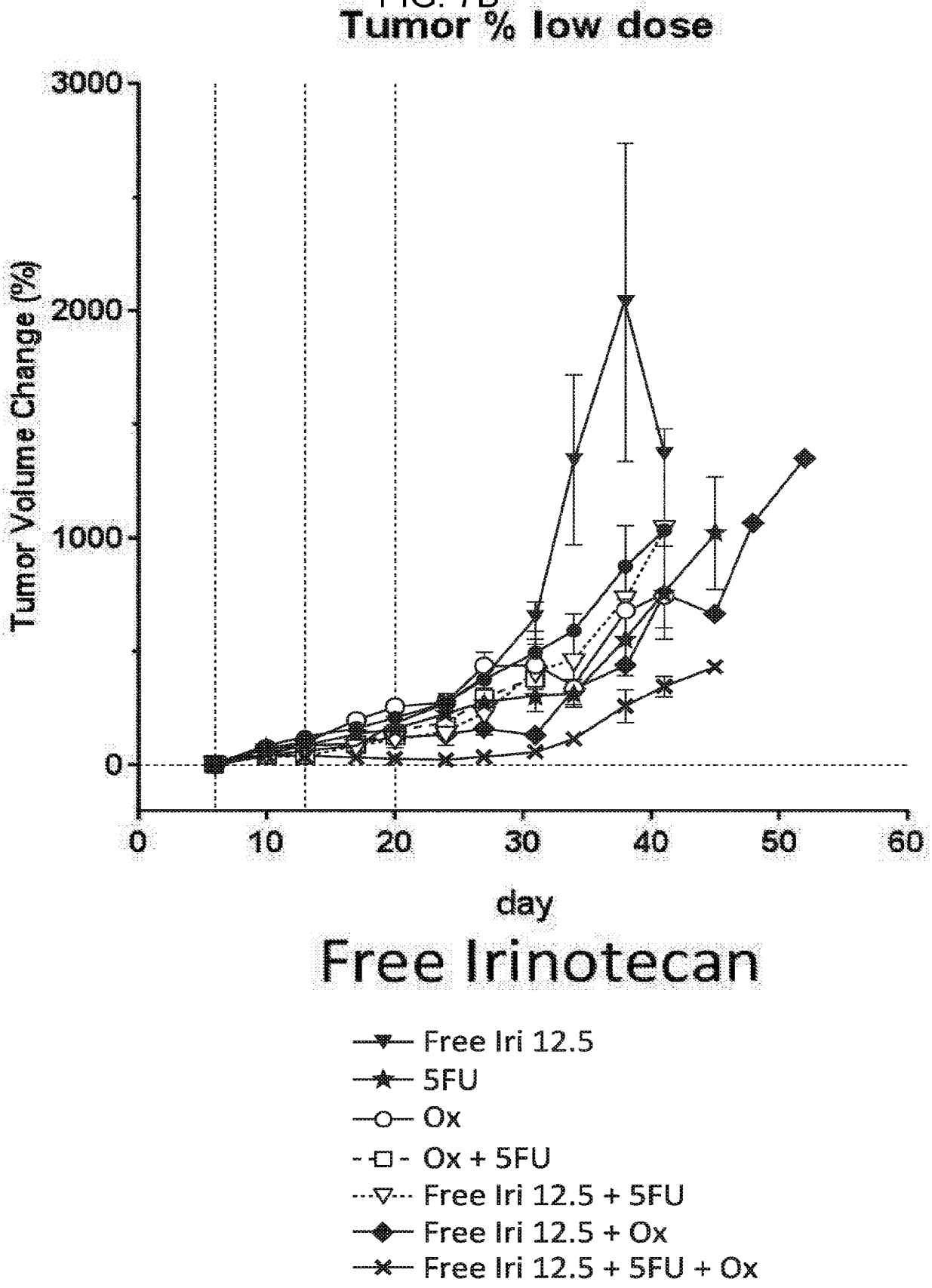

Tumor % low dose
Tumor Volume Change (%)
3000
2000
1000
0
0
10
20
30
40
50
60
day
Free Irinotecan
Free Iri 12.5
5FU
Ox
Ox + 5FU
Free Iri 12.5 + 5FU
Free Iri 12.5 + Ox
Free Iri 12.5 + 5FU + Ox

FIG. 8A

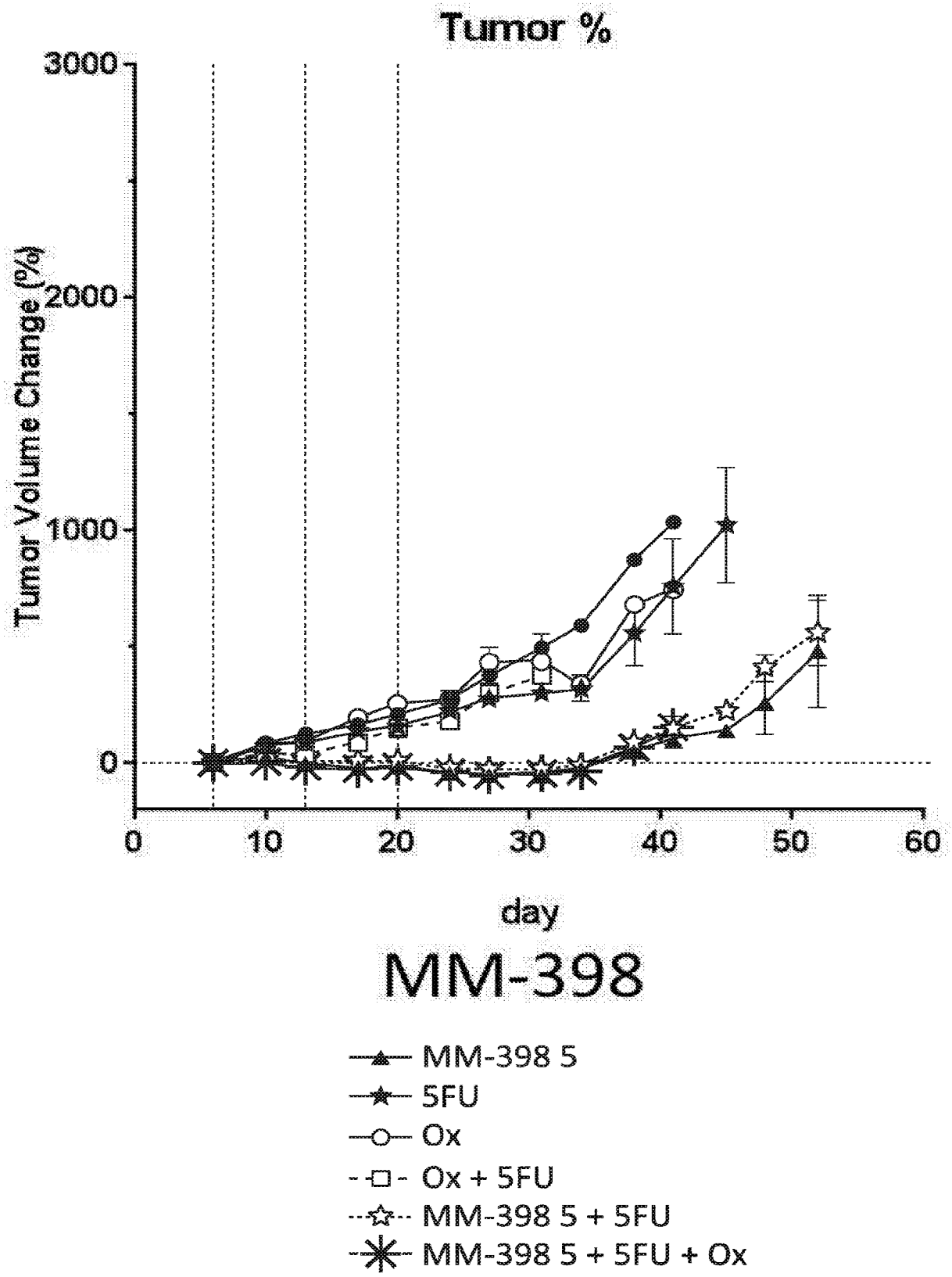
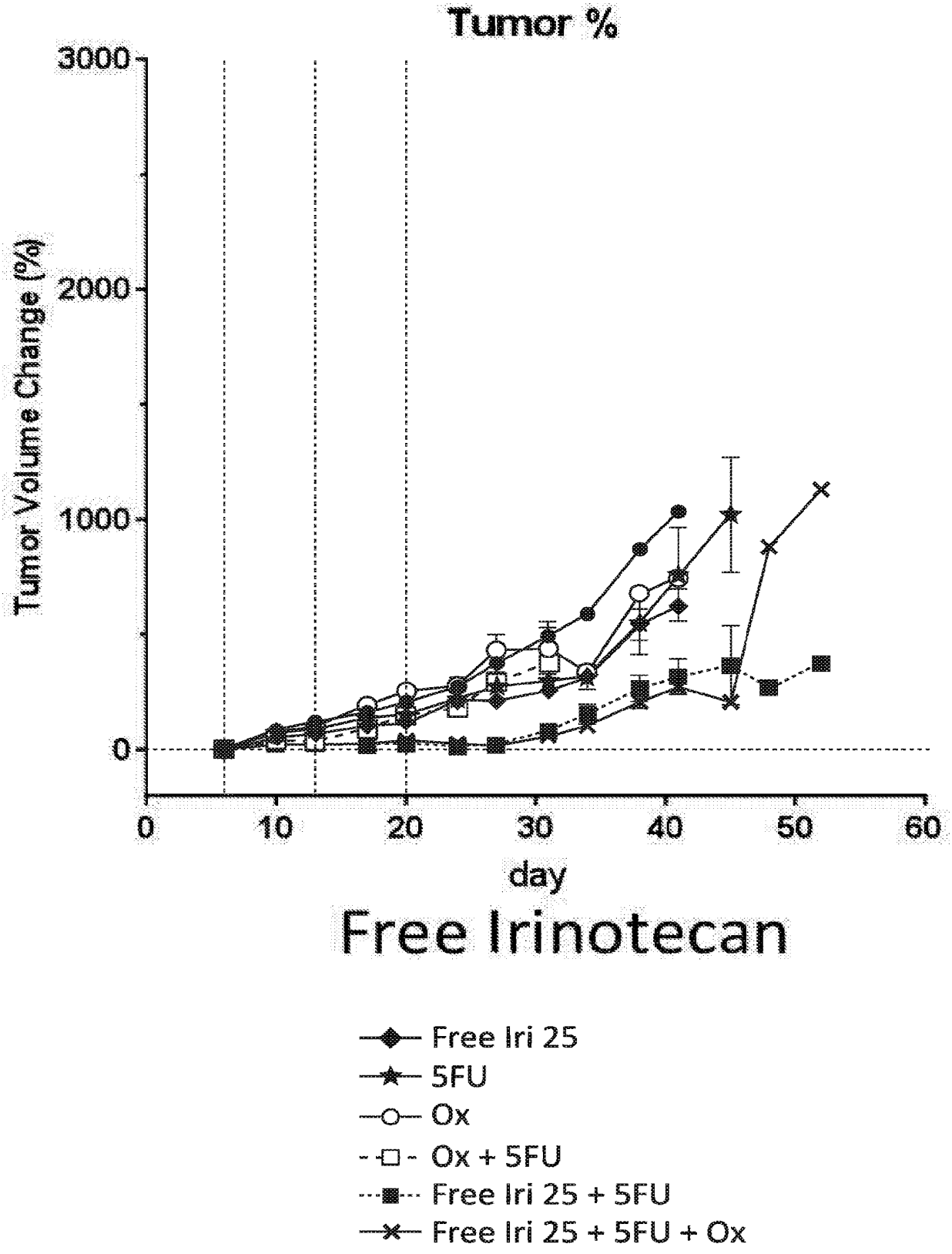

Tumor %
Tumor Volume Change (%)
3000
2000
1000
0
0
10
20
30
40
50
60
day
MM-398
MM-398 5
5FU
Ox
Ox + 5FU
MM-398 5 + 5FU
MM-398 5 + 5FU + Ox

FIG. 8B


Tumor %
Tumor Volume Change (%)
3000
2000
1000
0
0
10
20
30
40
50
60
day
Free Irinotecan
Free Iri 25
5FU
Ox
Ox + 5FU
Free Iri 25 + 5FU
Free Iri 25 + 5FU + Ox

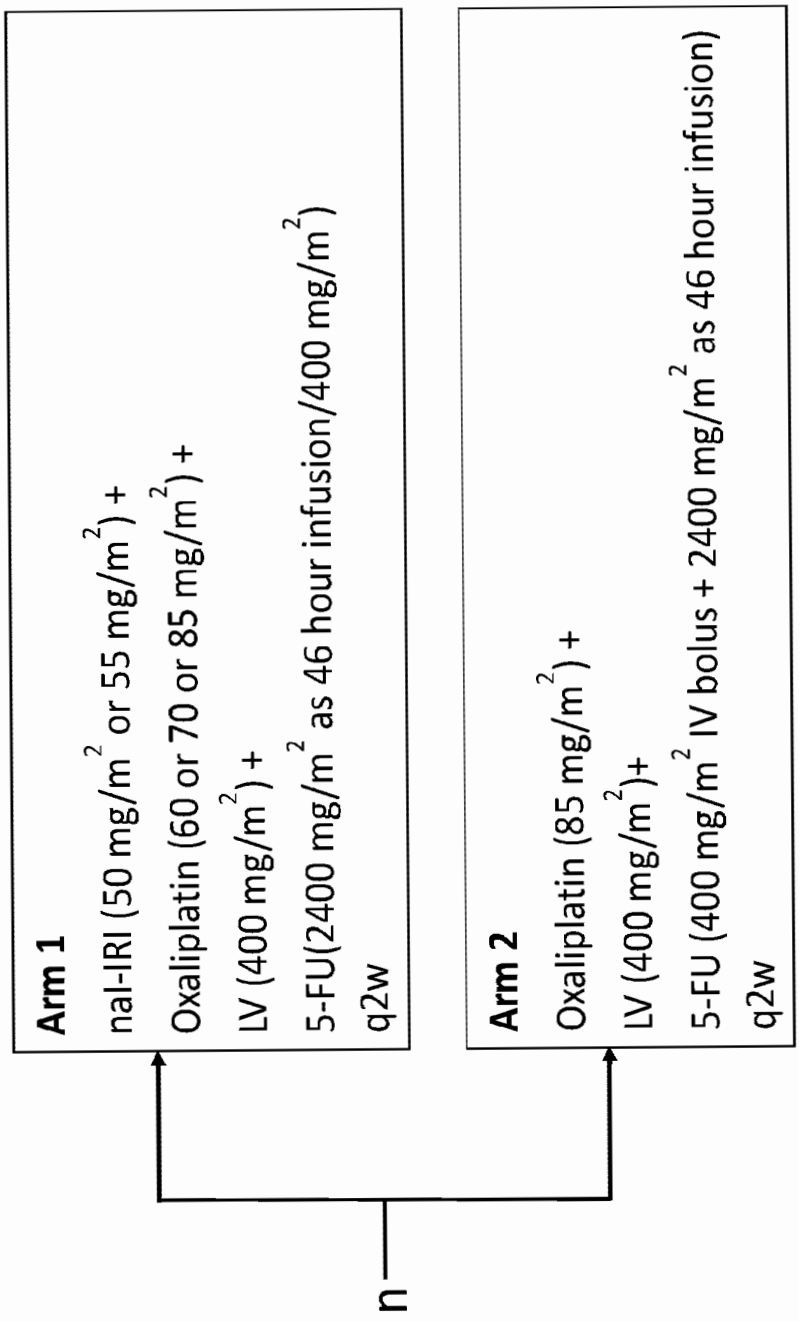

Arm 1
nal-IRI (50 mg/m2 or 55 mg/m2) +
Oxaliplatin (60 or 70 or 85 mg/m2) +
LV (400 mg/m2) +
5-FU(2400 mg/m2 as 46 hour infusion/400 mg/m2)
q2w
Arm 2
Oxaliplatin (85 mg/m2) +
LV (400 mg/m2)+
5-FU (400 mg/m2 IV bolus + 2400 mg/m2 as 46 hour infusion)
q2w
n

FIG. 9

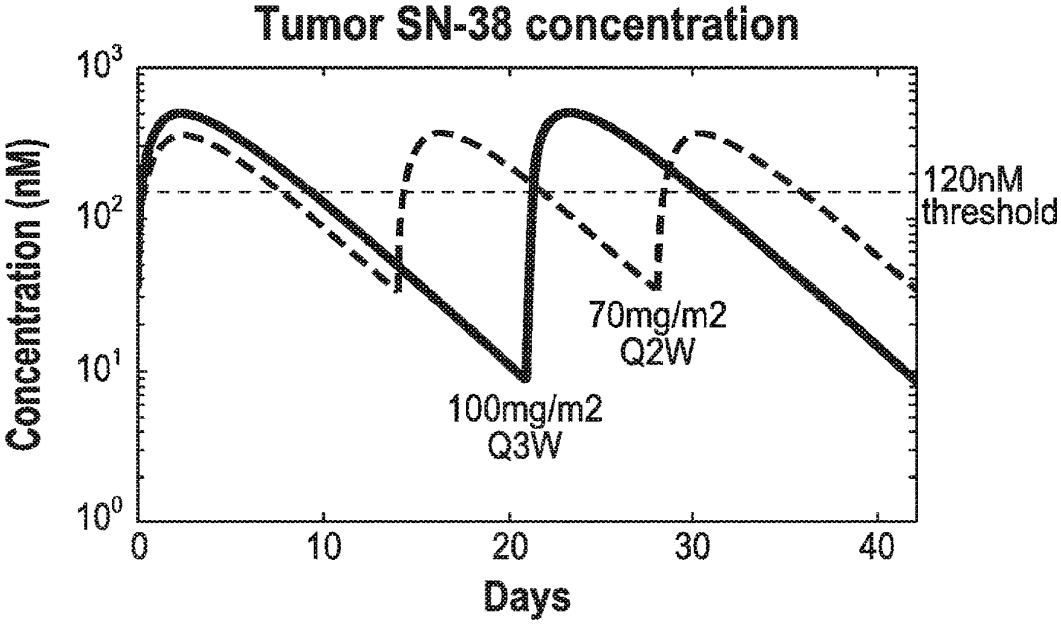


FIG. 10A

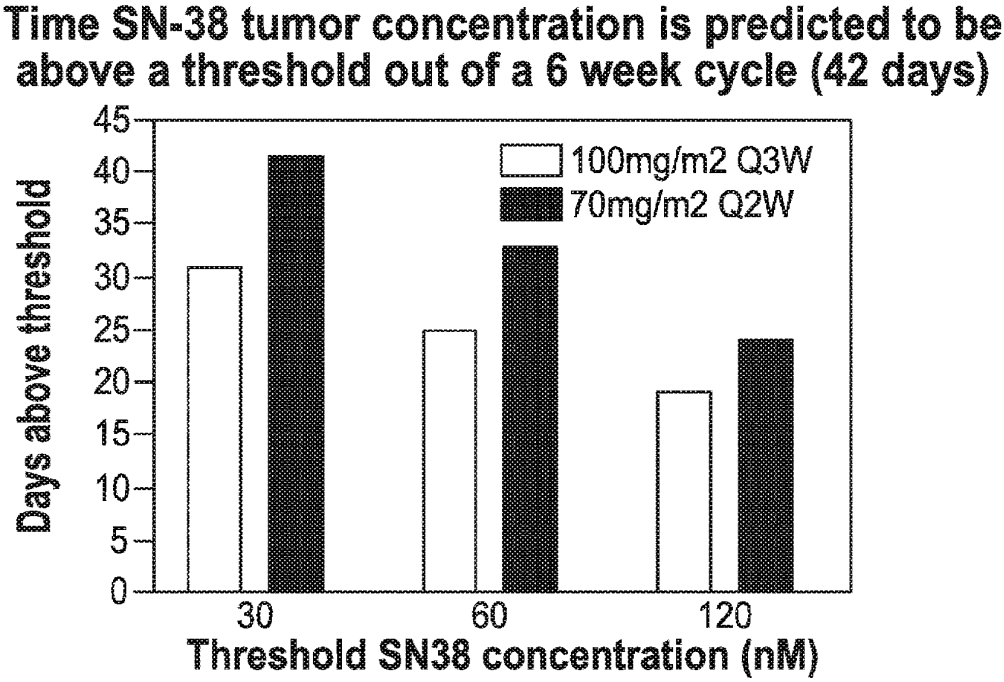
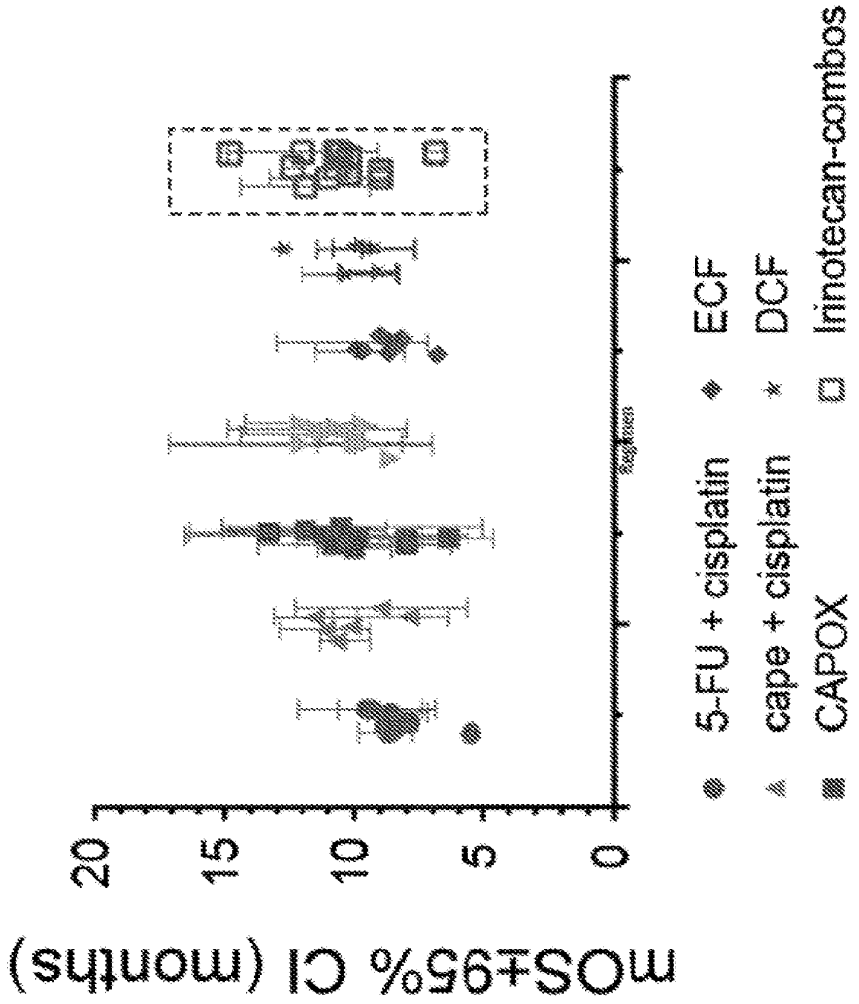


FIG. 10B

FIG. 11


mOS±95% CI (months)
20
15
10
5
0
5-FU + cisplatin
cape + cisplatin
CAPOX
FOLFOX
ECF
DCF
Irinotecan-combos

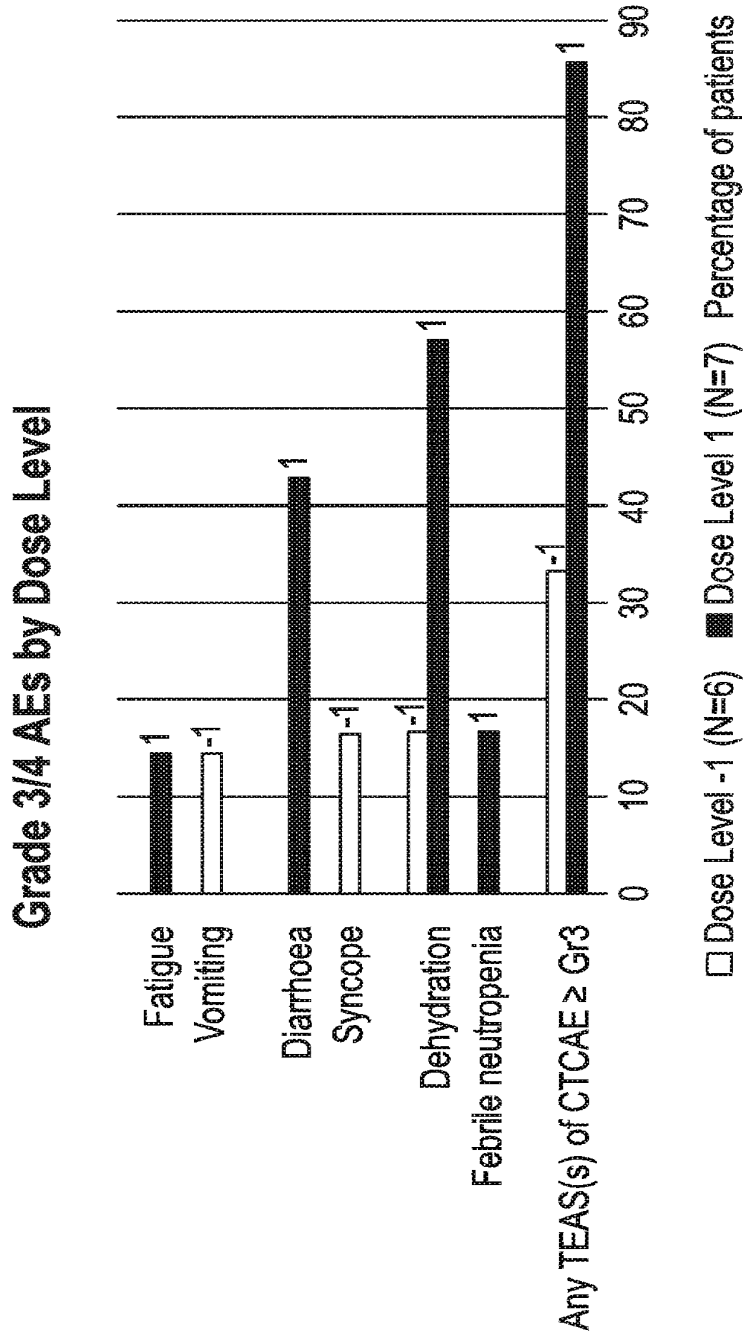

Grade 3/4 AEs by Dose Level
Fatigue
Vomiting
Diarrhoea
Syncope
Dehydration
Febrile neutropenia
Any TEAS(s) of CTCAE ≥ Gr3
1
-1
1
-1
-1
1
1
-1
1
0
10
20
30
40
50
60
70
80
90
Dose Level -1 (N=6)
Dose Level 1 (N=7)
Percentage of patients

FIG. 12

Patient Disposition

- All patients in dose level -1 are still on treatment

Reason for treatment Termination	Dose Level 1 (N=7)	Dose Level -1 (N=6)
Total Discontinuations	7	0
Investigator Decision	2	0
AE	1	0
Symptomatic Deterioration	1	0
pCR	1	0

FIG. 13

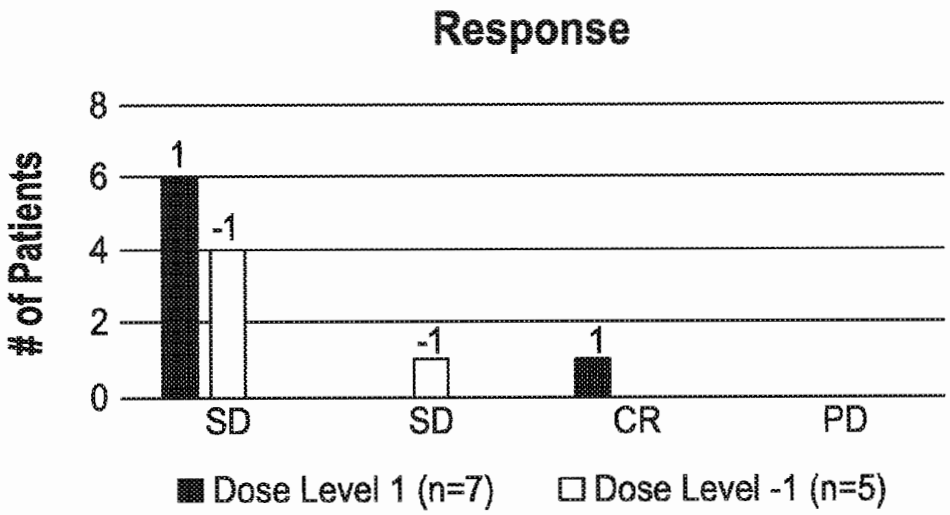


FIG. 14

Primary system organ class Preferred term	Dose Level -1 (N=6)	Dose Level 1 (N=7)	Total (N=13)
Subjects With Any TEAE(s) of CTCAE ≥ Gr 3	**2 (33.3)**	**6 (85.7)**	**8 (61.5)**
Blood and lymphatic system disorders	**1 (16.7)**	**0**	**1 (7.7)**
Febrile neutropenia	1 (16.7)	0	1 (7.7)
Metabolism and nutrition disorders	**1 (16.7)**	**5 (71.4)**	**6 (46.2)**
Dehydration	1 (16.7)	4 (57.1)	5 (38.5)
Decreased appetite	0	2 (28.6)	2 (15.4)
Hypokalaemia	0	2 (28.6)	2 (15.4)
Hypoalbuminaemia	0	1 (14.3)	1 (7.7)
Nervous system disorders	**1 (16.7)**	**0**	**1 (7.7)**
Syncope	1 (16.7)	0	1 (7.7)
Gastrointestinal disorders	**0**	**4 (57.1)**	**4 (30.8)**
Diarrhoea	0	3 (42.9)	3 (23.1)
Enteritis	0	1 (14.3)	1 (7.7)
Faecal incontinence	0	1 (14.3)	1 (7.7)
Large intestinal obstruction	0	1 (14.3)	1 (7.7)
Vomiting	0	1 (14.3)	1 (7.7)
General disorders and administration site conditions	**0**	**1 (14.3)**	**1 (7.7)**
Fatigue	0	1 (14.3)	1 (7.7)
Infections and infestations	**0**	**2 (28.6)**	**2 (15.4)**
Clostridium difficile colitis	0	1 (14.3)	1 (7.7)
Neutropenic sepsis	0	1 (14.3)	1 (7.7)
Pneumonia	0	1 (14.3)	1 (7.7)
Vascular disorders	**0**	**1 (14.3)**	**1 (7.7)**
Orthostatic hypotension	0	1 (14.3)	1 (7.7)
Uncoded SOC	**1 (16.7)**	**2 (28.6)**	**3 (23.1)**
Uncoded: Upper GI bleed	1 (16.7)	0	1 (7.7)
Uncoded: Hypertension	0	1 (14.3)	1 (7.7)
Uncoded: Small Bowel Oedema	0	1 (14.3)	1 (7.7)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/053293

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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

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

[X] See patent family annex.

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

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/053293

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/053293

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2017/053293

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2017/053293

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WO 2017031442 A1	23-02-2017	TW 201713312 A	16-04-2017
		US 2017049767 A1	23-02-2017
		WO 2017031442 A1	23-02-2017
		WO 2017031445 A1	23-02-2017
WO 2017172678 A1	05-10-2017	NONE	

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(22) International Filing Date: 28 May 2004 (28.05.2004)

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(26) Publication Language: English

(30) Priority Data:
60/474,480 30 May 2003 (30.05.2003) US

(71) Applicant *(for all designated States except US)*: **GENENTECH, INC.** [US/US]; 1 DNA Way, South San Francisco, California 94080-4990 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FYFE, Gwendolyn** [US/US]; 167 Carl Street, San Francisco, California 94117 (US). **HOLMGREN, Eric** [US/US]; 2124 Dartmouth Street, Palo Alto, California 94306 (US). **MASS, Robert D.** [US/US]; 106 Elinor Avenue, Mill Valley, California 94941 (US). **NOVOTNY, William** [US/US]; 637 Lombardy Way, Emerald Hills, California 94062 (US).

(74) Agents: **CUI, Steven X.** et al.; GENENTECH, INC., 1 DNA Way, South San Francisco, California 94080-4990 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** TREATMENT WITH ANTI-VEGF ANTIBODIES

(57) **Abstract:** This invention concerns in general treatment of diseases and pathological conditions with anti-VEGF antibodies. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer using an anti-VEGF antibody, preferably in combination with one or more additional anti-tumor therapeutic agents.

WO 2005/000900 A1

TREATMENT WITH ANTI-VEGF ANTIBODIES

This application claims priority to United States Provisional Application Serial No. 60/474,480, filed May 30, 2003, the disclosure of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates in general to treatment of human diseases and pathological conditions. More specifically, the invention relates to anti-angiogenesis therapy of cancer, either alone or in combination with other anti-cancer therapies.

BACKGROUND OF THE INVENTION

Cancer remains to be one of the most deadly threats to human health. In the U.S., cancer affects nearly 1.3 million new patients each year, and is the second leading cause of death after heart disease, accounting for approximately 1 in 4 deaths. It is also predicted that cancer may surpass cardiovascular diseases as the number one cause of death within 5 years. Solid tumors are responsible for most of those deaths. Although there have been significant advances in the medical treatment of certain cancers, the overall 5-year survival rate for all cancers has improved only by about 10% in the past 20 years. Cancers, or malignant tumors, metastasize and grow rapidly in an uncontrolled manner, making timely detection and treatment extremely difficult. Furthermore, cancers can arise from almost any tissue in the body through malignant transformation of one or a few normal cells within the tissue, and each type of cancer with particular tissue origin differs from the others.

Current methods of cancer treatment are relatively non-selective. Surgery removes the diseased tissue; radiotherapy shrinks solid tumors; and chemotherapy kills rapidly dividing cells. Chemotherapy, in particular, results in numerous side effects, in some cases so severe as to limit the dosage that can be given and thus preclude the use of potentially effective drugs. Moreover, cancers often develop resistance to chemotherapeutic drugs.

Thus, there is an urgent need for specific and more effective cancer therapies.

Angiogenesis is an important cellular event in which vascular endothelial cells proliferate, prune and reorganize to form new vessels from preexisting vascular network. There are compelling evidences that the development of a vascular supply is essential for

normal and pathological proliferative processes (Folkman and Klagsbrun (1987) *Science* 235:442-447). Delivery of oxygen and nutrients, as well as the removal of catabolic products, represent rate-limiting steps in the majority of growth processes occurring in multicellular organisms. Thus, it has been generally assumed that the vascular compartment is necessary, not only for organ development and differentiation during embryogenesis, but also for wound healing and reproductive functions in the adult.

Angiogenesis is also implicated in the pathogenesis of a variety of disorders, including but not limited to, tumors, proliferative retinopathies, age-related macular degeneration, rheumatoid arthritis (RA), and psoriasis. Angiogenesis is essential for the growth of most primary tumors and their subsequent metastasis. Tumors can absorb sufficient nutrients and oxygen by simple diffusion up to a size of 1-2 mm, at which point their further growth requires the elaboration of vascular supply. This process is thought to involve recruitment of the neighboring host mature vasculature to begin sprouting new blood vessel capillaries, which grow towards, and subsequently infiltrate, the tumor mass. In addition, tumor angiogenesis involve the recruitment of circulating endothelial precursor cells from the bone marrow to promote neovascularization. Kerbel (2000) *Carcinogenesis* 21:505-515; Lynden et al. (2001) *Nat. Med.* 7:1194-1201.

While induction of new blood vessels is considered to be the predominant mode of tumor angiogenesis, recent data have indicated that some tumors may grow by co-opting existing host blood vessels. The co-opted vasculature then regresses, leading to tumor regression that is eventually reversed by hypoxia-induced angiogenesis at the tumor margin. Holash et al. (1999) *Science* 284:1994-1998.

In view of the remarkable physiological and pathological importance of angiogenesis, much work has been dedicated to the elucidation of the factors capable of regulating this process. It is suggested that the angiogenesis process is regulated by a balance between pro- and anti- angiogenic molecules, and is derailed in various diseases, especially cancer. Carmeliet and Jain (2000) *Nature* 407:249-257.

Vascular endothelial cell growth factor (VEGF), which is also termed VEGF-A or vascular permeability factor (VPF), has been reported as a pivotal regulator of both normal and abnormal angiogenesis. Ferrara and Davis-Smyth (1997)*Endocrine Rev.* 18:4-25; Ferrara (1999) *J. Mol. Med.* 77:527-543. Compared to other growth factors that contribute to the processes of vascular formation, VEGF is unique in its high specificity for endothelial cells

within the vascular system. VEGF is essential for embryonic vasculogenesis and angiogenesis. Carmeliet et al. (1996) *Nature* 380:435-439; Ferrara et al. (1996) *Nature* 380:439-442. Furthermore, VEGF is required for the cyclical blood vessel proliferation in the female reproductive tract and for bone growth and cartilage formation. Ferrara et al. (1998) *Nature Med.* 4:336-340; Gerber et al. (1999) *Nature* Med. 5:623-628.

In addition to being an angiogenic factor in angiogenesis and vasculogenesis, VEGF, as a pleiotropic growth factor, exhibits multiple biological effects in other physiological processes, such as endothelial cell survival, vessel permeability and vasodilation, monocyte chemotaxis and calcium influx. Ferrara and Davis-Smyth (1997), *supra.* Moreover, recent studies have reported mitogenic effects of VEGF on a few non-endothelial cell types, such as retinal pigment epithelial cells, pancreatic duct cells and Schwann cells. Guerrin et al. (1995) *J. Cell Physiol.* 164:385-394; Oberg-Welsh et al. (1997) *Mol. Cell. Endocrinol.* 126:125-132; Sondell et al. (1999) *J. Neurosci.* 19:5731-5740.

Substantial evidence also implicates VEGF's critical role in the development of conditions or diseases that involve pathological angiogenesis. The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol.*. 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer.* 73:931-934 (1996); and Dvorak *et al. Am J. Pathol.* 146:1029-1039 (1995)). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Aiello *et al. N. Engl. J. Med.* 331:1480-1487 (1994)). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez *et al. Invest. Ophtalmo. Vis. Sci.* 37:855-868 (1996)).

Given its central role in promoting tumor growth, VEGF provides an attractive target for therapeutic intervention. Indeed, a variety of therapeutic strategies aimed at blocking VEGF or its receptor signaling system are currently being developed for the treatment of neoplastic diseases. Rosen (2000) *Oncologist* 5:20-27; Ellis et al. (2000) *Oncologist* 5:11-15; Kerbel (2001) *J. Clin. Oncol.* 19:45S-51S. So far, VEGF/VEGF receptor blockade by monoclonal antibodies and inhibition of receptor signaling by tyrosine kinase inhibitors are the best studied approaches. VEGFR-1 ribozymes, VEGF toxin conjugates, and soluble VEGF receptors are also being investigated.

The anti-VEGF antibody "Bevacizumab (BV)", also known as "rhuMAb VEGF" or "Avastin™", is a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. (1997) *Cancer Res.* 57:4593-4599. It comprises mutated human IgG1 framework regions and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF to its receptors. Approximately 93% of the amino acid sequence of Bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the sequence is derived from the murine antibody A4.6.1. Bevacizumab has a molecular mass of about 149,000 daltons and is glycosylated. Bevacizumab is being investigated clinically for treating various cancers, and some early stage trials have shown promising results. Kerbel (2001) *J. Clin. Oncol.* 19:45S-51S; De Vore et al. (2000) *Proc. Am. Soc. Clin. Oncol.* 19:485a; Johnson et al. (2001) *Proc. Am. Soc. Clin. Oncol.* 20:315a; Kabbinavar et al. (2003) *J. Clin. Oncol.* 21:60-65.

SUMMARY OF THE INVENTION

The present invention concerns methods of using anti-VEGF antibody for treating diseases and pathological conditions. In particular, the invention provides an effective approach for treating cancers, partially based on the unexpected results that adding anti-VEGF antibody to a standard chemotherapy results in statistically significant and clinically meaningful improvements among cancer patients.

Accordingly, in one aspect, the invention provides a method of treating cancer in a human patient, comprising administering to the patient effective amounts of an anti-VEGF antibody and an anti-neoplastic composition, wherein said anti-neoplastic composition comprises at least one chemotherapeutic agent.

The cancer amendable for treatment by the present invention include, but not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer, lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer (including gastrointestinal cancer), pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal

cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome. Preferably, the cancer is selected from the group consisting of breast cancer, colorectal cancer, rectal cancer, non-small cell lung cancer, non-Hodgkins lymphoma (NHL), renal cell cancer, prostate cancer, liver cancer, pancreatic cancer, soft-tissue sarcoma, kaposi's sarcoma, carcinoid carcinoma, head and neck cancer, melanoma, ovarian cancer, mesothelioma, and multiple myeloma. More preferably, the cancer is colorectal cancer. The cancerous conditions amendible for treatment of the invention include metastatic cancers. The method of the present invention is particularly suitable for the treatment of vascularized tumors.

Any chemotherapeutic agent exhibiting anticancer activity can be used according to the present invention. Preferably, the chemotherapeutic agent is selected from the group consisting of alkylating agents, antimetabolites, folic acid analogs, pyrimidine analogs, purine analogs and related inhibitors, vinca alkaloids, epipodopyyllotoxins, antibiotics, L-Asparaginase, topoisomerase inhibitor, interferons, platinum cooridnation complexes, anthracenedione substituted urea, methyl hydrazine derivatives, adrenocortical suppressant, adrenocorticosteroides, progestins, estrogens, antiestrogen, androgens, antiandrogen, and gonadotropin-releasing hormone analog. More preferably, the chemotherapeutic agent is selected from the group consisting of 5-fluorouracil (5-FU), leucovorin (LV), irenotecan, oxaliplatin, capecitabine , paclitaxel and doxetaxel. Two or more chemotherapeutic agents can be used in a cocktail to be administered in combination with administration of the anti-VEGF antibody. One preferred combination chemotherapy is fluorouracil-based, comprising 5-FU and one or more other chemotherapeutic agent(s). Suitable dosing regimens of

combination chemotherapies are known in the art and described in, for example, Saltz et al. (1999) *Proc ASCO* 18:233a and Douillard et al. (2000) *Lancet* 355:1041-7.

In one aspect, the present invention provides a method for increasing the duration of survival of a human patient having cancer, comprising administering to the patient effective amounts of an anti-VEGF antibody composition and an anti-neoplastic composition, wherein said anti-neoplastic composition comprises at least one chemotherapeutic agent, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition effectively increases the duration of survival.

In another aspect, the present invention provides a method for increasing the progression free survival of a human patient having cancer, comprising administering to the patient effective amounts of an anti-VEGF antibody composition and an anti-neoplastic composition, wherein said anti-neoplastic composition comprises at least one chemotherapeutic agent, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition effectively increases the duration of progression free survival.

Furthermore, the present invention provides a method for treating a group of human patients having cancer, comprising administering to the patient effective amounts of an anti-VEGF antibody composition and an anti-neoplastic composition, wherein said anti-neoplastic composition comprises at least one chemotherapeutic agent, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition effectively increases the response rate in the group of patients.

In yet another aspect, the present invention provides a method for increasing the duration of response of a human patient having cancer, comprising administering to the patient effective amounts of an anti-VEGF antibody composition and an anti-neoplastic composition, wherein said anti-neoplastic composition comprises at least one chemotherapeutic agent, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition effectively increases the duration of response.

The invention also provides a method of treating a human patient susceptible to or diagnosed with colorectal cancer, comprising administering to the patient effective amounts of an anti-VEGF antibody. The colorectal cancer can be metastatic. The anti-VEGF antibody treatment can be further combined with a standard chemotherapy for colorectal cancer such as the Saltz (5-FU/LV/irinotecan) regimen described by Saltz et al. (1999).

In one preferred embodiment, the invention provides a method of treating a human patient or a group of human patients having metastatic colorectal cancer, comprising administering to the patient effective amounts of an anti-VEGF antibody composition and an anti-neoplastic composition, wherein said anti-neoplastic composition comprises at least one chemotherapeutic agent, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition results in statistically significant and clinically meaningful improvement of the treated patient as measured by the duration of survival, progression free survival, response rate or duration of response. Preferably, the anti-neoplastic composition is a fluorouracil based combination regimen. More preferably the combination regimen comprises 5-FU+leucovorin, 5-FU+leucovorin+irinotecan (IFL), or 5-FU+leucorvin+oxaliplatin (FOLFOX).

The invention provides an article of manufacture comprising a container, a composition within the container comprising an anti-VEGF antibody and a package insert instructing the user of the composition to administer to a cancer patient the anti-VEGF antibody composition and an anti-neoplastic composition comprising at least one chemotherapeutic agent.

The invention also provides a kit for treating cancer in a patient comprising a package comprising an anti-VEGF antibody composition and instructions for using the anti-VEGF antibody composition and an anti-neoplastic composition comprising at least one chemotherapeutic agent for treating cancer in a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents Kaplan-Meier estimates of survival. The median duration of survival (indicated by the dotted lines) was 20.3 months in the group given irinotecan, fluorouracil, and leucovorin (IFL) plus bevacizumab, as compared with 15.6 months in the group given IFL plus placebo, corresponding to a hazard ratio for death of 0.66 ($P<0.001$).

Figure 2 represents Kaplan-Meier estimates of progression-free survival. The median duration of progression-free survival (indicated by the dotted lines) was 10.6 months in the group given irinotecan, fluorouracil, and leucovorin (IFL) plus bevacizumab, as compared with 6.2 months in the group given IFL plus placebo, corresponding to a hazard ratio for progression of 0.54 ($P<0.001$).

Figures 3A-3C provide analysis of duration of survival by different subgroups of patients divided by baseline characteristics.

Figure 4 represents Kaplan-Meier estimates of survival comparing the group given 5-FU/LV plus placebo vs. the group given 5-FU/LV plus bevacizumab (BV).

Figure 5 represents Kaplan-Meier estimates of progression-free survival comparing the group given 5-FU/LV plus placebo vs. the group given 5-FU/LV plus bevacizumab (BV).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. DEFINITIONS

The terms "VEGF" and "VEGF-A" are used interchangeably to refer to the 165-amino acid vascular endothelial cell growth factor and related 121-, 189-, and 206- amino acid vascular endothelial cell growth factors, as described by Leung *et al. Science*, 246:1306 (1989), and Houck *et al. Mol. Endocrin.*, 5:1806 (1991), together with the naturally occurring allelic and processed forms thereof. The term "VEGF" is also used to refer to truncated forms of the polypeptide comprising amino acids 8 to 109 or 1 to 109 of the 165-amino acid human vascular endothelial cell growth factor. Reference to any such forms of VEGF may be identified in the present application, e.g., by "VEGF (8-109)," "VEGF (1-109)" or "$VEGF_{165}$." The amino acid positions for a "truncated" native VEGF are numbered as indicated in the native VEGF sequence. For example, amino acid position 17 (methionine) in truncated native VEGF is also position 17 (methionine) in native VEGF. The truncated native VEGF has binding affinity for the KDR and Flt-1 receptors comparable to native VEGF.

An "anti-VEGF antibody" is an antibody that binds to VEGF with sufficient affinity and specificity. Preferably, the anti-VEGF antibody of the invention can be used as a therapeutic agent in targeting and interfering with diseases or conditions wherein the VEGF activity is involved. An anti-VEGF antibody will usually not bind to other VEGF homologues such as VEGF-B or VEGF-C, nor other growth factors such as PlGF, PDGF or bFGF. A preferred anti-VEGF antibody is a monoclonal antibody that binds to the same epitope as the monoclonal anti-VEGF antibody A4.6.1 produced by hybridoma ATCC HB 10709. More preferably the anti-VEGF antibody is a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. (1997) Cancer Res. 57:4593-4599, including but not limited to the antibody known as bevacizumab (BV; Avastin™).

A "VEGF antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with VEGF activities including its binding to one or more VEGF receptors. VEGF antagonists include anti-VEGF antibodies and antigen-binding fragments thereof, receptor molecules and derivatives which bind specifically to VEGF thereby sequestering its binding to one or more receptors, anti-VEGF receptor antibodies and VEGF receptor antagonists such as small molecule inhibitors of the VEGFR tyrosine kinases.

Throughout the present specification and claims, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

A "native sequence" polypeptide comprises a polypeptide having the same amino acid sequence as a polypeptide derived from nature. Thus, a native sequence polypeptide can have the amino acid sequence of naturally-occurring polypeptide from any mammal. Such native sequence polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence" polypeptide specifically encompasses naturally-occurring truncated or secreted forms of the polypeptide (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide.

A polypeptide "variant" means a biologically active polypeptide having at least about 80% amino acid sequence identity with the native sequence polypeptide. Such variants include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the polypeptide. Ordinarily, a variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the native sequence polypeptide.

The term "antibody" is used in the broadest sense and includes monoclonal antibodies (including full length or intact monoclonal antibodies), polyclonal

antibodies, multivalent antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments (see below) so long as they exhibit the desired biological activity.

Unless indicated otherwise, the expression "multivalent antibody" is used throughout this specification to denote an antibody comprising three or more antigen binding sites. The multivalent antibody is preferably engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

"Antibody fragments" comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment (Ward *et al., Nature* 341, 544-546 (1989)) which consists of a VH domain; (vii) isolated CDR regions; (viii) $F(ab')_2$ fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (*e.g.* single chain Fv; scFv) (Bird *et al., Science* 242:423-426 (1988); and Huston *et al., PNAS (USA)* 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (see, *e.g.*, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995); and US Patent No. 5,641,870).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally

occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen. Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) or Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all

of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan *et al. Nature Biotechnology* 14:309-314 (1996): Sheets *et al. PNAS (USA)* 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks *et al., J. Mol. Biol.*, 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, *e.g.,* mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al., Bio/Technology* 10: 779-783 (1992); Lonberg *et al., Nature* 368: 856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild *et al., Nature Biotechnology* 14: 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized *in vitro).* See, *e.g.*, Cole *et al., Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner *et al., J. Immunol.*, 147 (1):86-95 (1991); and US Pat No. 5,750,373.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks *et al. Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas *et al. Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier *et al. Gene* 169:147-155 (1995); Yelton *et al. J. Immunol.* 155:1994-2004 (1995); Jackson *et al., J. Immunol.* 154(7):3310-9 (1995); and Hawkins *et al, J. Mol. Biol.* 226:889-896 (1992).

An "isolated" polypeptide is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to greater than 95% by weight of polypeptide as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes the polypeptide *in situ* within recombinant cells since at least one component of the polypeptide's natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

A "functional antigen binding site" of an antibody is one which is capable of binding a target antigen. The antigen binding affinity of the antigen binding site is not necessarily as strong as the parent antibody from which the antigen binding site is derived, but the ability to bind antigen must be measurable using any one of a variety of methods known for evaluating antibody binding to an antigen. Moreover, the antigen binding affinity of each of the antigen binding sites of a multivalent antibody herein need not be quantitatively the same. For the multimeric antibodies herein, the number of functional antigen binding sites can be evaluated using ultracentrifugation

analysis as described in Example 2 below. According to this method of analysis, different ratios of target antigen to multimeric antibody are combined and the average molecular weight of the complexes is calculated assuming differing numbers of functional binding sites. These theoretical values are compared to the actual experimental values obtained in order to evaluate the number of functional binding sites.

An antibody having a "biological characteristic" of a designated antibody is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen.

In order to screen for antibodies which bind to an epitope on an antigen bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed.

An "agonist antibody" is an antibody which binds to and activates a receptor. Generally, the receptor activation capability of the agonist antibody will be at least qualitatively similar (and may be essentially quantitatively similar) to a native agonist ligand of the receptor. An example of an agonist antibody is one which binds to a receptor in the TNF receptor superfamily and induces apoptosis of cells expressing the TNF receptor. Assays for determining induction of apoptosis are described in WO98/51793 and WO99/37684, both of which are expressly incorporated herein by reference.

A "disorder" is any condition that would benefit from treatment with the antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the

therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy *in vivo* can, for example, be measured by assessing the duration of survival, time to disease progression (TTP), the response rates (RR), duration of response, and/or quality of life.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer (including gastrointestinal cancer), pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular

proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

The term "mammalian host" as used herein refers to any compatible transplant recipient. By "compatible" is meant a mammalian host that will accept the donated graft. Preferably, the host is human. If both the donor of the graft and the host are human, they are preferably matched for HLA class II antigens so as to improve histocompatibility.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.* At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

The term "anti-neoplastic composition" refers to a composition useful in treating cancer comprising at least one active therapeutic agent capable of inhibiting or preventing tumor growth or function, and/or causing destruction of tumor cells. Therapeutic agents suitable in an anti-neoplastic composition for treating cancer include, but not limited to, chemotherapeutic agents, radioactive isotopes, toxins, cytokines such as interferons, and antagonistic agents targeting cytokines, cytokine receptors or antigens associated with tumor cells. For example, therapeutic agents useful in the present invention can be antibodies such as anti-HER2 antibody and anti-CD20 antibody, or small molecule tyrosine kinase inhibitors such as VEGF receptor inhibitors and EGF receptor inhibitors. Preferably the therapeutic agent is a chemotherapeutic agent.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic

analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, e.g., Agnew, *Chem Intl. Ed. Engl.* 33:183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2- ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A

and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol- Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6- thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-11); topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON· toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Ralf and H-Ras; ribozymes such as a VEGF expression inhibitor (e.g., ANGIOZYME® ribozyme) and a HER2 expression inhibitor; vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell *in vitro* and/or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells in S

phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami *et al.* (WB Saunders: Philadelphia, 1995), especially p. 13.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); epidermal growth factor; hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and -beta; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-alpha; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, -beta and -gamma colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-alpha or TNF-beta; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, beta-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human patient, preferably within a pocket between the skin and

underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example,- by pinching or drawing the skin up and away from underlying tissue.

An "angiogenic factor" is a growth factor which stimulates the development of blood vessels. The preferred angiogenic factor herein is Vascular Endothelial Growth Factor (VEGF).

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the polypeptide. The label may be itself be detectable (*e.g.,* radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the polypeptide nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the polypeptide where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

II. PRODUCTION OF ANTI-VEGF ANTIBODIES

A. Antibody Preparation

(i) VEGF Antigen

Means for preparing and characterizing antibodies are well known in the art. A description follows as to exemplary techniques for the production of anti-VEGF antibodies

used in accordance with the present invention. The VEGF antigen to be used for production of antibodies may be, e.g., the $VEGF_{165}$ molecule as well as other isoforms of VEGF or a fragment thereof containing the desired epitope. Other forms of VEGF useful for generating anti-VEGF antibodies of the invention will be apparent to those skilled in the art.

Human VEGF was obtained by first screening a cDNA library prepared from human cells, using bovine VEGF cDNA as a hybridization probe. Leung *et al.* (1989) *Science*, 246:1306. One cDNA identified thereby encodes a 165-amino acid protein having greater than 95% homology to bovine VEGF; this 165-amino acid protein is typically referred to as human VEGF (hVEGF) or $VEGF_{165}$. The mitogenic activity of human VEGF was confirmed by expressing the human VEGF cDNA in mammalian host cells. Media conditioned by cells transfected with the human VEGF cDNA promoted the proliferation of capillary endothelial cells, whereas control cells did not. Leung *et al.* (1989) *Science, supra.*

Although a vascular endothelial cell growth factor could be isolated and purified from natural sources for subsequent therapeutic use, the relatively low concentrations of the protein in follicular cells and the high cost, both in terms of effort and expense, of recovering VEGF proved commercially unavailing. Accordingly, further efforts were undertaken to clone and express VEGF via recombinant DNA techniques. (*See, e.g.*, Ferrara (1995) *Laboratory Investigation* 72:615-618, and the references cited therein).

VEGF is expressed in a variety of tissues as multiple homodimeric forms (121, 145, 165, 189, and 206 amino acids per monomer) resulting from alternative RNA splicing. $VEGF_{121}$ is a soluble mitogen that does not bind heparin; the longer forms of VEGF bind heparin with progressively higher affinity. The heparin-binding forms of VEGF can be cleaved in the carboxy terminus by plasmin to release a diffusible form(s) of VEGF. Amino acid sequencing of the carboxy terminal peptide identified after plasmin cleavage is Arg_{110}-Ala_{111}. Amino terminal "core" protein, VEGF (1-110) isolated as a homodimer, binds neutralizing monoclonal antibodies (such as the antibodies referred to as 4.6.1 and 3.2E3.1.1) and soluble forms of VEGF receptors with similar affinity compared to the intact $VEGF_{165}$ homodimer.

Several molecules structurally related to VEGF have also been identified recently, including placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. Ferrara and Davis-Smyth (1987) Endocr. Rev., *supra*; Ogawa *et al.* (1998) *J. Biological Chem.* 273:31273-31281; Meyer *et al.* (1999) *EMBO J.*, 18:363-374. A receptor tyrosine kinase, Flt-4 (VEGFR-3), has been identified as the receptor for VEGF-C and VEGF-D. Joukov et al.

(1996) *EMBO. J.* 15:1751; Lee et al. (1996) *Proc. Natl. Acad. Sci.* USA 93:1988-1992; Achen et al. (1998) *Proc. Natl. Acad. Sci.* USA 95:548-553. VEGF-C has recently been shown to be involved in the regulation of lymphatic angiogenesis. Jeltsch et al. (1997) *Science* 276:1423-1425.

Two VEGF receptors have been identified, Flt-1 (also called VEGFR-1) and KDR (also called VEGFR-2). Shibuya et al. (1990) *Oncogene* 8:519-527; de Vries et al. (1992) *Science* 255:989-991; Terman et al. (1992) *Biochem. Biophys. Res. Commun.* 187:1579-1586. Neuropilin-1 has been shown to be a selective VEGF receptor, able to bind the heparin-binding VEGF isoforms (Soker et al. (1998) *Cell* 92:735-45). Both Flt-I and KDR belong to the family of receptor tyrosine kinases (RTKs). The RTKs comprise a large family of transmembrane receptors with diverse biological activities. At present, at least nineteen (19) distinct RTK subfamilies have been identified. The receptor tyrosine kinase (RTK) family includes receptors that are crucial for the growth and differentiation of a variety of cell types (Yarden and Ullrich (1988) *Ann. Rev. Biochem.* 57:433-478; Ullrich and Schlessinger (1990) *Cell* 61:243-254). The intrinsic function of RTKs is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a variety of cellular responses (Ullrich & Schlessinger (1990) *Cell* 61:203-212). Thus, receptor tyrosine kinase mediated signal transduction is initiated by extracellular interaction with a specific growth factor (ligand), typically followed by receptor dimerization, stimulation of the intrinsic protein tyrosine kinase activity and receptor trans-phosphorylation. Binding sites are thereby created for intracellular signal transduction molecules and lead to the formation of complexes with a spectrum of cytoplasmic signaling molecules that facilitate the appropriate cellular response. (e.g., cell division, differentiation, metabolic effects, changes in the extracellular microenvironment) see, Schlessinger and Ullrich (1992) *Neuron* 9:1-20. Structurally, both Flt-1 and KDR have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert domain. Matthews et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:9026-9030; Terman et al. (1991) *Oncogene* 6:1677-1683.

(ii) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to

conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme

hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The

hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al., Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al., Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al., Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iv) Humanized and human antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*,

Nature, 321:522-525 (1986); Riechmann *et al., Nature*, 332:323-327 (1988); Verhoeyen *et al., Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al., J. Immunol.*, 151:2296 (1993); Chothia *et al., J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al., Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al., J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and Duchosal *et al.* *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al., J. Mol. Biol.*, 227:381 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991); Vaughan *et al. Nature Biotech* 14:309 (1996)).

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.* , *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(vi) Multispecific antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (*i.e.* bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-$p185^{HER2}$/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-$p185^{HER2}$, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-

CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell ahesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (*e.g.* FcγRI, FcγRII or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection *in vitro* or *in vivo* such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-$p185^{HER2}$/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al.*, *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al., EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al., Methods in Enzymology*, 121:210 (1986). According to another approach described in W096/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al., Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the VEGF receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This

method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

(vii) Effector function engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al., J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (*i.e.,* a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used

include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugate antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al. Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.* avidin) which is conjugated to a cytotoxic agent (*e.g.* a radionucleotide).

(ix) Immunoliposomes

The antibody disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized

phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon *et al. J. National Cancer Inst.*81(19)1484 (1989)

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.* a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the antibody by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature*, 312: 604-608 (1984)).

(xi) Antibody-salvage receptor binding epitope fusions.

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.* by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.*, by DNA or peptide synthesis).

The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

(xii) Other covalent modifications of the antibody

Covalent modifications of the antibody are included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or

carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

Removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by Hakimuddin, *et al. Arch. Biochem. Biophys.* 259:52 (1987) and by Edge *et al. Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al. Meth. Enzymol.* 138:350 (1987).

Another type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

B. Vectors, Host Cells and Recombinant Methods

The anti-VEGF antibody of the invention can be produced recombinantly, using techniques and materials readily obtainable.

For recombinant production of an anti-VEGF antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal sequence component

The antibody of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody.

(ii) Origin of replication component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal

DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

(iii) Selection gene component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.,* ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.,* the gene encoding D-alanine racemase for *Bacilli.*

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.,* kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al., Nature*, 282:39 (1979)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al., Bio/Technology*, 9:968-975 (1991).

(iv) Promoter component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody nucleic acid. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter , β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antibody.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-

6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.,* the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

(v) Enhancer element component

Transcription of a DNA encoding the antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus

early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv (1982) *Nature* 297:17-18 on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription termination component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(vii) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such

as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)) ; baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)) ; mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for

inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

(viii) Culturing the host cells

The host cells used to produce the antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(ix) Antibody purification

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the

foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the anti-VEGF antibody and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*,from about 0-0.25M salt).

III. PHARMACEUTICAL FORMULATIONS

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as

phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-VEGF antibody formulations are described in WO 97/04801, expressly incorporated herein be reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, VEGF (*e.g.* an antibody which binds a different epitope on VEGF), VEGFR, or ErbB2 (e.g., Herceptin®) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine, growth inhibitory agent and/or small molecule VEGFR antagonist. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules.

Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. THERAPEUTIC USES OF ANTI-VEGF ANTIBODIES

It is contemplated that, according to the present invention, the anti-VEGF antibodies may be used to treat various neoplasms or non-neoplastic conditions characterized by pathological angiogenesis. Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

The antibodies of the invention are preferably used in the treatment of tumors in which angiogenesis plays an important role in tumor growth, including cancers and benign tumors. Examples of cancer to be treated herein include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer (including gastrointestinal cancer), pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma,

breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome. More particularly, cancers that are amenable to treatment by the antibodies of the invention include breast cancer, colorectal cancer, rectal cancer, non-small cell lung cancer, non-Hodgkins lymphoma (NHL), renal cell cancer, prostate cancer, liver cancer, pancreatic cancer, soft-tissue sarcoma, kaposi's sarcoma, carcinoid carcinoma, head and neck cancer, melanoma, ovarian cancer, mesothelioma, and multiple myeloma. More preferably, the methods of the invention are used to treat colorectal cancer in a human patient.

The present invention encompasses antiangiogenic therapy, a novel cancer treatment strategy aimed at inhibiting the development of tumor blood vessels required for providing nutrients to support tumor growth. Because angiogenesis is involved in both primary tumor growth and metastasis, the antiangiogenic treatment provided by the invention is capable of inhibiting the neoplastic growth of tumor at the primary site as well as preventing metastasis of tumors at the secondary sites, therefore allowing attack of the tumors by other therapeutics.

Combination Therapies

It is contemplated that when used to treat various diseases such as tumors, the antibodies of the invention can be combined with other therapeutic agents suitable for the same or similar diseases. When used for treating cancer, antibodies of the present invention may be used in combination with conventional cancer therapies, such as surgery, radiotherapy, chemotherapy or combinations thereof.

In certain aspects, other therapeutic agents useful for combination cancer therapy with the antibody of the invention include other anti-angiogenic agents. Many anti-angiogenic agents have been identified and are known in the arts, including those listed by Carmeliet and

Jain (2000). Preferably, the anti-VEGF antibody of the invention is used in combination with another VEGF antagonist or a VEGF receptor antagonist such as VEGF variants, soluble VEGF receptor fragments, aptamers capable of blocking VEGF or VEGFR, neutralizing anti-VEGFR antibodies, low molecule weight inhibitors of VEGFR tyrosine kinases and any combinations thereof. Alternatively, or in addition, two or more anti-VEGF antibodies may be co-administered to the patient.

In some other aspects, other therapeutic agents useful for combination tumor therapy with the antibody of the invention include antagonist of other factors that are involved in tumor growth, such as EGFR, ErbB2 (also known as Her2) ErbB3, ErbB4, or TNF. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the VEGF antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the VEGF antibody. However, simultaneous administration or administration of the VEGF antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-VEGF antibody.

Chemotherapeutic Agents

In certain aspects, the present invention provides a method of treating cancer, by administering effective amounts of an anti-VEGF antibody and one or more chemotherapeutic agents to a patient susceptible to, or diagnosed with, cancer. A variety of chemotherapeutic agents may be used in the combined treatment methods of the invention. An exemplary and non-limiting list of chemotherapeutic agents contemplated is provided herein under "Definition".

As will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents will be generally around those already employed in clinical therapies wherein the chemotherapeutics are administered alone or in combination with other chemotherapeutics. Variation in dosage will likely occur depending on the condition being treated. The physician administering treatment will be able to determine the appropriate dose for the individual subject.

By way of example only, standard chemotherapy treatments for metastatic colorectal cancer are described herein below.

In one preferred embodiment, the methods of the invention are used to treat colorectal cancer including metastatic colorectal cancer. Colorectal cancer is the third most common cause of cancer mortality in the United States. It was estimated that approximately 129,000 new cases of colorectal cancer would be diagnosed and 56,000 deaths would occur due to colorectal cancer in the United States in 1999, Landis et al., Cancer J Clin. 49:8-31 (1999). Approximately 70% of colorectal cancer patients present with disease that is potentially curable by surgical resection, August et al., Cancer Metastasis Rev 3:303-24 (1984). However, the prognosis for the 30% who present with advanced or metastatic disease and for the 20% who relapse following resection is poor. The median survival for those with metastatic disease is 12–14 months, Advanced Colorectal Cancer Meta-Analysis Project, J Clin Oncol 10:896-903 (1992).

The standard treatment for metastatic colorectal cancer in the United States has been until recently chemotherapy with 5-fluorouracil (5-FU) plus a biochemical modulator of 5-FU, leucovorin, Advanced Colorectal Cancer Meta-Analysis Project, J Clin Oncol 10:896-903 (1992); Moertel *N Engl J Med* 330:1136-42 (1994). The combination of 5-FU/leucovorin provides infrequent, transient shrinkage of colorectal tumors but has not been demonstrated to prolong survival compared with 5-FU alone (Advanced Colorectal Cancer Meta-Analysis Project, *J Clin Oncol* 10:896-903 (1992)), and 5-FU has not been demonstrated to prolong survival compared with an ineffective therapy plus best supportive care, Ansfield et al. *Cancer* 39:34-40 (1977). The lack of a demonstrated survival benefit for 5-FU/leucovorin may be due in part to inadequately sized clinical trials. In a large randomized trial of patients receiving adjuvant chemotherapy for resectable colorectal cancer, 5-FU/leucovorin demonstrated prolonged survival compared with lomustine (MeCCNU), vincristine, and 5-FU (MOF; Wolmark et al. *J Clin Oncol* 11:1879-87 (1993).

In the United States, 5-FU/leucovorin chemotherapy is commonly administered according to one of two schedules: the Mayo Clinic and Roswell Park regimens. The Mayo Clinic regimen consists of an intensive course of 5-FU plus low-dose leucovorin (425 mg/m 2 5-FU plus 20 mg/m 2 leucovorin administered daily by intravenous [IV] push for 5 days, with courses repeated at 4- to 5-week intervals), Buroker et al. J Clin Oncol 12:14-20 (1994). The Roswell Park regimen consists of weekly 5-FU plus high-dose leucovorin (500–600 mg/m 2 5-FU administered by IV push plus 500 mg/m 2 leucovorin administered as a 2-hour infusion weekly for 6 weeks, with courses repeated every 8 weeks), Petrelli et al., J Clin Oncol 7:1419-

26 (1989). Clinical trials comparing the Mayo Clinic and Roswell Park regimens have not demonstrated a difference in efficacy but have been underpowered to do so, Buroker et al., J Clin Oncol 12:14-20 (1994); Poon et al., J Clin Oncol 7:1407-18 (1989). The toxicity profiles of the two regimens are different, with the Mayo Clinic regimen resulting in more leukopenia and stomatitis and the Roswell Park regimen resulting in more frequent diarrhea. Patients with newly diagnosed metastatic colorectal cancer receiving either regimen can expect a median time to disease progression of 4–5 months and a median survival of 12–14 months, Petrelli et al., J Clin Oncol 7:1419-26 (1989); Advanced Colorectal Cancer Meta-Analysis Project, J Clin Oncol 10:896-903 (1992); Buroker et al., J Clin Oncol 12:14-20 (1994); Cocconi et al., J Clin Oncol 16:2943-52 (1998).

Recently, a new first-line therapy for metastatic colorectal cancer has emerged. Two randomized clinical trials, each with approximately 400 patients, evaluated irinotecan in combination with 5-FU/leucovorin, Saltz et al., Proc ASCO 18:233a (1999); Douillard et al., Lancet 355:1041-7 (2000). In both studies, the combination of irinotecan/5-FU/leucovorin demonstrated statistically significant increases in survival (of 2.2 and 3.3 months), time to disease progression and response rates as compared with 5-FU/leucovorin alone. The benefits of irinotecan came at a price of increased toxicity: addition of irinotecan to 5-FU/leucovorin was associated with an increased incidence of National Cancer Institute Common Toxicity Criteria (NCI-CTC) Grade 3/4 diarrhea, Grade 3/4 vomiting, Grade 4 neutropenia, and asthenia compared with 5-FU/leucovorin alone. There is also evidence showing that single-agent irinotecan prolongs survival in the second-line setting, Cunningham et al., Lancet 352:1413-18 (1998); Rougier et al., Lancet 352:1407-12 (1998). Two randomized studies have demonstrated that irinotecan prolongs survival in patients who have progressed following 5-FU therapy. One study compared irinotecan to best supportive care and showed a 2.8-month prolongation of survival; the other study compared irinotecan with infusional 5-FU and showed a 2.2-month prolongation of survival. The question of whether irinotecan has more effect on survival in the first- or second-line setting has not been studied in a well-controlled fashion.

Dosage and Administration

The antibodies and chemotherapeutic agents of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal,

intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-VEGF antibody and one or more chemotherapeutic agents. The present invention contemplates administration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. In a combination therapy regimen, the compositions of the present invention are administered in a therapeutically effective or synergistic amount. As used herein, a therapeutically effective amount is such that co-administration of anti-VEGF antibody and one or more other therapeutic agents, or administration of a composition of the present invention, results in reduction or inhibition of the targeting disease or condition. A therapeutically synergistic amount is that amount of anti-VEGF antibody and one or more other therapeutic agents necessary to synergistically or significantly reduce or eliminate conditions or symptoms associated with a particular disease.

Depending on the type and severity of the disease, about 1 μg/kg to 50 mg/kg (*e.g.* 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to about 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms

occurs. However, other dosage regimens may be useful. In a preferred aspect, the antibody of the invention is administered every two to three weeks, at a dose ranged from about 5mg/kg to about 15 mg/kg. More preferably, such dosing regimen is used in combination with a chemotherapy regimen as the first line therapy for treating metastatic colorectal cancer. In some aspects, the chemotherapy regimen involves the traditional high-dose intermittent administration. In some other aspects, the chemotherapeutic agents are administered using smaller and more frequent doses without scheduled breaks ("metronomic chemotherapy"). The progress of the therapy of the invention is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

Efficacy of the Treatment

The main advantage of the treatment of the present invention is the ability of producing marked anti-cancer effects in a human patient without causing significant toxicities or adverse effects, so that the patient benefited from the treatment overall. The efficacy of the treatment of the invention can be measured by various endpoints commonly used in evaluating cancer treatments, including but not limited to, tumor regression, tumor weight or size shrinkage, time to progression, duration of survival, progression free survival, overall response rate, duration of response, and quality of life. Because the anti-angiogenic agents of the invention target the tumor vasculature and not necessarily the neoplastic cells themselves, they represent a unique class of anticancer drugs, and therefore may require unique measures and definitions of clinical responses to drugs. For example, tumor shrinkage of greater than 50% in a 2-dimensional analysis is the standard cut-off for declaring a response. However, the anti-VEGF antibody of the invention may cause inhibition of metastatic spread without shrinkage of the primary tumor, or may simply exert a tumouristatic effect. Accordingly, novel approaches to determining efficacy of an anti-angiogenic therapy should be employed, including for example, measurement of plasma or urinary markers of angiogenesis and measurement of response through radiological imaging.

In one embodiment, the present invention can be used for increasing the duration of survival of a human patient susceptible to or diagnosed with a cancer. Duration of survival is defined as the time from first administration of the drug to death. In a preferred aspect, the anti-VEGF antibody of the invention is administered to the human patient in combination with one or more chemotherapeutic agents, thereby the duration of survival of the patient is

effectively increased as compared to a chemotherapy alone. For example, patient group treated with the anti-VEGF antibody combined with a chemotherapeutic cocktail of at least two, preferably three, chemotherapeutic agents may have a median duration of survival that is at least about 2 months, preferably between about 2 and about 5 months, longer than that of the patient group treated with the same chemotherapeutic cocktail alone, said increase being statistically significant. Duration of survival can also be measured by stratified hazard ratio (HR) of the treatment group versus control group, which represents the risk of death for a patient during the treatment. Preferably, a combination treatment of anti-VEGF antibody and one or more chemotherapeutic agents significantly reduces the risk of death by at least about 30% (i.e., a stratified HR of about 0.70), preferably by at least about 35% (i.e., a stratified HR of about 0.65), when compared to a chemotherapy alone.

In another embodiment, the present invention provides methods for increasing progression free survival of a human patient susceptible to or diagnosed with a cancer. Time to disease progression is defined as the time from administration of the drug until disease progression. In a preferred embodiment, the combination treatment of the invention using anti-VEGF antibody and one or more chemotherapeutic agents significantly increases progression free survival by at least about 2 months, preferably by about 2 to about 5 months, when compared to a treatment with chemotherapy alone.

In yet another embodiment, the treatment of the present invention significantly increases response rate in a group of human patients susceptible to or diagnosed with a cancer who are treated with various therapeutics. Response rate is defined as the percentage of treated patients who responded to the treatment. In one aspect, the combination treatment of the invention using anti-VEGF antibody and one or more chemotherapeutic agents significantly increases response rate in the treated patient group compared to the group treated with chemotherapy alone, said increase having a Chi-square p-value of less than 0.005.

In one aspect, the present invention provides methods for increasing duration of response in a human patient or a group of human patients susceptible to or diagnosed with a cancer. Duration of response is defined as the time from the initial response to disease progression. In a combination treatment of the invention using anti-VEGF antibody and one or more chemotherapeutic agents, a statistically significant increase of at least 2 months in duration of response is obtainable and preferred.

Safety of the Treatment

The present invention provides methods of effectively treating cancers without significant adverse effects to the human patient subject to treatment. The clinical outcomes of the treatment according to the invention are somewhat unexpected, in that several adverse events thought to be associated with anti-angiogenic therapies are not observed during the course of treatments according to the present invention. For example, previous clinical studies suggested that treatment with anti-VEGF antibodies may cause thrombosis (fatal in certain case), hypertension, proteinuria and epistaxis (bleeding). However, combination therapy of the invention using anti-VEGF antibody combined with a chemotherapy cocktail comprising at least two, preferably three, chemotherapeutic agents does not significantly increase incident occurrences of these adverse events, when compared with the chemotherapy alone. Thus, the treatment of the present invention unexpectedly contains side effects at acceptable level, at the same time significantly improve anticancer efficacy.

V. ARTICLES OF MANUFACTURE

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-VEGF antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package inserts with instructions for use, including for example a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, *e.g.* doxorubicin, or epirubicin, or instructing the user of the composition to administer the anti-VEGF antibody composition and an antineoplastic composition to a patient.

Deposit of Materials

The following hybridoma cell line has been deposited under the provisions of the Budapest Treaty with the American Type Culture Collection (ATCC), Manassas, VA, USA:

Antibody Designation	ATCC No.	Deposit Date
A4.6.1	ATCC HB-10709	March 29, 1991

The following examples are intended merely to illustrate the practice of the present invention and are not provided by way of limitation. The disclosures of all patent and scientific literatures cited herein are expressly incorporated in their entirety by reference.

VI. EXAMPLES

Example 1. Addition of an Anti-VEGF Antibody to Bolus Irinotecan/Fluorouracil/Leucovorin (IFL) in First Line Metastatic Colorectal Cancer

A multicenter, Phase III, randomized, active-controlled trial was conducted to evaluate the efficacy and safety of bevacizumab when added to standard first-line chemotherapy used to treat metastatic colorectal cancer. The trial enrolled over 900 patients with histologically confirmed, previously untreated, bi-dimensionally measurable metastatic colorectal cancer.

Methods and Materials

Anti-VEGF Antibody bevacizumab

The anti-VEGF antibody "Bevacizumab (BV)", also known as "rhuMAb VEGF"or "Avastin™", is a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. (1997) Cancer Res. 57:4593-4599. It comprises mutated human IgG1 framework regions and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF to its receptors. U.S. Pat No. 6,582,959; WO 98/45331. Approximately 93% of the amino acid sequence of bevacizumab, including most of the framework regions, is derived from human

IgG1, and about 7% of the sequence is derived from the murine antibody A4.6.1. Bevacizumab has a molecular mass of about 149,000 daltons and is glycosylated.

Identities of the polypeptide and sites of glycosylation were deduced from the amino acid composition and peptide map. The size and charge characteristics of the molecule and the purity of the clinical lots were demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or capillary electrophoresis non-gel sieving, isoelectric focusing, as well as ion-exchange and size-exclusion chromatography. The activity of bevacizumab was quantified by a binding enzyme-linked immunosorbent assay or a kinase receptor assay for recombinant human VEGF.

bevacizumab was produced by recombinant DNA technology, using a genetically engineered Chinese hamster ovary cell line. The protein was purified from the cell culture medium by routine methods of column chromatography and filtration. The final product was tested for quality, identity, safety, purity, potency, strength, and excipient/chemical composition according to U.S. Food and Drug Administration guidelines. The purity of bevacizumab is >95%. bevacizumab is supplied as a clear to slightly opalescent, sterile liquid ready for parenteral administration.

Patient Selection

Eligible patients had histologically confirmed metastatic colorectal carcinoma, with bidimensionally measurable disease. Other inclusion criteria included an age of at least 18 years, an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 (Oken et al. (1982) *Am. J. Clin. Oncol.* 5:649-55), a life expectancy of more than three months, and written informed consent. Adequate hematologic, hepatic, and renal function (including urinary excretion of no more than 500 mg of protein per day) was also required.

Exclusion criteria included prior chemotherapy or biologic therapy for metastatic disease (adjuvant or radiosensitizing use of fluoropyrimidines with or without leucovorin or levamisole more than 12 months before study entry was permitted), receipt of radiotherapy within 14 days before the initiation of study treatment, major surgery within 28 days before the initiation ofstudytreatment, clinically significant cardiovascular disease, clinically detectable ascites, pregnancy or lactation, regular use of aspirin (more than 325 mg per day) or other nonsteroidal andinflammatory agents, preexisting bleeding diatheses or coagulopathy or the need for full-dose anticoagulation, and known central nervous system metastases.

Study Design

Eligible patients were assigned to treatment with the use of a dynamic randomization algorithm that was designed to achieve overall balance between groups; randomization was stratified according to study center, baseline ECOG performance status (0 vs. 1), site of primary disease (colon vs. rectum), and number of metastatic sites (one vs. more than one). Initially, patients were randomly assigned in a 1:1:1 ratio to receive IFL plus placebo, IFL plus bevacizumab, or fluorouracil and leucovorin plus bevacizumab (Table 1), each of which was to continue until disease progression or unacceptable adverse effects occurred or for a maximum of 96 weeks.

Table 1. First-Line Treatment Regimens*

Treatment	Starting Dose	Schedule
Irinotecan Fluorouracil Leucovorin Placebo	125 mg/m^2 of body-surface area 500 mg/m^2 20 mg/m^2	Once weekly for 4wk; cycle repeated every 6 wk Every 2 wk
Irinotecan Fluorouracil Leucovorin Bevacizumab	125 mg/m^2 500 mg/m^2 20 mg/m^2 5 mg/kg of body weight	Once weekly for 4wk; cycle repeated every 6 wk Every 2 wk
Fluorouracil Leucovorin Bevacizumab	500 mg/m^2 500 mg/m^2 5 mg/kg	Once weekly for 4wk; cycle repeated every 8 wk Every 2 wk

*Treatment with fluorouracil, leucovorin, and bevacizumab was discontinued after the safety of adding bevacizumab to the regimen of irinotecan, fluorouracil, and leucovorin was confirmed. Confirmation occurred after the randomization of 313 patients. All drugs were given intravenously.

An interim analysis was scheduled to be performed after 300 patients underwent randomization, at which time an unblinded, independent data-monitoring committee was to assess the safety of IFL plus bevacizumab, on the basis of all the available safety information, including the number of deaths in each group, but in the absence of information related to tumor response. If the data-monitoring committee found no untoward adverse events attributable to the addition of bevacizumab to IFL, the enrollment of patients in the group assigned to receive fluorouracil and leucovorin plus bevacizumab was to be discontinued, and additional patients would be randomly assigned in a 1:1 ratio to receive either IFL plus placebo or IFL plus bevacizumab. However, if the data-monitoring committee concluded that the safety profile of IFL plus bevacizumab was unacceptable,

assignment to that treatment was to be discontinued, and patients would instead be randomly assigned in a 1:1 ratio to receive either the combination of fluorouracil and leucovorin plus bevacizumab or IFL plus placebo.

Tumor responses and progression were determined with the use of the Response Evaluation Criteria in Solid Tumors. Therasse et al. (2000) *J. Natl. Cancer Inst.* 92:205-16. At the time of disease progression, the treatment assignment was revealed and patients could be offered second-line treatment. Such patients in the group assigned to bevacizumab-containing treatment had the option to continue bevacizumab during this second-line treatment. No crossovers were allowed in the group given IFL plus placebo. Patients assigned to a treatment containing bevacizumab who had no signs of progressive disease at the end of the 96-week study period could continue to receive bevacizumab in a separate extension study. Patients in a group receiving bevacizumab who had a confirmed complete response or unacceptable adverse effects from chemotherapy could discontinue chemotherapy and receive bevacizumab alone.

Bevacizumab (or placebo) was administered concomitantly with chemotherapy. Doses of bevacizumab and chemotherapy were recalculated if a patient's weight changed by at least 10 percent during the study. Standard intracycle and intercycle dose modifications of irinotecan and fluorouracil (according to the package insert)[°] were permitted in patients with treatment-related adverse events. The doses of leucovorin and bevacizumab were not altered.

In the analysis of survival and subsequent treatment, all patients were followed until death, loss to follow-up, or termination of the study.

Assessments

After the baseline evaluation, tumor status was assessed every 6 weeks for the first 24 weeks of the study and then every 12 weeks for the remainder of therapy. All complete and partial responses required confirmation at least four weeks after they were first noted.

Safety was assessed on the basis of reports of adverse events, laboratory-test results, and vital sign measurements. Adverse events were categorized according to the Common Toxicity Criteria of the National Cancer Institute, version 2, in which a grade of 1 indicates mild adverse events, a grade of 2 moderate adverse events, a grade of 3 serious adverse events, and a grade of 4 1ife-threatening adverse events. Prespecified safety measures included the incidence of all adverse events, all serious adverse events, and adverse events that have been

associated with bevacizumab - hypertension, thrombosis, bleeding of grade 3 or 4, and proteinuria - as well as diarrhea of grade 3 or 4, and changes from baseline in various laboratory values and vital signs.

To monitor the safety of the regimen of IFL plus placebo and of IFL plus bevacizumab, the incidence of death, serious adverse events, diarrhea of grade 3 or 4, bleeding of grade 3 or 4 from any source, and thrombosis was monitored during the study in an un-blinded fashion by the data-safety monitoring committee until the completion of recruitment or the time of the interim analysis of efficacy, whichever came first.

Statistical Analysis

The primary outcome measure was the duration of overall survival; survival was measured without regard to subsequent treatments. There was no crossover between groups, however. Survival analysis techniques such as the Kaplan-Meier method, log-rank test, and Cox proportional hazards model were used. Secondary outcome measures were progression-free survival, objective response rates (complete and partial responses), the duration of responses, and the quality of life.

For patients who were alive at the time of analysis, data on survival were censored at the time of the last contact. Progression-free survival was defined as the time from randomization to progression or death during the study, with death during the study defined as any death that occurred within 30 days after the last dose of bevacizumab or chemotherapy. For patients without disease progression at the time of the final analysis, data on progression-free survival were censored at the last assessment of tumor status or on day 0 if no further assessment was performed after baseline. Patients without adequate follow-up data were categorized as having no response.

To detect a hazard ratio of 0.75 for death in the group given IFL plus bevacizumab as compared with the control group, approximately 385 deaths were required. All calculations were performed with the log-rank test and involved two-sided P values, with an alpha value of 0.05, a statistical power of 80 percent, and one interim analysis of efficacy.

Interim analyses were conducted in an un-blinded fashion. An interim analysis of safety was conducted after the random assignment of approximately 100 patients to each group. A second interim analysis of safety and efficacy was performed after 193 deaths had occurred (half the number of required events).

Efficacy analyses were performed according to the intention-to-treat principle. Safety analyses included all patients who received at least one dose of study medication.

Results

Characteristics of the Patients

During a period of about twenty months, 923 patients underwent randomization at 164 sites in the United States, Australia, and New Zealand. After 313 patients had been randomly assigned to one of the three groups -100 to IFL plus placebo, 103 to IFL plus bevacizumab, and 110 to fluorouracil, leucovorin, and bevacizumab - assignment to the group given fluorouracil, leucovorin, and bevacizumab was halted (the results in this group are not reported). This step was required by the protocol after the first formal interim analysis of safety concluded that the regimen of IFL plus bevacizumab had an acceptable safety profile and that assignment to this group could continue.

The intention-to-treat analysis of the primary end point of overall survival included 411 patients in the group given IFL plus placebo and 402 patients in the group given IFL plus bevacizumab. Table 2 shows selected demographic and baseline characteristics, which were well balanced between the groups. Similar numbers of patients in each group had previously undergone surgery or received radiation therapy or adjuvant chemotherapy for colorectal cancer.

Treatment

The median duration of therapy was 27.6 weeks in the group given IFL plus placebo and 40.4 weeks in the group given IFL plus bevacizumab. The percentage of the planned dose of irinotecan that was given was similar in the two groups (78 percent in the group given IFL plus placebo and 73 percent in the group given IFL plus bevacizumab).

As of the date of data cutoff, 33 patients in the group given IFL plus placebo and 71 in the group given IFL plus bevacizumab were still taking their assigned initial therapy. The rates of use of second-line therapies that may have affected survival, such as oxaliplatin or metastasectomy, were well balanced between the two groups. In both groups, approximately 50 percent of patients received some form of second line therapy; 25 percent of all patients received oxaliplatin, and less than 2 percent of patients underwent metastasectomy.

Table 2. Selected Demographic and Baseline Characteristics.*

Characteristic	IFL plus Placebo (N=411)	IFL plus Bevacizumab (N=402)
Sex (%)		
MALE	60	59
FEMALE	40	41
MEAN AGE (YR)	59.2	59.5
Race (%)		
White	80	79
Black	11	12
Other	9	9
Location of center(%)		
United States	99	99
Australia or New Zealand	<1	<1
ECOG performance status (%)		
0	55	58
1	44	41
2	<1	<1
Type of cancer (%)		
Colon	81	77
Rectal	19	23
Number of metastatic sites (%)		
1	39	37
>1	61	63
Prior cancer therapy (%)		
Adjuvant chemotherapy	28	24
Radiation therapy	14	15
Median duration of metastatic disease (mo)	4	4

*There were no significant differences between groups. IFL denotes irinotecan, fluorouracil, and leucovorin, and ECOG Eastern Cooperative Oncology Group.

Efficacy

The median duration of overall survival, the primary end point, was significantly longer in the group given IFL plus bevacizumab than in the group given IFL plus placebo (20.3 months vs. 15.6 months), which corresponds to a hazard ratio for death of 0.66

(P<0.001) (Table 3 and Fig. 1), or a reduction of 34 percent in the risk of death in the bevacizumab group. The one-year survival rate was 74.3 percent in the group given IFL plus bevacizumab and 63.4 percent in the group given IFL plus placebo (P<0.001). In the subgroup of patients who received second-line treatment with oxaliplatin, the median duration of overall survival was 25.1 months in the group given IFL plus bevacizumab and 22.2 months in the group given IFL plus placebo.

The addition of bevacizumab to IFL was associated with increases in the median duration of progression-free survival (10.6 months vs. 6.2 months; hazard ratio for progression, 0.54, for the comparison with the group given IFL plus placebo; P<0.001); response rate (44.8 percent vs. 34.8 percent; P=0.004); and the median duration of response (10.4 months vs. 7.1 months; hazard ratio for progression, 0.62; P=0.001) (Table 3). Figure 2 shows the Kaplan-Meier estimates of progression free survival. Treatment effects were consistent across prespecified subgroups, including those defined according to age, sex, race, ECOG performance status, location of the primary tumor, presence or absence of prior adjuvant therapy, duration of metastatic disease, number of metastatic sites, years since the diagnosis of colorectal cancer, presence or absence of prior radiotherapy, baseline tumor burden, and serum concentrations of albumin, alkaline phosphatase, and lactate dehydrogenase.

Table 3. Analysis of Efficacy*

End Point	IFL plus Placebo	IFL plus Bevacizumab	P Value
Median survival (mo)	15.6	20.3	<0.001
Hazard ratio for death		0.66	
One-year survival rate (%)	63.4	74.3	<0.001
Progression-free survival (mo)	6.2	10.6	<0.001
Hazard ratio for progression		0.54	
Overall response rate (%)	34.8	44.8	0.004
Complete response	2.2	3.7	
Partial response	32.6	41.0	
Median duration of response (mo)	7.1	10.4	0.001
Hazard ratio for relapse		0.62	

* IFL denotes irinotecan, fluorouracil, and leucovorin.

Safety

Table 4 presents the incidence of selected grade 3 or 4 adverse events during the assigned treatment, without adjustment for the median duration of therapy (27.6 weeks in the group given IFL plus placebo and 40.4 weeks in the group given IFL plus bevacizumab). The incidence of any grade 3 or 4 adverse events was approximately 10 percentage points higher among patients receiving IFL plus bevacizumab than among patients receiving IFL plus placebo, largely because of an increase in the incidence of grade 3 hypertension (requiring treatment) and small increases in the incidence of grade 4 diarrhea and leukopenia. However, there was no significant difference in the incidence of adverse events leading to hospitalization or to the discontinuation of study treatment or in the 60-day rate of death from any cause.

Table 4. Selected Adverse Events. *

Adverse Event	IFL plus Placebo (N=397)	IFL plus Bevacizumab (N=393)
	percent	
Any grade 3 or 4 adverse event	74.0	84.9**
Adverse event leading to hospitalization	39.6	44.9
Adverse event leading to discontinuation of treatment	7.1	8.4
Adverse event leading to death	2.8	2.6
Death within 60 days	4.9	3.0
Grade 3 or 4 leukopenia	31.1	37.0
Hypertension		
Any	8.3	22.4**
Grade 3	2.3	11.0**
Any thrombotic event	16.2	19.4
Deep thrombophletitis	6.3	8.9
Pulmonary embolus	5.1	3.6
Grade 3 or 4 bleeding	2.5	3.1
Proteinuria		
Any	21.7	26.5
Grade 2	5.8	3.1
Grade 3	0.8	0.8
Gastrointestinal perforation	0.0	1.5

* Data were not adjusted for differences in the median duration of therapy between the group given irinotecan, fluorouracil, and leucovorin (IFL) plus placebo and the group given IFL plus bevacizumab (27.6 weeks vs. 40.4 weeks).

** $P<0.01$. Only patients who received at least one study-drug treatment are included.

Phase 1 and 2 trials had identified hemorrhage, thromboembolism, proteinuria, and hypertension as possible bevacizumab-associated adverse effects. However, in the present

study, only the incidence of hypertension was clearly increased in the group given IFL plus bevacizumab, as compared with the group given IFL plus placebo. All episodes of hypertension were manageable with standard oral antihypertensive agents (e.g., calcium-channel blockers, angiotensin-converting-enzyme inhibitors, and diuretics). There were no discontinuations of bevacizumab therapy, hypertensive crises, or deaths related to hypertension in the bevacizumab group.

Rates of grade 2 or 3 proteinuria (there were no episodes of grade 4 proteinuria or nephrotic syndrome) and grade 3 or 4 bleeding from any cause were similar in the two groups, although all three cases of grade 4 bleeding were in the group given IFL plus bevacizumab. The incidence of all venous and arterial thrombotic events was 19.4 percent in the group given IFL plus bevacizumab and 16.2 percent in the group given IFL plus placebo (P=0.26).

Gastrointestinal perforation occurred in six patients (1.5 percent) receiving IFL plus bevacizumab. One patient died as a direct result of this event, whereas the other five recovered (three of them were able to restart treatment without subsequent complications). Of the six patients with a perforation, three had a confirmed complete or partial response to IFL plus bevacizumab. Factors other than the study treatment that may have been associated with gastrointestinal perforation were colon surgery within the previous two months in two patients and peptic-ulcer disease in one patient.

The results of this phase III study provide direct support for a broadly applicable use of antiangiogenic agents in the treatment of cancer. The addition of bevacizumab, an anti-VEGF antibody, to IFL chemotherapy conferred a clinically meaningful and statistically significant improvement in cancer patients as measured by, for example, overall survival, progression-free survival, response rate and duration of response. The increase of 4.7 months in the median duration of survival attributable to bevacizumab is as large as or larger than that observed in any other phase 3 trial for the treatment of colorectal cancer. Goldberg et al. (2004) *J. Clin. Oncol.* 22:23-30. The median survival of 20.3 months in the bevacizumab - treated population occurred in spite of the limited availability of oxaliplatin for second-line therapy during this trial.

As compared with IFL alone, the regimen of IFL plus bevacizumab increased progression-free survival from a median of6.2 months to 10.6 months, the overall response rate from 34.8 percent to 44.8 percent, and the median duration of response from 7.1 months to 10.4 months. These improvements are clinically meaningful. It was not predicted that the

absolute improvement in the response rate of 10 percent with IFL plus bevacizumab would have been associated with an increase in survival of this magnitude. This observation suggests that the primary mechanism of bevacizumab is the inhibition of tumor growth, rather than cytoreduction.

This clinical benefit was accompanied by a relatively modest increase in side effects of treatment, which were easily managed. There was an absolute increase of approximately 10 percent in the overall incidence of grade 3 and 4 adverse effects, attributable largely to hypertension requiring treatment, diarrhea, and leukopenia. The 60-day rates of death from any cause, hospitalization, and discontinuation of treatment were not significantly increased by the addition of bevacizumab to IFL.

Previous phase 1 and 2 clinical trials suggested that treatment with bevacizumab alone or with chemotherapy resulted in an increased incidence of thrombosis, bleeding, proteinuria, and hypertension. Kabbinavar et al. (2003) *J. Clin. Oncol.* 21:60-65; Yang et al. (2003) New Engl. J. Med. 349:427-34. With the exception of hypertension, an excess of these side effects was not found as compared with their incidence in the group given IFL plus placebo - thus highlighting the importance of randomized, placebo-controlled studies for the evaluation of safety as well as efficacy. One new potential adverse effect that occurred was gastrointestinal perforation. This complication was uncommon and had variable clinical presentations. Severe bowel complications, particularly in patients with neutropenia, have been reported with IFL and other chemotherapy regimens for colorectal cancer and in one series, fistulas were reported in over 2 percent of patients treated with fluorouracil-based regimens. Saltz et al. (2000) *New Engl. J. Med.* 343:905-914; Rothenberg et al. (2001) *J. Clin. Oncol.* 19:3801-7; Tebbutt et al. (2003) *Gut* 52:568-73. No such events occurred in the group given IFL plus placebo, whereas six cases were observed in the group given IFL plus bevacizumab (1.5 percent), sometimes in the setting of overall tumor responses. Although three of these six patients were able to restart treatment without subsequent complications, one patient died and two discontinued therapy permanently as a result of this complication.

While previous animal studies and early phase clinical trials have suggested uses of anti-angiogenic therapy for treating cancer, the present study showed for the first time that using an angiogenic inhibitor, such as an anti-VEGF antibody, indeed results in statistically significant and clinically meaningful benefits for cancer patients.

Example 2. Addition of Bevacizumab to Bolus 5-FU/Leucovorin in First-Line Metastatic Colorectal Cancer

This randomized, phase II trial compared bevacizumab plus 5-fluorouracil and leucovorin (5-FU/LV) versus placebo plus 5-FU/LV as first-line therapy in patients considered non-optimal candidates for first-line irinotecan.

PATIENTS AND METHODS

Patient Eligibility

Patients with histologically confirmed, previously untreated, measurable metastatic colorectal cancer were eligible if, in the judgment of the investigator, they were not optimal candidates for first-line irinotecan-containing therapy and had at least one of the following characteristics: age above 65 years, ECOG PS of 1 or 2, serum albumin equal or less than 3.5 g/dL, or prior radiotherapy to abdomen or pelvis. Patients were excluded if they had undergone major surgical procedures or open biopsy, or had experienced significant traumatic injury, within 28 days prior to study entry; anticipated need for major surgery during the course of the study; were currently using or had recently used therapeutic anticoagulants (except as required for catheter patency), thrombolytic therapy or chronic, daily treatment with aspirin (≥ 325 mg/day) or nonsteroidal anti-inflammatory medications; had a serious, non-healing wound, ulcer, or bone fracture; had a history or evidence of CNS metastases; were pregnant or lactating; or had proteinuria or clinically significant impairment of renal function at baseline. All patients provided written informed consent for their participation.

Study Design and Treatments

An interactive voice response system was used to randomly assign eligible patients to one of two treatment groups: 5-FU/LV plus placebo or 5-FU/LV plus bevacizumab. A dynamic randomization algorithm was utilized to achieve balance overall and within each of the following categories: study center, baseline ECOG performance status (0 vs. ≥ 1), site of primary disease (colon vs. rectum), and number of metastatic sites (1 vs. > 1). The 5-FU/LV treatment, comprising LV 500mg/m^2 over 2 hours and 5-FU 500mg/m^2 as a bolus midway through the LV infusion (Roswell Park regimen; Petrelli et al. (1989) *J. Clin. Oncol.* 7:1419-1426), was administered weekly for the first 6 weeks of each 8-week cycle. Chemotherapy was continued until study completion (96 weeks) or disease progression. Bevacizumab 5mg/kg or placebo was administered every 2 weeks. Patients in the bevacizumab arm who had a confirmed complete response or experienced unacceptable toxicity as a result of chemotherapy

treatment were allowed to discontinue 5-FU/LV and continue receiving bevacizumab alone as first-line treatment. At the time of disease progression, patients were unblinded to their treatment assignment and could receive any second-line treatment at the discretion of the investigator. Only patients who had been randomized to the bevacizumab group could receive bevacizumab as a component of second-line treatment. After completing the study, patients were followed for any subsequent treatment and survival every 4 months until death, loss to follow-up, or termination of the study.

Study Assessments

Patients underwent an assessment of tumor status at baseline and at completion of every 8-week cycle using appropriate radiographic techniques, typically spiral CT scanning. Tumor response, or progression, was determined by both the investigator and an independent radiology facility (IRF) utilizing the Response Evaluation Criteria in Solid Tumors. Therasse et al. (2000). The IRF assessment was performed without knowledge of the treatment assignment or investigator assessment. In addition, patients completed the Functional Assessment of Cancer Therapy—Colorectal (FACT-C), Version 4, a validated instrument for assessing quality of life (QOL) in colorectal cancer patients, at baseline and prior to each treatment cycle until disease progression. Ward et al. (1999) *Qual. Life Res.* 8:181-195.

Safety was assessed from reports of adverse events, laboratory test results, and vital sign measurements. Adverse events and abnormal laboratory results were categorized using the National Cancer Institute Common Toxicity Criteria (NCI-CTC), Version 2. Prespecified safety measures included four adverse events of special interest (hypertension, proteinuria, thrombosis, and bleeding) based on findings of previous clinical trials of bevacizumab.

Statistical Analysis

The primary outcome measure was duration of overall survival. Secondary outcome measures included progression-free survival, objective response rate (complete and partial), response duration, and change in the FACT-C QOL score. Survival duration was defined as the time from randomization to death. For patients alive at the time of analysis, duration of survival was censored at the date of last contact. Progression-free survival was defined as the time from randomization to the earlier of disease progression or death on study, defined as death from any cause within 30 days of the last dose of study drug or chemotherapy. For patients alive without disease progression at the time of analysis, progression-free survival was censored at their last tumor assessment, or day 1 (the first day of study treatment) if no postbaseline assessment was performed. In the analysis of objective response, patients

without tumor assessments were categorized as nonresponders. Disease progression and response analyses were based on the IRF assessments. Change in quality of life was analyzed as time to deterioration in QOL (TDQ), defined as the length of time from randomization to a the earliest of a ≥ 3-point decrease from baseline in colon-cancer specific FACT-C subscale score (CCS), disease progression, or death on study. TDQ was also determined for the TOI-C (sum of CCS, physical and functional well-being) and total FACT-C for changes from baseline of 7 and 9 points, respectively.

To detect a hazard ratio of 0.61 for death in the 5-FU/LV/bevacizumab group relative to the 5-FU/LV/placebo group, approximately 133 deaths were required. A two-tailed, log-rank test at the 0.05 level of significance with 80% power and two interim analyses were assumed in the calculations. Interim analyses were conducted by an unblinded, independent Data Monitoring Committee (DMC). A safety interim analysis was conducted after 44 deaths and a second safety and efficacy interim analysis was conducted after 89 deaths. The interim efficacy analysis was governed by a formal group sequential stopping rule based on an O'Brien-Fleming spending function. Kaplan-Meier methodology was applied to estimate the median survival, progression free survival, and duration of response time for each treatment group. Hazard ratios for the bevacizumab group relative to the placebo group were determined using the stratified Cox proportional hazards model. A two-sided stratified log rank test was used to compare the two groups. Stratified analyses included baseline ECOG performance status, site of primary disease, and the number of metastatic sites. Objective response rates were compared by the Chi-squared test. As exploratory analyses, the Cox proportional hazards model was used to estimate the effect of risk factors on modifications of treatment effect for duration of survival and progression-free survival. Efficacy analyses were performed on the intent-to-treat population, defined as all randomized patients. Safety analyses included all patients who received at least one dose of study drug.

RESULTS

Patient Characteristics

In a period of twenty three months, 209 patients were randomized at 60 sites in the United States and Australia/New Zealand. For the intent-to-treat analysis of the primary endpoint (overall survival), there were 105 patients in the 5-FU/LV/placebo group and 104 in the 5-FU/LV/bevacizumab group. Selected demographic and baseline characteristics similar

to those described in Example 1 were reasonably balanced between treatment groups. Low serum albumin (≤ 3.5 g/dL) at baseline was less common in the bevacizumab group than in the placebo group.

Treatment

The median duration of therapy was 23 weeks in the 5-FU/LV/placebo group and 31 weeks in the 5-FU/LV/bevacizumab group, and the 5-FU dose intensity (percentage of planned 5-FU doses actually received) in the two groups was similar (92% vs. 84%) during the treatment course. As of the date of date cut-off, 1 patient in the 5-FU/LV/placebo group and 7 in the 5-FU/LV/bevacizumab group remained on the assigned initial therapy. Subsequent therapies, which may have influenced survival, were used in approximately 50% of patients in both groups, although more patients in the 5-FU/LV/placebo group were treated with the active agents irinotecan and oxaliplatin.

Efficacy

Overall survival, the primary endpoint, was longer in the 5-FU/LV/bevacizumab group (median, 16.6 months) than in the 5-FU/LV/placebo group (median, 12.9 months), demonstrating a trend toward significance. The hazard ratio of death was estimated to be 0.79 (95% CI, 0.56 to 1.10; P = 0.16; Table 5 and Figure 4). The addition of bevacizumab to 5-FU/LV was associated with increases in median progression-free survival (9.2 vs. 5.5 months; hazard ratio = 0.50; 95% CI, 0.34 to 0.73; P = 0.0002, Table 5 and Figure 4), response rate (26.0% vs. 15.2%, P = 0.055), and median duration of response (9.2 months vs. 6.8 months; hazard ratio = 0.42; 95% CI, 0.15 to 1.17; P = 0.088). A further analysis of treatment effect on overall survival by baseline characteristics showed that patients with low serum albumin (≤ 3.5 g/dL) at baseline appeared to derive a significant survival benefit (hazard ratio = 0.46; 95% CI, 0.29 to 0.74; P = 0.001).

Table 5. Summary of Efficacy Analysis

Efficacy Parameter	**5-FU/LV/ Placebo (N = 105)**	**5-FU/LV/ Bevacizumab (N = 104)**	***P*-value**
Median survival (months)	12.9	16.6	
Hazard ratio		0.79	0.160
95% CI		0.56 to 1.10	

Progression-free survival (months)	5.5	9.2	
Hazard ratio		0.50	0.0002
95% CI		0.34 to 0.73	
Overall response rate (%)	15.2	26.0	0.055
Complete response	0	0	
Partial response	15.2	26.0	
Duration of response (months)	6.8	9.2	
Hazard ratio		0.42	0.088
95% CI		0.15 to 1.17	

5-FU/LV = 5 fluorouracil/leucovorin

Bevacizumab treatment had no detrimental effect on quality of life, and the TDQ results suggest a possible beneficial effect. The median TDQ as measured by the CCS score was 3.0 months in the 5-FU/LV/placebo group and 3.1 months in the 5-FU/LV/bevacizumab group (hazard ratio = 0.79, P = 0.188). The median TDQ for placebo-treated and bevacizumab-treated patients as measured by secondary TDQ measures was 2.3 and 3.2 months (TOI-C; hazard ratio = 0.71, P = 0.048) and 2.6 and 3.6 months (total FACT-C; hazard ratio = 0.66, P = 0.016).

Safety

A total of 204 patients (104 5-FU/LV/placebo and 100 5-FU/LV/bevacizumab) who received at least one dose of study drug comprised the safety population. A 16% increase (71% versus 87%) in total grade 3 and 4 toxicities was observed for patients receiving bevacizumab. Adverse events leading to death or study discontinuation were similar in the two groups, as were adverse events known to be associated with 5-FU/LV (specifically, diarrhea and leukopenia). Two patients, both in the 5-FU/LV/bevacizumab group, experienced a bowel perforation event. These events occurred at day 110 and day 338 of treatment, and both were determined to be associated with a colonic diverticulum at surgical exploration. One patient died as a result of this complication. Previous clinical trials had suggested hemorrhage, thromboembolism, proteinuria, and hypertension as possible bevacizumab-associated toxicities; however, in this study, no increases were seen in venous thrombosis, ≥ grade 3 bleeding, or clinically significant (≥ grade 3) proteinuria. Arterial thrombotic events (myocardial infarction, stroke, or peripheral arterial thrombotic event) occurred in 10 patients in the 5-FU/LV/bevacizumab group, compared to 5 patients in the 5-FU/LV/placebo group.

The 5-FU/LV/placebo group had a higher 60-day all-cause mortality compared to the 5-FU/LV/bevacizumab group (13.5% vs. 5.0%). Death due to disease progression in the first 60 days was similar (5.8% vs. 4.0%) in the two groups. In the 5-FU/LV/placebo group, deaths within the first 60 days not due to disease progression were attributed to the following: heart failure (1), sepsis (3), diarrhea (2), respiratory failure (1), and pulmonary embolus (1). In the 5-FU/LV/bevacizumab group, the single early death not due to disease progression was attributed to a myocardial infarction.

The results of this clinical trial further demonstrate that bevacizumab, a humanized monoclonal antibody against VEGF, provides important clinical benefit when added to first-line chemotherapy for the treatment of metastatic colorectal cancer. When compared with 5-FU/LV alone, the addition of bevacizumab prolonged median survival by 3.7 months, progression-free survival by 3.7 months, and response duration by 2.4 months, and increased the response rate by 11%.

These results should be viewed in the context of the study population. Specifically selected were patients who were poor candidates for first-line irinotecan-containing therapy, either because of a low likelihood of benefit or a high likelihood of treatment-associated toxicities. A careful analysis of the pivotal irinotecan trials showed that clinical benefit from this agent was confined to patients with a normal ECOG performance status (PS = 0).21, 22 Advanced age, prior pelvic radiation therapy, impaired performance status, and low serum albumin have all been reported to increase irinotecan-associated toxicities. 23-27 Patients with these characteristics are in need of alternative therapeutic options. A retrospective subset analysis from a smaller randomized phase II trial was previously conducted evaluating bevacizumab and 5-FU/LV in CRC and noted bevacizumab provided a substantial treatment effect in the subset of patients with baseline PS 1 or 2 (median survival, 6.3 months vs. 15.2 months), in the subset aged ≥ 65 years (11.2 months vs. 17.7 months), and in the subset with serum albumin < 3.5 (8.1 months versus 14.1 months). These results encouraged us to design the current trial, specifically including a poor-prognosis study population and powering the trial to detect a large treatment effect on survival. We were largely successful in enrolling a population different from that in the concurrently conducted pivotal trial of IFL/placebo versus IFL/bevacizumab. Compared with the pivotal trial, patients in the present trial had a higher median age (72 vs. 61 years) and substantially more patients had a performance status > 0 (72% vs. 43%) and albumin ≤ 3.5 mg/dL (46% vs. 33%).

Despite this high-risk study population, the regimen of 5-FU/LV/bevacizumab appeared to be well tolerated. The well-described bevacizumab-associated adverse event of grade 3 hypertension was seen in 16% of the 5-FU/LV/bevacizumab group versus 3% in the 5-FU/LV/placebo group. No cases of grade 4 hypertension occurred. Proteinuria of any grade was seen in 38% of the 5-FU/LV/bevacizumab group versus 19% of the 5-FU/LV/placeb group; however, only a single patient in the bevacizumab group developed grade 3 proteinuria, and there were no cases of grade 4 proteinuria. No increases in grade 3 or 4 bleeding or venous thrombotic events were seen in bevacizumab-treated patients. There was an imbalance in the incidence of arterial thrombotic events: 10% in the 5-FU/LV/bevacizumab group compared with 4.8% in the 5-FU-/LV placebo group. A similar imbalance was noted in the pivotal bevacizumab trial (1.0% in the IFL/placebo group and 3.3% in the IFL/bevacizumab group). The more advanced age of the population included in the present study may have contributed to a higher overall incidence of this adverse event, however the imbalance in both studies is noteworthy. Large, observational safety trials may be required to further define the incidence and potential risk factors for these, and other, uncommon adverse events associated with bevacizumab therapy.

In summary, these data demonstrate that bevacizumab, when combined with bolus 5-FU/LV, provides substantial clinical benefit for patients with previously untreated metastatic colorectal cancer who are deemed to be poor candidates for irinotecan-containing therapy. Together with the pivotal trial results, these data strengthen the evidence that bevacizumab-based, 5-FU/LV-containing therapy should be considered a standard option for the initial treatment of metastatic colorectal cancer.

WHAT IS CLAIMED IS:

1. A method of treating cancer in a human patient, comprising administering to the patient effective amounts of an anti-VEGF antibody and an anti-neoplastic composition, wherein said anti-neoplastic composition comprises at least one chemotherapeutic agent.

2. The method of claim 1, wherein the cancer is selected from the group consisting of breast cancer, colorectal cancer, rectal cancer, non-small cell lung cancer, non-Hodgkins lymphoma (NHL), renal cell cancer, prostate cancer, liver cancer, pancreatic cancer, soft-tissue sarcoma, kaposi's sarcoma, carcinoid carcinoma, head and neck cancer, melanoma, ovarian cancer, mesothelioma, and multiple myeloma.

3. The method of claim 1, wherein the cancer is metastatic.

4. The method of claim 1, wherein the patient is previously untreated.

5. The method of claim 1, wherein the chemotherapeutic agent is selected from the group consisting of alkylating agents, antimetabolites, folic acid analogs, pyrimidine analogs, purine analogs and related inhibitors, vinca alkaloids, epipodopyyllotoxins, antibiotics, L-Asparaginase, topoisomerase inhibitor, interferons, platinum cooridnation complexes, anthracenedione substituted urea, methyl hydrazine derivatives, adrenocortical suppressant, adrenocorticosteroides, progestins, estrogens, antiestrogen, androgens, antiandrogen, and gonadotropin-releasing hormone analog.

6. The method of claim 5, wherein the chemotherapeutic agent is selected from the group consisting of 5-fluorouracil (5-FU), leucovorin, irinotecan, oxaliplatin, capecitabine , paclitaxel and doxetaxel.

7. The method of claim 1, wherein the anti-neoplastic composition comprises a combination of at least two chemotherapeutic agents.

8. The method of claim 7, wherein the anti-neoplastic composition comprises 5-FU and leucovorin.

9. The method of claim 7, wherein the anti-neoplastic composition comprises 5-FU, leucovorin and irinotecan.

10. The method of claim 1, wherein upon completing treatment with the anti-VEGF antibody and the anti-neoplastic composition, the patient receives further chemotherapeutic treatment with at least one chemotherapeutic agent.

11. The method of claim 10, wherein the chemotherapeutic agent used in further chemotherapeutic treatment is selected from the group consisting of 5-FU, leucovorin, irinotecan, oxaliplatin, capecitabine, paclitaxel and doxetaxel.

12. The method of claim 11, wherein the chemotherapeutic agent is oxaliplatin.

13. The method of claim 1, wherein said anti-VEGF antibody binds the same epitope as the monoclonal anti-VEGF antibody A4.6.1 produced by hybridoma ATCC HB 10709.

14. The method of claim 1, wherein the anti-VEGF antibody is a human antibody.

15. The method of claim 1, wherein the anti-VEGF antibody is a humanized antibody.

16. The method of claim 15, wherein the anti-VEGF antibody is a humanized A4.6.1 antibody or fragment thereof.

17. The method of claim 1, wherein the anti-VEGF antibody is administered intravenously.

18. The method of claim 1, wherein the anti-VEGF antibody is administered to the patient at about 5mg/kg every 2 to 3 weeks.

19. The method of claim 1, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition effectively increases the duration of survival of the human patient.

20. The method of claim 19, wherein the duration of survival of the patient is increased by at least about 2 months when compared to another patient treated with the anti-neoplastic composition alone.

21. The method of claim 1, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition effectively increases the duration of progression free survival of the human patient.

22. The method of claim 21, wherein the progression free survival of the patient is increased by at least about 2 months when compared to another patient treated with the anti-neoplastic composition alone.

23. The method of claim 1, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition effectively increases the response rate in a group of human patients.

24. The method of claim 23, wherein the response rate of the group of human patients is significantly increased with a Chi-square p-value of less than 0.005 when compared to another group of patients treated with the anti-neoplastic composition alone.

25. The method of claim 1, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition effectively increases the duration of response of the human patient.

26. The method of claim 25, wherein the duration of response of the patient is increased by at least about 2 months when compared to another patient treated with the anti-neoplastic composition alone.

27. A method of treating a human patient susceptible to or diagnosed with colorectal cancer, comprising administering to the patient effective amounts of an anti-VEGF antibody.

28. The method of claim 27, wherein the colorectal cancer is metastatic.

29. The method of claim 27, wherein said anti-VEGF antibody binds the same epitope as the monoclonal anti-VEGF antibody A4.6.1 produced by hybridoma ATCC HB 10709.

30. The method of claim 27, wherein the anti-VEGF antibody is a human antibody.

31. The method of claim 27, wherein the anti-VEGF antibody is a humanized antibody.

32. The method of claim 31, wherein the anti-VEGF antibody is a humanized A4.6.1 antibody or fragment thereof.

33. The method of claim 27, wherein the anti-VEGF antibody is administered by intravenous infusion.

34. The method of claim 27, wherein the anti-VEGF antibody is administered to the patient at about 5mg/kg every 2 to 3 weeks.

35. The method of claim 27, further comprising administering to the patient one or more chemotherapeutic agents.

36. The method of claim 35, wherein the chemotherapeutic agent is selected from the group consisting of alkylating agents, antimetabolites, folic acid analogs, pyrimidine analogs, purine analogs and related inhibitors, vinca alkaloids, epipodopyyllotoxins, antibiotics, L-Asparaginase, topoisomerase inhibitor, interferons, platinum cooridnation complexes, anthracenedione substituted urea, methyl hydrazine derivatives, adrenocortical suppressant, adrenocorticosteroides, progestins, estrogens, antiestrogen, androgens, antiandrogen, and gonadotropin-releasing hormone analog.

37. The method of claim 35, wherein the chemotherapeutic agent is selected from the group consisting of 5-fluorouracil, leucovorin, irinotecan, oxaliplatin, capecitabine, paclitaxel and doxetaxel.

38. A method of treating a human patient or a group of human patients having metastatic colorectal cancer, comprising administering to the patient effective amounts of an anti-VEGF antibody composition and an anti-neoplastic composition, wherein said anti-neoplastic composition comprises a fluorouracil based combination of chemotherapeutic agents, whereby the co-administration of the anti-VEGF antibody and the anti-neoplastic composition results in statistically significant and clinically meaningful improvement of the treated patient as measured by the duration of survival, progression free survival, response rate or duration of response.

39. The method of claim 38, wherein the anti-neoplastic composition comprises 5-FU, leucovorin and irinotecan.

40. The method of claim 39, wherein the anti-neoplastic composition comprises the regimen having 500 mg/m^2 5-FU, 20mg/m^2 leucovorin and 125 mg/m^2 irinotecan and is administered to the patient in repeating 6-week cycles consisting of weekly administrations for 4 weeks followed by 2 weeks of rest, and wherein the anti-VEGF antibody is administered to the patient at 5 mg/kg every other week.

41. The method of claim 38, wherein the anti-neoplastic composition comprises 5-FU and leucovorin.

42. The method of claim 41, wherein the 5-FU and leucovorin are administered to the patient at 500 mg/m^2 each in repeating 8 week cycles consisting of weekly administrations for

4 weeks followed by 2 weeks of rest, and wherein the anti-VEGF antibody is administered to the patient at 5 mg/kg every other week.

43. The method of claim 41 for human patients considered non-optimal candidates for first-line irinotecan therapy.

44. The method of claim 38, wherein the anti-neoplastic composition comprises 5-FU, leucovorin and oxaliplatin.

45. An article of manufacture comprising a container, a composition within the container comprising an anti-VEGF antibody and a package insert instructing the user of the composition to administer to a cancer patient the anti-VEGF antibody composition and an anti-neoplastic composition comprising at least one chemotherapeutic agent.

46. A kit for treating cancer in a human patient comprising a package comprising an anti-VEGF antibody composition and instructions for using the anti-VEGF antibody composition and an anti-neoplastic composition comprising at least one chemotherapeutic agent for treating cancer in a patient.

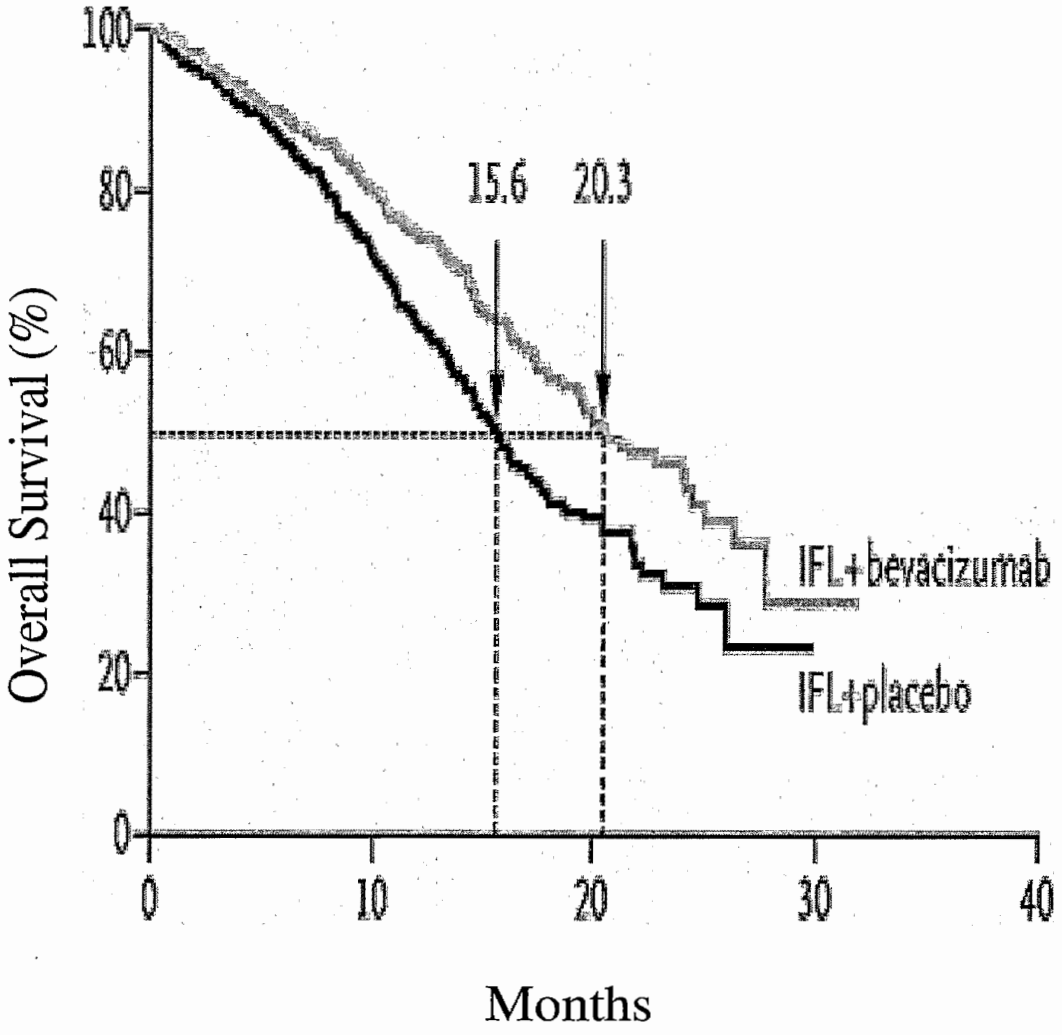
100
80
60
40
20
0
Overall Survival (%)
15.6
20.3
IFL+bevacizumab
IFL+placebo
0
10
20
30
40
Months

Figure 1

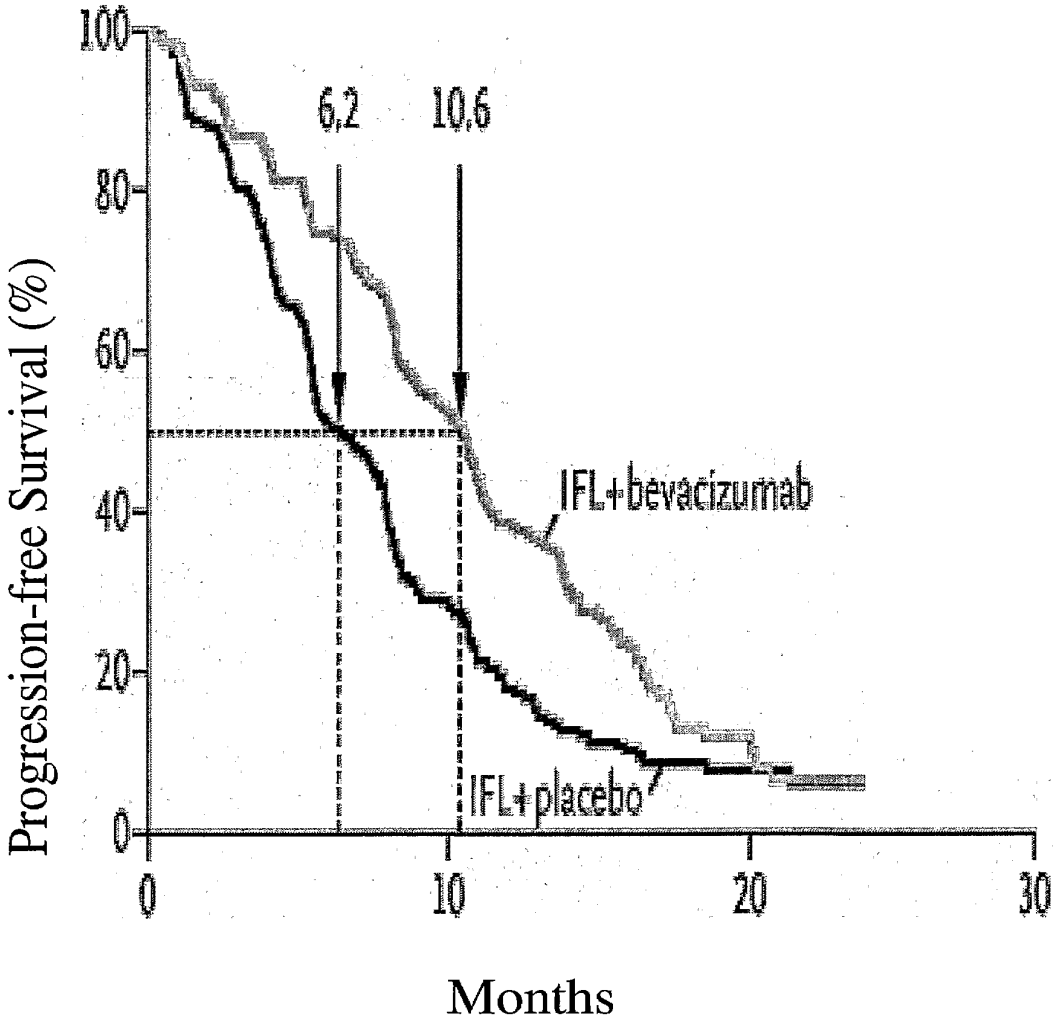
100
80
60
40
20
0
Progression-free Survival (%)
6.2
10.6
IFL+bevacizumab
IFL+placebo
0
10
20
30
Months

Figure 2

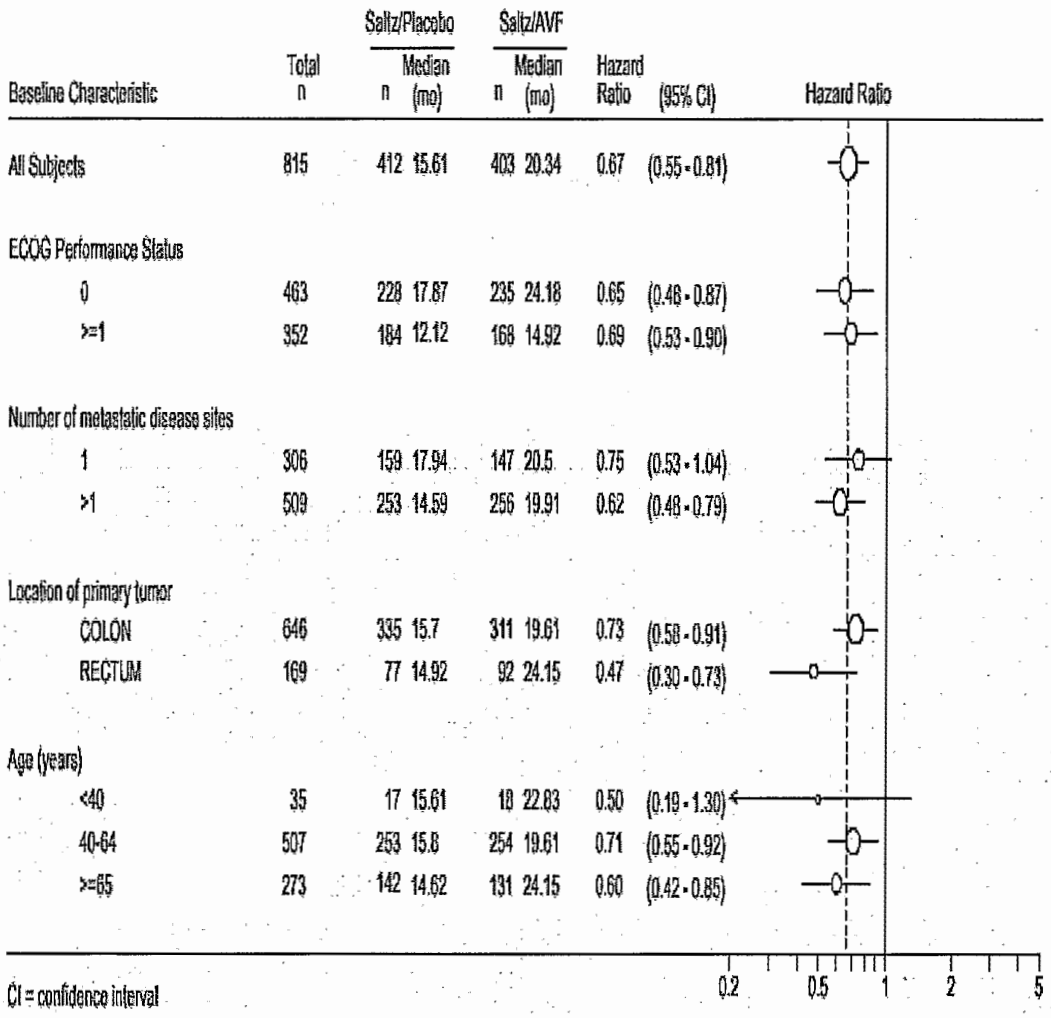
Saltz/Placebo
Saltz/AVF
Baseline Characteristic
Total n
n
Median (mo)
n
Median (mo)
Hazard Ratio
(95% CI)
Hazard Ratio
All Subjects 815 412 15.61 403 20.34 0.67 (0.55 - 0.81)
ECOG Performance Status
0 463 228 17.87 235 24.18 0.65 (0.46 - 0.87)
>=1 352 184 12.12 168 14.92 0.69 (0.53 - 0.90)
Number of metastatic disease sites
1 306 159 17.94 147 20.5 0.75 (0.53 - 1.04)
>1 509 253 14.59 256 19.91 0.62 (0.48 - 0.79)
Location of primary tumor
COLON 646 335 15.7 311 19.61 0.73 (0.58 - 0.91)
RECTUM 169 77 14.92 92 24.15 0.47 (0.30 - 0.73)
Age (years)
<40 35 17 15.61 18 22.83 0.50 (0.19 - 1.30)
40-64 507 253 15.8 254 19.61 0.71 (0.55 - 0.92)
>=65 273 142 14.62 131 24.15 0.60 (0.42 - 0.85)
CI = confidence interval
0.2
0.5
1
2
5

Figure 3A

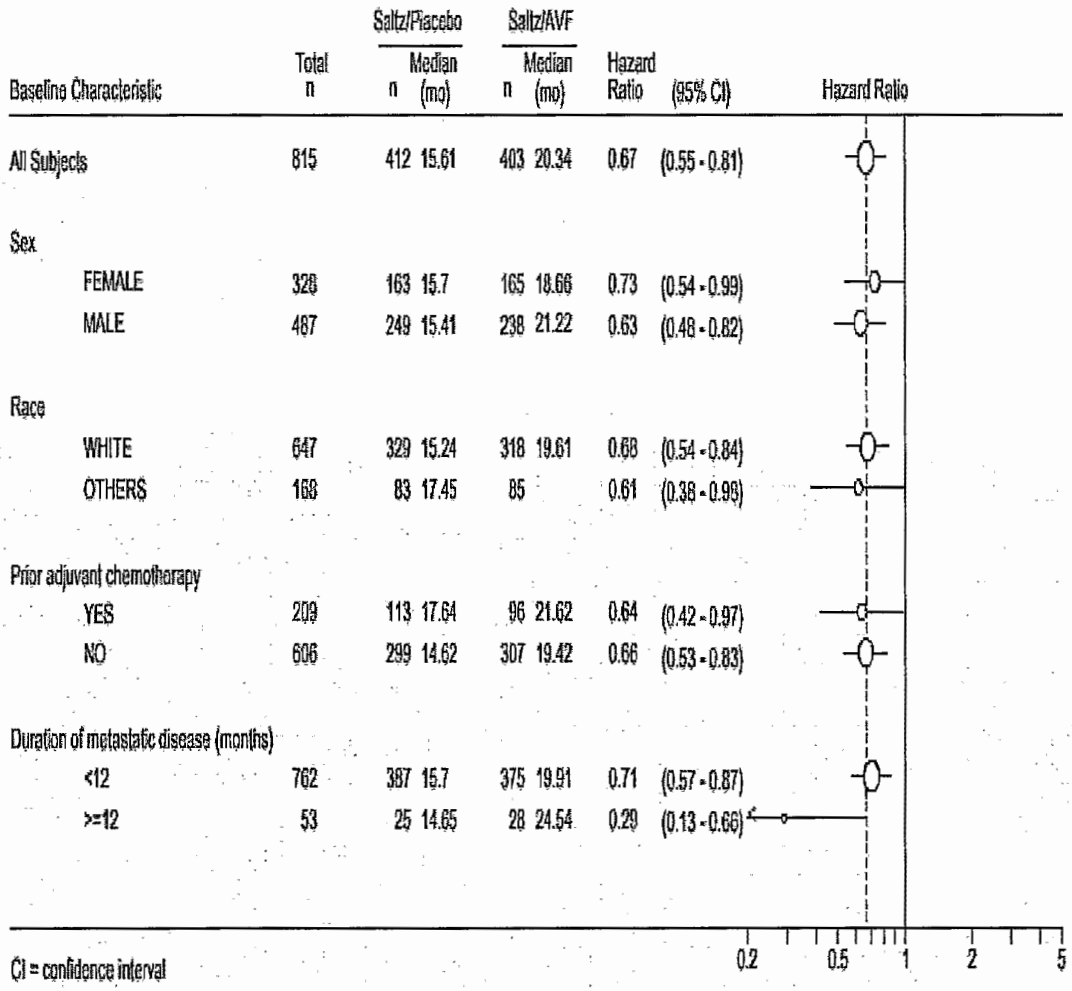
Saltz/Placebo
Saltz/AVF
Baseline Characteristic
Total n
n Median (mo)
n Median (mo)
Hazard Ratio (95% CI)
Hazard Ratio
All Subjects 815 412 15.61 403 20.34 0.67 (0.55 - 0.81)
Sex
FEMALE 326 163 15.7 165 18.66 0.73 (0.54 - 0.99)
MALE 487 249 15.41 238 21.22 0.63 (0.48 - 0.82)
Race
WHITE 647 329 15.24 318 19.61 0.68 (0.54 - 0.84)
OTHERS 168 83 17.45 85 0.61 (0.38 - 0.96)
Prior adjuvant chemotherapy
YES 209 113 17.64 96 21.62 0.64 (0.42 - 0.97)
NO 606 299 14.62 307 19.42 0.66 (0.53 - 0.83)
Duration of metastatic disease (months)
<12 762 387 15.7 375 19.91 0.71 (0.57 - 0.87)
>=12 53 25 14.65 28 24.54 0.29 (0.13 - 0.66)
CI = confidence interval
0.2
0.5
1
2
5

Figure 3B

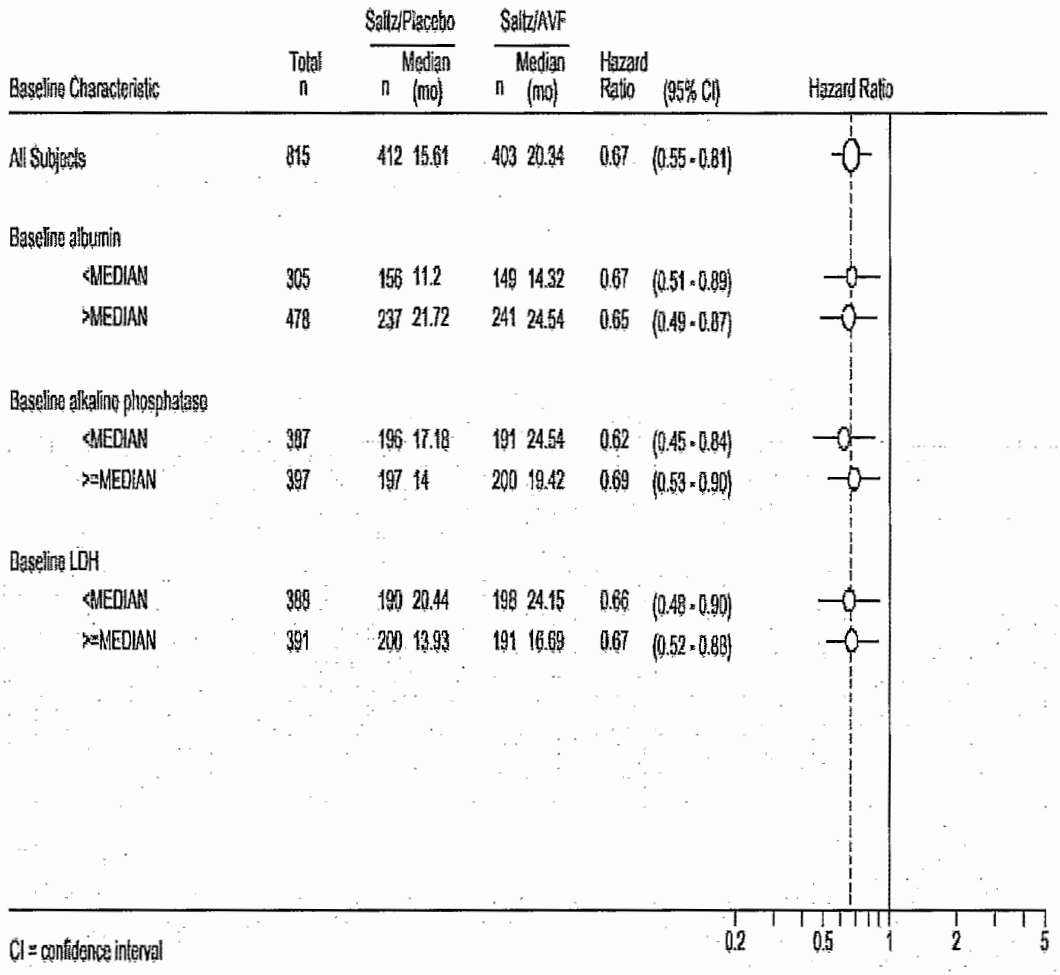
Saltz/Placebo
Saltz/AVF
Baseline Characteristic
Total n
n Median (mo)
n Median (mo)
Hazard Ratio (95% CI)
Hazard Ratio
All Subjects 815 412 15.61 403 20.34 0.67 (0.55 - 0.81)
Baseline albumin
<MEDIAN 305 156 11.2 149 14.32 0.67 (0.51 - 0.89)
>MEDIAN 478 237 21.72 241 24.54 0.65 (0.49 - 0.87)
Baseline alkaline phosphatase
<MEDIAN 387 196 17.18 191 24.54 0.62 (0.45 - 0.84)
>=MEDIAN 397 197 14 200 19.42 0.69 (0.53 - 0.90)
Baseline LDH
<MEDIAN 389 190 20.44 198 24.15 0.66 (0.48 - 0.90)
>=MEDIAN 391 200 13.93 191 16.69 0.67 (0.52 - 0.88)
CI = confidence interval
0.2
0.5
1
2
5

Figure 3C

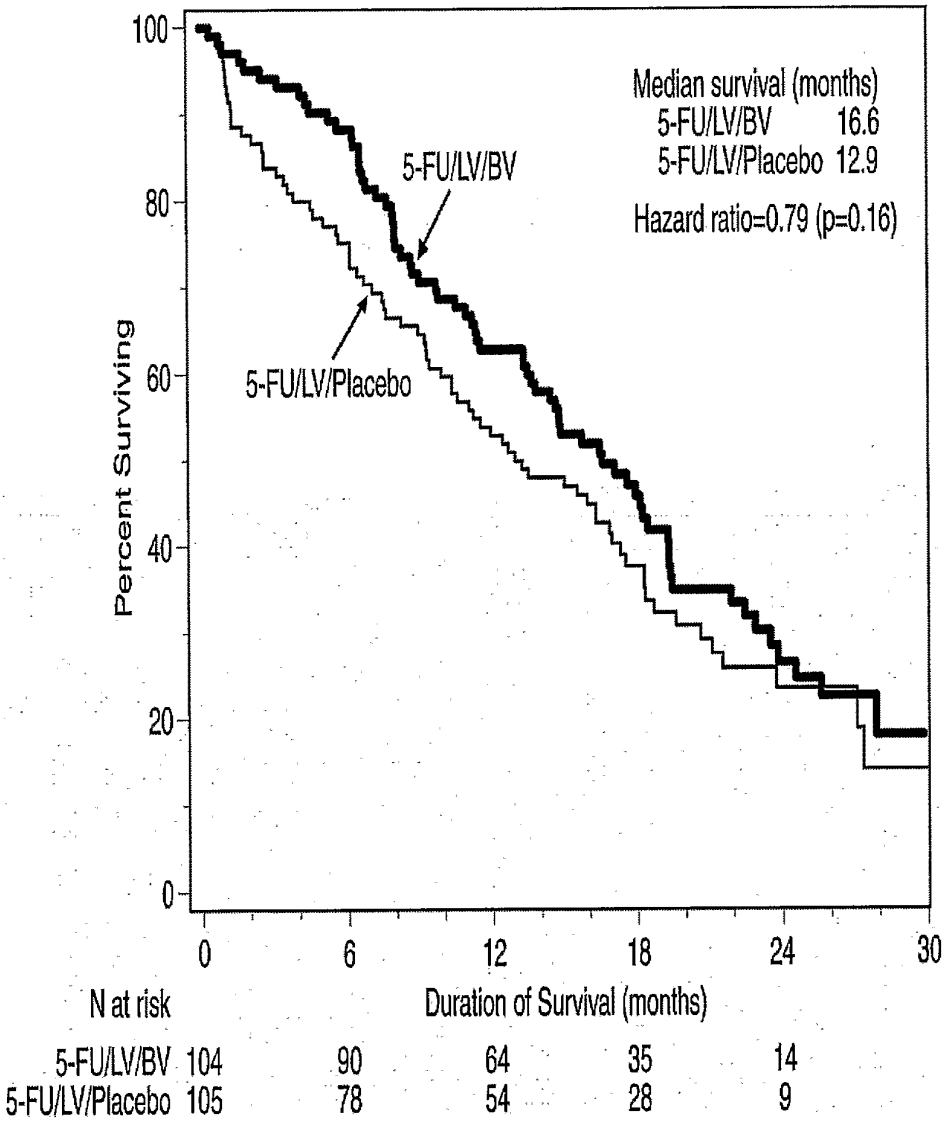
Median survival (months)
5-FU/LV/BV 16.6
5-FU/LV/Placebo 12.9
Hazard ratio=0.79 (p=0.16)
5-FU/LV/BV
5-FU/LV/Placebo
Percent Surviving
100
80
60
40
20
0
0
6
12
18
24
30
Duration of Survival (months)
N at risk
5-FU/LV/BV 104 90 64 35 14
5-FU/LV/Placebo 105 78 54 28 9

Figure 4

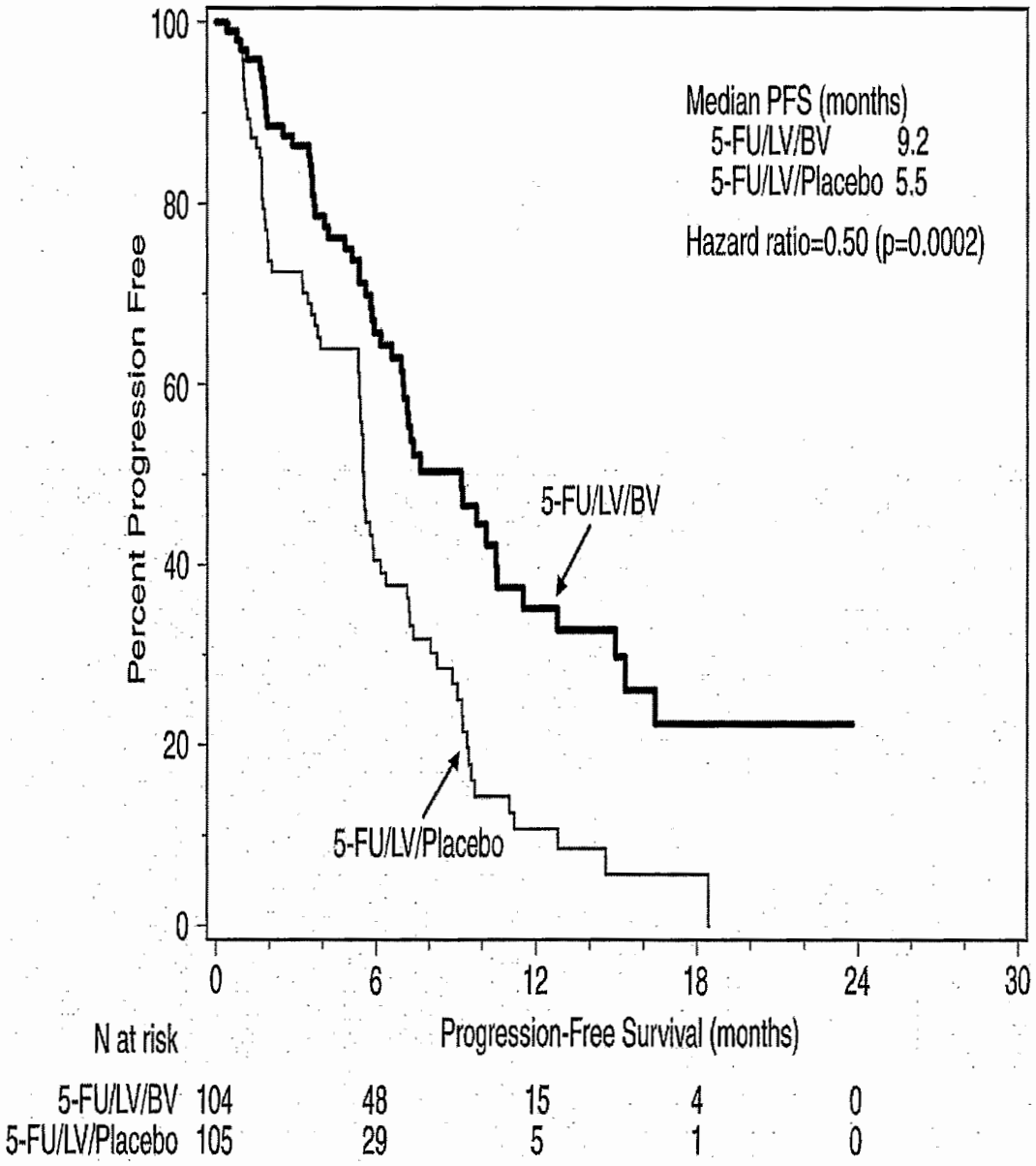
Median PFS (months)
5-FU/LV/BV 9.2
5-FU/LV/Placebo 5.5
Hazard ratio=0.50 (p=0.0002)
5-FU/LV/BV
5-FU/LV/Placebo
Percent Progression Free
100
80
60
40
20
0
0
6
12
18
24
30
Progression-Free Survival (months)
N at risk
5-FU/LV/BV 104 48 15 4 0
5-FU/LV/Placebo 105 29 5 1 0

Figure 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/017078

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/24 A61K39/395 A61P35/00 A61P35/04

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARGOLIN K ET AL: "Phase Ib trial of intravenous recombinant humanized monoclonal antibody to vascular endothelial growth factor in combination with chemotherapy in patients with advanced cancer: Pharmacologic and long-term safety data" JOURNAL OF CLINICAL ONCOLOGY, vol. 19, no. 3, 1 February 2001 (2001-02-01), pages 851-856, XP002302377 ISSN: 0732-183X abstract page 852, left-hand column, paragraph 1 - right-hand column, paragraph 2 table 1 table 2 ----- -/--	1-46

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2004	16/11/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Irion, A

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KABBINAVAR FAIROOZ ET AL: "Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer." JOURNAL OF CLINICAL ONCOLOGY : OFFICIAL JOURNAL OF THE AMERICAN SOCIETY OF CLINICAL ONCOLOGY. 1 JAN 2003, vol. 21, no. 1, 1 January 2003 (2003-01-01), pages 60-65, XP002302378 ISSN: 0732-183X abstract page 60, right-hand column, paragraph 2 page 62, left-hand column, paragraph 2 - right-hand column, paragraph 3 table 3 page 64, left-hand column, paragraph 4 - right-hand column, paragraph 4	1-46
P,X	D'ORAZIO A ET AL: "Adding a humanized antibody to vascular endothelial growth factor (Bevacizumab, Avastin(TM)) to chemotherapy improves survival in metastatic colorectal cancer" CLINICAL COLORECTAL CANCER 2003 UNITED STATES, vol. 3, no. 2, 2003, pages 85-88, XP009038755 ISSN: 1533-0028 the whole document	1-46
P,X	FERRARA NAPOLEONE ET AL: "Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer." NATURE REVIEWS. DRUG DISCOVERY. MAY 2004, vol. 3, no. 5, May 2004 (2004-05), pages 391-400, XP002302380 ISSN: 1474-1776 page 396, left-hand column, paragraph 2 - right-hand column, paragraph 1; figure 2	1-46
P,X	BRAUN ADA H ET AL: "New systemic frontline treatment for metastatic colorectal carcinoma" CANCER, vol. 100, no. 8, 15 April 2004 (2004-04-15), pages 1558-1577, XP009038748 ISSN: 0008-543X the whole document	1-46

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	GOLDBERG RICHARD M ET AL: "A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer." JOURNAL OF CLINICAL ONCOLOGY : OFFICIAL JOURNAL OF THE AMERICAN SOCIETY OF CLINICAL ONCOLOGY. 1 JAN 2004, vol. 22, no. 1, 1 January 2004 (2004-01-01), pages 23-30, XP001203533 ISSN: 0732-183X the whole document	1-46
T	HURWITZ HERBERT ET AL: "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer." THE NEW ENGLAND JOURNAL OF MEDICINE. 3 JUN 2004, vol. 350, no. 23, 3 June 2004 (2004-06-03), pages 2335-2342, XP009038752 ISSN: 1533-4406 the whole document	1-46
A	WO 98/45331 A (CHEN YVONNE MAN YEE ; BACA MANUEL (US); GENENTECH INC (US); WELLS JAME) 15 October 1998 (1998-10-15) the whole document	1-46
A	SALTZ L B ET AL: "IRINOTECAN PLUS FLUOROURACIL AND LEUCOVORIN FOR METASTATIC COLORECTAL CANCER" NEW ENGLAND JOURNAL OF MEDICINE, THE, MASSACHUSETTS MEDICAL SOCIETY, WALTHAM, MA, US, vol. 343, no. 13, 28 September 2000 (2000-09-28), pages 905-914, XP008033436 ISSN: 0028-4793 the whole document	1-46
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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O,A	H. HURWITZ ET AL: "Bevacizumab (a monoconal antibody to vascular endothelial growth factor) prolongs survival in first-line colorectal cancer (CRC): Results of a phase III trial of bevacizumab in combination with bolus IFL (irinotecan, 5-fluoruracil, leucovorin) a first-line therapy in subjects with metastatic CRC" 'Online! May 2003 (2003-05), ASCO AMERICAN SOCIETY OF CLINICAL ONCOLOGY , XP002302471 Retrieved from the Internet: URL:http://www.asco.org/hurwitz_no3646> 'retrieved on 2004-10-25! abstract	1-46

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/017078

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. [X] Claims Nos.: 1-44
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-44 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

[redacted] Information on patent family members

International Application No
PCT/US2004/017078

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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(71) Applicant *(for all designated States except US)*: **HERMES BIOSCIENCES, INC.** [US/US]; Suite B, 61 Airport Boulevard, South San Francisco, CA 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HONG, Keelung** [US/US]; 1360 De Haro Street, San Francisco, CA 94107 (US). **DRUMMOND, Daryl, C.** [US/US]; 247 Edgewood Drive, Pacifica, CA 94041 (US). **KIRPOTIN, Dmitri, B.** [RU/US]; 435 43rd Avenue, Apartment 101, San Francisco, CA 94121 (US).

(74) Agent: **HAILE, Lisa, A.**; DLA Piper Rudnick Gray Cary US LLP, Suite 1100, 4365 Executive Drive, San Diego, CA 92121-2133 (US).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: LIPOSOMES USEFUL FOR DRUG DELIVERY

(57) Abstract: The present invention provides liposome compositions containing substituted ammonium and/or polyanion, and optionally with a desired therapeutic or imaging entity. The present invention also provide methods of making the liposome compositions provided by the present invention.

WO 2005/107712 A1

LIPOSOMES USEFUL FOR DRUG DELIVERY

STATEMENT OF PRIORITY

[0001] This Application claims benefit of priority of the United States Provisional Patent Application No. 60/567,921 filed on May 3, 2004, which is incorporated herein by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] This invention relates generally to the field of liposomes, and more specifically to liposome compositions useful for delivery of therapeutic or diagnostic entities.

BACKGROUND OF THE INVENTION

[0003] Liposomes, or lipid bilayer vesicles, have been used or proposed for use in a variety of applications in research, industry, and medicine, particularly for the use as carriers of diagnostic or therapeutic compounds *in vivo*. See, for example: Lasic, D. *Liposomes: from physics to applications.* Elsevier, Amsterdam, 1993. Lasic, D, and Papahadjopoulos, D., eds. *Medical Applications of Liposomes.* Elsevier, Amsterdam, 1998. Liposomes are usually characterized by having an interior space sequestered from an outer medium by a membrane of one or more bilayers forming a microscopic sack, or vesicle. Bilayer membranes of liposomes are typically formed by lipids, *i.e.* amphiphilic molecules of synthetic or natural origin that comprise spatially separated hydrophilic and hydrophobic domains. See Lasic D., 1993, *supra*. Bilayer membranes of the liposomes can be also formed by amphiphilic polymers and surfactants (polymerosomes, niosomes). A liposome typically serves as a carrier of an entity such as, without limitation, a chemical compound, a combination of compounds, a supramolecular complex of a synthetic or natural origin, a genetic material, a living organism, a portion thereof, or a derivative thereof, that is capable of having a useful property or exerting a useful activity. For this purpose, the liposomes are prepared to contain the desired entity in a liposome-incorporated form. The process of incorporation of a desired entity into a liposome is often referred to as "loading". The liposome-incorporated entity may be completely or partially located in the interior space of the liposome, within the bilayer membrane of the liposome, or associated with the exterior surface of the liposome membrane. The incorporation of entities into liposomes is also referred to as encapsulation or entrapment,

[0004] and these three terms are used herein interchangingly with the same meaning. The intent of the liposomal encapsulation of an entity is often to protect the entity from the destructive environment while providing the opportunity for the encapsulated entity to exert its activity mostly at the site or in the environment where such activity is advantageous but less so in other sites where such activity may be useless or undesirable. This phenomenon is referred to as delivery. For example, a drug substance within the liposome can be protected from the destruction by enzymes in the body, but become released from the liposome and provide treatment at the site of disease.

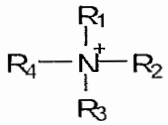
[0005] Ideally, such liposomes can be prepared to include the desired compound (i) with high loading efficiency, that is, high percent of encapsulated entity relative to the amount taken into the encapsulation process; (ii) high amount of encapsulated entity per unit of liposome bilayer material; (iii) at a high concentration of encapsulated entity, and (iv) in a stable form, *i.e.*, with little release (leakage) of an encapsulated entity upon storage or generally before the liposome appears at the site or in the environment where the liposome-entrapped entity is expected to exert its intended activity.

[0006] Therefore, there is a need in the art to provide various liposome compositions that are useful for delivery of a variety of compounds, especially therapeutic, diagnostic, or imaging entities.

SUMMARY OF THE INVENTION

[0007] The present invention is based on the discovery that substituted ammonium and polyanion are useful for loading and retaining entities inside liposomes. Accordingly the present invention provides methods and liposome compositions useful for delivery of a variety of entities, especially therapeutic entities, that is, entities useful in the diagnosis, prognosis, testing, screening, treatment, or prevention of an undesirable condition, *e.g.*, a disease, in living organism ,such as a human, a plant, or an animal.

[0008] In one embodiment, the present invention provides a composition comprising a liposome in a medium, wherein the inside of the liposome contains a substituted ammonium



(I)

wherein each of R_1, R_2, R_3, and R_4 is independently a hydrogen or an organic group having, inclusively, in totality up to 18 carbon atoms, wherein at least one of R_1, R_2, R_3, and R_4 is an organic group, wherein the organic group is independently a hydrocarbon group having up to 8 carbon atoms, and is an alkyl, alkylidene, heterocyclic alkyl, cycloalkyl, aryl, alkenyl, or cycloalkenyl group or a hydroxy-substituted derivative thereof, optionally including within its hydrocarbon chain a S, O, or N atoms, forming an ether, ester, thioether, amine, or amide bond, wherein at least three of R_1, R_2, R_3, and R_4 are organic groups, or the substituted ammonium is a sterically hindered ammonium, such as, for example, where at least one of the organic groups has a secondary or tertiary carbon atom directly linked to the ammonium nitrogen atom. Preferably, the substituted ammonium compound encapsulated into liposomes has a negative logarithm of the acidic (deprotonation) dissociation constant (pKa) of at least about 8.0, at least about 8.5, at least about 9.0, at least 9.5, or at least 10.0, as determined in an aqueous solution at ambient temperature.

[0009] In another embodiment, the present invention provides a composition comprising a liposome in a medium, wherein the inner space of the liposome contains a polyanion and wherein the polyanion is a polyanionized polyol or a polyanionized sugar. The liposome preferably contains a transmembrane gradient capable of effecting the loading of an entity into the liposome. In one embodiment, the transmembrane gradient is a gradient of an ammonium, a quarternary ammomium, or a primary, secondary, or tertiary substituted ammonium compound having in a diluted aqueous solution at ambient temperature a negative logarithm of the acidic (deprotonation) dissociation constant (pKa) of at least about 8.0, at least about 8.5, at least about 9.0, at least 9.5, or at least 10.0. The liposome optionally contains an entrapped entity, for example, a therapeutic, a detectable marker, or a globally cationic organic molecule.

[00010] In yet another embodiment, the composition provided by the present invention further comprises an entity encapsulated in the liposomes of the present invention. Preferably, the entity is encapsulated within the inner space of the liposome. For example, the inner space of the liposome further comprises an anti-neoplastic therapeutic and wherein the toxicity level of the composition to a subject is at least equal to or less than

the toxicity level of the anti-neoplastic therapeutic administered to the subject without the composition.

[0010] In yet another embodiment, the composition provided by the present invention is a liposome composition comprising a camptothecin compound. The composition has an anticancer activity at least two times, four times, or ten times higher than the camptothecin compound similarly administered in the absence of the composition, while the toxicity of the composition does not exceed, is at least two times, or at least four times lower than the toxicity of the camptothecin compound similarly administered in the absence of the composition. In a one embodiment, the camptothecin compound is a pro-drug, and is contained in the liposome of at least 0.1 mg, at least 0.2 mg, at least 0.3 mg, at least 0.5 mg, or at least 1 mg per 1 mg of the liposome membrane materials, e.g., lipids. The camptothecin compound is preferably encapsulated in the liposome substantially within the inner space of the liposome. In one instance, the camptothecin compound is irinotecan (CPT-11).

[0011] In yet another embodiment, the composition provided by the present invention is a liposome composition of a vinca alkaloid or a derivative thereof. The composition has the 24-hour drug retention within the liposome after 24 hours exposure in the blood of a mammal *in vivo* of at least 50%, at least 60%, or at least 70% of the original drug load. The vinca alkaloid or a derivative thereof is preferably encapsulated in the liposome substantially within the inner space of the liposome. One example of the mammal is a rat. Exemplary vinca alkaloids and derivatives are vincristine, vinblastine, and vinorelbine.

[0012] In still another embodiment, the present invention provides a method of encapsulating an entity into a liposome. The method comprises contacting the liposomes of the present invention with an entity, *e.g.*, therapeutic or detectable entity. Preferably, the contacting is performed under the conditions when the concentration of substituted ammonium or a polyanion of the present invention in the medium is lower than that in the inner space of the liposomes. In one embodiment, the liposome composition is contacted with an entity in an aqueous medium.

[0013] In still another embodiment, the present invention provides a method of encapsulating an entity into a liposome. The method comprises contacting the liposome-containing composition of the present invention with a pre-entity, wherein the pre-entity

is capable of being converted to an entity under a condition, and providing the condition inside the liposome whereby converting the pre-entity to the entity inside the liposome. In one case, the entity is an organic compound, and the pre-entity is a basic derivative thereof.

[0014] In still another embodiment, the present invention provides a kit for making liposome-encapsulated entities. The kit comprises a container with a liposome of the present invention, and, optionally, a container containing an entity, and/or instructions for a user, *e.g.* to encapsulate an entity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 shows blood pharmacokinetics of the liposome lipid (circles) and the drug (triangles) after i.v. bolus administration of CPT-11-loaded liposomes to a rat. The liposomes are loaded using TEA-Pn method (See Example 9).

[0016] Figure 2 shows the dynamics of drug-to-liposome lipid ratio in the blood of a rat *in vivo* following i.v. bolus administration of the liposome loaded with CPT-11 using TEA-Pn method (See Example 9).

[0017] Figure 3 shows antitumor efficacy of freeCPT-11 and liposomal CPT-11 against BT-474 human breast cancer xenografts in nude mice. "Control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 10).

[0018] Figure 4 shows the dynamics of the animals' body weights during the treatment of BT-474 tumor-bearing nude mice with free CPT-11 or liposomal CPT-11. "Control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 10).

[0019] Figure 5 shows the dynamics of drug-to-liposome lipid ratio in the blood of a rat *in vivo* following i.v. bolus administration of the liposome loaded with CPT-11 using TEA-SOS method. (See Example 14).

[0020] Figure 6 shows antitumor efficacy of free and liposomal CPT-11 against HT-29 human colon cancer xenografts in nude mice. The on-panel caption indicates the drug loading method and the administered dose per injection. "Saline control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 15).

[0021] Figure 7 shows the dynamics of the animals' body weights during the treatment of HT-29 tumor-bearing nude mice with free or liposomal formulations of CPT-11. The error bars represent standard deviation of the data. "Saline control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 15).

[0022] Figure 8A shows blood pharmacokinetics of the liposome lipid after i.v. bolus administration of Topotecan-loaded liposomes to a rat. The on-panel caption indicates the drug loading method and the drug content of the liposomes. (See Example 24).

[0023] Figure 8B shows the dynamics of drug-to-liposome lipid ratio in the blood of a rat *in vivo* following i.v. bolus administration of the liposomes loaded with Topotecan The on-panel caption indicates the drug loading method and the drug content of the liposomes. (See Example 24).

[0024] Figure 9 shows the *in vitro* cytotoxicity of free, liposomal, or HER2-targeted immunoliposomal Topotecan (TEA-Pn method) against SKBr-3 breast carcinoma cells. (See Example 27).

[0025] Figure 10 shows the *in vitro* cytotoxicity of free, liposomal, or HER2-targeted immunoliposomal Topotecan (TEA-SOS method) against SKBr-3 breast carcinoma cells. (See Example 32).

[0026] Figure 11 shows antitumor efficacy of various Topotecan (TPT) formulations against BT-474 human breast cancer xenografts in nude mice. "Saline control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 29).

[0027] Figure 12 shows the dynamics of the animals' body weights during the treatment of BT-474 tumor-bearing nude mice with free Topotecan (TPT), liposomal Topotecan (Ls-TPT), or anti-HER2 immunoliposomal Topotecan (F5 ILs-TPT). "Control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 29).

[0028] Figure 13A shows antitumor efficacy of Topotecan formulations against BT-474 human breast cancer xenografts in nude mice. Free Topotecan (Free TPT) or liposomal Topotecan (Ls-TPT) were administered at one-eighth of their maximum

tolerated doses. Error bars represent standard deviation of the data. "Control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 31).

[0029] Figure 13B shows antitumor efficacy of Topotecan formulations against BT-474 human breast cancer xenografts in nude mice. Free Topotecan (Free TPT) or liposomal Topotecan (Ls-TPT) were administered at one-fourth of their maximum tolerated doses. Error bars represent standard deviation of the data. "Control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 31).

[0030] Figure 13C shows antitumor efficacy of Topotecan formulations against BT-474 human breast cancer xenografts in nude mice. Free Topotecan (Free TPT) or liposomal Topotecan (Ls-TPT) were administered at one-half of their maximum tolerated doses. Error bars represent standard deviation of the data. "Control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 31).

[0031] Figure 13D shows antitumor efficacy of Topotecan formulations against BT-474 human breast cancer xenografts in nude mice. Free Topotecan (Free TPT) or liposomal Topotecan (Ls-TPT) were administered at their maximum tolerated doses. Error bars represent standard deviation of the data. "Control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 31).

[0032] Figure 14 shows the dynamics of the average body weights during the treatment of BT-474 tumor-bearing nude mice with free Topotecan (Free TPT) or liposomal Topotecan (Ls-TPT) administered at their maximum tolerated doses. "Control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 31).

[0033] Figure 15 shows the cytotoxicity of free 6-(3-aminopropyl)-ellipticine (Free AE), liposomal 6-(3-aminopropyl)-ellipticine (Ls-AE) , or HER2-targeted immunoliposomal 6-(3-aminopropyl)-ellipticine (F5 ILs-AE)) against BT-474 breast carcinoma cells *in vitro*. (See Example 35).

[0034] Figure 16 shows the *in vitro* cytotoxicity of free 6-(3-aminopropyl)-ellipticine (Free APE), liposomal 6-(3-aminopropyl)-ellipticine (Ls-APE), or EGFR-targeted immunoliposomal 6-(3-aminopropyl)-ellipticine (C225-ILs-APE) against breast

carcinoma cells with low (MCF-7) or high (MDA-MB468) expression of EGF receptor. (See Example 36).

[0035] Figure 17 shows blood pharmacokinetic attributes of the liposomally formulated 6-(3-aminopropyl)ellipticine (APE): blood pharmacokinetics of the liposome lipid (Panel A, open circles), the drug (Panel A, filled circles), and the dynamics of drug-to-liposome lipid ratio (Panel B) after i.v. bolus administration of APE liposomes to a rat. (See Example 37).

[0036] Figure 18 shows blood pharmacokinetic attributes of vinorelbine formulated into liposomes (Ls-VRB), and anti-HER2 immunoliposomes (F5-ILs-VRB): blood pharmacokinetics of the liposome lipid (Panel A), the drug (Panel B), and the dynamics of drug-to-liposome lipid ratio (Panel C) after i.v. bolus administration of vinorelbine liposomes to a rat. (See Example 43).

[0037] Figure 19 shows blood pharmacokinetics of the liposome lipid after i.v. bolus administration of vinorelbine-loaded liposomes to a rat. The liposomes are loaded using pre-entrapped triethylammonium dextransulfate (DS-TEA), ammonium dextransulfate (DS-A), or ammonium sulfate (S-A). (See Example 44).

[0038] Figure 20 shows the dynamics of drug-to-liposome lipid ratio in the blood of a rat *in vivo* following i.v. bolus administration of the liposomes loaded with vinorelbine using pre-entrapped triethylammonium dextransulfate (DS-TEA), ammonium dextransulfate (DS-A), or ammonium sulfate (S-A). (See Example 44).

[0039] Figure 21 shows blood pharmacokinetics of the liposome lipid after i.v. bolus administration of vinorelbine-loaded liposomes to a rat. The liposomes are loaded using pre-entrapped triethylammonium sucroseoctasulfate (TEA-SOS) and have the mean size as indicated at the on-panel caption. (See Example 45).

[0040] Figure 22 shows the dynamics of drug-to-liposome lipid ratio in the blood of a rat *in vivo* following i.v. bolus administration of vinorelbine-loaded liposomes. The liposomes are loaded using pre-entrapped triethylammonium sucrooctasulfate (TEA-SOS) and have the mean size as indicated at the on-panel caption. (See Example 45).

[0041] Figure 23 shows blood pharmacokinetics of the liposome lipid in a rat after i.v. bolus administration of vinorelbine formulated into liposomes (Ls-VRB) or anti-HER2 immunoliposomes (F5-ILs-VRB) using TEA-SOS method. (See Example 46).

[0042] Figure 24 shows the dynamics of drug-to-liposome lipid ratio in the blood of a rat *in vivo* following i.v. bolus administration of vinorelbine formulated into liposomes (Ls-VRB) or anti-HER2 immunoliposomes (F5-ILs-VRB) using TEA-SOS method. (See Example 46).

[0043] Figure 25 shows the *in vitro* cytotoxicity of free vinorelbine (free VRB), liposomal vinorelbine (Ls-VRB), or HER2-targeted immunoliposomal vinorelbine (F5-Ils-VRB) against HER2-overexpressing human breast cancer cells MDA-MB-453. (See Example 48).

[0044] Figure 26 shows the *in vitro* cytotoxicity of free vinorelbine (free VRB), liposomal vinorelbine (Ls-VRB), or HER2-targeted immunoliposomal vinorelbine (F5-Ils-VRB) against HER2-overexpressing CaLu-3 human non-small cell lung cancer cells. (See Example 49).

[0045] Figure 27 shows the *in vitro* cytotoxicity of free vinorelbine (free VRB), liposomal vinorelbine (Ls VRB/SOS-TEA), or HER2-targeted immunoliposomal vinorelbine (F5-ILs VRB/SOS-TEA) against HER2-overexpressing human breast cancer cells SKBr-3. (See Example 50).

[0046] Figure 28 shows antitumor efficacy of the free vinorelbine (free VRB) or liposomal vinorelbine (Ls VRB) against HT-29 human colon cancer xenografts in nude mice. "Saline" designates the mice treated with drug- and liposome-free vehicle only. Error bars represent standard deviation of the data. (See Example 51).

[0047] Figure 29 shows the dynamics of the average body weights during the treatment of HT-29 tumor-bearing nude mice with free vinorelbine (free VRB), liposomal vinorelbine (Ls VRB), or vehicle only (saline). Error bars represent standard deviation of the data. (See Example 51).

[0048] Figure 30 shows antitumor efficacy of the free vinorelbine (free VRB) or liposomal vinorelbine (Ls VRB) in a syngeneic C-26 murine colon carcinoma model.

The dose of the drug per injection was as indicated on the on-panel caption. Error bars represent standard deviation of the data. "Saline" designates the mice treated with drug- and liposome-free vehicle only. (See Example 52).

[0049] Figure 31 shows the dynamics of the average body weights during the treatment of mice bearing syngeneic C-26 murine colon carcinoma tumors with various doses of free vinorelbine (free VRB), liposomal vinorelbine (Ls VRB), or with vehicle only (saline). The dose of the drug per injection was as indicated on the on-panel caption. (See Example 52).

[0050] Figure 32 shows antitumor efficacy of the free vinorelbine (Free drug) or scFv F5-conjugated, anti-HER2 immunoliposomal vinorelbine prepared by a TEA-SOS method (F5-ILs-VRB TEA-SOS), anti-HER2 immunoliposomal vinorelbine prepared y a TEA-Pn method (F5-ILs-VRB TEA-Pn) against HER2-overexpressing human breast carcinoma (BT-474) xenografts in nude mice. "Saline control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 53).

[0051] Figure 33 shows the dynamics of the average body weights during the treatment of mice bearing HER2-overexpressing human breast carcinoma (BT-474) xenografts with free vinorelbine, scFv F5-conjugated, anti-HER2 immunoliposomal vinorelbine prepared using a TEA-SOS method, anti-HER2 immunoliposomal vinorelbine prepared by a TEA-Pn method, or with vehicle only. For explanation of the symbols, see the caption to Figure 32. (See also Example 53).

[0052] Figure 34 shows antitumor efficacy of the free vinorelbine (Free drug) or scFv F5-conjugated, anti-HER2 immunoliposomal vinorelbine prepared using various amounts of PEG-lipid against HER2-overexpressing human breast carcinoma (BT-474) xenografts in nude mice. The error bars are standard deviation of the data. "Vehicle control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 54).

[0053] Figure 35 shows antitumor efficacy of the free vinorelbine (free NAV), liposomal vinorelbine (NAV Lip), or FC225Fab'-conjugated, anti-EGFR-immunoliposomal vinorelbine (C225-NAV Lip) against EGFR-overexpressing human glioblastoma (U87) xenografts in nude mice. "Saline" designates the mice treated with drug- and liposome-free vehicle only. (See Example 55).

[0054] Figure 36 shows blood pharmacokinetics of the liposome lipid and the dynamics of the drug/liposome lipid ratio in the blood of a rat after i.v. bolus administration of doxorubicin formulated into liposomes using triethylammonium sulfate method. (See Example 56).

[0055] Figure 37 shows antitumor efficacy of the liposomal doxorubicin (Ls-Dox), or scFv F5-conjugated, anti-HER2 immunoliposomal doxorubicin (F5 ILs-Dox) prepared using various amounts of PEG-lipid against HER2-overexpressing human breast carcinoma (BT-474) xenografts in nude mice. The on-panel caption shows the amount of PEG-lipid expressed in mol.% of liposome phospholipids. "Saline control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 57).

[0056] Figure 38 shows blood pharmacokinetics of liposomal vinblastine in a rat. (See Example 58).

[0057] Figure 39 shows the dynamics of the drug/liposome lipid ratio in the blood of a rat after i.v. bolus administration of liposomal vinblastine. (See Example 58).

[0058] Figure 40 shows the *in vitro* cytotoxicity of free vincristine (Free VCR), liposomal vincristine (Ls-VCR), or HER2-targeted immunoliposomal vincristine (F5-ILs-VCR) against HER2-overexpressing human breast cancer cells SKBr-3. (See Example 61).

[0059] Figure 41 shows blood pharmacokinetics of the liposome lipid in a rat after i.v. bolus administration of vincristine formulated into liposomes of different average size (indicated on the on-panel caption). (See Example 62).

[0060] Figure 42 shows the dynamics of the drug/liposome lipid ratio in the blood of a rat after i.v. bolus administration of vincristine formulated into liposomes of different average size (indicated on the on-panel caption). (See Example 62).

[0061] Figure 43 shows antitumor efficacy of the free vincristine (free VCR), liposomal vincristine prepared by triethylammonium citrate method (Ls-VCR Citrate), liposomal vincristine prepared by triethylammonium sucrooctasulfate method (Ls-VCR SOS), or scFv F5-conjugated, anti-HER2 immunoliposomal vincristine prepared by triethylammonium sucrooctasulfate method (F5 ILs-VCR SOS) against HER2-

overexpressing human breast carcinoma (BT-474) xenografts in nude mice. "Saline control" designates the mice treated with drug- and liposome-free vehicle only. (See Example 64).

[0062] Figure 44 shows the dynamics of the average body weights during the treatment of mice bearing HER2-overexpressing human breast carcinoma (BT-474) xenografts with free vincristine (free VCR), liposomal vincristine prepared by triethylammonium citrate method (Ls-VCR Citrate), liposomal vincristine prepared by triethylammonium sucrooctasulfate method (Ls-VCR SOS), scFv F5-conjugated, anti-HER2 immunoliposomal vincristine prepared by triethylammonium sucrooctasulfate method (F5 ILs-VCR SOS), or with vehicle only (saline control). (See Example 64).

[0063] Figure 45 shows antitumor efficacy of the free vincristine (vincristine), liposomal vincristine (nt-vcr), or C225 Fab'-conjugated, anti-EGFR immunoliposomal vincristine (C225-vcr) against EGFRvIII-overexpressing human brain cancer (U87) xenografts in nude mice. "Saline" designates the mice treated with drug- and liposome-free vehicle only. (See Example 65).

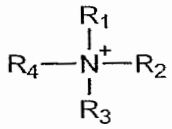
[0064] Figure 46 shows blood pharmacokinetics of CPT-11 and the dynamics of the percentage of CPT-11 present in the active (lactone) form in the blood of a rat after i.v. bolus administration of liposomal CPT-11. (See Example 69).

[0065] Figure 47 shows blood pharmacokinetics of CPT-11 and the dynamics of the percentage of CPT-11 present in the active (lactone) form in the blood of a rat after i.v. bolus administration of CPT-11 solution (free CPT-11). (See Example 69).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0066] The present invention relates in general to methods and liposome compositions useful for delivery a variety of entities, especially therapeutics and imaging agents. It is the discovery of the present invention that substituted ammonium and polyanion are useful for loading and retaining the entities, *e.g.*, compound, inside liposomes. Accordingly, the present invention provides liposome compositions and kits containing substituted ammonium and/or polyanion and methods of making these liposome compositions.

[0067] According to one feature of the present invention, it provides a composition of liposomes containing within its inner space one or more substituted ammonium compounds of a formula



(I)

wherein each of R_1, R_2, R_3, and R_4 is independently a hydrogen or an organic group, and wherein at least one of R_1, R_2, R_3, and R_4 is an organic group, such as, an alkyl, alkylidene, heterocyclic alkyl, cycloalkyl, aryl, alkenyl, or cycloalkenyl group, a hydroxy-substituted derivative thereof, optionally including within its hydrocarbon chain a S, O, or N atoms, *e.g.*, forming an ether (including an acetal or ketal), ester, sulfide (thioether), amine, or amide bond therein. If less than three of R_1, R_2, R_3, and R_4 are organic groups, then, according to the invention, at least one, and preferably two, of the organic groups has a secondary or tertiary carbon atoms (*i.e.*, carbon atoms having 2 or 3 carbon-carbon bonds, respectively) directly linked to the ammonium nitrogen, *i.e.*, the substituted ammonium is a sterically hindered ammonium. Generally, the presence of titratable ammonium, such as unsubstituted ammonium ion (NH_4^+), as well as primary and secondary straight chain alkylammonium ions in the inner space of the liposome of the present invention is known to provide for enhanced encapsulation of weak amphiphilic bases, for example, *via* a mechanism of "active", "remote", or "transmembrane gradient-driven" loading (Haran, et al., *Biochim. Biophys. Acta*, 1993, v. 1152, p. 253-258; Maurer-Spurej, et al., *Biochim. Biophys. Acta*, 1999, v. 1416, p. 1-10). However these ammonia compounds possess hydrogen atoms that easily enter into reactions of nucleophilic substitution, and otherwise react chemically with the liposome-entrapped entities, and therefore are capable of impairing the chemical integrity of the entitites during or after the liposome loading (entrapment) process. Thus, it is desirable for an entrapped substituted ammonium compound to be more chemically inert, lacking chemical functions which are unstable or readily reactive with the liposome components, that may include an encapsulated entity. Unexpectedly, we discovered that liposome compositions comprising within their inner space a substituted tertiary and quaternary ammonium that do not have a substitutable hydrogen, or a sterically hindered primary or secondary ammonium, in which the access to an ammonium hydrogen atom is sterically hindered by a neighbor bulky organic group, such as having one or two secondary or

tertiary carbon atoms linked to the ammonium nitrogen, show not only outstanding entity-loading capacity, but also improved stability of the liposome-entrapped entity, *e.g.*, a drug, against premature release from the liposome in the living body.

[0068] In one embodiment, the liposome-entrapped substituted ammonium compound is pharmaceutically inert, that is, does not elicit an adverse physiological response when administered to a living subject, *e.g.* a human or an animal, within an amount of the liposome membrane material that is sufficient to deliver an effective dose of the liposome-entrapped entity. In another embodiment, the substituted ammonium of the present invention has an acceptable level of toxicity to a subject. Usually an acceptable level of toxicity means that the toxic dose, *e.g.*, a maximum tolerated dose (MTD), or a dose causing 50% lethality (LD50) of the substituted ammonium of the present invention is at least twice, at least four times, at least eight times, or at least ten times higher than the toxic dose of a liposome-entrapped entity, *e.g.*, drug, loaded inside the liposomes of the present invention. For example, triethylammonium sulfate has an acceptable level of toxicity according to the present invention since its LD50 is about 40 times higher than the LD50 of doxorubicin, an anti-cancer drug. The toxicity levels or physiological responses of substituted ammoniums, as well as of the entities of interest, if not already known, can be readily established via routine techniques well known by persons skilled in the biomedical art. See, for example, S.C. Gad. *Drug Safety Evaluation,* Wiley, New York, 2002. One method of quantifying the toxicity of free and/or liposomally formulated drug is described in Example 16 herein.

[0069] In one preferred embodiment, the substituting organic groups among R_1, R_2, R_3, or R_4 are of the size and physico-chemical properties sufficient to ensure that the substituted ammonium forms in aqueous environment substantially a true (molecular) solution, but not micelles, bilayers, or similar self-assembled structures. Therefore, the substituted ammonium of the present invention preferably has little or substantially no distribution into the bilayer portion of liposomes, therefore minimizing the risk of destabilization, solubilization, or permeabilization of the liposomes entrapping the substituted ammonium.

[0070] The organic group of the substituted ammonium is typically a hydrocarbon containing, inclusively, up to 8 carbon atoms, up to 6 carbon atoms, or up to 4 carbon

atoms, and in totality, the substituting groups contain, inclusively, up to 18, up to 16, up to 12, or up to 9 carbon atoms. These substituting hydrocarbon groups include any combination of interlinked primary, secondary, or tertiary carbon atoms, as well as cycloalkyl groups being linked at their termini directly to the ammonium nitrogen to form a heterocycle, or to a carbon atom of an ammonium hydrogen-substituting group. These substituted alkyl groups can also include heteroatoms, *e.g.*, oxygen, nitrogen, or sulfur in their carbon chains forming a functional group, *e.g.*, ether, acetal, amine, or sulfide group, as well as forming a functional group, *e.g.*, hydroxyl group, linked to the alkyl carbon chain. Examples of the organic group of the present invention include, without any limitation, alkyls, alkylidenes, heterocyclic alkyls, cycloalkyls, aryls, alkenyls, cycloalkenyls, or hydroxy-substituted derivatives thereof, *e.g.*, a hydroxy-substituted alkylidene forming a ring inclusive of N in the substituted ammonium.

[0071] In another embodiment, the substituted ammonium is: a heterocyclic ammonium, *i.e.* an ammonium wherein at least two of R_1, R_2, R_3, or R_4 form a ring; a sterically hindered primary ammonium; or a sterically hindered secondary ammonium. In general, a sterically hindered primary or secondary ammonium includes any substituted ammonium with one or two of the R_1, R_2, R_3, and R_4 substituted with alkyl groups that sterically crowd the molecule, *e.g.*, any substituted ammonium with one or two of the R_1, R_2, R_3, and R_4 substituted with one or two cycloalkyl groups or alkyl groups having at least one secondary or tertiary alkyl carbon atom linked to the nitrogen of the substituted ammonium. Examples of such heterocyclic, sterically hindered primary ammoniums, and sterically hindered secondary ammonium include, without any limitation, isopropylethylammonium, isopropylmethylammonium, diisopropylammonium, tert-butylethylammonium, dicychohexylammonium, protonized forms of morpholine, pyridine, piperidine, pyrrolidine, piperazine, *tert*-bulylamine, 2-amino-2-methylpropanol-1, 2-amino-2-methyl-propandiol-1,3, and *tris*-(hydroxyethyl)-aminomethane. These substituted ammonium compounds are generally commercially available in the form of various salts, or are readily prepared from their corresponding amines by neutralization with acids.

[0072] In yet another embodiment, the substituted ammonium is a tertiary or quaternary ammonium including, without any limitation, trimethylammonium, triethylammonium, tributylammonium, diethylmethylammonium,

diisopropylethylammonium, triisopropylammonium, N-methylmorpholinium, N-hydroxyethylpiperidinium, N-methylpyrrolidinium, and N, N'-dimethylpiperazinium, tetramethylammonium, tetraethylammonium, and tetrabutylammonium. These substituted ammonium compounds are generally commercially available in the form of various salts, or are readily prepared from their corresponding amines by neutralization with acids.

[0073] In yet another embodiment, the substituted ammonium compound according to the invention is a globally cationic compound, that is, under the conditions of the entity encapsulation, typically, in aqueous solution at a pH between about pH 2 and about pH 8, bears net positive charge, *e.g.* as a result of ionization (protonation) of the nitrogen atom.

[0074] In yet another embodiment, the substituted primary, secondary, or tertiary ammonium compound encapsulated into liposomes has a negative logarithm of the acidic (deprotonation) dissociation constant (pKa) of at least about 8.0, at least about 8.5, at least about 9.0, at least 9.5, or at least about 10.0, as determined in a diluted aqueous solution at ambient temperature (typically 25 °C). Parameter pKa is a well known characteristic of ammonium compounds that generally characterizes the strength of their basic properties, and methods for pKa determination are conventional and routine in the art. The pKa values for many amines and their protonated forms (ammoniums) are tabulated in reference books of chemistry and pharmacology. See, for example, IUPAC Handbook of Pharmaceutical Salts, ed. by P.H. Stahl and C.G Wermuth, Wiley-VCH, 2002; CRC Handbook of Chemistry and Physics, 82nd Edition, ed. by D.R.Lide, CRC Press, Florida, 2001, p. 8-44 to 8-56. Generally, higher pKa characterizes stronger bases. Exemplary substituted ammonium compounds, as well as unsubstituted ammonium (listed as their conjugated amine bases) have the following pKa values: pyrrolidine,11.31; piperidine,11.12; diisopropylamine,11.05; diethylamine,10.93; triethylamime,10.75; dimethylamine,10.73; tert-butylamine,10.68; cyclohexylamine,10.66; methylamine,10.66; ethylamine,10.65; propylamine,10.54; Isopropylamine,10.53; N-ethylpiperidine,10.45; dicyclohexylamine,10.4; N-methylpiperidine,10.38; diethylmethylamine,10.35; dimethylpropylamine,10.15; trimethylamine, 9.8; piperazine, 9.73 (I) ,5.33 (II); 2-amino-2-methylpropanol, 9.69; N,N'-dimethylpiperazine, 9.66 (I),5.2 (II); diethyl-(2-hydroxyethyl)amine, 9.58; ethanolamine, 9.5; N-hyrdoxyethylpyrrolidine, 9.44; diethanolamine, 9.28; ammonia, 9.27; dimethyl-(2-hydroxyethyl)amine, 8.83; 2-amino-2-methylpropanediol-1,3, 8.8; morpholine, 8.5; tris-(hydroxymethyl)-aminomethane, 8.3;

N-methylglucamine, 8.03; triethanolamine, 7.76; N-ethylmorpholine, 7.67; N-hydroxyethylmorpholine, 7.39; imidazole, 7.03; pyridine, 5.23. As a rule, substitution of alkyl or cycloalkyl group for a hydrogen in an ammonium compound increases pKa value. Notably, multiple hydroxyl or ether functions in the substituting alkyl groups, or the presence of aromaticity in a nitrogen-containing heterocyclic group reduce pKa value relative to similar substituted ammonia without hydroxyl or ether functions. The compounds with more than one ammonium group usually have pKa of the second and subsequent ammonium group much lower than of the first one. We unexpectedly discovered that substituted ammonia with higher pKa values, that is, formed by more strongly basic amines, were more effective than those formed from weaker amines in stabilizing the drug inside liposomes. For example, both IHP and SOS salts of triethylammonium (pKa = 10.75) were notably more effective than corresponding salts of triethanolammonium (pKa = 7.76) in stabilizing irinotecan within the liposomes in vivo (Example 73).

[0075] The substituted ammonium contained in the liposome composition of the present invention can be in any suitable form, *e.g.*, salt. Suitable salts include pharmaceutically acceptable salts. See, for example, P.H.Stahl, C.G. Wermuth (eds), *Handbook of Pharmaceutical Salts*, Wiley-VCH, Weinheim, 2002. In one embodiment, the substituted ammonium is a salt containing one or more polyanions of the present invention. Optimally the counter-ion (anion) in the substituted ammonium salt of the present invention renders the salt water soluble, is pharmaceutically inert, capable of forming precipitates or gels when in contact with a therapeutic or detectable entity, and/or is less permeable through the liposome membrane than the substituted ammonium or its non-dissociated amine form. In general, the substituted ammonium salt of the present invention forms a true solution in the intraliposomal, *e.g.* aqueous, space, and does not form a significant amount of a condensed phase such as micelle, bilayer, gel, or crystalline phase. The relative amount of a substituted ammonium and a salt-forming anion, e.g., polyanion, is at or near the point of stiochiometric equivalency, and typically has the pH on the range of 3-9, more often, pH 4-8, dependent, for example, on the dissociation constant of the conjugated base of the substituted ammonium ion.

[0076] In general, the substituted ammonium is contained inside, that is, in the inner (interior) space of the liposomes of the present invention. In one embodiment, the

substituted ammonium is partially or substantially completely removed from the outer medium surrounding the liposomes. Such removal can be accomplished by any suitable means known to one skilled in the art, *e.g.*, dilution, ion exchange chromatography, size exclusion chromatography, dialysis, ultrafiltration, precipitation, etc.

[0077] According to another feature of the present invention, it provides a composition of liposomes containing a polyanion. The polyanion of the present invention can be any suitable chemical entity with more than one negatively charged groups resulting in net negative ionic charge of more than two units within the liposome interior, *e.g.*, aqueous, space. The polyanion of the present invention can be a divalent anion, a trivalent anion, a polyvalent anion, a polymeric polyvalent anion, a polyanionized polyol, or a polyanionized sugar. Sulfate, phosphate, pyrophosphate, tartrate, succinate, maleate, borate, and citrate are, without limitation, the examples of such di- and trivalent anions. In one preferred embodiment, the polyanion of the present invention is a polyanionic polymer, having an organic (carbon) or inorganic backbone , and a plurality of anionic functional groups, i.e functional groups ionizable to a negative charge in a neutral aqueous solution, and integrated or appended to the backbone. A polymer is a natural or synthetic compound, usually of high molecular weight, consisting of repeated linked units, each a relatively light and simple molecule. Exemplary polyanionic polymers are polyphosphate, polyvinylsulfate, polyvinylsulfonate, anionized polyacrylic polymers, anionized, *e.g.*, polysulfonated polyamines, such as polysulfonated poly(ethylene imine); polysulfated, polycarboxylated, or polyphosphorylated polysaccharides; acidic polyaminoacids; polynucleotides; other polyphosphorylated, polysulfated, polysulfonated, polyborated, or polycarboxylated polymers. Such polyvalent anions and polymers are well known in the art and many are commercially available. A polymeric anion of the present invention is preferably a biodegradable one, that is, capable of breaking down to non-toxic units within the living organism. Exemplary biodegradable polymeric anion is polyphosphate.

[0078] In another preferred embodiment, the polyanion is a polyanionized polyol or a polyanionized sugar. A polyol is an organic molecule having a plurality of hydroxyl groups linked to, *e.g.*, linear, branched, or cyclic, carbon backbone. Thus, a polyol can be characterized in other terms as a polyhydroxylated compound. Preferably, a majority of carbon atoms in a polyol are hydroxylated. Polyols (polyatomic alcohols) are molecules

well known in the art. Both straight chain (linear or branched) and cyclic polyols can be used. Exemplary polyols of the present invention are, without limitation: ethyleneglycol; glycerol, treitol, erythritol, pentaerythritol, mannitol, glucitol, sorbitol, sorbitan, xylitol, lactitol, maltitol, fructitol, and inositol. A sugar usually comprises a cyclic acetal, a cyclic ketal, a ketone, or an aldehyde group, or an adduct thereof, within a group of interlinked predominantly hydroxylated carbon atoms. Sugars are often naturally occurring compounds. Hydrolysis of sugars in aqueous medium leads to units called monosaccharides. Typically, in an aqueous solution a monosaccharide sugar molecule of five or six carbon atoms forms a cyclic hemiacetal, a ring structure. Preferably, sugars of the present inventions are monosaccharides or disaccharides, that is, consist of one or two monosaccharide units, each having from three to seven, preferably from three to six carbon atoms. Exemplary sugars of the present invention are, without limitation, monosacharide hexoses, such as glucose (dextrose), galactose, mannose, fructose; monosaccharide pentoses, such as xylose, ribose, arabinose, and disaccharides, such as lactose, trehalose, sucrose, maltose, and cellobiose. Compounds comprised of several interlinked sugar units forming a ring (cyclodextrins) and their derivatives can be also used. Reduction of sugars is one method to obtain polyols. More stable "non-reducing" and non-metabolizable disaccharides, such as sucrose or trehalose, are preferred. Various polyols, monosaccharides, and disaccharides are commercially available.

[0079] A polyanionized polyol or sugar is a polyol or a sugar having its hydroxyl groups completely or partially modified or replaced with anionic groups (anionized). Thus, a polyanionized polyol or polyanionized sugar comprises a polyol moiety or a sugar moiety along with anionic groups linked thereto. Exemplary anionic groups include, without any limitation, carboxylate, carbonate, thiocarbonate, dithiocarbonate, phosphate, phosphonate, sulfate, sulfonate, nitrate, and borate. It is preferred that at least one anionic group of a polyanionized sugar or polyol is strongly anionic group, that is, is more than 50% ionized in the broad range of pH, *e.g.* , pH 3-12, preferably, pH 2-12, when in the aqueous medium, or, alternatively, has a log dissociation constant (pK_a) of 3 or less, preferably of 2 or less. Polyanionization of a polyol or a sugar can be achieved by a variety of chemical processes well known in the art. For example, reaction of polyols and/or sugars with sulfur trioxide or chlorosulfonic acid in pyridine or 2-picoline results in some or all hydroxyl groups esterified with sulfuric acid residues (sulfated), providing

for a polysulfated sugar or polyol. Exemplary sulfated sugar of the present invention is sulfated sucrose including, without limitation, sucrose hexasulfate, sucrose heptasulfate, and sucrose octasulfate (See Ochi. K., et al., 1980, *Chem. Pharm. Bull.*, v. 28, p. 638-641). Similarly, reaction with phosphorus oxychloride or diethylchlorophosphate in the presence of base catalyst results in polyphosphorylated polyols or sugars. Polyphosphorylated polyols are also isolated from natural sources. For example, inositol polyphosphates, such as inositol hexaphosphate (phytic acid) is isolated from corn. A variety of sulfated, sulfonated, and phosphorylated sugars and polyols suitable to practice the present invention are disclosed, *e.g.*, in U.S. Pat. 5,783,568 and U.S. Pat. 5,281,237, which are incorporated herein by reference. It was unexpectedly discovered that polyanionised polyhydroxylated compounds with only strong acid dissociation steps, *e.g.* the groups having pKa of less than about 3.0, preferably less than about 2.0, such as, for example, sulfate monoesters (pKa 1.0 or less), provide liposomal encapsulation with better drug retention than polyanionized polyhydroxylated compounds having also weakly acidic dissociation steps, such as phosphate monoesters (step 1, pKa about 1.5; step 2, pKa about 6.7; see Stahl and Wermuth, *Op. cit.*, 2002). Example 73 below illustrates this discovery. Complexation of polyols and/or sugars with more than one molecule of boric acid also results in a polyanionized (polyborated) product. Reaction of polyols and/or sugars with carbon disulfide in the presence of alkali results in polyanionized (polydithiocarbonated, polyxanthogenate) derivatives. A polyanionized polyol or sugar derivative can be isolated in the form of a free acid and neutralized with a suitable base, for example, with an alkali metal hydroxide, ammonium hydroxide, or preferably with a substituted amine, *e.g.*, amine corresponding to a substituted ammonium of the present invention, in a neat form or in the form of a substituted ammonium hydroxide providing for a polyanionic salt of a substituted ammonium of the present invention. Alternatively, a sodium, potassium, calcium, barium, or magnesium salt of a polyanionized polyol/sugar can be isolated and converted into a suitable form, e.g., a substituted ammonium salt form, by any known method, for example, by ion exchange.

[0080] The polyanion of the present invention usually has a charge density of at least two, three, or four negatively charged groups per unit, *e.g.*, per carbon atom or ring in a carbon chain or per monosaccharide unit in a sugar. The polyanionized sugar or cyclic polyol of the present invention preferably has at least 75% of available hydroxyl groups

polyanionized, and more preferably 100% of available hydroxyl groups polyanionized. In addition, polyanionization inside the liposomes of the present invention is usually at a level that is compatible with or facilitates the delivery and release of the entity entrapped inside the liposomes at the site of its intended action, but decreases the release of the entrapped entity prematurely, *i.e.*, before the liposome reaches its site of intended action.

[0081] According to the present invention, the degree of polyanionization inside the liposomes can be used to regulate the release characteristics, *e.g.*, release rate and kinetics of an entity entrapped inside the liposomes. In general, the degree of polyanionization can be assessed based on the amount of polyanionized sugar or polyol relative to the total amount of anion(s) or in the case of polyanion being the only kind of anion, the percentage of polyanionization with respect to the total polyanionization capacity of the polyanion, *e.g.*, polyanionized sugar or polyol or a mixture thereof inside the liposomes of the present invention. In one embodiment, polyanionized sugar or polyol is mixed with one or more of other anions and the less the amount of polyanionized sugar or polyol over the amount of other anion(s), the faster the entity is released from the liposomes.

[0082] Usually if an entrapped entity is released from the liposomes at the site of its intended activity too slowly, the desired entity release rate can be achieved by using a mixture of polyanionized sugar or polyol with one or more other monovalent or polyvalent anions, *e.g.*, chloride, sulfate, phosphate, etc. Alternatively, one can use mixtures of polyanionized sugar or polyols with various degrees of polyanionization. In one embodiment, the degree of polyanionization inside the liposomes of the present invention is between 0.1% to 99%, 10% to 90%, or 20% to 80% of the total anion(s) inside the liposomes, *e.g.*, with an entrapped entity.

[0083] In general, the liposome composition of the present invention can contain one or more polyanions of the present invention in any suitable form, *e.g.*, in the form of an acid or a salt comprising a polyanion and a cation. The amount of polyanion, *e.g.*, polyanionized sugar or polyol can be stoichiometrically equivalent to or different from the amount of the cation. In one embodiment, the liposome composition of the present invention contains one or more polyanion salts of a cation, wherein there is a cation concentration gradient or a pH gradient present across the liposome membrane. In another embodiment, the liposome composition of the present invention contains one or

more substituted ammonium polyanion salts of the present invention. In yet another embodiment, the liposome composition of the present invention contains the polyanion inside the liposomes while the polyanion in the medium containing the liposomes is partially or substantially removed by any suitable means known to one skilled in the art, *e.g.*, dilution, ion exchange chromatography, size exclusion chromatography, dialysis, ultrafiltration, absorption, precipitation, etc. In still another embodiment, the liposome with entrapped polyanion, e.g., polyanionized polyol or polyanionized sugar, has also a transmembrane gradient effective in retaining substances within the liposome. Examples of such transmembrane gradients are pH gradient, electrochemical potential gradient, ammonium ion gradient, substituted ammonium ion gradient, or solubility gradient. A substituted ammonium gradient typically includes a substituted form of ammonium ion comprising at least one C-N bond, such as, primary, quaternary, tertiary, or quaternary ammonium. Methods of creating transmembrane gradients are routine in the art of liposomes.

[0084] According to yet another feature of the present invention, the liposome composition of the present invention contains one or more substituted ammoniums and/or polyanions of the present invention and a chemical or biological entity, *e.g.*, therapeutics or detectable entity. For example, the entity contained in the liposome composition of the present invention can be a therapeutic agent, ink, dye, magnetic compound, fertilizer, lure, biocatalyst, taste or odor modifying substance, bleach, or any entity that is detectable by any suitable means known in the art, *e.g.*, magnetic resonance imaging (MRI), optical imaging, fluorescent/luminescent imaging, or nuclear imaging techniques. Conveniently, an entity contained in or loadable to the liposome composition of the present invention is a weakly basic and membrane-permeable (lipophilic) entity, *e.g.*, an amine-containing or nitrogen base entity.

[0085] In one embodiment, the entity contained in the liposome composition of the present invention is a therapeutic agent.

[0086] In another embodiment, the entity contained in the liposome composition is an anticancer entity. A partial listing of some of the commonly known commercially approved (or in active development) antineoplastic agents by classification is as follows.

[0087] Structure-Based Classes: Fluoropyrimidines--5-FU, Fluorodeoxyuridine, Ftorafur, 5'-deoxyfluorouridine, UFT, S-1 Capecitabine; pyrimidine Nucleosides--Deoxycytidine, Cytosine Arabinoside, 5-Azacytosine, Gemcitabine, 5-Azacytosine-Arabinoside; . Purines--6-Mercaptopurine, Thioguanine, Azathioprine, Allopurinol, Cladribine, Fludarabine, Pentostatin, 2-Chloro Adenosine; Platinum Analogues--Cisplatin, Carboplatin, Oxaliplatin, Tetraplatin, Platinum-DACH, Ormaplatin, CI-973, JM-216; Anthracyclines/Anthracenediones--Doxorubicin, Daunorubicin, Epirubicin, Idarubicin, Mitoxantrone; Epipodophyllotoxins--Etoposide, Teniposide; Camptothecins--Irinotecan, Topotecan, Lurtotecan, Silatecan, 9-Amino Camptothecin, 10,11-Methylenedioxy Camptothecin, 9-Nitro Camptothecin, TAS 103, 7-(4-methyl-piperazino-methylene)-10,11-ethylenedioxy-20(S)-camptothecin, 7-(2-N-isopropylamino)ethyl)-20(S)-camptothecin; Hormones and Hormonal Analogues--Diethylstilbestrol, Tamoxifen, Toremefine, Tolmudex, Thymitaq, Flutamide, Bicalutamide, Finasteride, Estradiol, Trioxifene, Droloxifene, Medroxyprogesterone Acetate, Megesterol Acetate, Aminoglutethimide, Testolactone and others; Enzymes, Proteins and Antibodies--Asparaginase, Interleukins, Interferons, Leuprolide, Pegaspargase, and others; Vinca Alkaloids--Vincristine, Vinblastine, Vinorelbine, Vindesine; Taxanes--Paclitaxel, Docetaxel.

[0088] Mechanism-Based Classes: Antihormonals--See classification for Hormones and Hormonal Analogues, Anastrozole; Antifolates--Methotrexate, Aminopterin, Trimetrexate, Trimethoprim, Pyritrexim, Pyrimethamine, Edatrexate, MDAM; Antimicrotubule Agents--Taxanes and Vinca Alkaloids; Alkylating Agents (Classical and Non-Classical)--Nitrogen Mustards (Mechlorethamine, Chlorambucil, Melphalan, Uracil Mustard), Oxazaphosphorines (Ifosfamide, Cyclophosphamide, Perfosfamide, Trophosphamide), Alkylsulfonates (Busulfan), Nitrosoureas (Carmustine, Lomustine, Streptozocin), Thiotepa, Dacarbazine and others; Antimetabolites--Purines, pyrimidines and nucleosides, listed above; Antibiotics--Anthracyclines/Anthracenediones, Bleomycin, Dactinomycin, Mitomycin, Plicamycin, Pentostatin, Streptozocin; topoisomerase Inhibitors--Camptothecins (Topo I), Epipodophyllotoxins, m-AMSA, Ellipticines (Topo II); Antivirals--AZT, Zalcitabine, Gemcitabine, Didanosine, and others; Miscellaneous Cytotoxic Agents--Hydroxyurea, Mitotane, Fusion Toxins, PZA, Bryostatin, Retinoids, Butyric Acid and derivatives, Pentosan, Fumagillin, and others.

[0089] In addition to the above, an anticancer entity include without any limitation, any topoisomerase inhibitor, vinca alkaloid, *e.g.,* vincristine, vinblastine, vinorelbine, vinflunine, and vinpocetine, microtubule depolymerizing or destabilizing agent, microtubule stabilizing agent, *e.g.,* taxane, aminoalkyl or aminoacyl analog of paclitaxel or docetaxel, *e.g.,* 2'-[3-(N,N-Diethylamino)propionyl]paclitaxel, 7-(N,N-Dimethylglycyl)paclitaxel, and 7-L-alanylpaclitaxel, alkylating agent, receptor-binding agent, tyrosine kinase inhibitor, phosphatase inhibitor, cycline dependent kinase inhibitor, enzyme inhibitor, aurora kinase inhibitor, nucleotide, polynicleotide, and farnesyltransferase inhibitor.

[0090] In another embodiment, the entity contained in the liposome composition of the present invention is a therapeutic agent of anthracycline compounds or derivatives, camptothecine compounds or derivatives, ellipticine compounds or derivatives, vinca alkaloinds or derivatives, wortmannin, its analogs and derivatives, or pyrazolopyrimidine compounds with the aurora kinase inhibiting properties.

[0091] In yet another embodiment, the entity contained in the liposome composition of the present invention is an anthracycline drug, doxorubicin, daunorubicin, mitomycin C, epirubicin, pirarubicin, rubidomycin, carcinomycin, N-acetyladriamycin, rubidazone, 5-imidodaunomycin, N-acetyldaunomycine, daunoryline, mitoxanthrone; a camptothecin compound, camptothecin, 9-aminocamptothecin, 7-ethylcamptothecin, 10-hydroxycamptothecin, 9-nitrocamptothecin, 10,11-methylenedioxycamptothecin, 9-amino-10,11-methylenedioxycamptothecin, 9-chloro-10,11-methylenedioxycamptothecin, irinotecan, topotecan, lurtotecan, silatecan, (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin, 7-(4-methylpiperazinomethylene)-10,11-methylenedioxy-20(S)-camptothecin, 7-(2-N-isopropylamino)ethyl)-(20S)-camptothecin; an ellipticine compound, ellipticine, 6-3-aminopropyl-ellipticine, 2-diethylaminoethyl-ellipticinium and salts thereof, datelliptium, retelliptine.

[0092] In yet another embodiment, the entity contained in the liposome of the present invention is a pharmaceutical entity including, without limitaion any of the following: antihistamine ethylenediamine derivatives (bromphenifamine, diphenhydramine); Anti-protozoal: quinolones (iodoquinol); amidines (pentamidine); antihelmintics (pyrantel);

anti-schistosomal drugs (oxaminiquine); antifungal triazole derivatives (fliconazole, itraconazole, ketoconazole, miconazole); antimicrobial cephalosporins (cefazolin, cefonicid, cefotaxime, ceftazimide, cefuoxime); antimicrobial beta-lactam derivatives (aztreopam, cefmetazole, cefoxitin); antimicrobials of erythromycine group (erythromycin, azithromycin, clarithromycin, oleandomycin); penicillins (benzylpenicillin, phenoxymethylpenicillin, cloxacillin, methicillin, nafcillin, oxacillin, carbenicillin); tetracyclines; other antimicrobial antibiotics, novobiocin, spectinomycin, vancomycin; antimycobacterial drugs: aminosalicyclc acid, capreomycin, ethambutol, isoniazid, pyrazinamide, rifabutin, rifampin, clofazime; antiviral adamantanes: amantadine, rimantadine; quinidine derivatives: chloroquine, hydroxychloroquine, promaquine, qionone; antimicrobial qionolones: ciprofloxacin, enoxacin, lomefloxacin, nalidixic acid, norfloxacin, ofloxacin; sulfonamides; urinary tract antimicrobials: methenamine, nitrofurantoin, trimetoprim; nitroimidazoles: metronidazole; cholinergic quaternary ammonium compounds (ambethinium, neostigmine, physostigmine); anti-Alzheimer aminoacridines (tacrine); anti-Parkinsonal drugs (benztropine, biperiden, procyclidine, trihexylhenidyl); anti-muscarinic agents (atropine, hyoscyamine, scopolamine, propantheline); adrenergic dopamines (albuterol, dobutamine, ephedrine, epinephrine, norepinephrine, isoproterenol, metaproperenol, salmetrol, terbutaline); ergotamine derivatives; myorelaxants or curane series; central action myorelaxants; baclophen, cyclobenzepine, dentrolene; nicotine; beta-adrenoblockers (acebutil, amiodarone); benzodiazepines (ditiazem); antiarrhythmic drugs (diisopyramide, encaidine, local anesthetic series--procaine, procainamide, lidocaine, flecaimide), quinidine; ACE inhibitors: captopril, enelaprilat, fosinoprol, quinapril, ramipril; antilipidemics: fluvastatin, gemfibrosil, HMG-coA inhibitors (pravastatin); hypotensive drugs: clonidine, guanabenz, prazocin, guanethidine, granadril, hydralazine; and non-coronary vasodilators: dipyridamole.

[0093] According to the present invention, the entity contained in the liposome composition of the present invention can also be a pre-entity, *e.g.,* a pro-drug or an agent that is capable of being converted to a desired entity upon one or more conversion steps under a condition such as a change in pH or an enzymatic cleavage of a labile bond. Such conversion may occur after the release of the pro-drug from the liposome interior at the intended site of the drug/liposome action. However, the pre-entity can be converted into

the desired active entity inside the liposomes of the present invention prior to the use of the liposomes as a delivery vehicle, *e.g.*, administration to a patient. For example, an entity can be modified into a pre-entity so that it is easier to be loaded into the liposomes and then it can be converted back into the desired entity once it is inside the liposomes of the present invention. In this manner, according to the present invention, the entities that are generally not amenable to "active", "remote" or other gradient-based loading methods, can be effectively loaded into liposomes, *e.g.*, into the liposome interior space, in their native, unmodified form.

[0094] Globally cationic compounds, that is, compounds capable of attaining a net positive ionic charge under the liposome loading conditions, especially the compounds containing a titratable amine, are known to effectively load into liposomes exhibiting transmembrane ion gradients. If an entity of interest is an organic compound and is not not a globally cationic compound having a titratable amine, a derivative thereof having the requisite ionic properties can be prepared by a suitable modification, *e.g.*, according to the methods described in Woodle et al., in WO 96/25147. For example, an amine group can be introduced by esterification of a hydroxyl group of the entity with an amino acid. Alternatively, a hydrophobic group can be introduced into a water-soluble compound to aid in its partition into the liposome membrane and subsequent traversing of the membrane to the intraliposomal compartment, *i.e.*, inside the liposomes. Another useful modification to create a liposome-loadable pre-entity is the formation of a carbonyl group adduct, *e.g.*, a hydrazone, an oxime, an acetal, or a ketal. A modified amino-containing group can be hydrolyzed or otherwise chemically split from the modified compound after the loading of the modified compound into the liposomes according to the present invention. Typical processes to intraliposomally regenerate the entity from a pre-entity are hydrolysis, photolysis, radiolysis, thiolysis, ammonolysis, reduction, substitution, oxidation, or elimination. These processes can be effected, without limitation, by the change of pH or by an enzymatic action. For example, paclitaxel or docetaxel, a non-ionic entities, are converted into their 2'-(diethylaminopropionyl)- or 7'-(diethylaminopropionyl) esters, which are weak bases (pre-entities). After loading into the liposomes by any known method, including, without limitation, "active", "remote", "transmembrane-gradient-based" or "solubility gradient based" methods, and/or the methods of the present invention, the intraliposomal 2'-(diethylaminopropionyl)-

paclitaxel is converted into original paclitaxel by stimulating its hydrolysis through the increase of pH to above pH 7.0. Thus, a liposome encapsulating a neutral taxane molecule within its interior space is obtained with the drug/lipid ratio of over 0.05 mole per mole of the liposome lipid, without the help of hydrophilic covalent modifications of the taxane molecule (*e.g.* by attachment of PEG), cyclodextrine taxane compexes, or taxane-solubilizing, micelle-forming surfactants.

[0095] According to the present invention, the liposomes contained in the liposome composition of the present invention can be any liposome known or later discovered in the art. In general, the liposomes of the present invention can have any liposome structure, *e.g.*, structures having an inner space sequestered from the outer medium by one or more lipid bilayers, or any microcapsule that has a semi-permeable membrane with a lipophilic central part where the membrane sequesters an interior. A lipid bilayer can be any arrangement of amphiphilic molecules characterized by a hydrophilic part (hydrophilic moiety) and a hydrophobic part (hydrophobic moiety). Usually amphiphilic molecules in a bilayer are arranged into two dimensional sheets in which hydrophobic moieties are oriented inward the sheet while hydrophilic moieties are oriented outward. Amphiphilic molecules forming the liposomes of the present invention can be any known or later discovered amphiphilic molecules, *e.g.*, lipids of synthetic or natural origin or biocompatible lipids. Liposomes of the present invention can also be formed by amphiphilic polymers and surfactants, *e.g.*, polymerosomes and niosomes. For the purpose of this disclosure, without limitation, these liposome-forming materials also are referred to as "lipids".

[0096] According to the present invention, the liposomes contained in the liposome composition of the present invention can also be targeting liposomes, *e.g.*, liposomes containing one or more targeting moieties or biodistribution modifiers on the surface of the liposomes. A targeting moiety can be any agent that is capable of specifically binding or interacting with a desired target. In one embodiment, a targeting moiety is a ligand. The ligand, according to the present invention, preferentially binds to and/or internalizes into, a cell in which the liposome-entrapped entity exerts its desired effect (a target cell). A ligand is usually a member of a binding pair where the second member is present on or in a target cells or in a tissue comprising the target cell. Examples of ligands suitable for the present invention are: the folic acid, protein, *e.g.*, transferrin, growth factor, enzyme,

peptide, receptor, antibody or antibody fragment, such as Fab', Fv, single chain Fv, single-domain antibody, or any other polypeptide comprising antigen-binding sequences (CDRs) of an antibody molecule. A ligand-targeted liposome wherein a targeting moiety is an antibody or a target antigen-binding fragment thereof is called an immunoliposome. In a preferred embodiment, the liposome carrying a targeting moiety, *e.g.*, a ligand, is internalized by a target cell. In yet another embodiment, a targeting moiety is a ligand that specifically interacts with a tyrosine kinase receptor such as, for example, EGFR, HER2, HER3, HER4, PD-GFR, VEGFR, bFGFRor IGFR receptors. In still another embodiment, the targeting moiety specifically interacts with a growth factor receptor, an angiogenic factor receptor, a transferrin receptor, a cell adhesion molecule, or a vitamin receptor.

[0097] According to another embodiment of the present invention, the liposomes contained in the liposome composition exhibit a transmembrane concentration gradient of a substituted ammonium and/or polyanion of the present invention. Preferably, the higher concentration is in the interior (inner) space of the liposomes. In addition, the liposome composition of the present invention can include one or more trans-membrane gradients in addition to the gradient created by the substituted ammonium and/or polyanion of the present invention. For example, the liposomes contained in the liposome composition of the present invention can additionally include a transmembrane pH gradient, ion gradient, electrochemical potential gradient, and/or solubility gradient.

[0098] According to yet another embodiment of the present invention, the liposome composition of the present invention can be provided in a kit comprising a container with the liposomes, and optionally, a container with the entity and an instruction, *e.g.*, procedures or information related to using the liposome composition in one or more applications. Such instruction can be provided via any medium, *e.g.*, hard paper copy, electronic medium, or access to a database or website containing the instruction.

[0099] The liposome membrane composition of the present invention can be made by any suitable method known to or later discovered by one skilled in the art. In general, a variety of lipid components can be used to make the liposomes of the present invention. Lipid components usually include, but are not limited to (1) uncharged lipid components, *e.g.*, cholesterol, ceramide, diacylglycerol, acyl(poly ethers) or alkylpoly(ethers); (2)

neutral phospholipids, *e.g.*, diacylphosphatidylcholines, sphingomyelins, and diacylphosphatidylethanolamines, (3) anionic lipids, *e.g.*, diacylphosphatidylserine, diacylphosphatidylglycerol, diacylphosphatidate, cardiolipin, diacylphosphatidylinositol, diacylglycerolhemisuccinate, diaclyglycerolhemigluratate, cholesterylhemisuccinate, cholesterylhemiglutarate, and the like; (4) polymer-conjugated lipids, *e.g.*, N-[methoxy-(poly(ethylene glycol)diacylphosphatidylethanolamine, poly(ethylene glycol)-diacylglycerol, poly(ethylene glycol)-ceramide; and (5) cationic lipids, *e.g.*, 1,2,-diacyl-3-trimethylammonium-propane (DOTAP), dimethyldioctadecylammonium bromide (DDAB), and 1,2-diacyl-sn-glycero-3-ethylphosphocholine. Monoacyl- substituted derivatives of these lipids, as well as di- and monoalkyl-analogs can be also employed.

[0100] Various lipid components can be selected to fulfill, modify or impart one or more desired functions. For example, phospholipid can be used as principal vesicle-forming lipid. Inclusion of cholesterol is useful for maintaining membrane rigidity and decreasing drug leakage. Polymer-conjugated lipids can be used in the liposomal formulation to increase the lifetime of circulation via reducing liposome clearance by liver and spleen, or to improve the stability of liposomes against aggregation during storage, in the absence of circulation extending effect. While inclusion of PEG-lipids in the amount 1 mol% or above of the liposome lipid is asserted to have a several-fold prolongation of the liposome blood circulation time (see, e.g., U.S. Pat. 5,013,556), we have surprisingly discovered that liposomes of the present invention are quite long-circulating, and the addition of PEG-lipid to the liposome composition only extended the circulation longevity for less than two-fold, if at all. In addition, charge-modulating (titratable) lipids can be used to help delivery of liposome encapsulated entities to cytosolic or nuclear targets via facilitating some classes of entities escaping the confines of endosomal pathway.

[0101] In one embodiment, the liposomes of the present invention include lecithin, cholesterol, and an amphipathic polymer. The lecithin included in the liposomes of the present invention can be a natural lecithin, a hydrogenated natural lecithin, a synthetic lecithin, 1,2-distearoyl-lecithin, dipalmitoyl lecithin, dimyristoyl lecithin, dioleolyl lecithin, 1-stearoyl-2-oleoyl lecithin, or 1-palmitoyl-2-oleoyl lecithin whereas the amphipathic polymer can be a polyethylene glycol-lipid derivative, *e.g.*, polyethylene glycol phosphatidylethanolamine, polyethylene glycol-diacylglycerol, or

polyethyleneglycol-ceramide derivative, where the poly(ethylene glycol) portion has molecular weight from about 250 to about 20,000, most commonly from about 500 to about 5,000. In another embodiment, the lecithin and cholesterol ratio in the liposomes of the present invention is about 3:2 by mole. In yet another embodiment, the amphipathic polymer is at least 0.1 mole% of the liposome-forming lipid in the liposomes of the present invention. In yet another embodiment, the amount of an amphipathic polymer is between 0.1 mole% and 1 mole% of the liposome-forming lipid in the liposomes of the present invention. Preferably, the amphipathic polymer is a neutral polymer, i.e. possesses under the drug loading conditions the net ionic charge of zero, for example, PEG-diacylglycerol, PEG-dialkylglycerol, or PEG-ceramide. It was unexpectedly discovered that inclusion of ionically neutral amphipathic lipids up to PEG-lipid content of about 5.7 mol.% of total lipid afford high efficiency liposome loading of, *e.g.*, vinca alkaloids, such as vinorelbine, while in the case of anionically charged PEG-DSPE the loading efficiency noticeably declined at the PEG-lipid content of 1.6 mol.% or more (Example 72).

[0102] In still another embodiment, the liposomes of the present invention contain a camptothecin derivative, *e.g.*, a camptothecin prodrug such as irinotecan and is comprised of lecithin and cholesterol, *e.g.*, at a ratio of about 3:2 by mole, and an amphipathic polymer, *e.g.*, at an amount of at least 0.1 mole % or less than 1% of the liposome-forming lipid.

[0103] Liposomes of the present invention can be made by any method that is known or will become known in the art. See, for example, G. Gregoriadis (editor), *Liposome Technology*, vol. 1-3, 1st edition, 1983; 2nd edition, 1993, CRC Press, Boca Raton, FL. Examples of methods suitable for making liposome composition of the present invention include extrusion, reverse phase evaporation, sonication, solvent (*e.g.*, ethanol) injection, microfluidization, detergent dialysis, ether injection, and dehydration/rehydration. The size of liposomes can be controlled by controlling the pore size of membranes used for low pressure extrusions or the pressure and number of passes utilized in microfluidisation or any other suitable methods. In one embodiment, the desired lipids are first hydrated by thin-film hydration or by ethanol injection and subsequently sized by extrusion through membranes of a defined pore size; most commonly 0.05 μm, 0.08 μm, or 0.1 μm.

[0104] Liposome compositions containing the substituted ammonium and/or polyanion of the present invention inside the liposomes can be made by any suitable methods, *e.g.*, formation of liposomes in the presence of the substituted ammonium and/or polyanion of the present invention, *e.g.*, in the form of salt. The substituted ammonium and/or polyanion outside of the liposomes can be removed or diluted either following liposome formation or before loading or entrapping a desired entity. Alternatively, liposome composition containing the substituted ammonium and/or polyanion of the present invention can be made via ion exchange method directly or via an intermediate free acid step having a gradient of substituted ammonium of the present invention, *e.g.*, substituted ammonium salt of polyanionized sugar or polyol. Such liposomes can be neutralized using the amine or its salt with a volatile acid, *e.g.*, carbonate. The resulting liposome solution can be used directly or alternatively the salt contained therein can be removed if desired, *e.g.*, by evaporation and crystallization followed by dissolution in an aqueous medium.

[0105] Preferably, the liposome composition of the present invention has a transmembrane concentration gradient of the substituted ammonium and/or polyanion, *e.g.*, the concentration of the substituted ammonium and/or polyanion salt inside the liposome is higher, usually at least 100 times higher, than the concentration of the substituted ammonium and/or polyanion in the medium outside the liposome.

[0106] In one embodiment, the concentration of the substituted ammonium and/or polyanion salt inside the liposome is at least 100 times higher than the concentration of the substituted ammonium and/or polyanion salt in the medium outside the liposome and is at least at a concentration of about 10mM, 50mM, 0.1M, 0.2M, 0.5M, 0.6M, 0.7M, or 1.0M, wherein molarity is calculated based on the substituted ammonium. In another embodiment, the concentration of the substituted ammonium and/or polyanion salt inside the liposome is at least 100 times higher than the concentration of the substituted ammonium and/or polyanion salt in the medium outside the liposome and is at a concentration of about 0.65M or about 1.0M.

[0107] In addition, the liposome composition of the present invention usually has a pH outside which is compatible with or helpful for maintaining the stability of a desired entity during the loading process, along with the high loading efficiency, e.g., above 90%

entrapment. For example, pH in the range of 4-7, or pH 4.5-6.5, is preferred. In particular, according to the present invention, loading of a camptothecin compound, *e.g.*, topotecan or irinotecan, is best accomplished at the pH of the outer medium in the range between about 4.0 and about 7.0, more preferably between about pH 5.0 and pH 6.5. Loading of a vinca derivative, e.g., vincristine, vinorelbine, or vinblastine is best accomplished at pH about 5.0-7.0, more preferably at pH about 6.5.

[0108] According to the present invention, a desired entity can be loaded or entrapped into the liposomes by incubating the desired entity with the liposomes of the present invention in an aqueous medium at a suitable temperature, *e.g.*, a temperature above the component lipids' phase transition temperature during loading while being reduced below the phase transition temperature after loading the entity. The incubation time is usually based on the nature of the component lipids, the entity to be loaded into the liposomes, and the incubation temperature. Typically, the incubation times of few minutes to several hours are sufficient. Because high entrapment efficiencies of more than 85%, typically more than 90%, are achieved, there is usually no need to remove unentrapped entity. If there is such a need, however, the unentrapped entity can be removed from the composition by various mean, such as, for example, size exclusion chromatography, dialysis, ultrafiltration, adsorption, or precipitation. It was unexpectedly found that maintaining of the low ionic strength during the incubation of an entity, such as, in particular, a camptothecin derivative or a vinca alkaloid derivative, with the liposomes of the present invention, followed by the increase in ionic strength at the end of the incubation, results in higher loading efficiency, better removal of unentrapped drug, and better liposome stability against aggregation. Typically, the incubation is conducted, *e.g.*, in an aqueous solution, at the ionic strength of less than that equivalent to 50 mM NaCl, or more preferably, less than that equivalent to 30 mM NaCl. Following the incubation, a concentrated salt, *e.g.*, NaCl, solution may be added to raise the ionic strength to higher than that of 50 mM NaCl, or more preferably, higher than that of 100 mM NaCl. Without being bound by a theory, we hypothesize that the increase of ionic strength aids dissociation of the entity from the liposome membrane, leaving substantially all entity encapsulated within the liposomal interior space.

[0109] In general, the entity-to-lipid ratio, *e.g.*, drug load ratio obtained upon loading an entity depends on the amount of the entity entrapped inside the liposomes, the

concentration of entrapped substituted ammonium and/or polyanion, *e.g.*, salt, the physicochemical properties of the entrapped entity and the type of counter-ion (anion), *e.g.*, polyanion used. Because of high loading efficiencies achieved in the compositions and/or by the methods of the present invention, the entity-to-lipid ratio for the entity entrapped in the liposomes is over 80%, over 90%, and typically more than 95% of the entity-to-lipid ratio calculated on the basis of the amount of the entity and the liposome lipid taken into the loading process (the "input" ratio). Indeed, practically 100% (quantitative) encapsulation is common. The entity-to lipid ratio in the liposomes can be characterized in terms of weight ratio (weight amount of the entity per weight or molar unit of the liposome lipid) or molar ratio (moles of the entity per weight or molar unit of the liposome lipid). One unit of the entity-to-lipid ratio can be converted to other units by a routine calculation, as exemplified below. The weight ratio of an entity in the liposomes of the present invention is typically at least 0.05, 0.1, 0.2, 0.35, 0.5, or at least 0.65 mg of the entity per mg of lipid. In terms of molar ratio, the entity-to-lipid ratio according to the present invention is at least from about 0.02, to about 5, preferably at least 0.1 to about 2, and more preferably, from about 0.15 to about 1.5 moles of the drug per mole of the liposome lipid. In one embodiment, the entity-to-lipid ratio, *e.g.*, drug load ratio of camptothecin derivatives is at least 0.1, *e.g.*, 0.1 mole of camptothecin derivative per one mole of liposome lipid, and preferably at least 0.2. In another embodiment, the entity-to-lipid ratio, *e.g.*, drug load is at least about 300 mg entity (*e.g.*, vinca alkaloid or a derivative thereof) per mg of liposome-forming lipid. In yet another embodiment, the entity-to-lipid ratio, *e.g.*, drug load is at least about 500 mg entity (*e.g.* camptothecin derivative or camprothecin prodrug) per mg of liposome-forming lipid. Surprisingly, the invention afforded stable and close to quantitative liposomal encapsulation of a camptothecin derivative drug, e.g., irinotecan, at the drug-to-lipid ratio of over 0.8 mmol of the entity per 1 g of liposome lipid, over 1.3 mmol of entity per 1 g of liposome lipid, and even at high as 1.7 mmol entity per 1 g liposome lipid (see Example 74).

[0110] If the liposome comprises a phospholipid, it is convenient to express the entity content in the units of weight (mass) amount of the drug per molar unit of the liposome phospholipid, e.g., mg drug / mmol of phospholipid. However, a person skilled in the art would appreciate that the drug content can be equivalently expressed in a manner independent of the presence of phospholipids in a liposome, and furthermore, can be

equivalently expressed in terms of a molar amount of the drug per unit (mass or molar) of the liposome lipid content. For example, a liposome containing 3 molar parts of distearoylphosphatidylcholine (DSPC, molecular weight 790), 2 molar parts of cholesterol (molecular weight 387), and 0.015 molar parts of poly(ethylene glycol)-derivatized distearoylphosphatidylethanolamine (PEG-DSPE, molecular weight 2750), and containing a drug doxorubicin (molecular weight 543.5) at the drug/lipid ratio of 150 mg/mmol phospholipid, the same drug content can be equivalently expressed in terms of mg drug/mg total lipid as follows:

(a) Calculate the molar amounts of liposome lipid components normalized to the molar unit of liposome phospholipids (DSPC and PEG-DSPE in this example) by dividing the molar quantity of a component by the total of the molar quantities of the liposome phospholipids:

DSPC 3/(3+0.015) = 0.99502

Cholesterol 2/(3+0.015) = 0.66335

PG-DSPE 0.015/(3+0.015) = 0.00498

(b) Calculate the mass amount of total liposome lipid corresponding to a unit molar amount of liposome phospholipid and the components molecular weights:

Total lipid, mg/mmol phospholipid = 0.99502x790 + 0.66335x387 + 0.00498x2750 = 1056.48

(c) Calculate the mass amount of drug per mass unit of total lipid by dividing the drug content expressed in mass units per molar unit of phospholipid by the number obtained in step (b):

Doxorubicin, mg/mg total lipid = 150/1056.48 = 0.14198.

(d) Calculate the molar amount of the drug per unit mass of total lipid by dividing the number obtained in step (c) by the drug molecular weight (in this case, 543.5) :

Doxorubicin, mmol/g total lipid = 0.14198/543.5x1000 = 0.261.

(e) Calculate the molar part of phospholipids in the liposome lipid matrix:

Phospholipid molar part = (total moles of phospholipids)/(total moles amount of lipids) = (3+0.015)/(3+2+0.015) = 0.6012.

(f) Calculate the molar ratio of doxorubicin to total lipid.

[0111] Doxorubicin, mol/mol of total lipid = (Phospholipid molar part)x(Doxorubicin, g/mole phospholipid)/(Doxorubicin molecular weight) = 0.6012x150/543.5 = 0.166

[0112] Thus, the relationship between drug-to-lipid and drug-to-phospholipid ratio expressed in various units is readily established. As used herein, a "lipid" includes, without limitation, any membrane-forming components of the liposome membrane, such as, for example, polymers and/or detergents.

[0113] The liposome entrapped substituted ammonium and/or polyanion salt solution of the present invention usually has an osmotic strength (osmolality) which helps to keep the liposomes stable against osmotic damage (swelling and/or burst) without sacrificing the loading capacity of the liposomes. In one embodiment, the osmolality of the liposome composition of the present invention is in the range of 0.1 to 1.5 mol/kg or, preferably, 0.2 to 1.0 mol/kg. Surprisingly, we found that liposomes of the present invention are stable against adverse effect of high intraliposomal osmotic strength on the drug loading. Intraliposomal osmolarities of as high as 0.727 mol/kg were well tolerated, resulting in practically quantitative loading of a drug up to the theoretical maximum of stoichiometric exchange of intraliposomal substituted ammonium ions for molecules of the drug (in the case of irinotecan, one drug molecule per one substituted ammonium ion), even though the osmolarity of the extraliposomal aqueous medium during the co-incubation of the drug and the liposomes was close to the physiological value of about 0.3 mol/kg (Example 74).

[0114] In general, the liposome composition of the present invention is quite stable during storage, *e.g.*, as measured by the percentage of entrapped entity released outside of the liposomes or still maintained inside of the liposomes after a certain time period from the initial loading of the entity inside the liposomes of the present invention. For example, the liposome composition of the present invention is stable at 4°C for at least 6 months, *e.g.*, less than 10% of entrapped entity is released 6 months after the initial loading of the entity. In one embodiment, the liposome composition of the present invention is stable at 4°C for at least 2 years, *e.g.*, less than 20% of entrapped entity is released 2 years after the initial loading of the entity.

[0115] It is advantageous for a liposome-entrapped entity to remain encapsulated in the liposome until the liposome reaches the site of its intended action, *e.g.*, in the case of a

liposomal antitumor drug administered in a patient, a tumor. The liposomes of the present invention showed surprising stability against the release (leakage) of the entrapped entity under in vivo conditions, e.g. in the blood of a mammal. The exposure time needed for 50% release of the entrapped entity, *e.g.* drug, from the liposomes (half-release time) in the blood of a rat *in vivo* was more than 24 hours. In particular, liposomes loaded with vinca alkaloid drugs, *e.g.*, vinblastine, vincristine, and vinorelbine, showed remarkable stability against drug leakage in vivo, with half-release time of at least 24 hours, or the amount of entity remaining encapsulated after 24 hours in the blood in vivo at least about 50% of the pre-administration value. Typically the half-release time over 33 hours , or the amount of encapsulated entity remaining encapsulated after 24 hours in the blood in vivo at least about 60%, was observed; and even the half-release time over 46 hours, or the amount of encapsulated entity after 24 hours in the blood in vivo at least about 70% of the pre-administration value, was common. Sometimes the half-release time for an encapsulated drug in the blood in vivo was over 93 hours, and even over 120 hours. The liposome loaded with camptothecin derivatives, such as topotecan and irinotecan, also showed exceptional in vivo stability in the blood, with 79-85% of the original drug load remaining encapsulated after 24 hours. Remarkably, the liposomes of the present invention, while having such low in vivo drug release rate in the blood circulation, showed substantial in vivo antitumor activity exceeding that of the free drug (i.e administered as a solution).

[0116] The liposomes of the present invention provided unexpected combination of the high efficiency of the entrapped therapeutic agent and low toxicity. In general, the activity of a therapeutic entity liposomally encapsulated according to the present invention, *e.g.*, the anti-neoplastic activity of a camptothecin derivative in a mammal, is at least equal to, at least two times higher, or at least four times higher than the activity of the therapeutic entity if it is administered in the same amount via its routine non-liposome formulation, *e.g.*, without using the liposome composition of the present invention, while the toxicity of the liposomally encapsulated entity does not exceed, is at least twice, at least three times, or at least four times lower than that of the same therapeutic entity administered in the same dose and schedule but in a free, non-encapsulated form. For example, it is generally known that liposomal encapsulation of anti-cancer camptothecin derivatives by the published methods of others results in the increased toxicity (lower

maximum tolerated dose, lower 50% lethality dose) compared to unencapsulated drug. See U.S. Pat. 6,355,268; U.S. Pat. 6,465,008; Colbern, et al. *Clinical Cancer Res.* 1998, v. 4, p. 3077-3082; Tardi, et al. *Cancer Res.*, 2000, v. 60, p.3389-3393; Emerson, et al. *Clinical Cancer Res.* 2000, v. 6, p.2903-2912. Liposomally encapsulated camptothecin pro-drugs, such as irinotecan (CPT-11), which is a water-soluble, cationic camptothecin pro-drug derivative, have substantially higher, e.g. at least 4 times, and even 10 times, higher antitumor activity assessed in an in vivo tumor model than the drug in the absence of a liposomal formulation, *e.g.*, in a free (solution) form. This is even more remarkable since a therapeutic compound, *e.g.*, a camptothecin pro-drug, requires enzymatic activation, *e.g.*, by the action of endogenous non-specific carboxylesterase, but according to the present invention is encapsulated substantially within the interior space of the liposome. On the other hand, surprisingly, the toxicity of camptothecin prodrug such as CPT-11 in the liposomal form (drug/lipid mass ratio over 0.1 , *e.g.*, 0.2-0.6 or more) according to the present invention was over 2 times, over 3 times, and even over 4 times lower that than of the free (unencapsulated) pro-drug CPT-11. Moreover, a prolonged drug release from the CPT-11 liposomes in vivo was achieved, with more than 50%, and even more than 70% (79-86%) of the original drug content still remaining in the liposomes 24 hours after administration into the bloodstream, and with half-release times in excess of 24 hours, typically in excess of 48 hours. The prolonged remanence of the drug in the liposome in vivo was associated with higher antitumor effect. Surprisingly, the slowest in vivo CPT-11 release and the highest antitumor activity was observed in the liposomes containing low-molecular polyanionized sugar derivative (sucrose octasulfate) rather than a polymeric anion (polyphosphate) (Example 15).

[0117] According to another embodiment of the present invention, the liposome composition of the present invention can be provided as a pharmaceutical composition containing the liposome composition of the present invention and a carrier, *e.g.*, pharmaceutically acceptable carrier. Examples of pharmaceutically acceptable carries are normal saline, isotonic dextrose, isotonic sucrose, Ringer's solution, and Hanks' solution. A buffer substance can be added to provide pH optimal for storage stability. For example, pH between about 6.0 and about 7.5, more preferably pH about 6.5, is optimal for the stability of liposome membrane lipids, and provides for excellent retention of the entrapped entities. Histidine, hydroxyethylpiperazine-ethylsulfonate (HEPES),

morpholipo-ethylsulfonate (MES), succinate, tartrate, and citrate, typically at 2-20 mM concentration, are exemplary buffer substances. Other suitable carriers include, *e.g.*, water, buffered aqueous solution, 0.4% NaCl, 0.3% glycine, and the like. Protein, carbohydrate, or polymeric stabilizers and tonicity adjusters can be added, e.g., gelatin, albumin, dextran, or polyvinylpyrrolidone. The tonicity of the composition can be adjusted to the physiological level of 0.25-0.35 mol/kg with glucose or a more inert compound such as lactose, sucrose, mannitol, or dextrin. These compositions may be sterilized by conventional, well known sterilization techniques, *e.g.*, by filtration. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous medium prior to administration.

[0118] The pharmaceutical liposome compositions can also contain other pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. Additionally, the liposome suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alpha-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

[0119] The concentration of the liposomes of the present invention in the fluid pharmaceutical formulations can vary widely, i.e., from less than about 0.05% usually or at least about 2-10% to as much as 30 to 50% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, liposome pharmaceutical compositions composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0120] The amount of liposome pharmaceutical composition administered will depend upon the particular therapeutic entity entrapped inside the liposomes, the disease state being treated, the type of liposomes being used, and the judgment of the clinician.

Generally the amount of liposome pharmaceutical composition administered will be sufficient to deliver a therapeutically effective dose of the particular therapeutic entity.

[0121] The quantity of liposome pharmaceutical composition necessary to deliver a therapeutically effective dose can be determined by routine in vitro and in vivo methods, common in the art of drug testing. See, for example, D.B.Budman, A.H.Calvert, E.K.Rowinsky (editors). *Handbook of Anticancer Drug Development*, LWW, 2003. Therapeutically effective dosages for various therapeutic entities are well known to those of skill in the art; and according to the present invention a therapeutic entity delivered via the pharmaceutical liposome composition of the present invention provides at least the same, or 2-fold, 4-fold, or 10-fold higher activity than the activity obtained by administering the same amount of the therapeutic entity in its routine non-liposome formulation. Typically the dosages for the liposome pharmaceutical composition of the present invention range between about 0.005 and about 500 mg of the therapeutic entity per kilogram of body weight, most often, between about 0.1 and about 100 mg therapeutic entity/kg of body weight.

[0122] Typically, the liposome pharmaceutical composition of the present invention is prepared as a topical or an injectable, either as a liquid solution or suspension. However, solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The composition can also be formulated into an enteric-coated tablet or gel capsule according to known methods in the art.

[0123] The liposome composition of the present invention can be administered in any way which is medically acceptable which may depend on the condition or injury being treated. Possible administration routes include injections, by parenteral routes such as intramuscular, subcutaneous, intravenous, intraarterial, intraperitoneal, intraarticular, intraepidural, intrathecal,_or others, as well as oral, nasal, ophthalmic, rectal, vaginal, topical, or pulmonary, *e.g.,* by inhalation. For the delivery of liposomally drugs formulated according to the invention, to tumors of the central nervous system, a slow, sustained intracranial infusion of the liposomes directly into the tumor (a convection-enhanced delivery, or CED) is of particluar advantage. See Saito, *et al.*, Cancer Research, vol.64, p. 2572-2579, 2004; Mamot, *et al.*, J. Neuro-Oncology, vol. 68, p. 1-9, 2004._ The compositions may also be directly applied to tissue surfaces. Sustained release, pH

dependent release, or other specific chemical or environmental condition mediated release administration is also specifically included in the invention, *e.g.*, by such means as depot injections, or erodible implants.

EXAMPLES

[0124] The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLE 1. Preparation of the solutions of substituted ammonium salts.

[0125] Trialkylammonium and dialkylammonium sulfate solutions useful for loading drugs (e.g., doxorubicin) into liposomes were prepared by diluting sulfuric acid with water to a concentration of 0.25 M and then titrating the sulfuric acid solution with one of a variety of amines. The substituted amines used in this example were triethylamine, trimethylamine, dimethylamine, diethylamine, or diethanolamine. After the addition of the amines, the resulting solution was diluted to a final concentration of 0.2 M of the substituted ammonium salt. Osmolality was determined using a dew point osmometer. The properties of resulting substituted alkylammonium sulfate salt solutions are shown in the Table 1 below.

Table 1. Properties of various dialkylammonium and trialkylammonium sulfate solutions

Salt	Osmolality, mmol/kg	pH
Dimethylammonium sulfate	472	5.65
Dimethylethanolammonium sulfate	509	5.72
Diethylammonium sulfate	519	5.85
Trimethylammonium sulfate	497	5.81
Triethylammonium sulfate	559	5.33

EXAMPLE 2. Preparation of liposomes with entrapped dialkylammonium and trialkylammonium salts, and loading of a substance into these liposomes.

[0126] Distearoylphosphatidylcholine (DSPC), cholesterol (Chol), and N-(methoxy-poly(ethylene glycol)-oxycarbonyl)-distearoylphosphatidylethanolamine (PEG-DSPE)

(prepared from poly(ethylene glycol) with mol. weight 2,000) were co-dissolved in chloroform in a molar ratio of 3:2:0.015, and the chloroform was removed at 55-60 °C by rotary evaporation. The dried lipid film was then hydrated in a solution of one of each dialkyl- or trialkylammonium sulfates listed in Example 1 at 60 °C for 30 min. The lipid suspension was extruded under pressure through two stacked polycarbonate track-etched membrane filters with the pore size of 0.1 μm (Corning Nuclepore). The liposome size determined by quasielastic light scattering method was approximately 110-120 nm. Unencapsulated trialkylammonium or dialkylammonium salts were removed from the external medium of the liposomes by gel filtration using a cross-linked dextran gel (Sephadex G-75, Amersham Pharmacia Biotechnology) column eluted with HEPES-buffered saline, pH 7.2-7.4, and the liposomes were collected in a void-volume fraction of the column. Doxorubicin hydrochloride USP (lyophilized powder containing 5 weight parts of lactose per 1 part of doxorubicin) was added to the liposomes at a concentration of 150 μg drug/μmol of liposome phospholipid. The mixture was incubated at 55 °C for 45 min, chilled on ice for 10 min, and unencapsulated drug was removed by gel filtration chromatography using a Sephadex G-75 column eluted with HEPES-buffered saline, pH 7.4. The presence of free doxorubicin (characterized by the appearance of a slower moving red colored band) was visually undetectable. The purified doxorubicin-loaded liposomes were analyzed for phospholipid and doxorubicin according to Examples 70 and 71 (spectrophotometric method), respectively. The resulting drug loading efficiencies are shown in Table 2.

Table 2. Loading of doxorubicin in liposomes with entrapped solutions of dialkyl- and trialkylammonium salts. Input drug/phospholipid ratio 150 μg/μmol.

Liposome-entrapped salt:	Drug/phospholipid ratio in liposomes (μg/μmol)	Entrapment Efficiency (%)
Trimethylammonium sulfate	140.74 ± 10.35	93.8 ± 5.7
Triethylammonium sulfate	163.81 ± 16.41	109.2 ± 11.6
Diethylammonium sulfate	158.16 ± 18.34	105.4 ± 7.8
Dimethylethanolammonium sulfate	155.08 ± 8.51	103.4 ± 11.6

EXAMPLE 3. Preparation of liposomes containing various dialkyl-, trialkyl-, and heterocyclic-substituted ammonium sulfate salts and loading of doxorubicin into these liposomes.

[0127] The substituted ammonium sulfate salt solutions were prepared as in Example 1 using commercially available alkyl-substituted, hydroxyalkyl-substituted and heterocyclic amines. Liposomes were formed as in Example 1, except that instead of the lipid film hydration step, the neat lipids were dissolved in ethanol (approximately 100 µl of ethanol for every 50 µmol of phospholipid) and mixed with the substituted ammonium salt solution at 60-65°C so that the resulting lipid dispersion contained about 10 vol.% of ethanol.

[0128] Doxorubicin loading was accomplished by adding doxorubicin solution (2 mg/ml in HEPES-buffered saline pH 6.5) to the liposomes at a ratio of 155 µg drug/µmol liposome phospholipid (PL) and heating at 58 °C for 45 min in a hot water bath. The resulting liposomes were separated from any residual unencapsulated doxorubicin and analyzed for drug and lipid content as in Example 1. The results are shown in Table 3.

Table 3. Loading doxorubicin into liposomes with entrapped sterically hindered substituted alkyl, dialkyl-, trialkyl- and heterocyclic ammonium salts solutions.

Amine used to prepare substituted ammonium salt	Osmolality, mmol/kg	drug load, mg/mmol phospholipid	Loading efficiency, %
Trimethylamine	497	149.4 ± 7.9	96.4 ± 4.9
Triethylamine	559	149.6 ± 6.9	96.5 ± 4.3
Dimethylethanolamine	509	163.1 ± 6.6	105.3 ± 4.5
Dimethylamine	472	158.6 ± 7.4	102.3 ± 4.9
Diethylamine	519	156.7 ± 13.0	101.1 ± 8.5
Diisopropylamine	533	159.9 ± 6.2	103.2 ± 4.1
Tris(hydroxymethyl)-minomethane	423	179.9 ± 15.3	116.1 ± 11.5
1-Piperidineethanol	506	153.5 ± 7.1	99.0 ± 4.5
4-Methylmorpholine	465	152.4 ± 9.8	98.3 ± 6.2
Piperidine	479	158.5 ± 12.5	102.3 ± 8.2
1-Methylpyrolidine	492	153.6 ± 12.3	99.1 ± 7.8
Dimethylpiperazine	378	158.0 ± 6.5	101.9 ± 4.3

EXAMPLE 4 . Preparation of triethylammonium polyphosphate (TEA-Pn) solution.

[0129] Linear sodium poly(phosphate) having 13-18 phosphate units per molecule (Phosphate glass; CALGON®, obtained from Sigma Chemical Company) was dissolved in water to a concentration of about 1.3 M phosphate. The solution was passed through a column packed with 120 mL of sulfonated polystyrene-divinylbenzene copolymer cation exchange resin beads (Dowex 50Wx8-200, Dow Chemical Co.) in the hydrogen form. The column was pre-equilibrated with aqueous 3 – 3.6 M HCl to bring the resin into hydrogen form, and washed with deionized water to neutral pH. Fifteen ml of the sodium polyphosphate solution was applied on the column and eluted with deionized H_2O. The column eluent was monitored using a conductivity detector. The column outflow corresponding to the conductivity peak was titrated with neat triethylamine to pH 5.5-6.0. The solution was analyzed for residual sodium by potentiometry using a sodium-sensitive glass electrode and for phosphate content using an inorganic phosphate assay as in Example 1. The solution having residual sodium content of less than 1 % was diluted to a

final phosphate concentration of 0.55 M. The solution typically has a TEA concentration of 0.52-0.55 M, pH of 5.5-6.0, and osmolality of 430-480 mmol/kg

EXAMPLE 5. Removal of unentrapped polyphosphate salts from liposome preparations.

[0130] Liposomes (120 nm in size) with entrapped fluorescent marker 8-hydroxypyrene trisulfonate were prepared according to Kirpotin, et al., *Biochemistry* **36**:66-75, 1997, and mixed with the solution of sodium polyphosphate. The mixture was loaded on size exclusion columns containing cross-linked dextran beads (Sephadex G-75), 6% agarose beads (Sepharose 6B-CL), or 4% agarose beads (Sepharose 4B-CL), all from Amersham Pharmacia, and eluted with MES-Dextrose buffer (pH 5.5). The effluents were assayed for phosphate content using the phosphate assay of Bartlett (1959), and for the liposome content by spectrofluorometry. Of the studied gel-chromarography carries, Sepharose CL-6B provided complete separation of the polyphosphate from the liposomes at the sample/column bed volume ratio of 13.

EXAMPLE 6. Preparing solution of triethylammonium sucroseoctasulfate (TEA-SOS).

[0131] Sodium sucrose octasulfate (equivalent weight 144.8) is the sodium salt of sucrose derivative in which all hydroxyl groups have formed sulfuric acid esters. Sucrose octasulfate (SOS) sodium salt was purchased from Toronto Research Chemicals, Toronto, Canada, p/n S699020. Six grams of sodium sucrose octasulfate was dissolved in 16.57 ml of deionized water to give a final concentration of about 2.5 N of the sulfate groups.. The solution was treated by ion exchange as in Example 4. The solution of sucroseoctasulfuric acid obtained as an ion exchange column effluent was then titrated with neat triethylamine to pH 5.7 (neutralization point), and the pH and osmolality of the solution were determined. The resulting solution had the calculated triethylammonium concentration of 0.643 M, pH 5.7, and the osmolality of 530 mmol/kg. The presence of residual sodium was undetectable by potentiometry (less than 0.1%).

EXAMPLE 7. Liposomes loaded with Irinotecan (CPT-11) using substituted ammonium salts: preparation and *in vitro* drug release in the presence of blood plasma.

[0132] In this example, sulfate, citrate, pyrophosphate, triphosphate, and linear polyphosphate (13-18 mer) were studied as anions in the liposome-entrapped substituted

ammonium salt solutions. Phosphate polymers were chosen because of their biodegradability and because polyphosphates are found naturally in the cells, as opposed to other synthetic polymeric anions (polyacrylate, dextran sulfate, and the like). Also, the viscosity of solutions of low molecular weight polyphosphates was lower than that of other polymers, making polyphosphates more process-friendly.

[0133] The following materials were used for preparation of salt solutions.

1. Sodium polyphosphate, NaO-$[PO_3Na]_n$-Na, n=13-18, purchased from Sigma (Product No. P-8510, "Phosphate Glass, Practical Grade", also known as sodium hexametaphosphate or by the brand name CALGON);

2. Pentasodium tripolyphosphate, $Na_5P_3O_{10}$, purchased from Sigma (Product No. T-5883); 3. Tetrasodium pyrophosphate decahydrate, $Na_4P_2O_7 \cdot 10H_2O$, purchased from Sigma (Product No. P-9146).

4. Ion exchange resins Dowex 50Wx4 (4% cross-linked sulfonated polystyrene resin, 100-200 mesh) purchased from Sigma (Product No. 50X4-200) or Dowex HCR-W2 (8% cross-linked sulfonated polystyrene resin 50-100 mesh) purchased from Sigma (Product No. I-8880) were used interchangeably. The resins were washed by decantation in the following order: three times with deionized water, twice with 1N HCl (3x excess over the resin by volume), three times with water, twice with 1N NaOH, three times with water, three times with 1N HCl, and three times with water. After the decantation, the resins were in H^+-form.

5. Trimethylamine (TMA), aqueous solution 40%, from Aldrich Chemical Co. (Product No. 43, 326-8). The concentration was established by acid titration to be around 5.9 N.

6. Triethylamine (TEA), 99%, HPLC Grade, from Fisher (Product No. 04884). The concentration by acid titration was 6.9-7.1 N.

[0134] Water was purified through reverse osmosis, ion exchange, and organic removal to achieve organic free "16-18 MOhm" quality.

[0135] Aqueous solutions of pyrophosphate, triphosphate, and polyphosphate salts were prepared by ion exchange method. Solutions of sodium polyphosphate (3g in 25 mL of water), pyrophosphate (4g in 27 mL of water), or polyphosphate (6.7g in 30 mL of water) were loaded on the column containing 100 mL (bed volume) of the ion exchange

resin prepared as above. The column was eluted with water, and fractions were collected. The fractions showing acidic pH (pH<3) were pooled. Triplicates of 0.5-mL aliquots of the pooled fraction containing the phosphate acid were diluted with 20 mL water and titrated with 0.100 N NaOH to the end point of pH 4.5-5.0 (Fisher analytical solution) to determine normality. The pooled fractions after ion exchange were titrated with trimethylamine (to obtain trimethylammonium salts) to pH 5.4-5.5. After titration, the solutions were diluted, if necessary, to obtain a final concentration of trimethylammonium close to 0.5 N.

[0136] Trimethylammonium and triethylammonium sulfates were prepared by diluting 1.39 mL of concentrated (17.9 M) sulfuric acid with 80 mL water, and titrating the diluted solution with neat triethylamine or aqueous trimethylamine under the control of a pH-meter to equivalence point (pH 5.1-5.5). The volume was adjusted to 100 mL with water.

[0137] Trimethylammonium citrate solution was prepared by dissolving 1.572 g of citric acid monohydrate ACS from Sigma (Product No. C-1909) in 20 mL of water, and titrating the solution with aqueous trimethylamine to the point of equivalence. The volume was adjusted to 25 mL with water.

[0138] The solutions were filtered through a 0.2-μm cellulose acetate filter using positive pressure. Osmolality and pH of the solutions was measured using a vapor pressure osmometer and glass-calomel electrode pH-meter, respectively. The normality of the anion in the phosphate solutions was determined by blue phosphomolybdate spectrophotometric assay (see Example 70) after acid hydrolysis (5 min. 100 °C, 3N H_2SO_4). Anion normality took into account only the acidic functional groups that are substantially ionized at pH 5.5. Cation normality was determined on the basis of the added trialkylammonium base. The obtained solutions had the following properties (Table 4):

Table 4. Properties of substituted ammonium salt solutions for CPT-11 loading into liposomes.

Salt	cation normality	anion normality	pH	Osmolality (mmol/kg)
TMA citrate	0.58	0.60	5.1	791
TMA sulfate	0.50	0.50	5.4	625
TMA pyrophosphate	0.44	0.54	5.4	651
TMA triphosphate	0.57	0.68	5.4	695
TMA polyphosphate	0.49	0.58	5.5	336
TEA sulfate	0.54	0.50	5.35	719

[0139] Cholesterol and DSPC were purchased from Avanti Polar Lipids, Alabaster, Alabama, USA. PEG-DSPE (PEG mol. weight 2,000) was from Shearwater Polymers, Huntsville, AL, USA. DSPC, cholesterol, and PEG-DSPE in the weight ratio of 3:1:0.1 (molar ratio approximately 3:2:0.03) were dissolved in chloroform (Fisher; Optima grade, stabilized with amylene) at 60 mg/mL of DSPC. The solution was dispensed into Pyrex tubes at 30 mg of DSPC (0.5 mL) per tube and slowly evaporated at reduced pressure using rotary evaporator at 60 °C. The lipid films were dried under vacuum (100 micron mercury, oil pump) for 30-60 minutes at room temperature.

[0140] Dry lipid films were hydrated by gentle shaking in the above aqueous salt solutions at 60 °C for 15-20 minutes. The lipids formed a milky suspension (multilamellar vesicles). This milky suspension was subjected to five cycles of freezing in the mixture of dry ice and isopropanol (-80 °C, 3 minutes) and thawing in a water bath at 60 °C for 3 minutes. Then, the lipid suspension was extruded 10 times (double-strokes) through two stacked polycarbonate membrane filters (Nucleopore, Whatman, pore size 0.1 μm) using a manually operated reciprocating extruder (Avanti Polar Lipids) heated at 60 °C.

[0141] The extruded liposomes were kept at 60 °C for five minutes and quenched in ice water (0-4 °C) for five minutes. Then, the liposomes were separated from the gradient-forming salt solution into the loading buffer MES-Dextrose (50 g/L of Dextrose ACS, 0.975 g/L of 2-(N-morpholino)-ethanesulfonic acid (MES), and sufficient amount

of 5M NaOH to bring the pH to 6.4) by gel-chromatography on Sephadex G-75. Liposomes appear in the void volume fraction (approximately 30% of the column bed volume).

[0142] CPT-11 (Irinotecan hydrochloride) preparation containing 0.860 mg of the CPT-11 base per 1 mg of the solid was dissolved in 0.001N HCl to make a stock solution of 16.5 mg/mL CPT-11 base. This solution was mixed with the liposomes in MES-Dextrose buffer to achieve the ratio of 150 µg CPT-11 per 1 µmol of liposome phospholipids. The mixture was incubated at 55 °C in a water bath, with occasional gentle shaking (approximately every five minutes) for 30 minutes, then quickly chilled in ice water (0-4 °C). The liposomes were separated from the unencapsulated drug by gel-chromatography on Sephadex G-75, using MES-Dextrose as eluent. The encapsulated drug was determined by a spectrophotometric assay (Example 71), and the phospholipids determined using an extraction assay (Example 70).

[0143] *In vitro* drug release from so obtained CPT-11-loaded liposomes in the presence of 50% human plasma was studied as follows. Frozen human donor plasma was thawed as 37 °C, centrifuged at 14,500g for 10 minutes, and filtered through a 0.45-µm cellulose acetate syringe filter. The liposome preparations with loaded CPT-11 were sterilized by passage through 0.2-µm surfactant-free cellulose acetate (SFCA) sterile syringe filter. 0.5-mL of the liposomes were mixed with 0.5 mL of plasma in sterile 1.5-mL copolymer Eppendorf tubes, sealed, and incubated on a rocking platform at 37 °C for 24 hours. Blank sample contained 0.5 mL of sterile MES-Dextrose instead of liposomes. The liposomes were isolated by gel-chromatography on a beaded cross-linked 2% agarose gel (Sepharose CL-2B, Pharmacia; 10 mL bed volume) using 144 mM NaCl, 5 mM HEPES-Na, pH 7.4 buffer (HBS-5). The liposomes appeared at the void volume fraction, while plasma proteins and released drug (if any) were retarded by the gel. The liposome fractions were assayed for CPT-11 and phospholipids, and the drug/phospholipids ratio (output ratio) was determined. Readings of the blank samples (plasma only) were subtracted from the readings of the liposome-containing samples. Percent of the drug remaining in the liposomes after the incubation was determined by dividing output drug/lipid ratio by the input drug/lipid ratio (drug/lipid ratio prior to incubation with plasma). The loading and release data are summarized in Table 5.

Table 5. Loading of CPT-11 into liposomes with tertiary alkylammonium salts and *in vitro* release of the drug in the presence of human plasma.

Entrapped salt solution	Before incubation with plasma		After incubation with plasma	
	drug/lipid ratio	encapsulation efficiency (%)	drug/lipid ratio	drug remaining encapsulated (%)
TMA sulfate	127.2 ± 5.6	84.8 ± 3.8	132.1 ± 6.9	103.8 ± 10.0
TMA pyrophosphate	136.2 ± 9.0	90.8 ± 6.0	132.3 ± 5.0	97.1 ± 10.1
TMA triphosphate	132.9	88.6	129.2	97.3
TMA-Pn	134.4 ± 9.3	89.6 ± 6.2	135.0 ± 7.4	100.4 ± 12.4
TEA sulfate	131.1 ± 6.5	87.4 ± 4.4	125.2 ± 5.0	95.5 ± 8.6

EXAMPLE 8. *In vivo* stability of the liposomes loaded with CPT-11 using pyrophosphate, triphosphate, polyphosphate, citrate, and sulfate trialkylammonium salts.

[0144] While camptothecin liposomes may show acceptable drug leakage in blood plasma *in vitro*, the drug may leak more quickly in the blood circulation in vivo. Therefore, a panel of CPT-11 liposome formulations was screened for drug stability in the blood circulation *in vivo* using a single time point assay in mice.

[0145] The liposomes were prepared and loaded with CPT-11 as described in Example 6, with the following modifications. Instead of using PEG-DSPE from Shearwater Polymers, we used similar PEG-DSPE from Avanti Polar Lipids. To afford quantification of the liposome lipid matrix in the blood/tissue samples, a non-exchangeable radioactive label, [^{3}H]-Cholesteryl hexadecyl ether ([^{3}H]-CHE; (Amersham, USA) was added to the chloroform solution of the lipids in the amount of 0.25 mCi/mmol of phospholipids. The lipid solutions were dispensed into Pyrex tubes at 12 mg of DSPC/tube, and lipid films were formed by rotary evaporation/vacuum drying. Lipid films were hydrated in 0.7 mL of the gradient-forming substituted ammonium salt solutions. Lipid concentration in the liposomes with entrapped phosphate-containing salts was determined by radioactivity scintillation counting. The preparations without entrapped phosphate-containing salts were also assayed for phospholipids without extraction as described in Example 70, and used as lipid radioactivity standards. Portions of the liposome-drug mixtures prepared for the loading were saved and assayed to

confirm the pre-loading ratio of the added CPT-11 to the liposome lipid prior to loading ("input ratio"). Volume-averaged mean and standard deviation of the liposome size distribution were determined by quasi-elastic light scattering (QELS) using Gaussian model. The properties of these liposomes are summarized in Table 6.

Table 6. Characterization of CPT-11 loading into [^{3}H]-CHE-labeled liposomes for *in vivo* stability study

Entrapped salt solution	drug/lipid ratio before loading	drug/lipid ratio after loading	loading efficiency (%)	liposome size, (mean±SD) nm
TMA citrate	159.2 ± 3.5	156.7 ± 3.6	98.5 ± 4.4	122.1 ± 25.3
TMA sulfate	156.1 ± 2.5	156.1 ± 3.1	100.0 ± 3.6	122.2 ± 28.4
TMA pyrophosphate	164.6 ± 5.8	156.6 ± 4.3	95.2 ± 6.0	121.1 ± 19.9
TMA triphosphate	163.6 ± 5.7	156.0 ± 3.2	95.3 ± 5.3	122.4 ± 12.9
TMA polyphosphate	170.5 ± 8.0	162.4 ± 4.0	95.3 ± 6.8	123.0 ± 12.7
TEA sulfate	153. ± 3.3	154.9 ± 4.9	101.0 ± 5.3	121.1 ± 18.0

[0146] Six-week-old female CD-1 mice (Charles River) received tail vein injections of these liposomal CPT-11 formulations at the dose of 10 mg/kg (0.2 mg CPT-11/mouse) in duplicate. Eight hours later, the mice were anesthetized and exsanguinated through open heart puncture. The blood was collected into heparinized syringes (10-20 µl of 1000 U/ml heparin USP) and transferred into weighed tubes containing 0.4 ml of the phosphate-buffered physiological saline solution (PBS) containing 0.04% EDTA (Gibco BRL), kept on ice. The tubes were weighed to determine weights of the blood samples, blood cells were separated by centrifugation at 9,000g for 5 minutes, and supernatants containing PBS-diluted plasma, were saved for drug and liposome lipid assays. CPT-11 was quantified by fluorometric assay (Example 71). Liposome lipid was quantified by quenching-corrected radioactivity scintillation counting. The liposome and phospholipid-radioactivity standards were counted in parallel with the plasma samples. Percent of the drug that remained encapsulated was calculated by dividing the drug/radioactivity ratio in the plasma samples by the drug/radioactivity ratio of the injected liposomes. Due to the fast elimination of free CPT-11 from the blood (see Example 69) and the known stability of the [^{3}H]-CHE against lipid exchange, the assays' readings were considered indicative of the blood content of liposomal CPT-11 and lipid. Percent of injected lipid dose (%

I.D.) remaining in the circulation was calculated assuming 100% of the injected bolus entered the circulation; blood volume being 6.3% of the mouse body weight, and hematocrit of 45%. The results are summarized in Table 7.

Table 7. *In vivo* stability of CPT-11-encapsulation and circulation longevity of CPT-11-loaded liposomes in mice at a single point (8 hours) post injection. %I.D., % of injected dose.

Liposome-entrapped salt	Drug/lipid ratio, % of pre-injection value	Liposome lipid, % I.D. in the blood
TMA citrate	80.2 ± 7.8	18.8 ± 3.4
TMA sulfate	70.1 ± 4.8	23.6 ± 1.8
TMA pyrophosphate	67.3 ± 9.2	23.2 ± 3.1
TMA triphosphate	70.6 ± 6.0	24.9 ± 8.2
TMA polyphosphate	107.5 ± 8.9	24.3 ± 3.4
TEA sulfate	76.6 ± 13.1	23.6 ± 0.1

[0147] All preparations showed the level of drug encapsulation after 8 hours in the blood *in vivo* at 70-80% of the pre-injection level, while the liposomes containing polyphosphate were the most stable (drug encapsulation remains at about 100%).

EXAMPLE 9. Blood pharmacokinetics of CPT-11 liposomes prepared using riethylammonium polyphosphate.

[0148] The formulation of liposomal CPT-11 using triethylammonium polyphosphate salt was prepared as outlined in Example 3. The lipids – DSPC, cholesterol, and N-(methoxy-poly(ethylene glycol) (M.w. 2000)-oxycarbonyl)-DSPE (PEG-DSPE) (all from Avanti Polar Lipids, Inc.) – were combined as dry powders in the molar ratio of 3:2:0.015 and dissolved in 100% ethanol (USP grade, approx. 0.15 mL/100 mg of the lipids) at 62–65 °C. For pharmacokinetic studies, ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE, obtained from Amersham Pharmacia) was added to the lipids in the amount of 0.5 mCi/mmol of phospholipids as a non-exchangeable radioactive lipid label. The aqueous solution of TEA-Pn (0.5 M triethylammonium, pH 5.7-6.2) was prepared as in Example 4. TEA-Pn solution (10 times the volume of added ethanol) was mixed with the lipid solution at 60–65 °C and stirred at this temperature until a homogeneous milky suspension of multilamellar vesicles was formed. This suspension was extruded 15 times through 2 stacked polycarbonate track-etched filters (Corning Nuclepore) with the pore size of 100

nm using argon pressure extruder (Lipex Biomembranes) at 60–65 °C, and resulting unilamellar liposomes were quickly chilled in ice and then let attain ambient temperature. Ethanol and unincorporated polyphosphate salt were removed by gel chromatography on Sepharose CL-4B column eluted with MES-Dextrose buffer (5 mM MES, 50 g/L dextrose, pH adjusted to 6.5 with NaOH).

[0149] A stock solution of CPT-11 (Irinotecan hydrochloride) containing 20 mg/mL Irinotecan base in water was added to the liposomes at a drug/lipid ratio of 150-200 mg/mmol phospholipids, and the mixture was incubated with occasional agitation for 45-60 minutes at 60–62 °C. The incubation mixture was quickly cooled down and incubated for 10 minutes at 0 °C, then allowed to attain ambient temperature. 1/20 of the volume of 2.88 M NaCl was added to adjust to physiological ionic strength and improve the removal of membrane-bound CPT-11 (as opposed to the drug encapsulated within the liposome interior). Unencapsulated drug was removed by gel chromatography on Sephadex G-25 or G-75 column (Amersham Pharmacia) eluted with HBS-6.5 buffer (5 mM 2-(4-(2-hydroxyethyl)-piperazino)-ethylsulfonic acid (HEPES), 144 mM NaCl, pH 6.5). Liposome fractions eluted in the void volume were combined, sterilized by 0.2 micron filtration, and stored at 4-6 °C before use. The liposomes were characterized by lipid concentration, drug concentration, and particle size as in Example 7. The liposomes had the average size of 108 nm and CPT-11 content of 139 ± 18 mg of CPT-11 base per mmol of phospholipids.

[0150] The longevity of the liposome lipid and liposome drug in the blood and the characteristics of drug release from the liposomes *in vivo* were studied in female Sprague-Dawley rats (190-210g) with indwelling central venous catheters. The rats were injected with a 0.2-0.3 mL bolus of ^{3}H-CHE-labeled Irinotecan liposomes (0.05 mmol phospholipids, or 7-8 mg CPT-11 per kg of the body weight). Blood samples (0.2-0.3 mL) were drawn at various times post injection using heparin-treated syringe. The withdrawn blood volume was replenished using phosphate buffered physiological saline. The blood samples were diluted with 0.3 ml of ice-cold PBS containing 0.04% EDTA, weighed, and the blood cells were separated by centrifugation. The supernatant fluids were collected and assayed for CPT-11 using the fluorometric procedure of Example 71, and for the liposome lipid label by scintillation radioactivity counting using conventional methods. The liposome preparations with known drug and ^{3}H-CHE-lipid concentration

were used as standards. Radioactivity standards contain equal amount of diluted rat plasma to account for quenching. The amount of CPT-11 and the liposome lipid in the blood was calculated assuming the blood volume in ml as 6.5% of the body weight in grams, and the hematocrit of 40%. The total amount of the lipid and the drug in the blood was expressed as % of injected dose (% I.D., %ID) and plotted against post-injection time. The percent of drug remaining in the liposomes was calculated by dividing the drug/lipid ratio in the blood samples by the drug/lipid ratio of the injected liposomes (taken as 100%). Because the plots generally showed good agreement with monoexponential kinetics (linearity in semi-logarithmic scale), blood half-lives of the drug, the lipid, and of the drug release from the liposomes, were calculated from the best fit of the data to monoexponential decay equation using the TREND option of the Microsoft EXCEL computer program (Microsoft Corp., USA). The results are shown on Figure 1. From the best fit parameters, the blood half-lives for lipid and drug were 16.4 hours and 6.61 hours, respectively. Under these conditions, free CPT-11 clears from the circulation very rapidly (see Example 69).

[0151] The blood drug/lipid ratio revealed biphasic character of the CPT-11 release from the liposomes (Figure 2). In the first 24 hours, the release of drug followed was linear over time (R=0.992) giving evidence for zero-order release kinetics. Only after about 75% of the drug was released at 24 hour time point, further release became non-linear. For 24 hours, the liposomes released the drug at a constant rate of about 3.6% of the initial load/hour. Thus, 50% of the drug was released over the period of approximately 14 hours. Zero-order release of the drug is an attractive quality in sustained release formulations, as the rate of drug release remains constant over time.

EXAMPLE 10. Antitumor efficacy of CPT-11 liposomes prepared using triethylammonium polyphosphate against breast cancer xenografts in nude mice

[0152] Antitumor efficacy of CPT-11 liposomes was studied in the model of human breast carcinoma BT-474, an estrogen-dependent ductal adenocarcinoma that over-expresses C-ErbB2 (HER2) receptor. . BT-474 cells were obtained from American Type Culture Collection (Rockville, MD). A BT-474 sub-line with higher tumor growth rate was established from a fast-growing xenograft tumor nodule raised as described below. The cells were propagated in vitro in RPMI-1460 medium with 10% fetal calf serum, 0.1

mg/mL streptomycin sulfate, and 100 U/ml Penicillin G in T-150 flasks, and split 1:3 every week. NCR *nu/nu* female mice (4-6 week old; Taconic Farms) were subcutaneously implanted (at the base of tail) with 60-day sustained-release 0.72-mg 17β-estradiol pellets (Innovative Research of America, Inc.), and in two days were inoculated subcutaneously in the upper back area with 0.1 mL suspension containing $2x10^7$ BT-474 cells in cell growth medium. The tumor progression was monitored by palpation and caliper measurements of the tumors along the largest (length) and smallest (width) axis twice a week. The tumor sizes were determined twice weekly from the caliper measurements using the formula (Geran, R.I., *et al.*, 1972 *Cancer Chemother. Rep.* 3:1-88):

$$\text{Tumor volume} = [(\text{length}) \times (\text{width})^2] / 2$$

[0153] At day 13 after inoculation, the tumor reached an average size of 200 mm^3 and the animals were randomly assigned to three groups of 13-15 animals.

[0154] Liposomal CPT-11 was prepared as in Example 8 (drug/phospholipid ratio 192 mg/mmol; average liposome size 86.8 nm). Free and liposomal CPT-11 were diluted with MES-dextrose vehicle to 5 mg/ml of CPT-11 base The animals were injected via the tail vein with free CPT-11, liposomal CPT-11, or vehicle only on days 14, 18, 21, and 25 post tumor inoculation. The drug-containing formulations were given at the dose of 50 mg CPT-11/kg per injection, which is the average of the doses reported in the literature for the CPT-11 studies in rodent tumor models.

[0155] To assess treatment-related toxicity, the animals were also weighted twice weekly. The observations were made until day 60 post inoculation, at which time the estrogen supplementation pellet was exhausted. Average tumor volumes across the groups were plotted together and compared over time. As shown in Figure 3, while free CPT-11 reduced the rate of tumor growth, in the group that received liposomal treatment the tumors regressed dramatically. While at day 36 in the control group the tumors reached the maximum allowable size averaging 3,500 mm^3, and at day 46 in the group treated with free drug the tumors were about 1,000 mm^3 at average, at the same time point none of the animals in the liposome-treated group had a palpable tumor.

[0156] The treatment related toxicity was assessed by the dynamics of animals' body weight (Figure 4). Neither group revealed any significant toxicity. The weight of the animals in the control group was consistently increasing. There was a slight decrease in the average body weight of the animals receiving liposomal CPT-11, by about 3.3%, on the day of the last treatment. This weight loss was reversed, however, and the animals reached their expected weight. This decrease in the mean body weight was not statistically significant by Student's t-test compared to pretreatment weight (p=0.274). Thus, all treatments were tolerated without significant toxicity.

[0157] Thus, the liposome formulation of CPT-11 obtained by loading of the drug *via* pre-entrapped sterically hindered substituted ammonium salt (triethylammonium) of a polyanionic, biodegradable polymer (polyphosphate) showed extended blood life, sustained release characteristics, and increased antitumor activity in the studied tumor model without an appreciable increase in toxicity.

EXAMPLE 11. Comparative assessment of CPT-11 loaded liposomes prepared using pre-entrapped triethylammonium salts: effect of liposome size, drug/lipid ratio, and the nature of pre-entrapped anion.

[0158] Two prototype formulations of CPT-11-loaded liposomes were prepared, one using the liposomes with pre-entrapped TEA-Pn, and the other with pre-entrapped TEA-SOS. Preparation of these liposomes included the following manufacturing steps.

[0159] 1) *Combining the lipids by co-dissolving in ethanol.* The lipid matrix composition consisted of 1,2-Distearoyl-SN-phosphatidylcholine (DSPC) (Mol. wt. 790) 3 molar parts (59.8 mol.%); Cholesterol (Chol) (Mol. weight 387) 2 molar parts (39.9 mol.%); and N-(omega-methoxy-poly(ethylene glycol)-oxycarbonyl)-1,2-distearoylphosphatidyl ethanolamine (Mol. weight 2787) (PEG-DSPE) 0.015 molar parts (approx. 0.3 mol.%). DSPC and PEG-DSPE were purchased from Avanti Polar Lipids, Birmingham, Alabama. Cholesterol (highest purity grade) was purchased from Calbiochem. Dry lipids were weighed with the accuracy of $\pm$0.1 mg in a borosilicate glass container and combined with absolute ethanol at the ratio suitable for the lipid dispersion step below. Because of the high transition temperature of DSPC (55°C) the dissolution was typically performed at 55-60°C until clear solution was obtained.

[0160] 2) *Preparing the TEA-Pn and TEA-SOS solutions.* Sodium polyphosphate (n=13-18) was from Sigma Chemical Co., p/n P 8510. Sodium sucrose octasulfate was purchased from Toronto Research Chemicals, Toronto, Canada, p/n S699020. The salts were weighed down and dissolved in water to provide 1.2-2.5 N solutions. Anion exchangers Dowex 50Wx8-200 or Dowex HCR-W2 in H^+-form (available from Sigma) were used to convert the sodium salts into free acids. Before the first use, the resins were washed by stirring with 3 volumes of the following solutions, followed by decanting: (1) 1.0-1.2 M aqueous HCl 2 times; (2) Water 2 times; (3) 1.0-1.2 M aqueous NaOH 2 times; (4) Water 2 times; (5) 1.0-1.2 M aqueous HCL, 2 times. The suspension of washed resin in water was packed under gravity flow in a suitable size chromatography column to have at least 8 mL of the packed resin for each mL of the sodium salt solutions. The resin was further equilibrated by passage of 2 column volumes of 3.0-3.6 M aqueous HCL, followed by 5 column volumes of water or until the conductivity of the eluate falls below 1micro-S. After use, the columns were regenerated by sequential passage of: 1.-1.2 M HCl - 3 column volumes; 3.0-3.6 M HCl - 2 column volumes; water - at least 5 column volumes, or until the conductivity of the eluate falls below 1 μS, and stored under 0.2-um filtered water at room temperature. The Pn and SOS sodium salt solutions were applied on the drained surface of the column (approximately 1 ml for each 4 ml of the packed resin volume) and allowed to flow under gravity at the rate of about 1-2 ml/min for the resin bed size of 75-150 mL. The column was eluted with water. The eluate was tested for conductivity. The fractions with 10 mS or higher conductivity were collected. If more concentrated solutions of polyacids are required, the collection can start at 20-50 mS, but at the expense of somewhat higher loss of the gradient-forming salt. In the case of polyphosphoric acid, the collected solution is kept refrigerated (0-4°C) until the amine titration step because of the hydrolytic instability of the phosphodiester bond at low pH. The collected eluates would have a pH of less than 0.7 (typically about 0.4) and conductivity of about 120-200 mS. Optionally, the amine titration step is performed without delay because the stability of polyphosphate at low pH. HPLC-grade triethylamine (99.5+% purity) from Fisher, p/n 04884 was used to titrate the acid solutions obtained from ion exchange. The normality of neat TEA was determined by potentiometric titration. 0.100-mL Aliquots of TEA (0.100 ml) were taken into 20 ml of water in triplicate. The aliquots are titrated with 0.1 N HCl standard solution to the pH end point (glass electrode) of 5.5-6.0. The calculated normality (7.07 N) was close to the

theoretical value of 7.17N. A measured volume of the polyphosphoric (Pn) acid or sucrose octasulfuric (SOS) acid solution was titrated with neat TEA under the control of pH (glass) electrode. Thorough stirring was required to disperse the amine. Titration endpoint was pH 5.6-6.2. The volume of added TEA was accurately recorded. The volume of titrated solution was measured, and the concentration of TEA was calculated on the basis of the added TEA volume and normality. Water was added as necessary to adjust the TEA concentration to required 0.55±0.05 N or 0.65±0.03 N, as indicated below. The amount of residual sodium in the obtained TEA-Pn or TEA-SOS solutions was determined by potentiometry using sodium-selective glass electrode (Corning). One mL of the solution was diluted with 19 mL of water, and the sodium concentration was determined using the increment method according to the electrode manufacturer's manual. The amount of residual sodium was less than 1 mM, typically less than 0.5 mM. The obtained TEA-Pn or TEA-SOS solutions were passed through 0.2 μm cellulose acetate sterile filter using positive pressure feed. The final pH and osmolality of the solutions was measured and recorded. We use pH calomel micro-combination all-glass electrode for pH measurements, and vapor pressure/dew point osmometer for osmolality measurements. The solutions were stored refrigerated until use.

[0161] 3). Preparing lipid dispersion in the gradient-forming buffer by mixing of ethanolic solution of the lipids with the gradient-forming buffer. The lipids were dispersed in the gradient-forming salt solution using ethanol mixing method. All steps were performed at 60-65°C. The lipids were dissolved in 100% Ethanol USP at a concentration of about 0.5-0.6 M of DSPC in a chemical resistant glass pear-shaped flask or tube. The gradient-forming salt solution (TEA-Pn or TEA-SOS) was pre-warmed to 60-65°C and added to the ethanolic lipid solution at once, and the components were thoroughly mixed by swirling and/or vortexing. The final amount of ethanol was about 10 vol.%. For preparations of the scale in excess of 0.1 mmol phospholipid, the resulting suspension was placed on a rotary evaporator at 60-65°C and vacuumized with rotation until the evolution of ethanol stopped, as manifested by the end of foam formation. For the scale of 0.1 mmol phospholipid or less, ethanol was not removed from the lipid dispersion at this step. The resulting lipid suspensions were kept at 60-65°C and used promptly for the extrusion step.

[0162] 4). *Sequential extrusion of the lipid dispersion through defined pore membranes.* For the lipid suspension volumes up to 1 mL we used a manually operated reciprocating extruder supplied by Avanti Polar Lipids. The extruder is charged with 19 mm track-etched filter membranes and thermostatted by virtue of a metal heating block. For the volumes from 1 to 10 mL, we used a thermostatted, gas pressure operated, unidirectional flow extruder from Lipex Biomembranes. The extruder is charged with 25 mm filter membranes. The lipid suspensions were repeatedly extruded at 60-65°C using manual feed or argon gas pressure, as appropriate, through a series of 2 stacked polycarbonate membrane filters (the filters from Corning-Nuclepore and Osmonics Corp. were equally suitable) having nominal pore sizes of 100 nm, 80 nm, or 50 nm. Where the effect of liposome size was of interest, the extrusion was stopped at 100 nm, 80 nm, or 50 nm step. The exact type of filters used and number of extrusions is indicated below for each experiment. The extruded liposomes were kept at 60-65°C for about 15 min. and quickly cooled down to 2-4°C in an ice bath. After about 15 min. in the ice bath, the liposomes were allowed to reach room temperature.

[0163] 5). *Removal of extraliposomal gradient-forming buffer and transfer of the liposomes into a drug-loading buffer.* Non-encapsulated gradient-forming salt was removed, and the liposomes were transferred into the drug loading buffer using size exclusion chromatography (SEC). Tangential flow filtration, hollow fiber dialysis, of other scalable step can be used in scale-up manufacture. It is advantageous to ensure the complete removal of the extraliposomal polyanion by treatment of the liposomes with an anion-exchange resin (*e.g.*, Dowex-1 or Dowex-2 quaternary ammonium cross-linked polystyrene beads). Drug-loading buffer contained 50 g/L anhydrous Dextrose USP, and 5 mM tissue-culture certified HEPES in water, adjusted to pH 6.5 with NaOH. The buffer was vacuum-filtered through 0.2 micron Nylon filter (Whatman). The extruded liposomes were chromatographed on a column with Sepharose CL-4B (Pharmacia) and eluted with the drug-loading buffer. The liposomes appeared in the void volume fraction and were collected, based on the eluate turbidity, in the volume of about 2x of that applied. The eluted liposomes were assayed for phospholipid concentration according to Example 70, particle size by QELS, and stored at 4-6°C.

[0164] 6) *Incubation of liposomes with the drug.* Stock solution of CPT-11 (Irinotecan Hydrochloride) was prepared immediately before mixing with the liposomes by

dissolving Irinotecan Hydrochloride in water to achieve concentration of 20 mg/mL drug base. The pH of the solution was between 4.0 and 5.0. The drug solution was filtered through 0.2 micron polyethersulfone (PES) sterile filter using positive pressure. Aliquots of the liposomes in the drug loading buffer produced at the step 5 above were mixed at room temperature with the stock Irinotecan solution to achieve the drug/lipid input ratio in the range of 0.15-0.55 g of drug for mmol of liposome phospholipid. Particular input drug/lipid ratios are indicated below, where appropriate. The pH of the mixtures was adjusted to 6.5 with 1 M NaOH, the mixtures in glass vials were incubated on the thermostatted water bath at 58-62°C with slow agitation for 30-45 min, quickly cooled down in ice-water bath (0-2°C), and left at this temperature for 15 min. Then the liposomes were allowed to warm up to room temperature for the next step (removal of unencapsulated drug and transfer into the storage buffer). This step resulted in the encapsulation efficiency of more than 95%, typically 98-100% in the whole range of studied drug/lipid ratios.

[0165] 7). *Removal of unencapsulated CPT-11, transfer of the liposomes into the storage buffer, final filtration, and storage.* Unencapsulated drug was removed and the liposomes were transferred into the storage buffer using size exclusion chromatography. The storage buffer contained 20 mM HEPES, 135 mM NaCl, pH 6.5 (adjusted with NaOH) in water, and was 0.2-micron vacuum-filtered before use. Gel-chromatography on Sephadex G-75 (Amersham Pharmacia Biotech) was performed essentially as described under Step 2 above. CPT-11 liposomes eluted from the column (void volume fraction) were assayed for liposome phospholipid and CPT-11 (by spectrophotometry, see Examples 70 and 71), and volume-weighted mean particle size by QELS. The drug concentration was adjusted, if necessary, to be in the range of 2.0-4.0 mg/mL. The liposomes were filtered through 0.2 micron polyethersulfone sterile filters and aseptically dispensed into sterile polypropylene vials (Corning Cryo-Vials) or PTFE-lined screw-cap borosilicate 4-mL glass vials to approximately 70-80% of the vial volume. The vials were aseptically closed (in air), labeled, and stored at 4-6°C.

EXAMPLE 12. Effect of drug/lipid ratio on the drug loading efficiency and in vivo drug retention of TEA-Pn-containing liposomes

[0166] Liposomes with entrapped aqueous 0.65N solution of TEA-Pn, pH 6.1, osmolality 531 mmol/kg, were prepared following the procedure of Example 11. The

lipid dispersion was extruded ten times through two stacked 100 nm pore size polycarbonate filters. Liposome lipid matrix also included [^{3}H]-CHE at 0.5 mCi/mmol phospholipid. The liposome size before drug loading was 98.5 ± 34.3 nm. The liposomes were loaded at initial drug-to-phospholipid ratios of 200, 300, 400, and 500 mg CPT-11/ mmol phospholipid. The drug and phospholipid amounts in the liposomes were determined by spectrophotometry according to Example 71, and by phospholipid extraction-digestion-blue phosphomolybdate assay of Example 72, respectively.

[0167] To evaluate *in vivo* drug release rate, the method of Example 8 was followed. The liposomes were injected *via* tail vein into 6-week-old female Swiss Webster mice (body weight 18-22 g) at a dose of 5 mg CPT-11/kg. At 8 and 24 hours post injection, the mice, in groups of 3, were anesthetized, and exsanguinated via open heart puncture. The blood was mixed with 0.4 mL of ice-cold 0.04% EDTA in PBS, the blood cells were separated by centrifugation, and the plasma concentration of CPT-11 was measured by spectrofluorometry as described in Example 71. Lipid was determined by measuring the amount of [^{3}H]-CHE using quenching-corrected liquid scintillation counting, and the amount of drug retained in the liposomes was calculated by dividing the determined drug/lipid ratio by the drug/lipid ratio in the injected liposomes. Because of the fast blood clearance of free CPT-11, resulting in low blood level, we assumed that all assayed drug was in the liposomal form.

[0168] The results are presented in Table 8. The differences between drug retention among the groups were not statistically significant. As a result of these studies, we concluded that increasing the drug load up to 500 mg/mmol will not adversely affect drug loading or *in vivo* stability. This ratio was adopted for further studies.

Table 8. The effect of drug/lipid ratio on the drug loading and *in vivo* drug retention in Irinotecan TEA-Pn liposomes (average ± standard deviation).

Drug/lipid ratio, mg/mmol phospholipid			Drug remaining in the liposomes, % of pre-injection value	
Input	Output	% loaded	After 8 hours	After 24 hours
200	208.4	104.2	54.6±9.9	9.72±2.23
300	286.3	95.4	85.2±14.3	14.52±2.51
400	348.8	87.2	81.5±18.3	17.31±6.14
500	518.9	103.8	66.8±19.6	13.47±1.44

EXAMPLE 13. Drug loading efficiency of CPT-11 loading into TEA-SOS-containing liposomes: effect of liposome size and in vivo drug retention in mice.

[0169] Liposomes with entrapped solutions containing prepared as in Example 11 using gradient forming solution having 0.643 N TEA-SOS, pH 5.7, osmolality 530 mmol/kg. Lipid dispersion was extruded ten times through two stacked polycarbonate filters with the pore size of 50 nm, 80 nm, or 100 nm. Liposome lipid matrix also included [^{3}H]-CHE at 1.5 mCi/mmol of liposome phospholipid. The liposome size was determined by dynamic light scattering. The liposomes were loaded with CPT-11 at initial drug-to-phospholipid ratios of approximately 550 mg Irinotecan/mmol of phospholipid. The drug loaded liposomes were sized by QELS and assayed as described in Examples 70 and 71.

[0170] Female Swiss Webster mice (8-10 weeks, average 27-30 grams) were injected via tail vein with these CPT-11 liposome formulations at a drug dose of 10 mg/kg. The mice were sacrificed at 24 h and the blood was collected and assayed for CPT-11 and liposome lipids as in Example 11. The results are summarized in Table 9.

Table 9. Irinotecan loading and in vivo drug retention in TEA-SOS liposomes.

Extrusion membrane pore size, nm	Liposome size, nm mean SD	Drug load, mg Irinotecan/mmol phospholipid	Drug remaining in the liposomes after 24 hours in mice, % of pre-injection value
50	87.6±28.1	579.3± 24.2	79.2±3.8
80	98.5±15.1	571.1 ± 69.7	82.6 ± 2.1
100	110.8±25.2	567.7 ± 37.7	86.2 ± 2.7

[0171] Surprisingly, the liposomes with triethylammonium salt of sucrose octasulfate, a non-polymeric polyanionized organic hydroxylated organic compound (sugar), provided dramatically better (4-5 fold) *in vivo* drug retention in liposomes compared with similar liposomes with a polyanionic polymer (polyphosphate).

EXAMPLE 14. Blood pharmacokinetics of CPT-11-loaded SOS-TEA liposomes in rats.

[0172] Liposomes (100 nm extrusion membrane pore size) were prepared as described in Example 12 The liposomes was administered intravenously at a dose of 10 mg CPT11/kg to two nine-week-old female Sprague Dawley rats (Harlan) (body weight about 200 g) with indwelling central venous catheter at a dose of 10 mg CPT-11/kg (17.6

μmol of phospholipids/kg). Blood samples were taken at prescribed time points and analyzed for drug and liposome lipid content as in Example 9. The data were expressed as the % injected lipid dose/ml of plasma and the % drug retained inside the liposome at each time point, plotted against post injection time, and half-lives for liposome lipid, as well as half-lives for drug release from the liposomes, were calculated by best fit to a monoexponential kinetic model (Fig. 5). The half-life of drug release from CPT-11 loaded TEA-SOS liposomes was 56.8 hours, much longer than that of the similar TEA-Pn liposomes.

EXAMPLE 15. Antitumor activity of free CPT-11, and CPT-11 encapsulated into TEA-Pn and TEA-SOS-containing liposomes in athymic nude mice bearing subcutaneous xenografts of human colon carcinoma (HT-29).

[0173] The liposomes were prepared as in Example 11 using TEA-Pn solution with 0.65 M TEA, pH 6.1, and osmolality 531 mmol/kg, or TEA-SOS solution with 0.643 M TEA, pH 5.7, and osmolality 530 mmol/kg. The extrusion included 10 passages through two stacked polycarbonate membranes with pore size 100 nm. The resulting TEA-Pn and TEA-SOS liposomes had the size of 112.3±15.5 nm and 120.5±42.5 nm, respectively (mean± SD of the size distribution). The liposomes were loaded with CPT-11 at the input drug/phospholipids ratio of 500 mg/mmol. The resulting liposomes had the drug content of 465.6 ± 26.5 (93% loading efficiency) and 499.9±22.5 mg (100% loading efficiency) of CPT-11/mmol phospholipid for the TEA-Pn and TEA-SOS formulations, respectively.

[0174] HT-29 cells were obtained from American Type Culture Collection, Rockville, MD, and propagated in DMEM medium supplemented with 10% fetal calf serum, 50 U/ml penicillin G, and 50 μg/mL of streptomycin sulfate at 37°C, 5% CO_2 as recommended by the supplier. NCR *nu/nu* homozygous athymic male nude mice (6 week old, weight at least 16 g) were obtained from Charles River. The mice were inoculated subcutaneously in the right flank with 0.1 mL of the suspension containing 5 x 10^6 cells suspended in the growth medium without antibiotics. Eleven days later the animals having tumors with the size between 150 mm^3 and 350 mm^3 were assigned to the treatment groups according to the following method. The animals were ranked according to the tumor size, and divided into 6 categories of decreasing tumor size. Six treatment groups of 11 animals/group were formed by randomly selecting one animal from each size category, so that in each treatment group all tumor sizes were equally represented.

Starting at day 13, the animals received four tail vein injections, at the intervals of 4 days, of the following preparations: 1) Control (HEPES-buffered saline pH 6.5); 2) Free CPT-11 50 mg/kg, administered as freshly prepared 5 mg/mL solution in unbuffered physiological saline; 3) TEA-Pn liposomal CPT-11 at 25 mg/kg per injection; 4) TEA-Pn liposomal CPT-11 at 50 mg/kg per injection; 5) TEA-SOS liposomal CPT-11 at 25 mg/kg per injection; 6) TEA-SOS liposomal CPT-11 at 50 mg/kg per injection. The animal weight and tumor size were monitored twice weekly as described in Example 10. The weight of tumor was subtracted from the animal weighing results to obtain animal body weight. The animals were observed for 60 days following tumor inoculation. When the tumors in the group reached 20% of the mouse body weight, the animals in the group were euthanized. There were complete tumor regressions in some groups without the signs of tumor regrowth at the end of study. The tissues from the tumor inoculation site from these animals were collected and preserved for pathological analysis for residual tumor cells.

[0175] The results of this study are shown in Figures 6 and 7. Free CPT-11 had only minor effect on the tumor growth. All liposomes had pronounced effect resulting in tumor regression later followed by regrowth in most animals. 50 mg/kg dose was more effective than 25 mg/kg dose in both TEA-Pn and TEA-SOS CPT-11 liposomes. Average tumor doubling times calculated from the tumor size data (Fig.7) were: control - 4.2 days; free drug, 50 mg/kg - 4.8 days; TEA-Pn liposomal drug, 25 mg/mg - 43.6 days; TEA-Pn liposomal drug, 50 mg/kg - 47.5 days; TEA-SOS liposomal drug at 25 mg/kg - 48.2 days, and TEA-SOS liposomal drug at 50 mg/kg - over 56 days (doubling time was not reached). Thus, liposomal CPT-11 prepared according to the present invention was at least about 10-fold more active than the free drug, given at the same dose and schedule. Unexpectedly, TEA-SOS CPT-11 liposomes were prominently more effective in reducing tumor growth than TEA-Pn CPT-11 liposomes administered at the same dose. While in the groups treated with free drug and TES-Pn liposomal drug at 50 mg/kg per injection there were no animals without tumor regrowth, in the groups receiving 25 mg/kg of each liposomal formulation, one animal (9.1%) was tumor-free at the end of study, and in the group receiving 50 mg/kg of TEA-SOS liposomal CPT-11 formulation, at the end of study 4 animals (36.4%) were tumor-free without signs of regrowth.

[0176] The drug manifested some toxicity. The animals receiving free CPT-11, but not liposomal CPT-11, experienced temporary morbidity (loss of alertness, humped posture, ruffled fur, decreased mobility) for about one hour after drug injection. The animals receiving free CPT-11 suffered permanent loss of about 6% of weight during treatment, and did not recover, The animals receiving both liposomal CPT-11 formulations experienced transient weight loss at the time between second and third injections averaging about 5% (at 25 mg/kg) or about 9% (at 50 mg/kg) of the pre-treatment value, and eventually attained normal weight. Therefore, the toxicity of liposomal drug was not more than that of the free (non-liposomal) drug, while the efficacy of the liposomal drug was substantially higher. The weight loss was reversed when the drug treatment was finished, and all animals recovered their weight without terminal morbidity or toxic deaths. Later on, the animals gained weight concomitantly with tumor regressions. In the saline control group, animals that developed large tumors experienced weight loss evidently due to tumor-related morbidity. Overall, the liposome drug formulation where the drug was loaded into the liposomes having pre-entrapped polyanionized sugar (sucrose octasulfate) proved to be the most efficacious while having less toxicity than the non-liposomal drug.

EXAMPLE 16. Toxicity of free and liposomal CPT-11 in mice.

[0177] Acute toxicities of free CPT-11 and liposome-encapsulated CPT-11 prepared according to the present invention were compared by determining the maximum tolerated dose (MTD) following single i.v. injection in regular (immunocompetent) mice.

[0178] The following materials were used:

[0179] 1) CPT-11 (Irinotecan Hydrochloride) preparation having Irinotecan Hydrochloride 98.9% by HPLC, and moisture 7.6%. In this study drug formulations were prepared on the "as is" basis, without correction for the moisture content or the Irinotecan base content.

[0180] 2) Liposomal CPT-11 (Ls-CPT-11) was prepared as in Example 11, using lipid matrix of DSPC 200 mol. parts, Cholesterol 133 mol. parts, PEG-DSPE 1 mol. part; entrapped solution TEA-SOS having 0.65 M TEA, pH 6.4; drug loaded into liposomes in 5 mM HEPES buffer, 5% dextrose, pH 6.5,at 60°C for 30 min at the input drug/lipid ratio

500 mg drug/mmol of phospholipid. Loading efficiency was >99%. Liposome size (volume average mean ± standard deviation by QELS): 101 ± 37 nm. Liposomes were formulated in the vehicle, 20 mM HEPES-Na, 135 mM NaCl; pH 6.5. Drug concentrations in the injected formulations were as stated in the Tables below.

[0181] 3) Free CPT-11 solution. Free drug stock solution was prepared by dissolving Irinotecan Hydrochloride in 5% aqueous dextrose at 22 mg/mL, and sterilized by 0.2-μm filtration. This stock solution was diluted with sterile 5% dextrose prior to injection.

[0182] 4) Animals. Female Swiss Webster mice, 6-8 week old, were from Harlan, USA.

[0183] MTD determination generally followed the protocol adopted by the United States National Cancer Institute Developmental Therapeutics Program. The protocol included the following three steps:

[0184] Step 1): Range-seeking step with the dose escalation factor of 1.8. The groups of two animals were injected into the tail vein with increasing doses of the free or liposomal Irinotecan, beginning with the dose of 60 mg/kg, and continuing with the dose escalation factor of 1.8, until acute mortality or terminal morbidity (within >1 day post injection) is observed in any of the animals. The dose one step below the mortality/terminal morbidity dose is recorded.

[0185] Step 2): Range-seeking step with the dose escalation factor of 1.15. The groups of two animals were injected into the tail vein with increasing doses of the free or liposomal Irinotecan, beginning with the dose recorded at Step 1, and continuing with the dose escalation factor of 1.15, until acute mortality or terminal morbidity (within >1 day post injection) is observed in any of the animals. The dose one step below the mortality/terminal morbidity dose is recorded at tentative MTD.

[0186] Step 3): Validation step. The group of 5 animals is injected i.v. (tail vein) with free or liposomal Irinotecan at tentative MTD determined at Step 2. The animals are followed for 7 days, the animal body weight is recorded twice weekly and compared with the pre-injection weight. General health of the animals is observed (alertness, grooming, feeding, excreta, skin, fur, and mucous membrane conditions, ambulation, breathing,

posture). If during the observation period there is no mortality, progressive morbidity, or weight loss in excess of 15% of the pre-injection body weight, the dose is considered to be validated as acute single injection MTD. If any of these effects occur, the experiment is repeated at the next lower dose by a factor 1.15.

[0187] To obtain additional statistics for validation step, the body weight dynamics of surviving animals was followed for up to 11 days post injection. The dose of more than 324 mg/kg of the liposomal Irinotecan was impossible to administer because of the concentration and injection volume limitations. The results are presented in Table 10.

Table 10. MTD seeking study of CPT-11 formulations in mice.

RESULTS

Step 1. increase dose by a factor of 1.8

drug	inj. Dose (mg/kg)	drug conc. (mg/ml)	Inj. volume (µl)	mouse #	Animal body weight, at day post injection: 0 (g)	1 (g)	2 (g)	4 (g)	5 (g)	6 (g)	7 (g)	11 (g)
Ls-CPT11	60	8	150	1	19.2	18.0	nd	20.3	20.6	20.6	20.0	19.7
				2	19.7	19.3	nd	20.6	20.4	19.6	19.7	20.7
	100	12	165	1	19.5	18.6	nd	19.6	20.0	20.1	19.4	19.9
				2	20.1	18.9	nd	20.2	21.5	22.2	21.8	22.5
	180	22	165	1	19.4	18.4	nd	18.9	19.7	20.5	19.5	20.5
				2	20.0	19.3	nd	19.6	20.6	21.4	21.6	21.7
	324	30.6	210	1	21.8	21.2	21.2	nd	20.2	nd	20.2	nd
				2	21.6	20.4	21.3	nd	20.8	nd	21.4	nd
free CPT11	60	8	150	1	20.6	20.4	nd	22.1	22.1	22.2	22.0	22.5
				2	19.5	19.1	nd	20.2	20.3	20.4	20.5	21.1
	100	12	165	1	19.3	died 1-2 min after injection						
				2	20.1	died 1-2 min after injection						
				3	19.9	died 1-2 min after injection						

After injection, all mice treated with free CPT11 were sick, short of breath for about 1h and then recovered
After injection, all mice treated with Ls-CPT11 were normal.

Step 2 increase dose by a factor of 1.15

drug	inj. Dose (mg/kg)	drug conc. (mg/ml)	Inj. volume (µl)	mouse #	Animal body weight, at day post injection: 0 (g)	1 (g)	2 (g)	5 (g)	7 (g)
free CPT11	60	8	150	3	19.9	20.0	20.9	19.9	21.3
				4	19.5	18.7	19.4	18.8	18.9
	70	8	175	5	20.9	20.0	20.6	19.3	20.4
				6	22.3	21.8	22.4	22.4	22.8
	80	8	200	7	20.6	19.9	20.1	19.9	20.9
				8	20.6	20.8	21.1	20.7	21.4
	90	12	150	9	22.3	died 1-2 min after injection			
				10	22.4	died 1-2 min after injection			
		8	225	11	20.6	died 1-2 min after injection			

Step 3. Validation

drug	inj. Dose (mg/kg)	drug conc. (mg/ml)	Inj. volume (µl)	mouse #	Animal body weight, at day post injection 0 (g)	3 (g)	5 (g)	7 (g)
free CPT11	80	8	200	1	20.2	19.3	20.0	21.7
				2	20.5	20.6	20.5	21.2
				3	20.7	20.6	20.8	21.9
				4	20.8	21.4	22.1	23.0
				5	21.9	21.9	21.6	21.5
Ls-CPT11	324	36.5	180	6	21.0	20.0	20.1	20.2
				7	20.4	20.4	20.2	19.2
				8	20.4	19.8	20.3	20.7
				9	20.9	19.9	20.5	21.5
				10	20.7	19.5	19.8	20.2

[0188] Thus, while the MTD of free CPT-11 was 80 mg/kg, the MTD of liposomal CPT-11, surprisingly, was not achieved even at the highest administered dose of 324 mg/kg. Therefore, liposome encapsulation of CPT-11 according to the present invention has reduced the drug toxicity at least 4 – fold.

EXAMPLE 17. Storage stability of CPT-11-loaded TEA-SOS liposomes against drug leakage.

[0189] Five batches of liposomal CPT-11 were prepared using the TEA-SOS method (Example 11), at the drug/lipid input ratio of 500-550 mg/mmol phospholipid. The liposomes were prepared using membrane extrusion through polycarbonate membrane with 80 nm or 100 nm pore size, as indicated in the table below. The liposomes were 0.2-µm filter sterilized and stored at 3.4-14.5 mg/mL of CPT-11 in 135 mM NaCl, 20 mM HEPES-Na, pH 6.5 (storage buffer), at 4-8°C. After the indicated storage time, the leaked drug was removed by gel-chromatography on Sephadex G-75 using the storage buffer as eluent. The drug and phospholipid concentrations in the liposomes before and after gel-chromatography were assayed using spectrophotometry method and acid digestion-blue phosphomolybdate method, respectively, as described in Examples 70 and 71. CPT-11 liposomes prepared according to the present invention were very stable. The leakage of CPT-11 from these liposomes during storage was less than 5% over 6 months (Table 10).

Table 11. Encapsulation stability of CPT-11 liposomes during storage (data are mean±SE).

Liposome Lot #	Extrusion pore size, nm	CPT-11 concentration, mg/ml	Storage time, months	% drug remaining encapsulated
1	80	3.44±0.06	6	99.02±3.77
2	80	7.88±0.19	6	102.38±4.78
3	100	4.57±0.06	6	96.38±4.69
4	100	4.62±0.11	6	95.72±4.36
5	80	14.52±0.42	3	103.4±5.92

EXAMPLE 18. Liposomes loaded with Topotecan.

[0190] Liposomes with entrapped TEA-Pn solution and TEA-SOS solution were prepared as in Example 11. Stock solution of Topotecan Hydrochloride (GlaxoSmithKline, PA, USA) was prepared immediately before mixing with the liposomes by dissolving Topotecan Hydrochloride in water at 15-20 mg/ml, counting on the actual Topotecan HCl content. The pH was adjusted to 3.0 with 1 N HCl. The drug solution was filtered through 0.2 micron polyethersulfone (PES) sterile filter using positive pressure. Aliquots of the TEA-Pn or TEA-SOS-containing liposomes in the drug-loading buffer were mixed at room temperature with the stock Topotecan HCl solution to achieve the drug/lipid input ratio in the range of 0.15-0.45 g/mmol of liposome phospholipid. Preferred ratio was 0.35 g of Topotecan HCl for mmol of liposome phospholipid. The mixtures in glass containers were incubated on the thermostatted water bath at 55-62°C with slow agitation for 30-60 min, quickly cooled down in ice-water bath (0-2°C) and left at this temperature for 5-15 min. This step resulted in the encapsulation efficiency of 89-90% (TEA-Pn gradient) or 97-100% (TEA-SOS gradient). Unencapsulated Topotecan was removed, and the liposomes were transferred into the storage buffer using size exclusion column chromatography. Before application on the column, the ionic strength of the liposome preparation was increased by mixing with 1/20 vol. of 2.88 M aqueous sodium chloride, and the mixture was incubated for about 15 min. We unexpectedly found that adjusting the ionic strength of the liposome medium from the low value during the loading (typically equivalent to less than 20 mM NaCl) to

the higher value of above 20 mM NaCl, and preferably to 50 mM NaCl and above, improved the removal of unencapsulated drug and increased the stability of Topotecan-loaded liposomes against aggregation, possibly by facilitating the removal of membrane-bound Topotecan, as opposed to the drug encapsulated in the liposome interior. The rest of the procedure followed Example 11, step 7. For the results, see Table 12 below.

EXAMPLE 19. Preparation of anti-HER2-immunoliposomal formulations of Topotecan.

[0191] Topotecan immunoliposomes specifically internalizable by cancer cells overexpressing HER2 (C-ErbB-2) surface receptor tyrosine kinase oncoprotein were prepared by conjugating Topotecan liposomes to anti-HER2 single chain human Fv antibody fragment, F5, selected from the phage display library for its high internalization into HER2-overexpressing cells (Poul, et al., 2000, J. Molecular Biology, v. 301, p.1149-1161). F5 is a 27-KDa protein that binds to extracellular domain of HER2 receptor with affinity of about 150 nM, causing rapid internalization (Neve, et al., 2001, Biophys. Biochim. Res. Commun. v. 280, p.274-279). For liposome conjugation, the method of U.S. Pat. No. 6,210,707 and of Nielsen, et al. (2002), Biochim. Biophys. Acta, v. 1591, p.109-118, were generally followed. A hydrophilic lipopolymer conjugate of F5 was first prepared. C-terminus of F5 amino acid chain had an added terminal terminal cysteine group (F5Cys). The F5Cys construct was expressed in E.coli and isolated from the bacterial lysate by Protein A column chromatography. Protein A eluted fractions were adsorbed on anion-exchange resin to remove pyrogens and host DNA, and treated with a thiol reducing agent to liberate the thiol group of the terminal cysteine. The reduced F5Cys was further purified by ion exchange chromatography using SP Sepharose Fast Flow (Amersham Pharmacia). The purified protein was conjugated to a thiol-reactive lipid-poly(ethylene glycol) linker, N-(3-(N-maleimido)propyonylamido)-poly(oxyethylene)-oxycarbonyl)-1,2-distearoylphosphatidyl ethanolamine (Mal-PEG-DSPE), a derivative of PEG with mol. weight 2,000 (Figure 4.1), commercially produced by Avanti Polar Lipids, Inc., Alabama, USA. The protein and the linker were incubated in aqueous buffer solution at the molar ratio of 1:4, and the un-reacted linker was quenched with 1 mM cysteine. During the reaction, terminal cysteine of F5Cys is covalently attached to maleimido group of the linker. The resulting F5-PEG-DSPE conjugate was water soluble in the form of micelles having high apparent molecular

weight (500-850 KDa), and was separated from unreacted protein (about 25%) by size exclusion chromatography. The amount of protein in the purified conjugate was determined by UV spectrophotometry at 280 nm, and the amount of the linker was assayed using a spectrophotometric method identical to that used for phospholipid quantification (see Example 70) The purified F5-PEG-DSPE conjugate was stable in water, fully immunoreactive, and was stable against denaturation and loss of reactivity for at least 1 hour at 65°C and at least 3 months at 37°C.

[0192] To prepare anti-HER2 immunoliposomal Topotecan, Topotecan-loaded liposomes of Example 18 were mixed with F5-PEG-DSPE in the aqueous saline buffer at the ratio of 15 microgram of protein per 1 micromole of phospholipid (about 45 F5 copies per liposome). The mixture was incubated for 40 min. at 60°C, chilled on ice, and chromatographed on a column with Sepharose CL-4B (cross-linked 4% agarose beads, Amersham Pharmacia) to remove residual micellar conjugate, unconjugated protein, and any traces of extraliposomal drug that may have been released during the incubation. The liposomes with membrane-incorporated F5-PEG-DSPE were eluted with 5 mM HEPES-144 mM NaCl buffer pH 7.4, collected in the void volume of the column, sterile-filtered and dispensed for storage (4-6°C). The amount of liposome-incorporated F5 was typically >80% of the added conjugate. It was determined by SDS-PAGE of the liposomes with quantification of the Coomassie-stained F5 band by densitometry. Drug and lipid concentrations in the immunoliposome preparations were determined similarly to non-targeted liposomes. The properties of Topotecan liposomes and F5-immunoliposomes (Examples 18-19) are summarized in Table 12.

Table 12. Characteristics of Topotecan liposomes and immunoliposomes.

Liposome-entrapped salt	F5 scFv attachment:	Drug/lipid ratio, g/mol phospholipid		% encapsulation	Liposome size, Mean±SD, nm
		Input	Output		
TEA-Pn	No	173.6	155.2±5.9	89.4±3.4%	96.4±38.7
TEA-Pn	Yes	173.6	156.2±5.2	90.0±3.0%	96.2±33.8
TEA-SOS	No	347.2	340.8±14.7	98.2±4.2%	99.1±32.6

EXAMPLE 20. Effect of loading buffer pH and drug/lipid ratio on the Topotecan loading into liposomes.

[0193] Liposomes (DSPC/Chol/PEG-DSPE, 3:2:0.015 molar ratio) with entrapped 0.5 N TEA-Pn, pH 6.2, osmolality 413 mmol/kg, were prepared using the ethanol injection method (Example 18), extruded through two stacked polycarbonate filters with 100 nm pore size 5 times and with 50 nm pore size 10 times. The loading buffer was 5 mM MES, 50 g/L Dextrose, adjusted to various pHs in the range 5.0-6.5. The liposome size was 73.1 ± 21.3 nm by QELS. The liposomes were loaded by mixing a Topotecan stock solution (20 mg/ml) with the liposomes in the loading buffer at the input drug-to-phospholipid ratio of 100 mg/mmol, incubating the mixture at 60 °C for 45 min, quenching on ice for 15 min and removing the unencapsulated drug using a Sephadex G-75 column eluted with 20 mM HEPES, 135 mM NaCl, pH 6.5. Topotecan and phospholipid were quantified by spectrophotometry (Examples 70 and 71). The results (Table 13) indicated that Topotecan loading was nearly quantitative in the range of pH 5.5-6.5.

Table 13. Effect of loading buffer pH on the % of Topotecan encapsulation into the liposomes with entrapped TEA-Pn.

Loading buffer pH	% encapsulation
5.0	50.1±2.1
5.5	97.2±8.1
6.0	115.5±15.0
6.5	102.1±8.1

[0194] The effect of drug to lipid ratio (0.15-0.45 mg/mmol phospholipid) on the loading efficiency was also studied. The liposomes with entrapped TEA-Pn (0.5 M TEA, pH 5.8, osmolality 480 mmol/kg) were prepared as above, except the final extrusion step was ten times through two stacked 0.08 μm polycarbonate filters. The loading was at pH 6.5. The liposome size was 93.1 ± 15.1 nm by QELS. The results (Table 14) showed that drug loading efficiency was over 85% over the whole range of drug/lipid ratios studied.

Table 14. Effect of drug/lipid ratio on the encapsulation efficiency of Topotecan into the liposomes containing TEA-Pn.

Topotecan/phospholipid ratio, mg/mmol		% encapsulation (mean±SE)
Input ratio	Output ratio (after loading)	
168.2	166.9±11.1	99.2±6.6
224.4	232.5±47.6	103.7±21.2
280.3	253.5±19.8	90.4 7.0
336.4	298.3±18.0	88.7±5.3
392.5	361.2±36.8	92.0±9.4
448.5	394.9±29.5	88.0±6.6

EXAMPLE 21. Topotecan liposome stability *in vitro* in the presence of plasma.

[0195] Liposomes (DSPC/Chol/PEG-DSPE, molar ratio 3:2:0.015) with entrapped 0.5 N TEA-Pn, pH 6.2, osmolality 413 mmol/kg, were prepared as described in Example 18. Liposomes with the size of 96.4 ± 29.3 nm were produced by extrusion ten times through two stacked 100 nm pore size polycarbonate filters. For quantitation of the liposome lipid in plasma, [^{3}H]-CHE was included in the lipid solution at 0.5 μCi/μmol of DSPC. Topotecan was loaded at pH 6.0, 58 °C for 45 min at a drug/phospholipid ratio of 150 mg/mmol. The efficiency of loading was 148.48 ± 10.26 μg Topotecan/μmol phospholipid (99.0 ± 6.8 %).

[0196] The liposomes were incubated with 50% human plasma in a multiwell microdialysis device (Spectra-Por MicroDialyzer 10-well, Spectrum, USA). Human donor plasma was diluted by the equal volume of HEPES-buffered saline (20 mM HEPES, 135 mM NaCl), pH 6.5, containing 0.02% sodium azide and charged into the lower reservoir of the dialyzer (32 mL). The wells (0.4 mL) were separated from the reservoir by a polycarbonate membrane with 30 nm pore size, to afford free passage of plasma proteins and small molecules but not the liposomes. The liposomes were mixed with calculated amounts of plasma and HEPES-buffered saline to achieve the concentration of 2.5 mM phospholipid and 50 vol.% of plasma. The device was incubated at 37°C, and the contents of the reservoir were stirred slowly. After 8 hours of incubation, the contents of the lower reservoir were changed for fresh 50% plasma. At the

given time points (see below) 50-μL aliquots were withdrawn from the wells, and chromatographed on the columns containing 2.2-2.4 mL of Sepharose CL-2B, eluent HEPES-buffered saline to separate the liposomes from plasma proteins and free drug. The liposomes were collected in the void volume fractions. Topotecan was quantified by fluorometry using excitation at 384 nm and emission at 524 nm after solubilization of the plasma samples in 90% aqueous isopropanol-0.1 N HCl, and the lipid was quantified by scintillation counting of [^{3}H]-CHE (quenching corrected). The determined drug-to-lipid ratio at time was compared to the initial ratio prior to incubation to obtain the % of Topotecan that remained encapsulated at each time point. After 8 hours of incubation, the amount of drug remaining in the liposome was about 55% of its initial value (Table 15).

Table 15. *In vitro* release of Topotecan from the liposomes loaded by TEA-Pn gradient in 50% human plasma at 37°C.

Incubation time ,hours	% drug remaining encapsulated
1	95.5±5.4
4	76.8±7.3
8	55.9±4.1
24	55.4±16.8

EXAMPLE 22. Topotecan liposomes with entrapped TEA-Pn gradient at various drug/lipid ratios: *in vivo* drug retention and circulation longevity in mice.

[0197] The liposomes (DSPC/Chol/PEG-DSPE at 3:2:0.015 molar ratio, containing [^{3}H]-CHE at 0.5 mCi/mmol DSPC) with encapsulated gradient-forming salt solution (0.5 N TEA-Pn, pH 6.2, osmolality 413 mmol/kg) were prepared as in Example 18 using extrusion 12 times through two stacked 100 nm pore size polycarbonate filters. The liposome size was 107.7 ± 19.1 nm by QELS. The liposomes in 5 mM HEPES, 50 g/L Dextrose, pH 6.5 were mixed with the aqueous stock solution of Topotecan (20 mg/ml) at drug/phospholipid ratios in the range130-360 μg /μmol, followed my incubating the mixture at 58 °C for 45 min, placing on ice for 15 min and removal of unencapsulated drug by Sephadex G-75 chromatography. Twelve-week old female FvB mice were injected with the liposomes via the tail vein at a dose of 5 mg Topotecan per kg body weight (approx. 0.2 mg Topotecan/animal) in triplicate. At indicated times, typically 8

hours or 24 hours post injection, the mice were anesthetized, exsanguinated, and the blood samples were assayed for the drug and the liposome lipid as in Example 8. The results are shown in Table 16. After 24 hours, about 6-32% of the initial drug load remained encapsulated. Higher loads of the drug (>200 mg/mmol phospholipid) resulted in longer drug retention.

Table 16. *In vivo* drug retention and circulation longevity of prototype Topotecan liposomes loaded using TEA-Pn gradient method to different drug/lipid ratios.

Encapsulated drug/phospholipid ratio, mg/mmol	Lipid remaining in circulation, % injected dose		Topotecan remaining encapsulated, % of initial load	
	After 8 hours	After 24 hours	After 8 hours	After 24 hours
127.2±10.9	36.1±2.0	18.7±8.1	51.7±7.1	6.72±2.5
207.2±21.6	32.1±5.2	9.84±1.88	75.6±13.0	13.8±3.5
301.3±24.5	34.4±3.2	8.04±4.25	79.2±4.2	25.6±4.4
360.3±35.6	33.6±2.4	8.68±4.96	73.5±7.0	32.3±9.8

EXAMPLE 23. *In vivo* drug retention and circulation longevity of Topotecan liposomes loaded using different entrapped ammonium and triethylammonium salts.

[0198] The liposomes composed of DSPE, cholesterol, and PEG-DSPE (3:1:0.1 by weight), also containing [^{3}H]-CHE at 0.22 mCi/mmol DSPE, were prepared as in Example 18, except that the extrusion step included 10 passages through 2 stacked 200-nm pore filters, 10 passages through 2 stacked 100-nm pore filters, and 20 passages through 2 stacked 50-nm pore filters. The liposomes contained the following salt solutions:

[0199] 0.5 N ammonium dextran sulfate solution (A-DS) was prepared from sodium dextran sulfate (M.w. 5000), purchased from Sigma, and converted into ammonium salt by the ion exchange procedure similar to that of Example 4. The solution of dextran sulfuric acid was immediately titrated with 12.4 M aqueous ammonia. The A-DS solution has pH 5.66, osmolality 208 mmol/kg.

[0200] 0.48 N ammonium sucrose octasulfate (A-SOS) was prepared similar to Example 6, but ammonium hydroxide was used for titration. The solution had pH 6.27, osmolality 258 mmol/kg.

[0201] 0.47 M triethylammonium sucrose octasulfate (TEA-SOS) was prepared as in Example 6. The solution has pH 6.6, osmolality 297 mmol/kg.

[0202] Topotecan was loaded into the liposomes in the aqueous solution of 10 mM MES-Na, 50 g/L dextrose, pH 6.5, by incubating the liposomes with the drug at 61-62°C and input drug/phospholipid ratio of 346±1 mg/mmol, for 40 min, followed by incubating on ice for 10 min. The liposomes were purified from unencapsulated drug by chromatography on Sephadex G-25, eluent – aqueous 2 mM Histidine, 144 mM NaCl, pH 6.6 (HCl).

[0203] Seven to nine week old female Swiss Webster mice were injected via the tail vein with these liposomal Topotecan formulations at the dose of 5 mg Topotecan per kg body weight (approx. 0.2 mg Topotecan/animal) in triplicate. After 8 hours or 24 hours post injection the blood was collected and analyzed for Topotecan and liposome lipid as in Example 22.

[0204] The results are presented in Table 17 below. While all three liposome formulations demonstrated very close liposome circulation longevity, having about 23-28% of the injected dose remaining in blood 24 hours post injection, unexpectedly the drug retention in TEA-SOS liposomes and in A-SOS liposomes was better than in A-DS liposomes both in terms of magnitude (about 2-fold improvement in drug retention) and statistical significance (statistical significance at 95% confidence level by 2-tailed non-paired Student's t-test $p=0.0257$ and $p=0.00995$, respectively; and by Mann's U-test the difference was significant with $\alpha=0.01$). Drug retention in TEA-SOS containing Topotecan liposomes was better than in A-SOS containing Topotecan liposomes.

Table 17. *In vivo* drug retention and circulation persistence of Topotecan liposomes prepared using TEA-SOS, ammonium-SOS (A-SOS), and ammonium dextran sulfate (A-DS.

Gradient	Drug/phospho-lipid ratio, mg/mmol	Loading efficiency, %	Liposome size, nm	Lipid remaining in circulation, % injected dose		Topotecan remaining encapsulated, % of initial load	
				After 8 hours	After 24 hours	After 8 hours	After 24 hours
A-DS	288.1±20.6	83.3±6.0	76.9±22.7	43.7±1.2	27.7±1.5	43.6±6.8	18.7±1.5
A-SOS	346.2±14.3	100.0±4.1	99.7±28.9	42.3±2.2	23.4±2.0	53.3±0.8	31.3±3.2
TEA-SOS	340.8±14.7	98.5±4.2	99.1±32.6	42.1±2.3	23.0±2.9	57.0±5.6	38.1±6.1

EXAMPLE 24. Drug and lipid plasma pharmacokinetics of liposomal Topotecan in rats

[0205] The circulation longevity and Topotecan release parameters were assessed in rats. The liposomes (DSPC/Cholesterol/PEG-DSPE molar ratio 3:2:0.015) were prepared by ethanol mixing/extrusion method and loaded with Topotecan using TEA-Pn gradient or TEA-Sucrose octasulfate (TEA-SOS) gradient as described in Example 18 and loaded at various drug/lipid ratios (15-450 mg/mmol phospholipid). For lipid matrix quantification, the liposome lipid contained [3H]-CHE at 0.5-1.5 mCi/mmol DSPC. Female Sprague Dawley rats (6-8 week old; body weight about 200 g) with indwelling central venous catheters were injected i.v. (via the catheter) with the Topotecan liposomes at the dose of 4 - 5 mg/kg of body weight. The catheter was flushed with saline. At selected times (up to 48 hours post injection) the blood samples (0.2-0.3 mL) were drawn via the catheter into heparinized syringes, mixed with 0.4 mL of cold phosphate buffered saline with 0.04% EDTA, blood cells were separated by centrifugation, and the supernatants (PBS-diluted plasma) were assayed for lipid by ^{3}H-CHE radioactivity counting (quenching corrected), and for Topotecan by fluorometry (Example 71). The assay results were corrected for plasma dilution, calculated from the weight of obtained blood sample and assuming a hematocryt of 40%. The total blood dose of the drug and lipid was estimated from the blood volume calculated as 6.5% of the body weight. The percent of Topotecan retained in the liposomes was calculated by comparing the drug/lipid ratio at a given time point to the drug/lipid ratio of the injected liposomes. Table 18 below summarizes blood half-lives of the lipid, the drug, and the half-lives for

drug release, as well as other properties of the liposomes. Pharmacokinetic (PK) curves are shown on Figures 8A (lipid) and 8B (drug/lipid ratio). In summary, the blood PK curves for both drug and lipid fit well to single exponent model (R^2 0.984-0.999). Despite their 90-100 nm size and very small amount of PEGylated lipid (0.3 mol.%), the liposomes unexpectedly showed good circulation longevity (plasma half-lives of the lipid component were in the range of 11-16 hours). The slowest release of Topotecan (half-time 22.9 hours) was observed with the liposomes loaded using the TEA-SOS method.

Table 18. Circulation half-life ($t_{1/2}$) of lipid, drug, and half-time for drug release from the prototype Topotecan liposomes in rats.

Entrapped salt, and concentration	Topotecan load, mg / mmol phospholipid	Liposome size, nm (mean±SD)	Injected dose, mg/kg	$t_{1/2}$ lipid, hours	$t_{1/2}$ drug, hours	$t_{1/2}$ of drug release, hours	No. of animals per group
TEA-Pn 0.5N	124.3±9.7	92.3±23.3	4	15.8	4.13	5.34	3
TEA-Pn 0.5N	360.3±35.6	107.7±19.1	5	12.8	6.06	9.97	2
TEA-SOS 0.643N	439.2±15.9	108.8±13.4	5	10.8	7.36	22.87	2

EXAMPLE 25. Drug stability against leakage during storage of Topotecan liposomes

[0206] The samples of several prototype formulations prepared for the above-described studies, were stored at 4-6°C for various times to assess the storage stability of the encapsulated Topotecan against drug leakage from the liposomes. The liposome samples were passed through Sephadex G-75 columns, eluted with 20 mM HEPES, 135 mM NaCl, pH 6.5, to remove extraliposomal drug, and analyzed for drug content by spectrophotometry and for lipid by [3H]-CHE radioactivity counting. The results (Table 19) indicate good retention of Topotecan in the liposomes during storage.

Table 19. Drug retention in prototype Topotecan liposomes during storage.

Liposome gradient-forming salt	Liposome size, mean±SD, nm	Initial drug load, mg drug/mmol phospholipid	Storage time, months	Drug load after storage as % of initial
TEA-Pn 0.500 N pH 6.2	96.4±29.3	148.5±10.3	8	101.6±5.5
TEA-Pn 0.500 N pH 6.2	107.7±19.1	127.2±10.9	6	94.6±6.2
TEA-Pn 0.500 N pH 6.2	107.7 ±19.1	207.2±21.6	6	113.9±9.4
TEA-Pn 0.500 N pH 6.2	107.7±19.1	301.3±24.5	6	112.9±9.3
TEA-SOS 0.643 N pH 5.6	108.8±13.4	439.2±15.9	2	97.8±9.4

EXAMPLE 26. *In vitro* uptake of liposomal and immunoliposomal Topotecan by HER2-overexpressing cancer cells.

[0207] This study addressed the capacity of Topotecan-loaded anti-HER2-immunoliposomes prepared according to the invention to deliver Topotecan specifically into HER2-overexpressing cells in cell culture. The (immuno)liposomes were prepared and loaded with Topotecan using TEA-Pn method of Example 19. HER-2 overexpressing human breast carcinoma cells (SKBr-3, ATCC) were grown in modified McCoy 5A medium (without tricine) supplemented with 10% fetal calf serum, 50 μg/mL streptomycin sulfate and 50 U/ml penicillin G (complete growth medium) in T-75 flasks at 37°C, 5% CO_2, to confluency. The cells were harvested by trypsinization, inoculated into 24-well cell culture plates at 150,000 cells/well in 0.5 mL of the complete growth medium, and allowed to acclimate overnight. The medium was replaced with 0.5 mL of complete growth medium containing Topotecan formulations at the selected concentration in the range of 0.01-0.1 mM phospholipid. Triplicate wells were used for each condition. Control wells were incubated in the absence of drug and/or liposomes (to obtain background readings for drug assay). The plates were incubated with slow agitation at 37°C, 5% CO_2 for 4-8 hours. The media were aspirated, and the cells were rinsed 4 times with 1 mL portions of cold Hanks' balance salt solution containing Ca and Mg salts. The cells were solubilized by adding 0.1 mL of 1% Triton X-100 in water, and the amount of drug in the cell lysates was determined by fluorometry (Example 71). The standard curve was obtained in the range of 10-2500 ng Topotecan/well, and fit to second order polynomial (to account for self-quenching at higher drug concentration) after

subtracting the cell autofluorescence background. When a microplate fluorometer was used, the filter selection was 400/30 nm for excitation, 530/25 nm for emission. Both cuvette- and microplate fluorometers gave the same results.

[0208] The results of two experiments are summarized in Table 20 below. There was prominent cellular uptake of HER2-targeted liposomal drug (50-300 times higher than of no-targeted liposomal Topotecan). Interestingly, uptake of free Topotecan was also significantly lower than of HER2-targeted immunoliposomal Topotecan. This may be explained by rapid hydrolysis of the camptothecin lactone ring of Topotecan molecule in the cell growth medium in the presence of serum, generating the carboxylate form of the drug which may have lower cell permeability and lower cytotoxicity. In summary, the ability of cell-targeted, internalizable, ligand-conjugated immunoliposomes to deliver Topotecan intracellularly was confirmed.

Table 20. *In vitro* cellular uptake of Topotecan liposomes and anti-HER2 immunoliposomes containing TEA-Pn (nd, not determined). For liposome characteristics see Table 12.

Liposome concentration, mM phospholipid	Topotecan concentration μg/mL	Exposure time, hours	Topotecan uptake by SK-Br-3 cells, ng/100,000 cells		
			Non-targeted liposomes	F5-Immunoliposomes	Free drug
0.1	15.5	4	1.45±0.09	163±5.7	nd
0.01	1.55	4	0.185±0.03	60.2±2.0	nd
0.033	5.0	8	3.62±2.03	169.6±13.7	5.56±0.91

EXAMPLE 27. Cytotoxicity of liposomal and immunoliposomal Topotecan against HER2-Overexpressing Cancer Cells *In Vitro*.

[0209] Once the capacity of anti-HER2 Topotecan immunoliposomes for intracellular drug delivery into HER2-overexpressing cancer cells was established (Example 26), it was important to ensure that the internalized liposomes can release the drug in its active form. To this end, *in vitro* cytotoxicity of the free Topotecan (i.e., Topotecan formulated as a solution), liposomal Topotecan, and anti-HER2-immunoliposomal Topotecan was studied. The liposomal Topotecan formulations were prepared, and SKBr-3 cells were grown and harvested as described in Example 26. The cells were inoculated into 96-well

cell culture plates at 5,000 cells in 0.1 mL of the complete growth medium, in triplicate, and left to acclimate overnight. Edge-most rows and columns of the plate were left empty. Sterile preparations of Topotecan liposomes, immunoliposomes, or free drug (freshly prepared by diluting Topotecan 20 mg/mL stock, pH 3, into unbuffered saline to 2 mg/mL) were diluted with complete drug medium to achieve concentrations starting from 90, 30, or 10 μg/mL and serially diluted down in the medium by the factor of 3. The media in the wells were replaced with 0.2 mL of drug/liposome dilutions , and incubated at 37°C, 5% CO_2, for specified time (4-6 hours). One well in each row was incubated with drug-free medium to serve as a non-treated control. The drug-containing media were aspirated from the wells, the cells were rinsed with 0.2 mL of drug-free medium, and 0.2 mL of fresh drug-free medium was added to all wells. The plates were incubated for 4 days at 37°C, 5% CO_2. Without medium change, 0.03 mL of the 2 mg/mL solution of a tetrazolium dye (Thiazolyl Blue, MTT) (Sigma Chemical Co.) in serum-free medium was added to each well. The plates were incubated for additional 2-3 hours at 37°C, 5% CO_2. The media were aspirated, and the wells were filled with 0.2 mL of 70 vol.% aqueous isopropanol, 0.075 N HCl, and agitated gently until the formazan dye dissolves (15-30 min). The optical density of the formazan solutions was determined using microplate photometer at 540 nm. The cell viability as % of non-treated control was calculated as the ratio of the optical density in the experimental wells to the optical density in the wells containing non-treated cells, corrected for background. The data were plotted against the drug concentration, and the IC50 dose was estimated graphically from intersection of the viability-concentration curve with the 50% viability line.

[0210] The results are presented in Figure 9. The drug dose resulting in 50% growth inhibition (IC_{50}) for free Topotecan or non-targeted liposomal Topotecan was in excess of 30 μg/mL; for F5-Immunoliposomal Topotecan, 0.15 μg/mL. These results are consistent with the targeted drug uptake data.

EXAMPLE 28. Comparative stability and plasma pharmacokinetics of liposomal and F5-immunoliposomal Topotecan in mice.

[0211] Topotecan liposomes containing radioactive lipid label [^{3}H]-CHE at 1.5 mCi/mmol of phospholipid were prepared according to Examples 11 and 19 using an ethanol lipid solution mixing-extrusion procedure under the following conditions: gradient-forming salt solution: 0.643 N triethylammonium sucrose octasulfate; polycarbonate membrane extrusion: 15 passages through 2 stacked PCTE filters, 80 nm pore size; Topotecan loading: drug/phospholipid input ratio 350 mg/mmol (calculated for Topotecan free base); F5 scFv conjugation was performed as described in Example 19. The liposomes had the following characteristics:

[0212] Size by QELS: weight average 101.2 nm; standard deviation, 20.1 nm.

[0213] Drug encapsulation: Topotecan liposomes (Topo-Ls) 359.3±27.4 mg/mmol phospholipid; Topotecan F5scFv-immunoliposomes (Topo-F5-ILs) 326.3±15.9 mg/mmol phospholipid.

[0214] The study was performed generally as in Example 22. The groups of nine male Swiss Webster mice (8-10 week old, 24-27 g) were injected via tail vein with Topo-Ls, Topo-F5ILs, or freshly prepared Topotecan 1 mg/mL in unbuffered saline, at the dose of 5 mg Topotecan base per kg of the body weight (equivalent to the lipid dose of 14-16 μmol of phospholipid/kg body weight). At 1 hour, 8 hour, or 24 hour post injection time points, 3 animals per time point were exsanguinated via open heart punction under Ketamine/Xylazine anesthesia, the blood was collected into tubes containing PBS-EDTA, and assayed for Topotecan (fluorometry) and liposome lipid (by radioactivity scintillation counting). The amounts of drug and lipid dose remaining in the blood at given time points were calculated from the administered dose being taken as 100%, assuming the blood amount per animal as 6.3% of the body weight, and packed blood cell fraction of 45%. The amount of drug remaining encapsulated in the liposomes at each time point was calculated for each animal individually by comparing drug/lipid radioactivity ratio of the plasma samples with that of the injected liposomes. The amount of free Topotecan in the plasma samples collected at 1 hour post injection was less than 1% of the injected dose (indeed, they were below the detection limit of our assay method); therefore, further time points of the free Topotecan group were not studied. Because of the fast blood clearance

and low blood levels of free Topotecan we assumed that essentially all Topotecan found in the blood at all time points represents liposomally encapsulated Topotecan.

[0215] The results are summarized in Table 21 below. Remarkably, the liposomes prepared according to the invention retained 79-85% of the original drug load even 24 hours after injection into the bloodstream of the animals. The differences between average plasma values of the lipid or drug between the liposome and immunoliposome groups were in the range of 1.8-13.6% and were close to, or within the range of, assay errors. Probabilities of the null hypothesis between the liposome and immunoliposome group with regard to drug or lipid values at each time point, calculated using Student's t-test, were in the range of 0.543-0.938. We conclude that the differences in residual blood levels of the drug or lipid between the two preparations were negligible and statistically indistinguishable.

Table 21. The amounts of liposome lipid, Topotecan, and of Topotecan remaining encapsulated in the liposomes in the plasma of mice at various time points post i.v. injection.

Time post injection	Lipid, % of injected dose	Drug, % of injected dose	Drug/Lipid, % of pre-injection value
F5-conjugated liposomal Topotecan (Topo-F5ILs):			
1 hour	57.58±4.95	55.45±7.23	96.14±7.32
8 hours	35.37±3.84	34.18±5.87	96.31±11.92
24 hours	15.51±11.84	12.30±9.02	79.36±8.03
Liposomal Topotecan (unconjugated) (Topo-Ls):			
1 hour	58.88±9.51	57.63±9.45	97.90±5.29
8 hours	39.61±1.99	38.82±1.49	98.06±4.44
24 hours	15.84±3.85	13.45±2.64	85.25±7.03

EXAMPLE 29. Antitumor efficacy of liposomal and anti-HER2-immunoliposomal Topotecan in BT-474 xenograft model.

[0216] In this study we used the first prototype Topotecan immunoliposomes that use triethylammonium-polyphosphate gradient for drug entrapment. The liposomes were

prepared generally following the methods of Examples 11 and 19. Lipid matrix components - DSPC (Avanti Polar Lipids; 3 mol. parts), Cholesterol (Calbiochem, 98.3%; 2 mol. parts) and methoxy-PEG(2000)-DSPE (Avanti Polar Lipids, 0.015 mol. parts) - were combined with 100% ethanol USP to give the solution containing 0.5 mM phospholipid at 60°C. The ethanol lipid solution was diluted at 60°C with the aqueous triethylammonium polyphosphate solution (0.608 M triethylamine, 0.65 N phosphate, pH 6.1, osmolality 531 mmol/kg), mixed thoroughly, and extruded 10 times through 2 stacked polycarbonate membranes with the pore size of 100 nm (Nuclepore, Corning) using thermostatted gas-pressure extruder (Lipex Biomembranes) at 60°C. The extruded liposomes were chilled on ice, and unencapsulated triethylammonium polyphosphate was removed by gel chromatography on Sepharose CL-4B using 5% dextrose-5 mM HEPES-Na buffer, pH 6.5, as eluent. The liposome size was 103.8±35.1 nm by QELS. The liposomes in this buffer were incubated with Topotecan hydrochloride at 60°C for 30 min. at the ratio of 0.35 mg Topotecan base per μmol of phospholipid. At the end of incubation, the liposomes were chilled on ice and chromatographed on Sephadex G-75, eluent 20 mM HEPES-Na, 135 mM NaCl, pH 6.5, to remove any unencapsulated drug. The drug content was determined by fluorometry, and the lipid content by phosphate assay as previously reported. Liposomal Topotecan so obtained has 365.4±23.1 mg Topotecan base per mmol of phospholipid. To prepare HER2-targeted Topotecan immunoliposomes, a portion of this liposomal Topotecan preparation was incubated with the purified conjugate of anti-HER2 scFv F5 and maleimido-PEG-DSPE linker generally as described in Example 19. Briefly, F5-PEG-DSPE conjugate in aqueous 10% sucrose-10 mM Na citrate solution, pH 6.5, was combined with Topotecan liposomes at the ratio of 15 mg protein per mmol of liposome phospholipid, and incubated at 60°C for 30 min. The incubation mixture was chilled on ice and chromatographed on Sepharose CL-4B, eluent 20 mM HEPES-Na, 135 mM NaCl, pH 6.5, to remove any unincorporated scFv conjugate. The drug-to-lipid ratio decreased by 14 % following this additional incubation.

[0217] The Topotecan liposome and immunoliposome formulations containing 1-2 mg/mL of Topotecan were passed through 0.2 micron sterile syringe filter, dispensed aseptically into polypropylene vials and stored at 4-6°C for up to 1 month before use.

[0218] Free Topotecan was freshly prepared by dissolving Topotecan Hydrochloride powder at 2 mg/mL in 5% dextrose and sterilized by passage through 0.2-micron syringe filter.

[0219] A HER2-overexpressing BT-474 human breast adenocarcinoma xenograft model was established as described in Example 10. At day 13 post tumor inoculation, the animals having tumors in the range of 120-350 cubic mm were selected and randomized into 3 treatment and 1 control group of 12 animals each. At days 14, 18, and 21 post tumor inoculation the mice were treated with i.v. (tail vein) injections of Topotecan formulations at the per injection dose of 5 mg/kg body weight, or with equal volume of physiological saline. General health of the animals was monitored daily. Tumor sizes and body weights were monitored twice weekly for up to day 53 post tumor inoculation. The animals whose tumors reached 20% of the body weight, or those with progressive weight loss reaching 20% or more were euthanized.

[0220] Figures 11 and 12 show the tumor growth and animal body weight data, respectively. Liposomal Topotecan formulations were more active in tumor growth suppression than the free drug, and F5-targeted liposomal formulation was more active than the non-targeted one. The average tumor sizes at the end of the observation period were significantly different among the treatment groups (p values by non-paired 2-tailed Student's t-test were 1.2×10^{-6} for free v. immunoliposomal drug, 0.000114 for free v. liposomal drug, and 0.00718 for liposomal v. immunoliposomal drug). Thus, liposomally encapsulated Topotecan was more active than the free drug, and anti-HER2 immunoliposomal Topotecan was more active than non-targeted liposomal drug. In the liposomal and immunoliposomal group, after initial regression, tumor regrowth occurred within 10 days of the last treatment. There was no tumor regression in the free drug group. It was noticed that the liposomal formulations of Topotecan at a given dose were more toxic than the free drug. There was gastrointestinal toxicity. The animals receiving liposomal Topotecan developed diarrhea and suffered body weight loss averaging about 14% at its peak. While in the non-targeted liposomal group the animals recovered, except one (12.5%) that had persistent 15% weight loss at the end of study, in the F5-targeted group five animals (41.6%) developed terminal morbidity and expired; and two more (16.7%) had persistent weight loss of about 15%. In the control group and free drug group, there was no weight loss or treatment-related morbidity.

EXAMPLE 30. Maximum tolerated dose (MTD) of free and liposomal Topotecan in mice given in 3 weekly i.v. injections.

[0221] This study used a liposome Topotecan formulation prepared as in to Example 29, except the triethylammonium polyphosphate solution was replaced with triethylammonium sucrose octasulfate solution having 0.65 M triethylammonium, pH 6.2; and for extrusion 80-nm polycarbonate membrane filters were used instead of 100-nm. Volume-weighted liposome size determined by quasi-elastic light scattering method in Gaussian approximation (QELS) was 95.1±19.6 nm (average±SD); drug/lipid ratio was 369.1±18.3 mg/mmol phospholipid. Five-six week old female Swiss-Webster mice (18-20 g) in the groups of two received three i.v. (tail vein) injections of free or liposomal Topotecan on a once-a-week schedule, starting from the dose of 2 mg/kg Topotecan base per injection and increasing to each subsequent group by the factor of 1.8 to the dose of 37.8 mg/kg. Immunoliposomal Topotecan was not included in this study. Animal body weight and general health was monitored daily. Progressive weight loss of more than 20% or natural death at any time in any of two animals in a group during the period of ten days since the beginning of treatment were considered indicative of the toxic dose. According to the animal mortality and weight data MTD was determined to fall within the range of 11.7-21 mg/kg for free Topotecan, and 2.0-3.6 mg/kg for liposomal (Prototype 2) Topotecan. In the second study, the mice received injections of the free, liposomal, or F5-immunoliposomal Topotecan (prepared from the liposomal Topotecan of this Example as described in Example 29) with the doses from 2.0 mg/kg (liposomal/immunoliposomal Topotecan) or 12 mg/kg (free Topotecan), and increased to each subsequent group by the factor of 1.15 until the dose next to the upper range of the established MTD interval was achieved. The highest dose that did not result in death or terminal morbidity in any of the animals was considered an MTD and was found to be 18.4 mg/kg for free Topotecan, 3.0 mg/kg for liposomal Topotecan, and 3.0 mg/kg for immunoliposomal Topotecan. Thus, liposomal Topotecan showed greater toxicity than the free drug.

EXAMPLE 31. Antitumor efficacy of liposomal Topotecan in BT-474 xenograft model at the range of 0.125-1.0xMTD

[0222] The Topotecan liposomes and F5-immunoliposomes of Example 30 were used in this study. BT-474 subcutaneous xenografts were raised in nude mice as in Example 29. At day 18 after tumor cell inoculation the animals with tumors (105-345 cubic mm,

average about 200 cubic mm) were randomized into treatment groups of 6 animals/group, and a control group of 8 animals/group. The animals received free or liposomal Topotecan at 1xMTD, 0.5xMTD, 0.25xMTD, or 0.125xMTD at three i.v. (tail vein) injections at day 19, 23, and 27 post tumor inoculation. The control group received injections of physiological saline. The tumor sizes and animal body weights were monitored as in Example 29. To obtain animal body weight measurements, the tumor weight (calculated from the tumor size assuming tumor density of 1.0) was subtracted from the total animal weight measurements. All drug formulations at MTD showed antitumor activity (Figures 13A-13D). There was no significant difference in efficacy between free and liposomal drug given at their respective MTD or at identical fractions (1/2, 1/4, or 1/8) thereof. Thus, liposome encapsulation of the drug using TEA-SOS gradient resulted in about 6-fold increase in antitumor activity, but also in the similar increase in drug toxicity. Dynamics of animal body weights revealed that all treatments were non-toxic except the treatment with free Topotecan at MTD which showed transient decrease in body weight (about 15% of the pre-treatment value) that later resolved (Figure 14).

EXAMPLE 32. Preparation and targeted *in vitro* cytotoxicity of Topotecan liposomes prepared using triethylammonium sucrooctasulfate entrapment method.

[0223] Liposomal Topotecan was prepared generally following the procedure of Example 18, using the entrapped solution of TEA-SOS having 643 mM TEA, pH 5.7, osmolality 530 mmol/kg, and drug/phospholipid ratio of 170 mg/mmol. The liposomes had 155 mg drug/mmol phospholipid; 90% loading efficiency, and particle size 105 nm. These liposomes were incubated with the micellar solution of F5-PEG-DSPE conjugate at about 30 scFv per liposomes (15 mg antibody/mmol phospholipid) at 60°C for 1 hour generally as described in Example 19. Antibody-conjugated liposomes were separated by SEC using Sepharose CL-4B and formulated into HBS-6.5 HEPES-buffered saline. There was no detectable change in drug/lipid ratio during the attachment of anti-HER2 scFv (F5).

[0224] The uptake of Topotecan formulations by cancer cells was determined as follows. HER2-overexpressing human breast adenocarcinoma cells (SK-Br-3, ATCC HTB-30) were plated into 24-well cell culture plates at 150,000 cells/well and acclimated

overnight. The cells were incubated (in triplicate) with F5-targeted and non-targeted liposomal Topotecan in complete growth medium at liposome concentrations of 0.1 mM and 0.01 mM for 4 hours at 37°C. The cells were rinsed 4 times with Hanks' Balanced Salt Solution, solubilized in 0.1% Triton X-100 - 70% acidified isopropanol mixture 1:10, and the amount of cell-associated Topotecan per well was determined by fluorometry. The results (mean ± standard error) are summarized in Table 22. The targeted liposomes delivered 100-300 times more drug into the targeted cells than nontargeted liposomes.

Table 22. Uptake of liposomal Topotecan by SK-Br-3 breast carcinoma cells.

Formulation	Topotecan uptake at 0.1 mM phospholipid, ng/well	Topotecan uptake at 0.01 mM phospholipid, ng/well
Non-targeted liposome	4.76 ± 0.24	0.607 ± 0.088
HER2-targeted liposome	533.8 ± 13.7	197.0 ± 4.6
Ratio: Targeted/Non-targeted	112.1 ± 8.6	324 ± 55

[0225] Cytotoxicity of these Topotecan formulations against SKBr-3 breast cancer cells was determined as described in Example 27. SKBr-3 cells were inoculated into 96-well plates at 5,000 cells/well, acclimated overnight, and incubated with increasing concentrations (0.004-30 μg/mL) of free, liposomal, or F5-immunoliposomal Topotecan in cell growth medium for 4 hours at 37°C. The drug-containing media were removed and the cells were allowed to grow in the drug-free medium for 72 hours. The quantity of viable cells per well was determined using Thiazolyl Blue(MTT) tetrazolium assay and expressed as % of that of control (non-treated) cells. The results are presented on Figure 10. Topotecan immunoliposomes were more cytotoxic (IC_{50} 0.15-0.5 μg/mL) than non-targeted Topotecan liposomes ($IC_{50} \geq 3.1$. μg/mL) and free Topotecan ($IC_{50} \geq 2.3$ μg/mL)

EXAMPLE 33. *In vivo* stability of Topotecan liposomes of different size.

[0226] The liposomes containing TEA-Pn were prepared as in Example 22 using extrusion 12 times through 100 nm pore size polycarbonate membranes or additionally 12 times through 50 nm pore size polycarbonate membranes. Topotecan (TPT) was added at a ratio of 150 μg /μmol phospholipid.. The loading was completed at 58 °C for 45 min a hot water bath, followed by quenching on ice. The efficiency of loading for the 50-nm- and 100-nm-extruded liposome was 126.80 ± 19.24 μg TPT/μmol PL (84.5 ±

12.8 %) and 148.48 ± 10.26 μg TPT/μmol PL (99.0 ± 6.8 %), respectively. Female Swiss Webster mice in the groups of three were injected intravenously with one of the two formulations of Ls-TPT at a dose of 5 mg TPT/kg. The mice were sacrificed after 6 h and the blood was collected. Plasma was analyzed for TPT and liposome lipid as described in Example 22. The results are presented in Table 23.

Table 23. *In vivo* stability of Ls-TPT of different sizes loaded using TEA-Pn entrapment method.

Liposome size, nm	Drug in plasma, % of injected dose	Liposome lipid in plasma, % of injected dose	Drug/lipid ratio, % of pre-injection value
74.2 ± 21.6	32.93 ± 1.97	45.7 ± 2.2	72.06 ± 5.51
96.4 ± 29.3	33.26 ± 3.56	37.6 ± 5.3	88.41 ± 15.68

EXAMPLE 34. Synthesis and liposome encapsulation of 6-(3-aminopropyl) ellipticine (6-APE).

[0227] 6-(3-aminopropyl)ellipticine was prepared from ellipticine in a two-step method based on the procedure by Werbel et al., *J. Med. Chem.* 1986, v.29, p.1321-1322. 501.4 mg of ellipticine base (NSC 71795) (Aldrich Chemical Co.) was stirred with approximately 100 mg of sodium hydride (Sigma; washed with anhydrous petroleum ether) in 5 ml of dry dimethylformamide (DMF) at room temperature for 30 min. To this mixture, a solution of 678 mg of N-bromopropylphtalimide (Aldrich) in 2 mL of dry DMF was added dropwise. The purple-colored reaction mixture was stirred under argon overnight, treated with 1 mL of water, and poured into 60 ml of water. The mixture was extracted twice with 25 mL of methylene chloride, the extract was dried over anhydrous sodium sulfate, and passed through a layer of neutral alumina. The alumina layer was rinsed twice with 10 mL of methylene chloride and the combined filtrate and rinses were brought to dryness in vacuum. The product was stirred overnight with 20 ml of absolute ethanol and 2 ml of anhydrous hydrazine at room temperature. The obtained slurry was filtered under vacuum, a yellow filtrate was diluted with 50 mL of 0.2 N NaOH and extracted with two portions (75 ml and 50 ml) of chloroform. The chloroform extract was dried over Na_2SO_4 and brought to dryness in vacuum. Crude product (yield 408 mg) was

chromatographed on silica 60 column eluted isocratically with chloroform-methanol mixture (7:3 by volume), saturated with dry trimethylamine. The fractions eluted in a second yellow-colored band, following un-reacted ellipticine, were shown to contain the desired compound in approximately 30% yield. The structure was confirmed by ^{1}H-NMR. TLC: R_f 0.29-0.31 (Silica 60; $CHCl_3$-MeOH 7:3 by volume, saturated with trimethylamine). Ellipticine, R_f 0.81-0.83. The obtained compound was converted into dihydrochloride salt by dissolving in anhydrous ethanol and titration with 6 N HCl solution in dry isopropanol. The orange crystals of 6-APE dihydrochloride (NSC 176328) were filtered out, rinsed with ether, and dried in vacuum. Yield of dihydrochloride 86%.

[0228] The liposomes were prepared by hydration of the neat lipid film of DSPC, Cholesterol, and PEG(M.w. 2,000)-DSPE (3:2:0.015 molar ratio) in a solution of trimethylammonium polyphosphate (TMA-Pn) at 0.5 M TMA, pH 5.6, at 60°C, followed by six cycles of rapid freezing (-78°C) and thawing (60°C), and extrusion ten times through two stacked 50-nm pore size polycarbonate filters. Unencapsulated TMA-Pn was removed using a Sepharose CL-4B column eluted with HEPES-Dextrose (5 mM HEPES, 5 % Dextrose, pH 5.5). The liposome size was 85.7 ± 32.1 nm.

[0229] Concentrated 6-APE solution (10 mg/ml) was added to the TMA-Pn-containing liposomes at a drug-to-phospholipid ratio of 100 µg APE/µmol phospholipid, the mixture was incubated at 58 °C for 45 min, and quickly cooled down on ice for 15 min. Unencapsulated drug was removed by gel chromatography on a Sephadex G-75 column eluted with HEPES-Dextrose buffer (5 mM HEPES-Na, 5 % dextrose, pH 6.5). Liposome-entrapped APE was then quantitated by spectrophotometry as in Example 71, and liposome phospholipid was determined using the extraction assay of Example 70. The drug encapsulation was practically quantitative.

EXAMPLE 35. Preparation of HER2-targeted immunoliposomal 6-APE and cytotoxicity of 6-APE formulations against HER2-overexpressing BT-474 breast cancer cells *in vitro*.

[0230] Liposomes with encapsulated 6-APE (Ls-APE) were prepared as in Example 34 above. Anti-HER2 immunoliposomes with encapsulated 6-APE (F5-ILs-APE) were prepared from Ls-APE by the method of Example 19. An MTT-based cell viability assay of Example 27 was used to determine the cytotoxicity of 6-APE delivered as a solution, Ls-APE, or as HER2-targeted F5-ILs-APE against HER2-overexpressing human breast

carcinoma cells (BT-474). The cells were exposed to drug-containing media for 6 hours, and post-incubated in drug-free medium for 3 days. The results are shown on Figure 15. The IC_{50} for free APE is 0.26 μg APE/ml, for F5-ILs-APE was 0.756 μg APE/ml, and for nontargeted Ls-APE was 51.0 μg APE/ml. There was a 67.5 fold difference in activity between targeted and nontargeted liposomal 6-APE, indicating a considerable targeted delivery effect.

EXAMPLE 36. EGFR-targeted immunoliposomal formulations of 6-APE and cytotoxicity against cancer cells *in vitro*.

[0231] 6-APE-loaded liposomes were prepared as described in Example 34. EGFR-targeted immunoliposomes were prepared by attachment of EGFR-specific Fab' antibody fragments as follows. An EGFR-specific IgG MAb C225 (cetuximab, ERBITUX™, Imclone Systems) was digested with pepsin to produce $(Fab')_2$ fragments. Purified $(Fab')_2$ fragments were reduced by treatment with 10-20 mM 2-mercaptoethylamine for 15 min at 37 °C, and Fab' fragments were purified by gel filtration using Sephadex G-25. The presence of reactive thiol groups was typically about 0.9 thiol groups per protein molecule (quantified using Ellmann's reagent). C225Fab' were covalently conjugated to an amphiphilic linker Mal-PEG-DSPE (Avanti Polar Lipids, AL) in aqueous solution at pH 6.2-6.5 and protein-linker molar ratio of 1:4 for 2-4 hours at room temperature, or overnight at 4-6°C, to produce C225Fab'-PEG-DSPE conjugate with the yield 30-50 % of the protein. This micelle-forming conjugate was separated from non-reacted protein by size exclusion column chromatography on 3% agarose - 4% polyacrylamide beaded gel (Ultrogel AcA34, obtained from Sigma Chemical Co.), eluted with HBS-6.5 buffer. The conjugate was recovered in void volume fractions. Immunoliposomal 6-APE was formed by incubating these liposomes with C225 Fab'-PEG-DSPE with drug-loaded liposomes at the ratio of 30 mg C225 protein/mmol liposome phospholipid for 30 min at 60°C, quenching on ice for 15 min, and purifying the immunoliposomes by gel chromatography on a Sepharose CL-4B column also eluted with HBS-6.5 buffer (the liposomes appear in or near the void volume of the column).

[0232] MDA-MB-468 EGFR-overexpressing human breast cancer cells and MCF-7 human breast cancer cells with low EGFR expression (ATCC, Rockville, MD) were cultured in their supplier-recommended growth media, and the cytotoxicity of free, liposomal, and anti-EGFR-immunoliposomal 6-APE against these cells was studied

according to the method of Example 27 The cells were incubated with drug-containing media for 6 hours, followed by 3 days post-incubation in the drug-free medium. The results are shown in Figure 16. In MDA-MB-468 cells IC_{50} for the free 6-APE was about 0.1 μg/ml, and for C225-ILs-APE about 0.9 μg/ml. In MCF-7 cells IC_{50} was about 0.1 for the free 6-APE was about 0.5 μg/ml , and for C225-ILs-APE about 14 μg/ml. IC_{50} of Ls-APE in both cell lines was >30 μg/ml. Thus, EGFR-targeted 6-APE-loaded immunoliposomes demonstrated antigen-specific cytotoxic activity in EGFR-overexpressing MDA-MB-468 breast cancer cells, but not in MCF-7 breast cancer cell that do not overexpress EGFR. In MCF-7 cells, the targeted and nontargeted 6-APE liposomes were equally active.

EXAMPLE 37. Pharmacokinetics of liposomal 6-APE in rats.

[0233] Liposomes with entrapped TEA-Pn solution (557 mM phosphate groups, 500 mM TEA, pH 5.8, osmolality 480 mmol/kg) and lipid composition of DSPC, cholesterol, and PEG-DSPE (molar ratio 3:2:0.015) were prepared as in Example 11 above. Ethanolic solution of the lipids was combined at 60°C with 10 volumes of the aqueous TEA-Pn solution, and extruded ten times through two stacked 80 nm pore size polycarbonate membranes. Unencapsulated TEA-Pn was removed using a Sepharose CL-4B column eluted with MES-Dextrose (5 mM MES-Na, 5 % Dextrose, pH 5.5). The liposome size was 92.3 ± 23.3 nm by QELS. A non-exchangeable radioactive lipid label [^{3}H]-CHE was included in the lipid matrix at 0.5 mCi/mmol phospholipid. The liposomes were loaded with 6-APE as described in Example 34.

[0234] The pharmacokinetic study followed the protocol of Example 9. Female Sim Albino rats (9 weeks, 200 g) were injected i.v. at a dose of 10 mg 6-APE/kg. Blood was drawn at prescribed time points and the plasma was analyzed for 6-APE by fluorometry. Plasma aliquots (0.05-0.2 ml) were mixed with 1-2 mL of 90% aqueous isopropanol-0.1 N HCl, and the 6-APE was quantified by fluorescence as in Example 71. The lipid was quantified by [^{3}H]-CHE radioactivity scintillation counting.

[0235] The results are shown in Figure17. The blood half-life ($t_{1/2}$) of the drug was 13.7 hours and of the liposome lipid 16.6 hours (panel A). The half-life of the drug release from liposomes was 77.9 hours, demonstrating remarkable encapsulation stability (panel B).

EXAMPLE 38. Synthesis and liposomal encapsulation of 2-(2-(N,N-diethylamino)ethyl)ellipticinium (2-DAE).

[0236] 2-(2-(N,N-diethylamino)ethyl-ellipticinium chloride (NSC 359449) is an anti-cancer ellipticine derivative which is prepared by alkylation of ellipticine with 2-(N,N-diethylamino)ethylchloride in methanol in the presence of triethylamine (see Werbel, L.M., Angelo, M., Fry, D.M., and Worth, D.F. *J. Med. Chem.* 1986, **29**:1321-1322). Liposomes containing entrapped TEA-Pn were prepared as described in Example 37. 2-DAE.2HCl was incubated with the TEA-Pn liposomes in 5 mM HEPES-Na, 5% Dextrose, pH 7.4, at a 2-DAE-to-phosholipid ratio of 100 µg/µmol. The amount of loaded drug was 88.2 µg APE/µmol PL (efficiency 88.2 %).

EXAMPLE 39. Pharmacokinetics of liposomal 2-DAE in rats.

[0237] Blood pharmacokinetics of liposomal 2-DAE (Example 38) was studied in rats as in Example 37. The $t_{1/2}$ of 2-DAE was 17.8 h and of the liposome lipid matrix, 18.2 h (A). The half-life of the drug release from liposomes in the blood was $t_{1/2}$ = 677 h (B). Thus, these liposomes were extraordinarily stable against drug leakage in the bloodstream.

EXAMPLE 40. Loading of vinorelbine into liposomes using TEA-Pn method. The effect of pH.

[0238] The liposomes were prepared by the ethanol injection method as in Example 11 using TEA-Pn solution of 0.608 M TEA, 0.65 M phosphate groups, pH 6.1, and osmolality 531 mmol/kg, and lipid suspension extrusion 15 times through two stacked 100 nm pore size polycarbonate membranes. The resulting liposome size was 108.3 ± 17.1 nm by QELS Vinorelbine (VRB) in the form of stock solution of vinorelbine bitartrate 10 mg/mL USP was added to the liposomes in aqueous 5 mM HEPES-Na, 5% dextrose, pH 6.5, at a drug-to-phospholipid ratio of 350 µg/µmol, the pH was adjusted to the desired value using 1-5 N NaOH. and the mixture was incubated at 58±2 °C for 30 min. The mixture was then chilled on ice for 15 min, and unencapsulated drug was removed by Sephadex G-75 gel filtration chromatography, eluting with HBS-6.5 buffer (20 mM HEPES-Na, 135 mM NaCl, pH 6.5). Aliquots of purified liposomes were then solubilized in acid isopropanol and analyzed for vinorelbine using spectrophotometry at 270 nm. Liposome phospholipid was quantified using the phosphate assay of Bartlett (1959) after methanol-chloroform extraction.

[0239] The calculated drug-to-lipid ratios after loading were as shown in Table 24. Vinorelbine loading was quantitative *(i.e.* practically 100%) and independent of pH in the studied range.

Table 24. Vinorelbine loading into liposomes with entrapped TEA-Pn at various pH values of external buffer

pH	Drug-to-phospholipid ratio (μg/μmol)	Loading efficiency (%)
4.5	351.2 ± 52.88	100.4 ± 15.2
5.0	347.6 ± 6.35	99.3 ± 1.8
5.75	355.2± 11.2	101.5 ± 3.2
6.25	377.0 ± 21.5	107.7 ± 6.6
7.0	374.3 ± 29.58	106.9 ± 9.0

EXAMPLE 41 Liposomal vinorelbine prepared by TEA-Pn method at various drug/lipid ratios: encapsulation efficiency and *in vivo* stability in mice.

[0240] Liposomes with entrapped TEA-Pn solution were prepared according to Example 40 except that [^{3}H]-CHE was included in the lipid matrix at 1.5 mCi/mmol phospholipid. The liposome size was 98.5 ± 34.3 nm by QELS. The liposomes were mixed with vinorelbine bitartrate USP in aqueous buffer of 5 mM HEPES-Na, 5% dextrose, pH 6.5 at the drug-to-phospholipid ratio of 150-450 mg VRB/mmol, and incubated at 58±2 °C for 30 min. No pH adjustment was made following the addition of the drug. The vinorelbine-loaded liposomes (Ls-VRB) were isolated and analyzed for the drug and phospholipid as in Example 40.

[0241] Female five-six week old Swiss Webster mice (Harlan Bioresearch) in the groups of three were injected intravenously with Ls-VRB-Pn at a dose of 5 mg VRB/kg. The lipid dose varied according to the degree of loading and can be determined from the above calculated drug-to-lipid ratios. At 8 hours or 24 hours post injection, the animals were anesthetized, exsanguinated, and the blood was collected on ice into weighed tubes containing known amounts of PBS with 0.04% EDTA . The blood cells were separated by centrifugation, and the supernatants were analyzed for liposome lipid by [^{3}H]-CHE, radioactivity scintillation counting and for vinorelbine using HPLC as follows. The samples were spiked with vinblastine (internal standard), extracted with diethyl ether,

evaporated, and the residues were dissolved in the mobile phase consisting of aqueous 50 mM triethylammonium acetate (pH 5.5) and acetonitrile (58:42 by volume) The samples were loaded on a C_{18} reverse phase silica column (Supelco C-18 column, 250 mm x 4 mm i.d., particle size of 5 μm) preceded by a C-18 guard column. The column was eluted isocratically with the above mobile phase at a flow rate of 1.0 ml/min. VRB was detected using an absorbance detector at 280 nm. Typical retention times for VRB and vinblastine (internal standard) were 9.1 min and 7.8 min, respectively.

[0242] The results are shown in Table 25. The loading efficiency decreased with the increase in drug/lipid ratio, from practically 100% at 150 mg/mmol to about 66% at 450 mg/mmol. It was noted that the addition of vinorelbine bitartrate at the ratios of over 250 mg vinorelbine per mmol phospholipid caused substantial acidification of the liposome suspension (pH <4.0) that lead to reduced loading efficiency. Thus, the need for pH control during the drug loading step was established. The amounts of liposome matrix detected in the blood after 8 hours were in the range of 30.4 ± 6.6 % of injected dose (%id) to 38.6 ± 5.2 %id without apparent relation to the absolute amount of injected lipid. After 24 hours there was still from 6.4 %ID to 14.8 %ID of the lipid matrix detectable in the blood. The amount of drug that remained encapsulated after 8 hours varied from 37% to 63%. However, as 24 hours post injection the drug levels dropped below detection limit of the employed analytical method.

Table 25. Encapsulation efficiency and *in vivo* drug retention of liposomal vinorelbine prepared at different drug/lipid ratios using TEA-Pn method (without loading buffer pH adjustment). The drug retention data are mean ± SD (N=3).

Vinorelbine/phospholipid ratio			% drug remaining encapsulated at 8 hours post injection
Input, mg/mmol, calculated	Output, mg/mmol, measured	Encapsulation efficiency, %	
150	156	104.0	36.6 ± 4.2
250	238	95.2	56.3 ± 1.3
350	260	74.3	65.9 ± 2.3
450	299	66.4	63.0 ± 4.1

EXAMPLE 42. Vinorelbine loading into liposomes using TEA-SOS method at various drug/lipid ratios.

[0243] TEA-SOS liposomes for drug loading were prepared as in Example 40 except that the TEA-SOS solution with 0.65 M TEA, pH 5.4, osmolality 521 mmol/kg was used instead of TEA-Pn solution, and the liposomes were extruded through 80 nm pore size polycarbonate membranes. The liposome size was 86.6 ± 12.9 nm by QELS. VRB was added to the liposomes in aqueous 5 mM HEPES-Na, 5% Dextrose, pH 6.5, at various drug-to-phospholipid ratios, and the mixture was subsequently incubated at 60 °C for 30 min. The VRB-loaded liposomes were then isolated and analyzed as in Example 40.

[0244] The calculated drug-to-lipid ratios in the VRB liposomes are shown in Table 26. Remarkably, as opposed to polymeric anion assisted loading, vinorelbine loading in the liposomes with polyanionized sugar (sucrose octasulfate) was practically quantitative independently of the drug/lipid ratio for up to 450 mg VRB/mmol phospholipid, and only slightly less (88%) at 550 mg VRB/mmol phospholipid.

Table 26. Dependence of vinorelbine loading into liposomes on drug-to-lipid ratio

Vinorelbine/phospholipid ratio, mg/mmol		Loading efficiency (%)
Total	Encapsulated into liposomes	
150	159.9 ± 11.5	106.6 ± 8.1
250	255. ± 12.4	102.2 ± 5.1
350	381.8 ± 16.3	109.1 ± 5.1
450	456.1 ± 29.5	101.4 ± 6.6
550	486.2 ± 26.0	88.4 ± 4.2

EXAMPLE 43. Preparation of HER2-targeted immunoliposomes loaded with vinorelbine by TEA-Pn method, and comparative blood pharmacokinetics of HER2-targeted and nontargeted vinorelbine liposomes in rats.

[0245] Anti-HER2 scFv F5-PEG-DSPE conjugate was prepared as in Example 19. HER2-targeted vinorelbine immunoliposomes were prepared by incubation of non-targeted vinorelbine liposomes (Example 41, loaded at drug/phospholipid ratio of 350 mg/mmol) with F5-PEG-DSPE conjugate (Example 19) in aqueous 20 mM HEPES-Na, 135 mM NaCl, pH 6.5 buffer at the protein/phospholipid ratio of 15 mg/mmol at 60 °C

for 30 min. Unincorporated F5 conjugate was removed by gel chromatography on a Sepharose 4B column eluted with the same buffer. Non-targeted liposomes (Ls-Pn-VRB) and HER2-targeted ones (F5-ILs-Pn-VRB) were administered i.v. to female Albino rats (8-9 weeks old; 200 g) at a dose of 5 mg VRB/kg. At various time points, blood was collected as described in Example 9, and analyzed for VRB and the liposome lipid as in Example 41. Blood half-life of the liposome lipids and the 50% drug release time were calculated from the lipid concentration-time plots or by drug/lipid ratio-time plots, respectively, by finding best fit to monoexponential kinetics using the MICROSOFT EXCEL (Microsoft Corp.) spreadsheet TREND function. The results (Figure18) indicated that both targeted and non-targeted vinorelbine liposomes had identical drug and lipid pharmacokinetics with lipid half-life of about 12.1 hours and 50% drug release time of about 4.3 hours.

EXAMPLE 44. Preparation and comparative in vivo stability of vinorelbine liposomes prepared using ammonium and substituted ammonium salts.

[0246] Ammonium dextran sulfate (DS-A) solution with pH 5.8, 0. 65 M NH_4^+, osmolality of 390 mmol/kg, and triethylammonium dextran sulfate solution (DS-TEA)with pH 6.0, 0. 65 M NH_4^+, osmolality 465 mmol/kg, were prepared from Dextran sulfate with mol. weight 10,000 (Sigma Chemical Co.) according to the method of Example 4, using titration with 12.4 M aqueous ammonia or neat triethylamine, respectively. Ammonium sulfate (S-A) aqueous solution 325 mM, pH 5.1, osmolality 703 mmol/kg, was prepared from analytical grade ammonium sulfate. All three solutions contained less than 1 % Na^+ of the total cation content. Liposomes entrapping these solutions were prepared using the ethanol mixing-extrusion method of Example 41 (DSPC/Cholesterol/PEG-DSPE 3:2:0.015 molar ratio). Radioactive lipid label [^{3}H]-CHE was included in the lipid matrix at 1.5 mCi/mmol phospholipid. Extrusion step consisted of 10 passages through two stacked 0.1 μm polycarbonate membranes. VRB was added to the liposomes in 5 mM HEPES-Na, 5% Dextrose, pH 6.5, at a drug-to-phospholipid ratio of 350 mg/mmol, the pH was adjusted to 6.5 using 1 N NaOH, and the mixture was incubated at 58-60C °C for 30 min. The reaction was then chilled on ice for 15 min, and unencapsulated drug was removed using Sephadex G-75 gel filtration chromatography, eluting with aqueous 20 mM HEPES-Na, 135 mM NaCl, pH 6.5. The purified, vinorelbine-loaded liposomes were analyzed for VRB spectrophotometrically and for

phospholipid using the phosphate assay of Bartlett (1959) (see Examples 70, 71). Blood pharmacokinetics of the liposomal lipid and drug was studied in rats as in Example 43.

[0247] The results are shown in Figures 19-20, and in Table 27. Liposomes loaded with triethylammonium dextransulfate were compared with those loaded using ammonium salt of dextran sulfate. Unexpectedly, those loaded using the triethylammonium salt were considerably more stable than those loaded using ammonium salt. The pharmacokinetics of the liposomal carrier itself was similar with the three different formulations and was thus primarily dependent on the lipid composition employed. Leakage of vinorelbine from Ls-VRB loaded using triethylammonium dextran sulfate was about three times slower than from those loaded using ammonium dextransulfate. The liposomes loaded using ammonium sulfate had the fastest drug leakage rate.

Table 27. Comparative *in vivo* stability of drug encapsulation into liposomes using entrapped ammonium and substituted ammonium salt.

Formulation, liposome-entrapped salt	Liposome size, nm, mean ± SD (by QELS)	Blood half-life of the lipid matrix, hours	Time to 50% drug release in the blood, hours
DS-TEA	120.8 ± 28.5	9.5 ± 3.3	66.3 ± 13.4
DS-A	107.8 ± 15.4	11.2 ± 0.6	22.9 ± 1.7
S-A	114.5. ± 15.6	10.7 ± 0.2	1.77 ± 0.16

EXAMPLE 45. Preparation and *in vivo* stability of vinorelbine loaded liposomes of various size.

[0248] [^{3}H]-CHE-labeled liposomes (1.5 mCi/mmol phospholipid) with entrapped solution of triethylammonium sucrose octasulfate (0.65 M TEA, pH 6.4, osmolality 502 mmol/kg) were prepared by the ethanol mixing-extrusion method of Example 11. The extrusion step contained 15 passages through two stacked polycarbonate membranes with the pore size of 0.05, 0.08, or 0.1 μm. Vinorelbine loading, isolation of vinorelbine liposomes, and liposome characterization followed the method of Example 40. Female Albino rats (8-9 weeks old; 200 g) were used to study liposome *in vivo* stability. Liposome lipid and drug pharmacokinetics was studied in rats as in Example 43.

[0249] The results are shown in Figures 21, 22, and in the Table 28 below. Liposomes extruded through 0.05, 0.08, and 0.1 μm polycarbonate filters were compared and shown to have similar drug and liposomal carrier pharmacokinetics, as well as a similar extent of contents leakage. The drug release from the liposomes in blood was characterized by the 50% release times in the range of approximately 40-80 hours, well above 24 hours.

Table 28. Characterization of vinorelbine liposomes.

Liposome size, nm, mean ± SD (by QELS)	Drug load, mg/mmol phospholipid	Loading efficiency, %	Blood half-life of the lipid matrix, hours	Time to 50% drug release in the blood, hours
87.6 ± 28.1	352.4 ± 13.9	100.7 ± 4.0	14.6 ± 0.7	39.7 ± 3.1
98.5 ± 15.1	322.6 ± 22.7	92.2 ± 6.5	13.0 ± 0.2	47.9 ± 3.8
109.6 ± 24.6	357.0 ± 10.5	102.0 ± 3.0	14.3 ± 0.3	78.0 ± 1.4

EXAMPLE 46 . Preparation of HER2-targeted vinorelbine liposomes using TEA-SOS entrapment method, and pharmacokinetics of HER2 scFv-targeted and non-targeted immunoliposomal vinorelbine in rats.

[0250] Liposomes were prepared, loaded with vinorelbine at the 350 mg/mmol drug-phospholipid ratio, and analyzed as described in Example 43, except that TEA-SOS solution of Example 45 was substituted for TEA-Pn solution. Extrusion step included 15 passages through 0.08 μm pore size polycarbonate filters. The liposome size was 95.0 ± 26.0 nm by QELS. F5scFv-linked anti-HER2 vinorelbine immunoliposomes were prepared from these vinorelbine liposomes, and blood pharmacokinetics of the liposomal lipid and drug of HER2-targeted and nontargeted liposome vinorelbine was studied in rats as described in Example 43. Circulation half-life of the liposome lipid was 11.4 hours and 10.3 hours, and the 50% drug release time was 30.9 hours and 30.3 hours for F5-ILs-VRB and Ls-VRB, respectively. Thus, the lipid and drug pharmacokinetics of Ls-VRB and F5-ILs-VRB was very close, indicating that the introduction of the scFv-PEG-DSPE conjugate neither affected clearance of the carrier itself nor resulted in increased leakage of the drug from the carrier while in the circulation (Figs. 23, 24).

EXAMPLE 47. Preparation and pharmacokinetic properties of vinorelbine liposomes comprising non-ionic lipid derivatives of poly(ethylene glycol).

[0251] Methoxy-PEG (Mol. weight 2,000)-derivative of synthetic C_{20}-ceramide (PEG-ceramide) was obtained from Northern Lipids, Inc., Canada.. Methoxy-PEG(Mol. weight 2,000)-distearoylglycerol (PEG-DSG) (SUNBRIGHT GS20) was from NOF Corp., Japan.

[0252] Liposomes having the lipid composition of DSPC, cholesterol, and PEG-lipid (PEG-ceramide or PEG-DSG) in the molar ratio of 3:2:0.3 and entrapped TEA-SOS solution (0.65 M TEA, pH 6.4, osmolality 502 mmol/kg) were prepared by the ethanol mixing/extrusion method of Example 11. The extrusion step included two passages through two stacked polycarbonate membrane filters 2 times using pore size 0.2 μm and 10 times using 0.08 μm pore size. The liposomes were loaded with vinorelbine at the drug/phospholipid ratio of 350 mg/mmol, characterized by size, drug, and lipid concentration, and their pharmacokinetics was studied in rats as in Example 46. Both formulations showed prolonged circulation time of the lipid matrix and slow release of the drug in vivo, with at least 50% of the drug remaining encapsulated after 24 hour in the blood *in vivo*, as shown in the Table 29 below.

Table 29 Characterization of vinorelbine liposomes with various PEG-lipids.

PEG-lipid	Liposome size, nm, mean ± SD (by QELS)	Drug load, mg/mmol phospholipid	Loading efficiency%	Blood half-life of the lipid matrix, hours	Time to 50% drug release in the blood, hours
PEG-ceramide	103.3 ± 30.9	291.4 ± 18.0	83.26 ± 5.14	14.0	102.7
PEG-DSG	101.3 ± 20.1	359.3 ± 7.2	102.7 ± 2.1	15.1	24.6

[0253] Remarkably, the increased PEGylation of these liposomes (PEG lipid content about 5.7 mol.% of the total lipid) had practically no effect on the liposome blood circulation longevity compared to the similar, size-matched liposomes having low PEGylation of about 0.3 mol. % of total lipid (Example 45, 109.6 nm, $t_{1/2}$=14.3 hours; 98.5 nm, $t_{1/2}$=13.0 hours).

EXAMPLE 48. Preparation of HER2-targeted liposomal vinorelbine and cytotoxicity of free, HER2-targeted, and non-targeted liposomal vinorelbine against MDA-MB-453 cells *in vitro*.

[0254] Vinorelbine-loaded liposomes (Ls-VRB) were prepared as in Example 42 (without [^{3}H]-CHE) using drug loading at pH 6.0 and 350 μg vinorelbine/μmol phospholipid. Anti-HER2 immunoliposomal vinorelbine (F5-ILs-VRB) was formed by incubating these liposomes with F5-PEG-DSPE conjugate as described in Example 19 and 42 above, except that [^{3}H]-CHE was not added. "Free" vinorelbine was prepared by dilution of vinorelbine bitartrate 10 mg/ml solution USP into the cell culture medium.

[0255] MDA-MB-453 are human breast adenocarcinoma cells (American Type Culture Collection, Rockville, MD) that moderately overexpresses HER2 receptor (about $3x10^4$ to $1x10^5$ copies/cell). Cytotoxicity of VRB delivered as the free drug, as nontargeted liposomal vinorelbine, or as HER2-targeted (F5)-immunoliposomal vinorelbine against MDA-MB-453 cells was determined as described in Example 27, except that the cells were plated in 96 well microtiter plates under the supplier-recommended growth conditions (Leibowitz L-15 with 10% fetal calf serum, no CO_2 supplementation) at a density of 10,000 cells/well, and the drug formulations were added in a series of 1:3 stepwise dilutions starting with 0.03-0.1 mg/ml. The cell viability data were plotted against drug concentration (Figure 25) and drug concentrations required to reduce the cell viability to 50% (IC_{50}) were estimated from the graphs. IC_{50} of F5-targeted vinorelbine liposome 0.06 μg/ml) was close to that of the free drug (0.07 μg/ml) and substantially lower than that of non-targeted liposomes (2.2 μg/ml). This represents a 37-fold enhancement in activity as a result of cancer cell-specific targeted delivery of the drug.

EXAMPLE 49. Cytotoxicity of free, HER2-targeted, and non-targeted liposomal vinorelbine against CaLu-3 cells *in vitro*.

[0256] The liposomes and methods of the previous example (Example 48) were used to study cytotoxicity of free vinorelbine, Ls-VRB, and F5-ILs-VRB in HER2-overexpressing human non-small cell lung carcinoma cells CaLu-3 (American Type Culture Collection, Rockville, MD). The cells were grown in RPMI-1460 medium with 10% fetal calf serum in the presence of 5% CO_2. The results are shown in Figure 26. The IC_{50} for free VRB was 1.2 μg/ml, 10 μg/ml for F5-ILs-VRB, and 50 μg/ml for

nontargeted Ls-VRB. This represents a 5-fold enhancement in liposome-encapsulated drug activity as a function of targeted delivery to the cells.

EXAMPLE 50. Cytotoxicity of free, HER2-targeted, and non-targeted liposomal vinorelbine against SKBr-3 cells *in vitro*.

[0257] The liposomes and methods of Example 48 and the were used to study cytotoxicity of free vinorelbine, Ls-VRB, and F5-ILs-VRB in HER2-overexpressing human breast carcinoma cells SKBr-3 (American Type Culture Collection, Rockville, MD), except that the cells were grown in the modified McCoy 5A medium with 10% fetal calf serum in the presence of 5% CO_2, plated at a density of 5,000 cells/well , and the drug was incubated with the cells for 6 h.

[0258] The results are shown in Figure 27. The IC_{50} for free VRB was 0.28 μg/ml, 0.17 μg/ml for F5-ILs-VRB, and 0.8 μg/ml for nontargeted Ls-VRB. This represents a 4.7-fold enhancement in drug activity as a function of targeted delivery.

EXAMPLE 51 *In vivo* antitumor efficacy of liposomal vinorelbine in HT29 human colon cancer xenografts in mice.

[0259] Small unilamellar vesicle liposomes (93.2 ± 26.4 nm by QELS) were prepared from distearoylphosphatidylcholine, cholesterol, and PEG-DSPE (3:2:0.045 molar ratio) by hydration from a concentrated ethanolic solution in an aqueous solution of triethylammonium sucroseoctasulfate (0.6 M triethylammonium, pH 5.7-6.2), followed by repeated extrusion through polycarbonate membranes (100 nm pore size), removal of extraliposomal polyanionic salt, and loading with vinorelbine by incubation with the liposomes in isoosmotic buffer pH 6.5, drug/lipid ratio of 325 mg VRB/mmol phospholipid, at 60 °C as described in Example 42.

[0260] Female BALB/c homozygous nude mice (6-8 weeks, weighing 17-20g) were injected subcutaneously in the flank area with $1x10^6$ of HT-29 human colon carcinoma cells (American Type Culture Collection, Rockville, MD). Starting with day 16 post-tumor inoculation, when the mean tumor diameter reached 5-8 mm, the mice were randomly divided into three groups of six animals each and treated with free or liposomal vinorelbine at a dose of 5 mg/kg through the tail-vein every three days for a total of four injections. For the control group, mice were treated with an equal volume of saline. The tumor size of each mice was measured using a caliper and the tumor volume was

calculated using the formula: (tumor length)x(tumor width)^2/2. To assess treatment-related toxicity, the animals were also weighed twice weekly. Liposomal vinorelbine was shown to be considerably more efficacious in suppressing the growth of HT-29 tumors that free vinorelbine, causing tumors to regress, while in the free drug group the tumors always continued to grow (Figure 28). There was little change in animals' body weight during the course of treatment indicating that the treatment was well tolerated, and that liposomalization did not increase the drug toxicity (Figure 29).

EXAMPLE 52 *In vivo* antitumor efficacy of liposomal vinorelbine against C-26 syngeneic murine colon cancer tumors.

[0261] Liposomal vinorelbine and free vinorelbine were prepared as in Example 48. Male BALB/c mice (6-8 weeks, weighing 17-20g) were inoculated subcutaneously with $2x10^5$ of C-26 murine colon carcinoma cells. At day 17 post-inoculation, when the mean tumor diameter reached 5-8 mm, mice were randomly divided into six treatment groups of five animals/group. The tumor bearing mice were injected through the tail-vein with free vinorelbine at 6 mg/kg, 8 mg/kg, or 12 mg/kg, and with liposomal vinorelbine at 4 mg/kg or 6 mg/kg every three days for a total of four injections. For the control group, mice were injected with equal volume of normal saline. Tumor sizes and animals body weights were followed as in Example 51. Liposomal vinorelbine even at 4 mg/kg, was considerably more efficacious in reducing the tumor growth than free drug at 12 mg/kg (Figure 30), The animal body weights in the course of treatment showed little change (<10% decrease) indicating that the toxicity of liposomal vinorelbine was not increased compared to that of free drug (Figure 31).

EXAMPLE 53 *In vivo* antitumor efficacy of HER2-targeted liposomal vinorelbine against BT-474 human breast cancer xenograft tumors in mice: effect of loading counter-ion.

[0262] VRB-loaded liposomes 99.5 ± 10.2 nm in size were prepared by the TEA-Pn method of Example 41 and TEA-SOS method of Example 42, respectively, except that [^{3}H]-CHE was not added. VRB was loaded at the drug/phospholipid ratio of 350 mg/mmol. HER2-targeted liposomal vinorelbine was formed by incubating these liposomes with F5-PEG-DSPE conjugate (see Example 19) as described in Example 43. BT-474 HER2-overexpressing human breast carcinoma xenografts were raised in homozygous nude mice as in Example 10. At day 25 post tumor cell inoculation, when

the tumors reached about 200 mm^3 in size (range 144-309 mm^3), the mice were randomized into four groups of eight animals/group, and treated i.v. with 5 mg/kg of free VRB, F5-ILs-VRB with Pn as a counter-ion, or F5-ILs-VRB with SOS as a counter-ion, at a dose of 5 mg/kg weekly for a total of three injections. The control group received equal volume of normal saline. The tumors and animal body weights were monitored as in Example 10. HER2-targeted liposomal vinorelbine loaded using sucrose octasulfate was noticeably more efficacious in reducing tumor growth than the same targeted construct loaded using poly(phosphate), and both immunoliposomal preparations were considerably more efficacious than free vinorelbine when administered at a dose of 5 mg VRB/kg (Figure 32). The drug-treated mice demonstrated little change in weight indicating that the treatment was well tolerated (Figure 33).

EXAMPLE 54. *In vivo* antitumor efficacy of HER2-targeted liposomal vinorelbine against BT-474 human breast cancer xenograft tumors in mice: effect of PEGylation.

[0263] The liposomes of DSPC and cholesterol in the molar ratio 3:2 were prepared according to Example 48 by hydration of the lipid matrix of DSPC, cholesterol, and PEG-distearoylglycerol with PEG mol. weight 2,000 (GS-20, NOF Corp., Japan) at a molar ratio 3:2:0.015 ("0.5%PEG") or 3:2:0.3 ("10%PEG") via ethanolic solution method in an aqueous triethylammonium sucroseoctasulfate, followed by membrane extrusion according to Example 48. VRB was loaded into the liposomes at the drug/phospholipid ratio of 350 mg/mmol. F5 immunoliposomal vinorelbine was formed by incubating these liposomes with F5-PEG-DSPE conjugate (Example 19) as described in Example 43. Nude mice with BT-474 xenografts were raised and treated i.v. with free VRB, F5-ILs-VRB-"0.5%PEG", or F5-ILs-VRB-"10%PEG" at 5 mg/kg as in Example 53. As shown in Figure 34 , F5-ILs-VRB with higher PEGylation provided with a non-ionic PEG lipid derivative PEG-DSG was noticeably more efficacious in reducing tumor growth than F5-ILs-VRB with lower amount of PEG-DSG, while both preparations were more active than the free drug.

EXAMPLE 55 *In vivo* antitumor efficacy of EGFR-targeted liposomal vinorelbine against U87 human brain cancer xenograft tumors in mice.

[0264] The liposomes (86.6 ± 12.9 nm in size by QELS) with encapsulated 0.65 M TEA-SOS solution were prepared and loaded with VRB according to Example 42. Anti-

EGFR-immunoliposomal VRB (C225Fab'-ILs-VRB) was prepared by incubation of VRB liposomes with the PEG-DSPE conjugate of an anti-EGFR antibody Fab' fragments as described in Example 36.

[0265] Male NCR *nu/nu* mice (5-6 weeks, weighing 17-20 g) were injected subcutaneously in the flank area with $1x10^7$ of U87 human glioblastoma cells (ATCC) suspended in the growth medium in a total volume of 150 µl. When the tumor reached an average size of 250 mm^3, mice were randomly divided into four groups of 10-12 animals. The mice were treated with three weekly i.v. injections of "free" VRB, nontargeted Ls-VRB, or C225Fab'-ILs-VRB at a dose of 5 mg VRB/kg.. The control group received an equal volume of saline. The tumor sizes and animal body weights were monitored as in Example 10. C225-Fab'-ILs-VRB was noticeably more efficacious in suppressing the growth of EGFR-overexpressing human brain cancer xenograft tumors than either non-targeted liposomal vinorelbine or free vinorelbine at an equal dose (Figure 35).

EXAMPLE 56. Preparation and pharmacokinetics of doxorubicin encapsulated in the liposomes using triethylammonium sulfate method.

[0266] Liposomes with various lipid matrix composition (as indicated in the table below) were formed as described in Example 2. N-Glutaryl-DSPE (Glu-DSPE) was from Avanti Polar Lipids, AL, USA. A neat lipid film was formed from the lipid solution in chloroform using rotary evaporation, trace volatiles were removed under vacuum (90 µm Hg, 2 hours), the lipid film was hydrated in a triethylammonium sulfate (TEA-SO_4) solution (0.65 N TEA), subjected to six cycles of rapid freeze and thaw, and extruded through two stacked 0.1 µm pore size polycarbonate filters ten times and through two stacked 0.05 µm pore size polycarbonate filters ten times. For lipid matrix quantification in the blood samples, [^{3}H]-CHE was included in the lipid matrix at 0.5-1.5 mCi/mmol phospholipid. The liposomes with entrapped TEA-SO_4 solution were loaded with doxorubicin according to Example 2. The liposomes in HEPES-buffered saline (20 mM HEPES-Na, 135 mM NaCl, pH 6.5) were incubated with doxorubicin hydrochloride (drug/phospholipid ratios of 140-170 mg/mmol) at 60 °C for 45 min followed by quenching on ice and removal of unencapsulated doxorubicin by gel chromatography. Doxorubicin was assayed by spectrophotometry (Example 71), and phospholipid was assayed by Bartlett method (Example 70). The properties of resulting liposomes are summarized in Table 30 below.

Table 30. Properties of liposomal doxorubicin at various lipid compositions.

Lipid composition (molar ratio)	Liposome size, nm (mean ± SD by QELS)	drug/phospholipid (mg/mmol)
DSPC/Chol/PEG-DSPE (3:2:0.015)	81.8 ± 27.3	163.6 ± 4.4
DSPC/Chol (3:2)	79.1 ± 27.9	137.0 ± 17.5
DSPC/Chol/Glu-DSPE (2.85:2:0.15)	83.6 ± 27.2	141.7 ± 10.4
DSPC/Chol/PEG-DSPE (2.7:2:0.3)	83.7 ± 23.1	175.0 ± 6.8

[0267] Blood pharmacokinetics of these doxorubicin-containing liposomes having lipid composition of DSPC/Chol/PEG-DSPE 2.7:2:0.3 was studied in rats at a single i.v. dose of 5 mg doxorubicin/kg as described in Example 9. The liposomes were long circulating (half-life of about 28 hours) (Figure 36). The stable doxorubicin-to-phospholipid ratio indicated that the formulation was remarkably stable against the drug leakage in the circulation, losing less than 25 % of the drug over a 48-hour time period.

EXAMPLE 57. Doxorubicin-loaded liposomes and anti-HER2 immunoliposomes prepared by TEA-sulfate method: preparation and in vivo antitumor efficacy against HER2-overexpressing human breast cancer xenografts.

[0268] Doxorubicin-loaded liposomes having various lipid compositions and properties (listed in the table below) were prepared as described in Example 56. Doxorubicin-loaded anti-HER2 immunoliposomes were prepared from doxorubicin-loaded liposomes by co-incubation with anti-HER2 scFv F5-PEG-DSPE conjugate (approx. 30 scFv/liposome) as described in Example 19. NCR *nu/nu* mice bearing the subcutaneous human breast tumor xenograft (BT-474) were raised, treated (in groups of 10-12 animals) with liposomal or anti-HER2 immunoliposomal doxorubicin at a dose of 5 mg/kg once weekly for a total of three weeks once the tumors reached an average size of 200 mm^3, and the tumor progression and animal body weights were monitored as described in Example 29. For non-targeted doxorubicin liposome formulations, the lipid compositions containing no PEG-DSPE, 0.5 mol.% PEG-DSPE, or 10 mol.% PEG-DSPE, were studied; for F5-immunoliposomal doxorubicin, the formulations with 0.5 mol.% PEG-DSPE and 10 mol.% PEG-DSPE were studied (here the quantity of PEG-DSPE is expressed as mol. % of liposome phospholipid). The results (Figure 37, Table 31) demonstrated that all doxorubicin treatments were effective in retarding the tumor growth. On the basis of tumor sizes at day 53 post inoculation, the differences in tumor

growth inhibition among all three non-targeted liposome groups did not raise to statistical significance (ANOVA p=0.081), but the immunoliposome doxorubicin was significantly more efficacious than non-targeted liposomal doxorubicin (ANOVA $p=5.5x10^{-10}$), the "10%PEG-DSPE" formulation being more efficacious than "0.5%PEG-DSPE" (Student's t-test, p=0.027). In the"10%PEG-DSPE" F5-ILs group, the tumors regressed to 1 mm^3 or less in 67% of animals, while in "0.5%PEG-DSPE" F5-ILs group only in 9%. In the control group (saline treatment) the tumors exceeded the acceptable size limit of 15% body weight at day 38-43.

Table 31. Liposomal doxorubicin *in vivo* antitumor efficacy study: liposome characteristics and treatment outcomes.

Lipid composition	Liposome size, nm (mean ± SD)	drug/phospholipid ratio, mg/mmol (mean ± SD)	Average tumor size at day 58, mm^3 (mean ± SEM)
DSPC/Chol/PEG-DSPE (3:2:0.015)	83.4 ± 23.3	136.7 ± 6.7	490 ± 74
DSPC/Chol (3:2)	80.5 ± 26.6	151.2 ± 1.9	587 ± 61
DSPC/Chol/PEG-DSPE (2.7:2:0.3)	81.0 ± 24.7	140.1 ± 4.2	365 ± 60
DSPC/Chol/PEG-DSPE (3:2:0.015) + F5 scFv-PEG-DSPE	not measured	140.7 ± 2.8	119 ± 39
DSPC/Chol/PEG-DSPE (2.7:2:0.3) + F5 scFv-PEG-DSPE	not measured	132.9 ± 2.2	15.5 ± 7.6

EXAMPLE 58 Preparation of liposomal vinblastine and blood pharmacokinetics of liposomal vinblastine in rats.

[0269] Liposomes with entrapped aqueous TEA-SOS solution (0.65 M TEA, pH 6.4, osmolality 502 mmol/kg) and size 99.5 ± 10.2 nm (mean± SD by QELS) were prepared by the method of Example 11 using extrusion 2 times through two stacked 0.2 μm polycarbonate membranes and ten times through two stacked 0.08 μm polycarbonate membranes. Vinblastine (VBL) in the form of vinblastine sulfate USP was added at a drug-to-phospholipid ratio of 150 mg/mmol. The pH of the drug-liposome mixture was adjusted to 6.5 using 1 N NaOH, and the mixture was subsequently incubated at 60 °C for 30 min. The reaction was then cooled on ice for 15 min and unencapsulated drug

removed using Sephadex G-75 gel filtration chromatography, eluting with 5 mM HEPES-Na, 135 mM NaCl, pH 6.5. The purified liposomes were then analyzed for VBL spectrophotometrically and for phospholipid by Bartlett method as in Examples 70 and 71. [^{3}H]-CHE was included in the formulation at a ratio of 1.5 mCi/mmol phospholipid. The liposomal vinblastine had 152.4 ± 12.0 mg VBL/mmol phospholipid (quantitative encapsulation).

[0270] Blood pharmacokinetics of the liposomal vinblastine in female Albino rats (8-9 weeks old; 200 g) at a dose of 5 mg VBL/kg was studied as described in Example 9. Vinblastine was quantified in blood plasma samples as described in Example 41 (using vinorelbine as internal standard). Vinblastine liposomes showed good circulation longevity (plasma half-life of the lipid component 12.8 ± 0.04 hours) (Figure 38) and very good stability against drug leakage from the liposomes with greater than 70 % of the initial vinblastine load remaining encapsulated after 24 h (Figure 39). The post-injection time to achieve release of 50% of the encapsulated drug was found to be 40.6 ± 1.2 hours.

EXAMPLE 59 Preparing liposomes loaded with vincristine using TEA-SOS method and the effect of pH on the loading efficiency.

[0271] Liposomes with the size of 86.6 ± 12.9 nm (by QELS), lipid composition of DSPC/Chol/PEG-DSPE in the molar ratio of 3:2:0.015 and entrapped aqueous TEA-SOS solution (0.65 M TEA, pH 5.4, osmolality 521 mmol/kg) were prepared by the method of Example 11 using extrusion step of 15 passages through two stacked 0.08 μm pore size polycarbonate membranes. Vincristine (VCR) was added to the liposomes in 5 mM HEPES-Na, 5% dextrose aqueous buffer, pH 6.5, as vincristine sulfate at a drug-to-phospholipid ratio of 350 μg vincristine/μmol phospholipid, the pH was adjusted to the indicated ratio using 1 N NaOH, the mixture was incubated at 60 °C for 30 min, chilled on ice for 15 min, and the liposomes were separated from unencapsulated drug using Sephadex G-75 gel filtration chromatography, eluting with HBS-6.5 (20 mM HEPES, 135 mM NaCl, pH 6.5). The purified liposomes were then analyzed for vincristine by spectrophotometry using absorbance at 265 nm after solubilization in acid isopropanol, and for the phospholipid content using the phosphate assay of Bartlett (1959).

[0272] The results are shown below in Table 32. The drug loading was in excess of 90% in the range of pH 4.5-7.5, and practically quantitative at pH 5.0-7.5. At pH 3.5,

which is the pH observed in the liposome mixture after addition of the drug, but without pH adjustment, the loading was considerably lower.

Table 32. pH-Dependence of vincristine loading into liposomes with entrapped TEA-SOS.

pH	Drug/phospholipid ratio, μg/μmol	Loading efficiency (%)
3.5	39.7 ± 4.9	11.3 ± 0.2
4.5	327.2 ± 20.6	93.5 ± 5.4
5.0	360.6 ± 5.8	103.0 ± 1.7
5.5	371.2 ± 30.2	106.1 ± 9.1
6.0	347.7 ± 20.4	99.3 ± 5.8
6.5	347.7 ± 20.9	99.4 ± 5.9
7.0	377.3 ± 22.2	107.8 ± 6.8
7.5	371.5 ± 24.9	106.1 ± 7.6

EXAMPLE 60. Preparing liposomes loaded with vincristine using TEA-SOS method: effect of the drug/lipid ratio on the loading efficiency.

[0273] SOS-TEA-containing liposomes were prepared as in Example 59 and loaded with vincristine sulfate at a drug-to-phospholipid ratio of 150-550 μg vincristine/μmol phospholipid at pH 6.5 according to the procedure of Example 59. The liposomes purified from unencapsulated drug were then analyzed for VCR by spectrophotometry and for the liposome phospholipid using the assay of Bartlett (1959). The drug loading efficiency was in excess of 90% over the whole studied range of drug/lipid ratios, and was practically quantitative between 150-450 μg vincristine/μmol phospholipid (Table 33).

Table 33. Vincristine loading into liposomes containing TEA-SOS at different drug-to-lipid ratios.

Input drug-to-phospholipid (μg/μmol)	Encapsulated drug-to-phospholipid (μg/μmol)	Loading efficiency (%)
150	163.6 ± 6.6	109.0 ± 4.8
250	251.1 ± 17.0	100.5 ± 6.8
350	347.7 ± 20.9	99.4 ± 5.9
450	452.0 ± 18.8	100.4 ± 4.2
550	521.6 ±24.9	94.8 ± 4.3

EXAMPLE 61. Preparing immunoliposomal vincristine and cytotoxicity of liposomal and immunoliposomal vincristine against cancer cells *in vitro*.

[0274] Liposomal vincristine (Ls-VCR) was prepared as described in Example 59 using the drug/phospholipid ratio of 350 mg/mmol. HER2-specific F5-immunoliposomal vincristine (F5-ILs-VCR) was prepared from the liposomal vincristine by co-incubation with anti-HER2 scFv F5-PEG-DSPE conjugate as described in Example 19. "Free" vincristine (VCR) solution was prepared by dilution of vincristine sulfate USP in water, followed by sterile filtration. Cytotoxicity of VCR, Ls-VCR, and F5-ILs-VCR against HER2-overexpressing human breast carcinoma cells SKBr-3 (ATCC) was determined by MTT-based cell viability assay using the procedure of Example 27, wherein the cells were inoculated into 96-well microtiter plates at 5,000 cells/well, acclimated overnight, and incubated with the drug-containing media for 4 hours, followed by post-incubation in a drug-free medium for 3 days. The results are shown on Figure 40. The IC_{50} was 75 ng/ml for free VCR, 11 ng/ml for F5-ILs-VCR, and 3 μg/ml for Ls-VCR. The targeted liposomal vincristine prepared according to the invention was 6.8 times more active than the free drug, and 273 times more active than non-targeted liposomal drug, showing substantial enhancement in anticancer activity as a function of cell-specific drug delivery.

EXAMPLE 62 Blood pharmacokinetics of Ls-VCR in rats.

[0275] Liposomes with entrapped SOS-TEA solution (0.65 M TEA, pH 5.8, osmolality 530 mmol/kg), and lipid composition of DSPC/Chol/PEG-DSPE (molar ratio 3:2:0.015), also containing [^{3}H]-CHE at 1.5 mCi/mmol phospholipid, were prepared by

the method of Example 11 using extrusion step of 10 passages through two stacked polycarbonate membranes with the pore size of 80 nm or 100 nm. The liposomes were loaded with VCR at pH 6.5, drug/phospholipid ratio of 350 mg/mmol, as described in Example 59. The VCR-loaded liposomes were administered i.v. to female albino rats (180-220 g) at a dose of 5 mg VCR/kg, and the blood pharmacokinetics of the drug and the liposome lipid was studied as described in Example 9. The amount of VCR in the blood samples was quantified by HPLC as described in Example 41, except that the volume ratio of aqueous triethylammonium acetate (pH 5.5) and acetonitrile in the mobile phase was 65:35. The typical retention time for VCR was 8.8 min. The results are shown in Figure 41 and Table 34. Both preparations had extensive circulation longevity (blood half-lives of 12-17 hours). Liposomal vincristine was remarkably stable against drug leakage in both preparations (half-release time over 120 hours) (Figure 42).

Table 34. Characteristics of liposomes loaded with vincristine at 350 mg/mmol phospholipid using TEA-SOS method.

Extrusion pore size, nm	Liposome size, nm (mean ± SD)	Drug load, mg/mmol phospholipid	$t_{1/2\beta}$ lipid, hours	$t_{1/2\beta}$ VCR, hours	$t_{1/2}$ VCR release, hours
80	101.2 ± 20.2	347.7 ± 20.93	17.5 ± 1.5	16.0 ± 2.0	>120
100	125.6 ± 32.0	366.8 ± 18.11	12.1 ± 0.7	12.0 ± 0.8	Not detectable

EXAMPLE 63. Blood pharmacokinetics of Ls-VCR in rats at various drug/lipid ratios.

[0276] Liposomes with entrapped SOS-TEA solution (0.65 M TEA, pH 6.4, osmolality 485 mmol/kg), and lipid composition of DSPC/Chol/PEG-DSPE (molar ratio 3:2:0.015) also containing [^{3}H]-CHE at 1.5 mCi/mmol phospholipid were prepared by the method of Example 11 using extrusion step of 10 passages through two stacked polycarbonate membranes with the pore size of 50 nm or 80 nm. The liposomes were loaded with VCR at pH 6.5 as described in Example 59 by adding a stock 20 mg/mL aqueous VCR sulfate solution at the calculated drug/lipid ratios of 100, 200, or 350 mg/mmol phospholipid. The efficiency of drug loading was over 96% for all preparations. The VCR-loaded liposomes were administered i.v. to female albino rats (8-9 week old, 190-220 g) at a dose of 5 mg VCR/kg, and the blood pharmacokinetics of the

drug and the liposome lipid was studied as described in Example 62. The results are shown in Table 35. Liposomal vincristine had good circulation longevity (blood half-life of the drug about 20-30 hours) and was exceptionally stable at all studied sizes and drug-to-lipid ratios (half-life of drug release over 93 hours).

Table 35. Characteristics of liposomes loaded with vincristine using TEA-SOS method at various drug/lipid ratios.

Extrusion pore size, nm	Liposome size, nm (mean ± SD)	VCR, mg/mmol phospholipid		$t_{1/2}$ lipid, hours	$t_{1/2}$ VCR, hours	$t_{1/2}$ drug release, hours
		added	encapsulated			
50	76.8 ± 27.2	100	96.1 ± 3.0	35.6 ± 2.7	30.3 ± 4.0	227 ± 96
		200	193.3 ± 3.9	20.8 ± 2.2	18.4 ± 0.7	244 ± 130
		350	375.2 ± 10.0	24.8 ± 0.9	19.6 ± 0.9	93.2 ± 6.7
80	101.6 ± 25.3	100	104.5 ± 2.1	33.0 ± 7.6	26.8 ± 4.8	153 ± 10

EXAMPLE 64. Preparation of HER2-targeted liposomal vincristine and antitumor efficacy of non-targeted and HER2-targeted liposomal vincristine against HER2-overexpressing human breast cancer xenografts in mice.

[0277] Vincristine-loaded liposomes (Ls-VCR-SOS) using TEA-SOS method were prepared according to Example 63 (with omission of [^{3}H]-CHE component) using 50 nm pore size membrane extrusion and drug loading at drug/phospholipid ratio of 100 mg/mmol. F5 immunoliposomal vincristine (F5-ILs-VCR) was formed by incubating Ls-VCR-SOS with anti-HER2 scFv F5-PEG-DSPE conjugate (Example 19) as described in Example 43. Vincristine-loaded liposomes using TEA-citrate (Ls-VCR-Cit) were prepared similarly to Ls-VCR-SOS liposomes, except that triethylammonium citrate solution (prepared by titrating aqueous citric acid with neat triethylamine to pH 5.1 and adjusting the concentration to 0.65 M triethylamine) was substituted for TEA-SOS solution. The treatment study design followed the method of Example 10. Subcutaneous xenograft tumors of BT-474 human breast carcinoma were raised in nude mice, and when the tumors reached the size of 250 mm^3 (range144-309 mm^3) the mice in the groups of eight to nine, were treated with free VCR, Ls-VCR, or F5-ILs-VCR at a weekly i.v. dose of 2 mg VCR/kg for a total of three weeks, starting at day 19 post tumor inoculation. The tumor sizes and animal body weights were monitored as described in Example 10. For the

control group, mice were treated with an equal volume of saline. The differences in tumor sizes between the treatment groups were statistically assessed at day 63 post tumor inoculation using Mann-Whitney test. The dynamics of average tumor size in the groups is shown in Figure 43. F5-ILs-VCR demonstrated maximum efficacy when compared to either Ls-VCR or free VCR, causing at day 63 complete tumor regressions in six out of eight animals (75%). Ls-VCR-Cit was also effective, causing complete tumor regressions still observed at day 63 in two out of nine animals (22%), however, it was less effective than F5-ILs-VCR ($p<0.005$). Ls-VCR-SOS and free VCR were equally effective ($p>0.2$) and less effective than either F5-ILs-VCR or Ls-VCR-Cit. Thus, surprisingly, with cell-targeted delivery, a liposomal drug encapsulated using a polyvalent anion of the present invention proved more efficacious than the drug liposomally encapsulated via non-binding anion. Animal body weight dynamics showed that all liposomal VCR preparations were less toxic than free VCR, causing less body weight loss during treatment (Figure 44).

EXAMPLE 65. Preparation of EGFR-targeted liposomal vincristine and antitumor efficacy of non-targeted and EGFR-targeted liposomal vincristine against EGFR-overexpressing human brain cancer xenografts in mice.

[0278] Vincristine-loaded liposomes (Ls-VCR) were prepared using TEA-SOS method as in Example 64. EGFR-targeted immunoliposomal vincristine was prepared by co-incubation of the liposomes with anti-HER2 Fab' C225Fab-PEG-DSPE conjugate as described in Example 36.

[0279] Male NCR *nu/nu* mice (5-6 week old , weighing 17-20 g) were injected subcutaneously in the flank area with 0.15 ml of the cell growth medium containing $1x10^7$ U87 human glioblastoma cells stably expressing epidermal growth factor receptor (HER1) mutant EGFRvIII. At day 11 when the mean tumor size reached 300-400 mm^3, the mice were randomly divided into four groups of 10-12 animals/group. Treatments with free VCR (vincristine sulfate 1 mg/mL in saline), Ls-VCR, or C225Fab-ILs-VCR at i.v. dose of 1.5 mg/kg were administered on days 11, 18, and 25 post tumor inoculation. Mice in the control group were similarly injected with an equal volume of normal saline. The tumor sizes and mouse body weights were monitored as in Example 10. The results are shown in Figure45. All animals treated with VCR formulations showed retardation of tumor growth compared to control animals. There was no significant difference between

the groups treated with free VCR and Ls-VCR. EGFR-targeted C225Fab-ILs-VCR was more efficacious than free or non-targeted liposomal VCR.

EXAMPLE 66. Preparation of liposomes with entrapped triethylammonium inositol hexaphosphate (TEA-IHP) solution.

[0280] A polyanionized polyol, inositol hexaphosphate (IHP) dodecasodium salt, was obtained from Sigma (St.Louis, MO). Aqueous solution containing 0.65 M triethylammonium and 0.681 M of phosphate groups, pH 6.5, and osmolality of 718 mmol/kg, was prepared by ion-exchange on the Dowex 50Wx8-200 cross-linked sulfonated polystyrene resin followed by titration with neat TEA and dilution with water according to the procedure of Example 4. The residual sodium content was less than 1% of the sum of cations. Dry lipids (150 μmol DSPC, 100 μmol Chol, 0.75 μmol PEG-DSPE) were dissolved in 0.5 ml of 100% ethanol USP at 60 °C and mixed with 4.5 ml of triethylammonium inositol hexaphosphate solution pre-heated to the same temperature. The ethanol was partially removed by rotary evaporation at 30-40 mm Hg and 40-45°C until the mixture showed no bubbling. The lipid suspension was then extruded at 60-65 °C 15 times through two stacked 0.1 μm pore size polycarbonate membranes. The resulting liposomes were 104.3 ± 39.0 nm in size by QELS. The unencapsulated triethylammonium IHP was removed by gel chromatography on a Sepharose 4B column, eluted with 5 mM HEPES-Na, 5% dextrose, pH 6.5 buffer, and the liposomes were quantified by phospholipid concentration using Bartlett's method with extraction according to Example 70.

EXAMPLE 67. Loading of drugs into liposomes with entrapped TEA-IHP solution.

[0281] The liposomes of Example 67 were loaded with CPT11 or vinorelbine Vinorelbine was loaded at a drug-to-phospholipid ratio of 175 or 350 g/mol, and CPT11 at a ratio of 250 or 500 g/mol. The drugs were added to the liposomes in the HEPES-dextrose buffer (Example 67) at the input drug/phospholipid ratios, indicated below (see Table 36). If necessary, the pH was adjusted to 6.5-6.8 using 1 N NaOH. The mixtures were incubated at 60 °C for 30 min, cooled down on ice for 15 min, and chromatographed on a Sephadex G-25 gel filtration column, eluted with 5 mM HEPES-Na, 145 mM NaCl, pH 6.5. Aliquots of the purified liposomes were solubilized in acidified methanol and analyzed by spectrophotometry (Example 71). Phospholipid was

quantified by the method of Bartlett (1959) with extraction (Example 70). Both drugs loaded quantitatively (i.e., practically 100%) into the liposomes, as shown below in Table 36.

Table 36. Properties of drugs loaded into liposomes with entrapped inositol hexaphosphate.

Drug	Input drug/lipid ratio, g/mol phospholipid	Encapsulated drug/lipid ratio, g/mol phospholipid	Loading efficiency, %
Vinorelbine	175	175.3 ± 8.0	100.2 ± 4.5
Vinorelbine	350	352.3 ± 11.8	100.6 ± 3.3
CPT-11	250	265.1 ± 11.2	106.1 ± 4.7
CPT-11	500	518.7 ± 27.8	103.7 ± 5.8

EXAMPLE 68. Chemical stability of free or liposomal CPT-11 in the presence of mouse plasma *in vitro*.

[0282] In the body, CPT-11, which is a pro-drug, undergoes chemical transformation to form an active drug metabolite known as SN-38. Both SN-38 and CPT-11 are also converted from their active lactone forms into an inactive products known as a SN-38 or CPT-11 carboxylates. In this Example the effect of liposomalization of CPT-11 in accordance with the present invention on the CPT-11 chemical conversion into these products in the presence of blood plasma was studied. Liposomes with entrapped triethylammonium sucroseoctasulfate (0.65 M TEA, pH 6.4, osmolality 485 mmol/kg) and lipid composition of DSPC, Cholesterol, and PEG-DSPE in a molar ratio of 3:2:0.015 were prepared according to Example 11, using extrusion ten times through two stacked 0.08 μm polycarbonate filters. The liposomes were 87.4 ± 19.2 nm in size by QELS. CPT-11 was loaded at approximately 500 mg of CPT-11 base/mmol liposome phospholipid by incubation in an aqueous 5 mM HEPES-Na, 5% dextrose, pH 6.5, at 60 °C for 30 min., followed by quenching on ice for 15 min. The CPT-11-loaded liposomes were then purified on a Sephadex G-75 column eluted with HEPES buffered saline (5 mM HEPES, 145 mM NaCl, pH 6.5). The resulting CPT-11 liposomes had 536.5 ± 20.1mg CPT-11/mmol of phospholipid. Free CPT-11 solution was prepared by freshly dissolving Irinotecan Hydrochloride USP at 1 mg/ml in 144 mM aqueous NaCl, acidified

to pH 3 with diluted HCl. Ten-µl aliquots of free or liposomal CPT-11 or free CPT-11 were mixed with 90 µl of heparin-stabilized mouse plasma (Harlan Bioproducts, USA) and incubated at 37 °C in a shaking water bath. At a given time point liposome samples, in triplicate, were chromatographed on Sepharose CL-4B size exclusion columns (2 ml bed volume) eluted with HBS-6.5, and the drug-containing fractions were detected by fluorescence. The first (void volume) and second (trailing) drug-containing peaks were collected and considered as the liposomally encapsulated and released drug fractions. The samples were extracted with 400 µl of ice-cold methanol by vortexing for 10 s followed by centrifugation at 14,100xg for 5 min. The supernatants were analyzed for CPT-11 and its conversion products by HPLC using modification of a method by Warner and Burke, *J Chromatogr., Ser. B Biomed. Sci. Appl.* 1997, vol. 691, p.161-71. 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 vol%B to 50 vol.%B in 14 min. The eluted products were detected by fluorescence with an excitation at 375 nm and emission at 500 nm. The retention times were 5.3 min (CPT-11 carboxylate), 6.8 min (SN-38 carboxylate), 9.3 min (CPT-11) and 11.0 min (SN-38). The results (Table 37) indicated that while free CPT-11 and CPT-11 released from the liposomes underwent conversion, intraliposomal CPT-11 was quite stable.

Table 37. Conversion of free and liposomal CPT-11 into SN-38 and carboxylate forms in mouse plasma *in vitro*.

Sample	Time, hours	CPT-11, %		SN-38, %	
		lactone	carboxylate	lactone	carboxylate
Free CPT-11	2	1.9 ± 0.4	35.2± 1.9	4.4± 0.1	58.4± 2.1
	12	<0.1	11.5± 0.9	9.9 ± 0.8	78.6 ± 1.3
	24	<0.1	<0.1	22.5 ± 9.8	77.5 ± 9.8
Ls-CPT-11	12	97.7 ± 0.1	<0.1	2.3 ± 0.1	<0.1
(encapsulated)	24	97.7 ± 0.1	<0.1	2.3 ± 0.1	<0.1
Ls-CPT-11	12	60.5 ± 10.4	25.0 ± 7.1	5.0 ± 0.3	9.5 ± 3.0
(released)	24	78.3 ± 6.7	14.0 ± 5.2	6.5 ± 0.5	1.2 ± 1.7

EXAMPLE 69. *In vivo* chemical stability of free or liposomal CPT-11 in rats.

[0283] Liposomal CPT-11 was prepared as in Example 68 using triethylammonium sucroseoctasulfate having 0.65 M TEA, pH 6.4, and osmolality 502 mmol/kg. The liposome size was 98.5 ± 18.4 nm, and CPT-11 encapsulation was 510.1 ± 16.5 mg CPT-11/mmol phospholipid. The liposomal and free CPT-11 was administered intravenously at the dose of 25 mg/kg into female Albino rats (180-220 g) with indwelling central venous catheters, and the blood samples were withdrawn at intervals over the period of 48 hours. The blood samples were mixed with ice-cold PBS containing 0.04% EDTA and quickly centrifuged to remove blood cells. Aliquots of the supernatant fluids were assayed for CPT-11, SN-38, and their carboxylate forms by HPLC as in Example 68 above. The results are shown in Figures 46 and 47. Whereas the free CPT-11 was cleared very rapidly, being undetectable after 30 min, the liposomal CPT-11 was persistent in the circulation ($t_{1/2}$ 15.2 hours) with 37.8 % of the drug in the blood at 24 h, and approximately 10 % of the drug still in the circulation after 48 h. There was no detectable conversion of the liposomal form of CPT-11 to either SN-38 or the carboxylate form of CPT-11. Free CPT-11, i.e. administered as a solution, cleared from the circulation quite fast (half-life of about 16 min), and there was appreciable conversion to the carboxylate form of the drug.

EXAMPLE 70. Quantification of the liposome phospholipid.

[0284] *Modified acid digestion - blue phosphomolybdate method I.* This method is modified after Bartlett (1959). 10-20 ml aliquots of liposomes are placed into heat-resistant glass tubes, digested by heating with 0.5 ml of 10 N sulfuric acid for 2 hours at 110-130°C, mineralized by addition of 50 ml of 9% hydrogen peroxide, and heated for additional 30 min. until no hydrogen peroxide is detected by an indicator paper strip. The digested samples at ambient temperature are diluted with 1 ml of 0.2% aqueous ammonium molydbate, mixed with 0.1 ml of 5% aqueous ascorbic acid, and incubated on a boiling water bath for 10 min. The absorbance of reduced phosphomolybdate complex is measured at 800 nm and compared to a standard curve concurrently produced using inorganic phosphate standard solutions.

[0285] *Modified acid digestion - blue phosphomolybdate method II.* This method is a modification of the method by Morrison (1964). 5 µl aliquots of liposomes having 1-10

mM phospholipid are mixed with 60 µl of concentrated sulfuric acid and 10 µl of 30% hydrogen peroxide in heat-resistant glass tubes. The mixtures are heated at 200-220°C for 10 min., diluted with 0.7 µl of deionized water, mixed with 10 µl of 10% aqueous sodium sulfite, incubated on a boiling water bath for 5 min, and chilled down to ambient temperature. 200 µl of 2% aqueous ammonium molybdate and 10 µl of 10% aqueous ascorbic acid are added, and the samples are incubated on a boiling water bath for 10 min. Samples are quickly chilled to ambient temperature, and the absorbance of reduced phosphomolybdate complex is determined at 825 nm against the blank sample. The amount of phospholipid is determined from the standard curve obtained in the same run using standard solutions having 2, 4, 6, 8, and 10 mM potassium dihydrogen phosphate.

[0286] *Extraction method.* 25-100 µl aliquots of liposomes are extracted 3 times with 200 µl portions of methanol-chloroform mixture (1:2 by volume). The organic phases are combined in a heat-resistant glass tube, and the solvents are removed in vacuum. The residues are treated with 10N sulfuric acid and further assayed for phosphorus according to the method I above.

[0287] Unless indicated otherwise, the analytical data are presented as the mean ± standard error of triplicate runs.

EXAMPLE 71. Quantification of drugs in the liposomes.

[0288] *Spectrophotometric quantification.* Aliquots of liposomes (10-50 µl) are mixed with 1 mL of 70 vol. % aqueous isopropanol containing 0.075-0.1 N HCl, and the absorbance against the blank sample is measured at the following wavelengths: doxorubicin, 485 nm; CPT-11 and topotecan, 372 nm; ellipticines, 306 nm, vinorelbine, 270 nm; vincristine and vinblastine, 265 nm. The amount of drug is determined by comparison to a concurrently run standard curve.

[0289] *Fluorometric quantification.* Aliquots of liposome-containing samples (e.g., blood plasma) are diluted with acidified isopropanol (0.02-0.1 ml aliquots: 1 mL of 70% isopropanol-0.075 N HCl; >0.1 ml aliquots: 90% isopropanol-0.1 N HCl to 1 mL). If protein precipitation occurs, the samples are incubated on ice 1-2 hours and clarified by centrifugation 10 min at 12,100xg. The fluorescence of the supernatants is measured at the following wavelengths: CPT-11, excitation 370 nm, emission 423-425 nm;

Topotecan, excitation 380-385 nm, excitation 520-525 nm; ellipticines, excitation 306 nm, emission 520 nm. The amount of drug is calculated from concurrently run standard curves after subtraction of the blank fluorescence.

EXAMPLE 72. Effect of lipopolymers on the loading efficiency of vinorelbine into liposomes.

[0290] Liposomes composed of DSPC 200 molar parts, cholesterol 133 molar parts, and poly(ethylene glycol)(mol. weight 2,000)-derivatized lipids PEG-DSPE (1-20 molar parts) or PEG-DSG (20 molar parts),and containing encapsulated 0.65 M TEA-SOS solution were prepared according to the method of Example 11, using 80 nm pore size membrane for extrusion step. The liposomes were loaded with vinorelbine at the drug/phospholipid ratio of 350 mg/mmol and purified from unencapsulated drug according to the method of Example 40. The liposomes were assayed for drug and lipid content as described in Examples 70, 71, and for the liposome size by QELS using volume-weighted Gaussian approximation. The results (Table 38) indicated that while anionic PEG derivative, PEG-DSPE, at the amount of more than 1 mole % of the liposome phospholipid (0.3 mole % of the total lipid), had negative effect on the drug loading efficiency, the neutral derivative, PEG-DSG, surprisingly, did not affect the loading efficiency even at 9.1 mole % of the liposome phospholipid (5.7 mole % of total lipid).

Table 38. Properties of vinorelbine liposomes prepared by TEA-SOS method at various amounts of PEG-lipid derivatives.

PEG-lipid	PEG-lipid amount, mol. % of total lipid	Liposome size, nm (mean SD)	Drug load, mg/mmol phospholipid	Loading efficiency, % encapsulation
PEG-DSPE	0.3	108 ± 32	359.5 ± 17.8	102.7 ± 5.2
PEG-DSPE	0.6	110 ± 18	346.6 ± 14.5	99.0 ± 4.1
PEG-DSPE	1.8	104 ± 35	332.0 ± 14.0	94.9 ± 3.8
PEG-DSPE	2.9	94 ± 33	259.8 ± 9.5	74.2 ± 2.0
PEG-DSPE	4.0	100 ± 36	155.4 ± 7.0	44.4 ± 0.9
PEG-DSPE	5.7	103 ± 31	61.2 ± 5.2	17.5 ± 0.3
PEG-DSG	5.7	97 ± 36	362.7 ± 14.2	103.6 ± 4.2

EXAMPLE 73. Effect of intraliposomal drug-trapping agent on the blood longevity of CPT-11 in mice.

[0291] Liposomes with entrapped 0.65 N solutions of triethylammonium (TEA) or triethanolammonium (TEOA) salts of inositol hexaphosphate (IHP, phytic acid) or sucrose octasulfate were prepared and loaded with CPT-11 at 500 g/mol phospholipid following general procedure of Example 66. The liosomes were administered intravenously to Swiss-Webster mice in the dose of 5 mg CPT-11/kg body weight. Twenty four hours later, the mice were anesthetized, and exsanguinated via open heart puncture. The blood was collected, analysed for CPT-11 content in the blood plasma by HPLC as described in Exampe 68, and the drug amount was expressed as % of injected dose remaining in the blood (%ID). TEOA-IHP was less effective in improving the blood longevity of the drug than TEA-IHP, TEOA-SOAS, and TEA-SOS (Table 39).

Table 39. CPT-11 remanence in the blood 24 hours following intravenous administration of CPT-11 liposomes in mice.

Intraliposomal drug-trapping agent	%ID remaining in the blood
TEOA-IHP	2.74 ± 0.54
TEA-IHP	5.86 ± 0.20
TEOA-SOS	7.03 ± 0.17
TEA-SOS	11.32 ± 0.46

EXAMPLE 74. Drug loading into liposomes containing 1.05 N diethylammonium sucrose octasulfate

[0292] Aqueous solution of 1.05 N diethylammonium sucrose octasulfate (DEA-SOS) pH 6.0, osmolarity 727 mmol/kg, was prepared using ion-exchange/titration method of Example 6 using neat diethylamine (99.5% purity). The lipid matrix of 3 molar parts DSPC, 2 molar parts Cholesterol, and 0.015 molar parts PEG2000-DSPE, was formulated into liposomes (volume-weighted average size 92.4 nm) in the presence of DEA-SOS solution, and CPT-11 was loaded in the liposomes as various drug/lipid input ratios using the method of Example 11. Non-encapsulated drug was removed by gel-chromatography, and the amount of encapsulated drug per unit lipid (drug/lipid output ratio) was

determined. Encapsulation efficiency was calculated as % of drug/lipid output ratio relative to input ratio. The results are shown in Table 40. The loading achieved it's maximum level of about 1.76 mol drug per mol phospholipid (1.67-1.70 mol drug/g total lipid), which is in good agreement with the amount (1.78 mol diethylammonium/mol phospholipid) based on the diethyammonium content of the liposomes, assuming stiochiometric exchange of intraliposomal diethylammonium ions for the drug molecules and estimated intraliposomal entrapped volume of approximately 1.7 l/mol phospholipid.

Table 40. Loading of CPT-11 in DSPC/Chol/PEG-DSPE liposomes containing 1.05 N DEA-SOS.

Drug/lipid input ratio, mol/g	Drug/lipid output ratio, mol/g	Encapsulation efficiency, %
1.25	1.247 ± 0.038	99.8 ± 3.0
1.50	1.534 ± 0.052	102.3 ± 3.5
1.80	1.669 ± 0.043	92.7 ± 2.4
2.06	1.690 ± 0.054	82.0 ± 2.6
2.20	1.704 ± 0.062	77.5 ± 2.8
2.42	1.685 ± 0.103	69.6 ± 4.3

[0293] Unless indicated otherwise, the analytical data are presented as the mean ± standard error of triplicate runs. The rat plasma pharmacokinetic data are the mean ± standard error of duplicate runs.

[0294] Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the scope of the invention should be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all cited articles and references, including patent applications and publications, are incorporated herein by reference for all purposes.

What is claimed is:

1. A composition comprising a liposome in a medium, said liposome having an interior space separated from the medium by a membrane comprising one or more lipids, wherein the interior space of said liposome contains a substituted ammonium having a formula

R_1-(R_2-)N^+(-R_3)-R_4

wherein N is an ammonium nitrogen atom, each of R_1, R_2, R_3, R_4 is independently a hydrogen atom or an organic group having each independently not more than 8 carbon atoms, and in totality not more than 18 carbon atoms inclusive, wherein at least one of R_1, R_2, R_3, R_4 is an organic group;

wherein the organic group is independently alkyl, alkylidene, heterocyclic alkyl, cycloalkyl, aryl, alkenyl, cycloalkenyl, or a hydroxy-substituted derivative thereof, optionally including a S, O, or N atoms forming an ether, ester, thioether, amine, or amide bond; and

wherein at least three of R_1, R_2, R_3, R_4 are the organic groups; or at least one of the organic groups has a secondary or tertiary carbon atom directly linked to the ammonium nitrogen atom; and

wherein said interior space also comprises an anion.

2. The composition of Claim 1 wherein the organic groups independently each have not more than 6 carbon atoms.

3. The composition of Claim 1 wherein the organic groups have in totality not more than 16 carbon atoms.

4. The composition of Claim 1 wherein the organic groups have in totality not more than 12 carbon atoms.

5. The composition of Claim 1 wherein said substituted ammonium forms a true solution in water.

6. The composition of Claim 1 wherein substantially all said substituted ammonium is contained within said interior space of the liposome.

7. The composition of Claim 1 wherein said substituted ammonium is pharmaceutically inert.

8. The composition of Claim 1 wherein said substituted ammonium is selected from the group consisting of isopropylethylammonium, isopropylmethylammonium, diisopropylammonium, *tert*-butylethylammonium, dicychohexylammonium, morpholinium, pyridinium, piperidinium, pyrrolidinium, piperazinium, *tert*-butylammonium, 2-ammonio-2-methylpropanol-1, 2-ammonio-2-methyl-propandiol-1,3, tris-(hydroxyethyl)-ammoniomethane, N,N'-diethyl-ethanolammonium, N,N',N"-tris-(2-hydroxyethyl)ammonium, N, N'-bis-(2-hydroxyethyl)ethylammonium, trimethylammonium, triethylammonium, diethylmethylammonium, diisopropylethylammonium, triisopropylammonium, N-methylmorpholinium, 1-(2-hydroxyethyl)piperidinium, 1-methylpyrrolidinium, 1, 4-dimethylpiperazinium, tetramethylammonium, tetraethylammonium, and tetrabutylammonium

9. The composition of Claim 1 wherein the concentration of the substituted ammonium in the interior space of the liposome is higher than the concentration of the substituted ammonium in the liposome-containing medium.

10. The composition of Claim 1 wherein the concentration of the substituted ammonium in the interior space is at least about a concentration of 0.05 M, 0.1 M, 0.2 M, 0.5 M, 0.6 M, or 0.7 M.

11. The composition of Claim 1 wherein the concentration of the substituted ammonium in the interior space is about 0.65 M.

12. The composition of Claim 1 wherein the anion is a divalent anion, a trivalent anion, a polyvalent anion, a polymeric anion, a polyanionized polyol, or a polyanionized sugar.

13. A composition comprising a liposome in a medium, said liposome having an interior space separated from the medium by a membrane comprising one or more lipids, wherein said interior space comprises an anion selected from the group consisting of a polyanionized polyol or polyanionized sugar, and wherein said liposome comprises a transmembrane gradient effective for retention of an entity within said liposome.

14. The composition of Claim 13 wherein said transmembrane gradient is an ion gradient, an ammonium ion gradient, a pH gradient, an electrochemical potential gradient, a solubility gradient, or a gradient of a substituted ammonium ion comprising at least one C-N bond.

15. The composition of any of the Claims 1 to14 wherein said anion comprises

(i) a polyol moiety selected from the group consisting of a monosaccharide, a disaccharide, a linear polyhydroxylated compound, a cyclic polyhydroxylated compound, an aliphatic alcohol having more than one hydroxyl group, an alicyclic alcohol having more than one hydroxyl group, or a heterocyclic alcohol having more than one hydroxyl group, and

(ii) linked to said polyol moiety, at least two strongly anionic functional groups.

16. The composition of Claim 15 wherein at least one of said strongly anionic functional groups is a sulfuric acid ester, a phosphoric acid ester, a boric acid ester, a sulfonic acid group, a phosphonic acid group, a thiocarbonic acid group, a dithiocarbonic acid group, a derivative thereof, or an analog thereof.

17. The composition of Claim 15 wherein said polyol moiety is selected from the group consisting of arabinose, ribose, xylose, glucose, galactose, sorbose, fructose, maltose, sucrose, lactose, trehalose, ethylene glycol, prolylene glycol, glycerol, treitol, erythritol, pentaerythritol, ribitol, arabitol, sorbitol, mannitol, lactitol, maltitol, fructitol, glucitol, xylitol, or inositol.

18. The composition of Claim 15 wherein said polyanion is polysulfated sucrose having from 3 to 8 sulfate groups per molecule.

19. The composition of Claim 15 wherein said polysulfated sucrose is sucrose octasulfate.

20. The composition of Claim 15 wherein said polyanion is inositol hexaphosphate.

21. The composition of Claim 15 wherein the concentration of said anion in said liposome interior is at least about 0.05, 0.1, 0.2, 0.5, 0.6, 0.7, or 1.0 gram-equivalent/L.

22. The composition of Claim 15 wherein the concentration of said anion in said liposome interior is about 0.65 gram-equivalent/L or about 1.0 gram-equivalent/L.

23. The composition of any of the Claims 1 - 22 further comprising an entity other than said substituted ammonium and said anion.

24. The composition of Claim 23 wherein the entity is a globally cationic entity, a therapeutic entity, or a detectable marker.

25. The composition of Claim 23 wherein said entity is present in said interior space at a concentration which exceeds the concentration of said entity in said medium.

26. The composition of Claim 23 wherein said medium is essentially free from said entity.

27. The composition of Claim 23 wherein molar ratio of said entity to the totality of said lipids is at least about 0.05, at least about 0.1, at least about 0.2, at least about 0.3, at least about 0.5,at least about 0.7, or at least about 1.0.

28. The composition of Claim 24 wherein said therapeutic entity is an antimicrobial therapeutic, antiviral therapeutic, or an anti-neoplastic therapeutic.

29. The composition of Claim 24 wherein said therapeutic entity is an aminoglycoside antibiotic or a fluoroquinolone derivative.

30. The composition of Claim 28 wherein said therapeutic entity is selected from a group consisting of: a topoisomerase inhibitor, a farnesyltransferase inhibitor, a tyrosine kinase inhibitor, a cyclin-dependent kinase inhibitor, a phosphatase inhibitor, an aurora kinase inhibitor, a microtubule depolymerizing agent, a microtubule stabilizing agent, an alkylating agent, an enzyme, an enzyme inhibitor, a histone deacetylase inhibitor, an antimetabolite, a receptor-binding agent, a

hormone, a hormone antagonist, a nucleotide, a polynucleotide, a derivative of any of the foregoing, a pro-drug of any of the foregoing, and an analog of any of the foregoing.

31. The composition of Claim 28 wherein said anti-neoplastic therapeutic is selected from a group consisting of an anthracycline compound, a camptothecin compound, a vinca alkaloid, an ellipticine compound, a taxane compound, a wortmannin compound, a pyrazolopyrimidine compound, a derivative of any of the foregoing, a pro-drug of any of the foregoing, and an analog of any of the foregoing.

32. The composition of Claim 24 wherein said therapeutic entity is selected from a group consisting of: doxorubicin, daunorubicin, mitomycin C, epirubicin, pirarubicin, rubidomycin, carcinomycin, N-acetyladriamycin, rubidazone, 5-imidodaunomycin, N-acetyldaunomycin, daunoryline, mitoxanthrone, camptothecin, 9-aminocamptothecin, 7-ethylcamptothecin, 10-hydroxycamptothecin, 9-nitrocamptothecin, 10,11-methylenedioxycamptothecin, 9-amino-10,11-methylenedioxycamptothecin, 9-chloro-10,11-methylenedioxycamptothecin, irinotecan, topotecan, lurtotecan, silatecan, (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin, 7-(4-methylpiperazinomethylene)-10,11-methylenedioxy-20(S)-camptothecin, 7-(2-N-isopropylamino)ethyl)-(20S)-camptothecin, vincristine, vinblastine, vinorelbine, vinflunine, vinpocetine, vindesine, ellipticine, 6-3-aminopropyl-ellipticine, 2-diethylaminoethyl-ellipticinium and salts thereof, datelliptium, retelliptine, paclitaxel, docetaxel, a derivative of any of the foregoing, a pro-drug of any of the foregoing, and an analog of any of the foregoing..

33. The composition of Claim 24 wherein said therapeutic entity is selected from a group consisting of: an antihistamine ethylenediamine derivative, brompheniramine, diphenhydramine, an anti-protozoal drug, quinolone, iodoquinol, an amidine compound, pentamidine, an antihelmintic

compound, pyrantel, an anti-schistosomal drug, oxaminiquine, an antifungal triazole derivative, fliconazole, itraconazole, ketoconazole, miconazole, an antimicrobial cephalosporin, cefazolin, cefonicid, cefotaxime, ceftazimide, cefuoxime, an antimicrobial beta-lactam derivative, aztreopam, cefmetazole, cefoxitin, an antimicrobial of erythromycine group, erythromycin, azithromycin, clarithromycin, oleandomycin, a penicillin compound, benzylpenicillin, phenoxymethylpenicillin, cloxacillin, methicillin, nafcillin, oxacillin, carbenicillin, a tetracycline compound, novobiocin, spectinomycin, vancomycin; an antimycobacterial drug, aminosalicyclc acid, capreomycin, ethambutol, isoniazid, pyrazinamide, rifabutin, rifampin, clofazimine, an antiviral adamantane compound, amantadine, rimantadine, a quinidine compound, quinine, quinacrine, chloroquine, hydroxychloroquine, primaquine, amodiaquine, mefloquine, an antimicrobial qionolone, ciprofloxacin, enoxacin, lomefloxacin, nalidixic acid, norfloxacin, ofloxacin, a sulfonamide; a urinary tract antimicrobial, nitrofurantoin, trimetoprim; a nitroimidazoles derivative, metronidazole, a cholinergic quaternary ammonium compound, ambethinium, neostigmine, physostigmine, an anti-Alzheimer aminoacridine, tacrine, an anti-Parkinsonal drug, benztropine, biperiden, procyclidine, trihexylhenidyl, an anti-muscarinic agent, atropine, hyoscyamine, scopolamine, propantheline, an adrenergic compound, dopamine, serotonin, albuterol, dobutamine, ephedrine, epinephrine, norepinephrine, isoproterenol, metaproperenol, salmetrol, terbutaline, a serotonin reuptake inhibitor, an ergotamine derivative, a myorelaxant of a curare series, a central action myorelaxant, baclophen, cyclobenzepine, dentrolene, nicotine, a nicotine receptor antagonist, a beta-adrenoblocker, acebutil, amiodarone, a benzodiazepine compound, ditiazem, an antiarrhythmic drug, diisopyramide, encaidine, a local anesthetic compound, procaine, procainamide, lidocaine, flecaimide, quinidine; an ACE inhibitor, captopril, enelaprilat, fosinoprol, quinapril, ramipril; an opiate derivative, codeine, meperidine, methadone, morphine, an antilipidemic, fluvastatin, gemfibrosil, an HMG-coA

inhibitor, pravastatin, a hypotensive drug, clonidine, guanabenz, prazocin, guanethidine, granadril, hydralazine, a non-coronary vasodilator, dipyridamole, an acetylcholine esterase inhibitor, pilocarpine, an alkaloid, physostigmine, neostigmine, a derivative of any of the foregoing, a pro-drug of any of the foregoing, and an analog of any of the foregoing.

34. The composition of Claim 24 wherein at least 90% of said entity remains encapsulated in the liposome after 6 months at 2 - 8 °C.

35. The composition of Claim 24 wherein at least 80% of said entity remains encapsulated in the liposome after 2 years at 2 - 8 °C.

36. The composition of Claim 24 wherein said entity is encapsulated in the liposomes of said composition at a first entity-to-lipid ratio, and wherein 24 hours following administration of said composition into the bloodstream of a mammal, said entity remains encapsulated in said liposomes at a second entity-to-lipid ratio wherein the second entity-to-lipid ratio is over 50%, at least 60%, or at least 70% of the first substance-to-lipid ratio.

37. The composition of Claim 36 wherein the entity is a vinca alkaloid, an analog thereof, or a derivative thereof.

38. The composition of Claim 36 wherein the entity is vinorelbine.

39. The composition of Claim 36 wherein said mammal is a rat.

40. A composition comprising a liposome comprising one or more lipids and an anti-neoplastic therapeutic entity encapsulated therein at an entity-to lipid molar ratio of at least about 0.10,

wherein said liposome composition has in vivo anti-neoplastic activity at least a four-fold higher than the anti-neoplastic activity of the entity in a free non-liposomal form, and

wherein the toxicity of said liposome composition administered to a mammal is equal or less than the toxicity of said entity administered to said mammal in a free non-liposomal form.

41. The composition of Claim 40 wherein the toxicity of said liposome composition administered to a mammal is at least two times less or at least three times less than the toxicity of said entity administered to said mammal in a free non-liposomal form.

42. The composition of Claim 40 wherein the toxicity of said liposome composition administered to a mammal is at least four times less than the toxicity of said entity administered to said mammal in a free non-liposomal form.

43. The composition of Claim 40 wherein the entity is a pro-drug.

44. The composition of Claim 40 wherein said entity is a camptothecin topoisomerase I inhibitor, a camptothecin prodrug, an analog thereof, or a derivative thereof.

45. The composition of Claim 44 wherein said camptothecin prodrug is irinotecan.

46. The composition of Claim 44 wherein said entity administered into the bloodstream of a mammal has a half-release time from said liposomes of at least 10 hours.

47. The composition of Claim 44 wherein said entity administered into the bloodstream of a mammal has a half-release time from said liposomes of at least 24 hours.

48. The composition of Claim 44 wherein said liposome comprises a polyanion selected from a group consisting of a polyanionized sugar and polyanionized polyol.

49. The composition of Claim 48 wherein said polyanion is sucrose octasulfate, or inositol hexaphosphate.

50. The composition of Claim 44 wherein said liposome comprises a biodegradable polyanionic polymer.

51. The composition of Claim 50 wherein the biodegradable polyanionic polymer is polyphosphate.

52. The composition of Claim 44 wherein said liposome contains a substituted ammonium compound having a formula

R_1-(R_2-)N^+(-R_3)-R_4

wherein N is an ammonium nitrogen atom, each of R_1, R_2, R_3, R_4 is independently a hydrogen atom or an organic group having each independently not more than 8 carbon atoms, and in totality not more than 18 carbon atoms inclusive, wherein at least one of R_1, R_2, R_3, R_4 is an organic group;

wherein the organic group is independently alkyl, alkylidene, heterocyclic alkyl, cycloalkyl, aryl, alkenyl, cycloalkenyl, or a hydroxy-substituted derivative thereof, optionally including a S, O, or N atoms forming an ether, ester, thioether, amine, or amide bond; and

wherein at least three of R_1, R_2, R_3, R_4 are the organic groups; or at least one of the organic groups has a secondary or tertiary carbon atom directly linked to the ammonium nitrogen atom.

53. The composition of any of the Claims 40-52 wherein said topoisomerase inhibitor is entrapped at a concentration of at least about 0.30 mole drug per mol. lipid.

54. The composition of any of the Claims 40-53 wherein said topoisomerase inhibitor is entrapped at a concentration between about 0.40 mole drug per mole lipid and about 1.7 mole drug per mole lipid.

55. The composition of Claims 40-54 wherein said mammal is a mouse.

56. The composition of any of the Claims 40-55 wherein said anti-tumor activity is determined in vivo in an HT-29 tumor model or a BT-474 tumor model.

57. The composition of Claims 46-47 wherein said mammal is a rat.

58. A composition for administering a vinca alkaloid drug, comprising liposomes in a medium, said liposomes having an interior space and a membrane separating said interior from said medium, said membrane comprising one or more lipids, said liposomes comprising a vinca alkaloid drug entrapped in the liposomes at a first drug/lipid ratio, wherein 24 hours following administration of said composition into the bloodstream of a mammal, the vinca alkaloid drug remains entrapped in aid liposomes at a second drug/lipid ratio which is over about 50% of said first drug/lipid ratio.

59. The composition of Claim 58 wherein said second drug/lipid ratio is at least about 60% of said first drug/lipid ratio.

60. The composition of Claim 58 wherein said second drug/lipid ratio is at least about 70% of said first drug/lipid ratio.

61. The composition of Claim 58-60 wherein said first drug/lipid molar ratio is at least 0.05, at least 0.1, or at least 0.2.

62. The composition of Claim 61 wherein said first drug/lipid mass ratio is at least about 0.05 mg/mmol, at least about 0.1 mg/mmol, or at least about 0.3 mg/mmol.

63. The composition of any of the Claims 58-62 wherein said liposomes comprise an inside/outside transmembrane ion gradient effective for retaining of the drug within the liposomes.

64. The composition of any of the Claims 58-63 wherein said interior space comprises a polyanion selected from the group of polymeric polyanion, polyanionized polyol, or polyanionized sugar.

65. The composition of Claim 63 or 64 wherein said interior space comprises ammonium ion or a substituted ammonium ion.

66. The composition of Claim 65 wherein the substituted ammonium ion is a primary ammonium, a secondary ammonium, a tertiary ammonium, or a quaternary ammonium ion.

67. The composition of Claim 63 or 64 wherein said interior space comprises a substituted ammonium ion having a formula

R_1-(R_2-)N^+(-R_3)-R_4

wherein N is an ammonium nitrogen atom, each of R_1, R_2, R_3, R_4 is independently a hydrogen atom or an organic group having each independently not more than 8 carbon atoms, and in totality not more

than 18 carbon atoms inclusive, wherein at least one of R_1, R_2, R_3, R_4 is an organic group;

wherein the organic group is independently alkyl, alkylidene, heterocyclic alkyl, cycloalkyl, aryl, alkenyl, cycloalkenyl, or a hydroxy-substituted derivative thereof, optionally including a S, O, or N atoms forming an ether, ester, thioether, amine, or amide bond; and

wherein at least three of R_1, R_2, R_3, R_4 are the organic groups; or at least one of the organic groups has a secondary or tertiary carbon atom directly linked to the ammonium nitrogen atom; and

wherein said interior space also comprises an anion.

68. The composition of Claim 65 wherein said polyanion is comprised in said interior space at a concentration of at least 0.05 gram-equivalent/L, at least 0.2 gram-equivalent/L, at least 0.5 gram-equivalent per liter, or at least 0.6 gram-equivalent/L.

69. The composition of any of the Claims 58-68 wherein said vinca alkaloid drug is vincristine, vinblastine, vinorelbine, vinflunine, vindesine, vinpocetine, an analog thereof, or a derivative thereof.

70. The composition of Claim 69 wherein said vesicle-forming lipid comprises phosphatidylcholine and cholesterol.

71. The composition of Claim 70 wherein said phosphatidylcholine is selected from the group consisting of a natural lecithin, a hydrogenated natural lecithin, a synthetic lecithin, 1,2-distearoyl-lecithin, 1,2-dipalmitoyl-lecithin, 1,2-dimyristoyl-lecithin, 1,2-dioleoyl-lecithin, 1-stearoyl-2-oleoyl-lecithin, and 1-palmitoyl-2-oleoyl lecithin.

72. The composition of Claim 70 wherein said phosphatidylcholine and said cholesterol are contained in molar ratio of about 3:2.

73. The composition of any of the Claims 58-68 wherein said lipids comprises a neutral PEG-lipid derivative or an anionic PEG-lipid derivative.

74. The composition of Claim 73 wherein said neutral PEG-lipid derivative is from about 0.1 mol. % to about 10 mol. % of the totality of said lipids.

75. The composition of Claim 73 wherein said neutral PEG lipid derivative is PEG-ceramide or PEG-diacylglycerol.

76. The composition of Claim 73 wherein said anionic PEG-lipid derivative is contained at less than 1 mol% of the total lipid.

77. The composition of Claim 76 wherein said anionic PEG-lipid derivative is N-(PEG)-diacyl-phosphatidylethanolamine.

78. The composition of any of the Claims 58-77 wherein said mammal is a rat.

79. A composition for administering a vinca alkaloid drug, comprising liposomes in a medium, said liposomes having an interior space and a membrane separating said interior from said medium, said membrane comprising lipids,

wherein said lipids comprise lecithin and cholesterol in the molar ratio of about 3:2; and

wherein said vinca alkaloid drug is contained in said liposomes at the drug/lipid ratio from about 0.15 mg/mmol lecithin to about 0.55 mg/mmol lecithin; and

wherein said interior space also contains a biodegradable polyanionic polymer, a polyanionized polyol, or a polyanionized sugar; and

wherein said polymer or sugar is contained in said interior space of the liposomes at a concentration from about 0.5 gram-equivalent/L to about 1.0 gram-equivalent/L; and

wherein said vinca alkaloid drug is vinorelbine, vincristine, or vinblastine.

80. The composition of Claim 79 wherein said polyanionized sugar is sucrose octasulfate.

81. The composition of Claim 80 also comprising an ionically neutral poly(ethylene glycol)-lipid derivative in the amount from about 0.1 mol.% to about 10 mol.% of the total lipid.

82. The composition of Claim 81 wherein said poly(ethylene glycol)-lipid derivative is poly(ethylene glycol)-dialkylglycerol, poly(ethylene glycol)-diacylglycerol, or poly(ethylene glycol)-ceramide, wherein molecular weight of poly(ethylene glycol) portion of said poly(ethylene glycol)-lipid derivative is from about 250 to about 10,000.

83. The composition of Claim 80 also comprising an anionic poly(ethylene glycol)-lipid derivative in the amount from about 0.1 mol.% to about 0.9 mol.% of the total lipid.

84. The composition of Claim 83 wherein said anionic poly(ethylene glycol)-lipid derivative is PEG-phosphatidylethianolamine, wherein

molecular weight of the poly(ethylene glycol) portion of said poly(ethylene glycol)-lipid derivative is from about 250 to about 10,000.

85. The composition of any of the Claims 79-84 wherein half-release time of said vinca alkaloid drug from said liposomes in the blood circulation of a mammal is over 24 hours.

86. The composition of Claim 85 wherein the mammal is a rat.

87. A composition comprising a liposome having an interior space and comprising a taxane compound wherein said taxane compound is contained essentially within the interior space of said liposome.

88. The composition of Claim 87 wherein said taxane is ionically neutral molecule.

89. The composition of Claim 88 wherein the molecular structure of said taxane does not comprise a hydrophilic polymer moiety.

90. The composition of any of the Claim 87-89 wherein the amount of said encapsulated taxane is at least 0.05 mole per mole of said lipid.

91. The composition of Claim 89 wherein the amount of said encapsulated taxane is at least 0.1 mole per mole of said lipid.

92. The composition of any of the claims 87-92 wherein said interior space is essentially free from a solubilizing aid selected from a micelle-forming surfactant compound and a cyclodextrin compound.

93. The composition of any of the Claims 87-92 wherein said taxane is paclitaxel or docetaxel.

94. The composition of any of the preceding claims wherein said lipids comprise a lipid derivatized with a hydrophilic polymer.

95. The composition of Claim 94 wherein said hydrophilic polymer-derivatized lipid comprises up to 20 mole % of the totality of said lipids.

96. The composition of Claim 94 wherein said hydrophilic polymer-derivatized lipid comprises less than 1 mole % of the totality of said lipids.

97. The composition of Claim 94 wherein said liposome has blood circulation longevity in a mammal of less than two times higher than the circulation longevity of an identically composed liposome except for the absence of said hydrophilic polymer-derivatized lipid.

98. The composition of Claim 97 wherein said mammal is a rat.

99. The composition of Claim 96 wherein said hydrophilic polymer-derivatized lipid comprises from about 0.1 mole % to about 0.9 mole % of the totality of said lipids.

100. The composition of any of the Claims 94-99 wherein said hydrophilic polymer is poly(ethylene glycol), or a derivative thereof.

101. The composition of any of the Claims 94-100 wherein said hydrophilic polymer-derivatized lipid is a poly(ethylene glycol)-derivatized phospholipid, a poly(ethylene glycol)-derivatized diacylglycerol, a poly(ethylene glycol)-derivatized dialkylglycerol, a poly(ethylene glycol)-derivatized ceramide, a poly(ethylene glycol)-derivatized fatty acid, a poly(ethylene glycol)-derivatized fatty alcohol, or a poly(ethylene glycol)-derivatized sterol.

102. The composition of any of the Claims 94-101 wherein said hydrophilic polymer is poly(ethylene glycol) having at least three ethylene glycol units.

103. The composition of any of the Claims 94-101 wherein said hydrophilic polymer is poly(ethylene glycol) having molecular weight from about 200 to about 10,000.

104. The composition of any of the Claims 94-101 wherein said hydrophilic polymer is poly(ethylene glycol) having molecular weight from about 500 to about 5,000.

105. The composition of any of the preceding claims wherein said liposome comprises a targeting moiety.

106. The composition of Claim 105 wherein said targeting moiety is a protein, a peptide, a polysaccharide, a polynucleotide, natural small molecule, a synthetic small molecule, a combination thereof, or a derivative thereof.

107. The composition of Claim 105 wherein said targeting moiety is a naturally, synthetically, or recombinantly produced protein comprising an antigen binding sequence of an antibody.

108. The composition of Claim 105 wherein said targeting moiety is an antibody, an antigen-binding fragment thereof, a single-chain protein comprising the antigen-binding polypeptide sequences of an antibody, a single-domain antibody, an analog of any of the foregoing, or a derivative of any of the foregoing.

109. The composition of Claim 105 wherein said targeting moiety is linked to said liposome membrane and exposed to said medium.

110. The composition of Claim 109 wherein said linked targeting moiety comprises a hydrophilic polymer that links said liposome membrane to said moiety.

111. The composition of Claim 110 wherein said hydrophilic polymer is poly(ethylene glycol).

112. The composition of Claim 111 wherein said poly(ethylene glycol) has molecular weight from about 250 to about 30,000.

113. The composition of any of the Claims 104-112 wherein said targeting moiety effects internalization of said liposome in a cell.

114. The composition of any of the Claims 104-112 wherein said targeting moiety selectively binds to a receptor tyrosine kinase, a growth factor receptor, an angiogenic factor receptor, a transferrin receptor, a cell adhesion molecule, or a vitamin receptor.

115. The composition of Claim 114 wherein said tyrosine kinase receptor is a growth factor receptor

116. The composition of Claim 115 wherein said tyrosine kinase growth factor receptor is an EGFR, ErbB-2 (HER-2), ErbB-3 (HER3), or ErbB-4 (HER4).

117. The composition of Claim 114 wherein said angiogenic factor receptor is bFGF receptor, or VEGF receptor.

118. The composition of Claim 114 wherein said cell adhesion molecule is an integrin.

119. The composition of any of the claims 113-118 wherein said cell is a malignant cell.

120. A method of encapsulating an entity into a liposome comprising a step of contacting the composition of any of the Claims 1 - 22 with the entity for the time sufficient for said substance to become encapsulated into said liposome.

121. The method of Claim 120 wherein at least a portion of said entity enters the liposome interior space.

122. The method of Claim 120 wherein at least 90% of said entity enters the liposome interior space.

123. The method of Claim 120 wherein the proportion of said entity that becomes encapsulated into the liposomes is at least 80%, at least 90%, or at least 95%.

124. The method of Claim 120 wherein the entity is a globally cationic entity, a therapeutic entity, or a detectable marker.

125. The method of Claim 120 wherein molar ratio of said entity to the totality of said lipids is at least about 0.05, about 0.1, about 0.2, or about 0.3.

126. The method of Claim 120 wherein said therapeutic entity is an antimicrobial therapeutic, antiviral therapeutic, or an anti-neoplastic therapeutic.

127. The method of Claim 120 wherein said therapeutic entity is an aminoglycoside antibiotic or a fluoroquinolone derivative.

128. The method of Claim 124 wherein said therapeutic entity is selected from a group consisting of: a topoisomerase inhibitor, a farnesyltransferase inhibitor, a tyrosine kinase inhibitor, a cyclin-

dependent kinase inhibitor, a phosphatase inhibitor, an aurora kinase inhibitor, a microtubule depolymerizing agent, a microtubule stabilizing agent, an alkylating agent, an enzyme, an enzyme inhibitor, a histone deacetylase inhibitor, an antimetabolite, a receptor-binding agent, a hormone, a hormone antagonist, a nucleotide, a polynucleotide, a derivative of any of the foregoing, a pro-drug of any of the foregoing, and an analog of any of the foregoing.

129. The method of Claim 124 wherein said therapeutic entity is selected from a group consisting of an anthracycline compound, a camptothecin compound, a vinca alkaloid, an ellipticine compound, a taxane compound, a wortmannin compound, a pyrazolopyrimidine compound, a derivative of any of the foregoing, a pro-drug of any of the foregoing, and an analog of any of the foregoing.

130. The method of Claim 124 wherein said therapeutic entity is selected from a group consisting of: doxorubicin, daunorubicin, mitomycin C, epirubicin, pirarubicin, rubidomycin, carcinomycin, N-acetyladriamycin, rubidazone, 5-imidodaunomycin, N-acetyldaunomycin, daunoryline, mitoxanthrone, camptothecin, 9-aminocamptothecin, 7-ethylcamptothecin, 10-hydroxycamptothecin, 9-nitrocamptothecin, 10,11-methylenedioxycamptothecin, 9-amino-10,11-methylenedioxycamptothecin, 9-chloro-10,11-methylenedioxycamptothecin, irinotecan, topotecan, lurtotecan, silatecan, (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin, 7-(4-methylpiperazinomethylene)-10,11-methylenedioxy-20(S)-camptothecin, 7-(2-N-isopropylamino)ethyl)-(20S)-camptothecin, vincristine, vinblastine, vinorelbine, vinflunine, vinpocetine, vindesine, ellipticine, 6-3-aminopropyl-ellipticine, 2-diethylaminoethyl-ellipticinium and salts thereof, datelliptium, retelliptine, paclitaxel, docetaxel, a derivative of any of the foregoing, a pro-drug of any of the foregoing, and an analog of any of the foregoing..

131. The method of Claim 124 wherein said therapeutic entity is selected from a group consisting of: an antihistamine ethylenediamine derivative, bromphenifamine, diphenhydramine, an anti-protozoal drug, quinolone, iodoquinol, an amidine compound, pentamidine, an antihelmintic compound, pyrantel, an anti-schistosomal drug, oxaminiquine, an antifungal triazole derivative, fliconazole, itraconazole, ketoconazole, miconazole, an antimicrobial cephalosporin, cefazolin, cefonicid, cefotaxime, ceftazimide, cefuoxime, an antimicrobial beta-lactam derivative, aztreopam, cefmetazole, cefoxitin, an antimicrobial of erythromycine group, erythromycin, azithromycin, clarithromycin, oleandomycin, a penicillin compound, benzylpenicillin, phenoxymethylpenicillin, cloxacillin, methicillin, nafcillin, oxacillin, carbenicillin, a tetracycline compound, novobiocin, spectinomycin, vancomycin; an antimycobacterial drug, aminosalicyclc acid, capreomycin, ethambutol, isoniazid, pyrazinamide, rifabutin, rifampin, clofazimine, an antiviral adamantane compound, amantadine, rimantadine, a quinidine compound, quinine, quinacrine, chloroquine, hydroxychloroquine, primaquine, amodiaquine, mefloquine, an antimicrobial qionolone, ciprofloxacin, enoxacin, lomefloxacin, nalidixic acid, norfloxacin, ofloxacin, a sulfonamide; a urinary tract antimicrobial, nitrofurantoin, trimetoprim; a nitroimidazoles derivative, metronidazole, a cholinergic quaternary ammonium compound, ambethinium, neostigmine, physostigmine, an anti-Alzheimer aminoacridine, tacrine, an anti-Parkinsonal drug, benztropine, biperiden, procyclidine, trihexylhenidyl, an anti-muscarinic agent, atropine, hyoscyamine, scopolamine, propantheline, an adrenergic compound, dopamine, serotonin, albuterol, dobutamine, ephedrine, epinephrine, norepinephrine, isoproterenol, metaproperenol, salmetrol, terbutaline, a serotonin reuptake inhibitor, an ergotamine derivative, a myorelaxant of a curare series, a central action myorelaxant, baclophen, cyclobenzepine, dentrolene, nicotine, a nicotine receptor antagonist, a beta-adrenoblocker, acebutil, amiodarone, a benzodiazepine compound, ditiazem, an antiarrhythmic drug, diisopyramide, encaidine, a local

anesthetic compound, procaine, procainamide, lidocaine, flecaimide, quinidine; an ACE inhibitor, captopril, enelaprilat, fosinoprol, quinapril, ramipril; an opiate derivative, codeine, meperidine, methadone, morphine, an antilipidemic, fluvastatin, gemfibrosil, an HMG-coA inhibitor, pravastatin, a hypotensive drug, clonidine, guanabenz, prazocin, guanethidine, granadril, hydralazine, a non-coronary vasodilator, dipyridamole, an acetylcholine esterase inhibitor, pilocarpine, an alkaloid, physostigmine, neostigmine, a derivative of any of the foregoing, a pro-drug of any of the foregoing, and an analog of any of the foregoing.

132. The method of any of the Claims 120-131 wherein said contacting is in an aqueous solution.

133. The method of Claim 132 wherein said aqueous solution has pH in the range of about 4 to about 7.

134. The method of Claim 132 wherein said aqueous solution has ionic strength equivalent or less than that of 50 mM sodium chloride.

135. The method of Claim 132 wherein said aqueous solution has ionic strength equivalent or less than that of 20 mM sodium chloride.

136. The method of Claim 132, wherein the aqueous solution has pH between about 6.0 and about 7.0, and the entity is a vinca alkaloid, an analog thereof, or a derivative thereof.

137. The method of Claim 136 wherein the pH of the aqueous solution of about 6.5, and the entity is vinorelbine.

138. The method of Claim 135 wherein the pH of the aqueous solution is between about 5 and about 7, and the substance is a camptothecin derivative.

139. The method of Claim 139 wherein the pH of the aqueous solution is between about 5.0 and about 6.5 , and the substance is Topotecan or Irinotecan.

140. The method of Claim 134 or 135, wherein following said contacting, the ionic strength of said aqueous solution is increased to more than that of 50 mM sodium chloride.

141. The method of Claim 140 wherein said ionic strength value is increased to at least that of 100 mM sodium chloride.

142. The method of Claim 141 wherein said ionic strength value is increased to at least that of 150 mM sodium chloride.

143. A method for preparing a liposomes containing an encapsulated entity, said process comprising the steps of:

(a) providing a pre-entity of said entity;

(b) encapsulating said pre-entity into a liposome having an interior space; and

(c) providing a condition inside the liposome to convert said encapsulated pre-entity into said entity within the interior space of said liposome to obtain said entity in a liposomally encapsulated form

wherein said entity is an organic compound or a compound comprising a coordination complex of a metal of the platinum group.

144. The method of Claim 143 wherein said encapsulating step comprises contacting the pre-entity with said liposome having a transmembrane

gradient for the time sufficient for encapsulation of said derivative into said liposome.

145. The method of Claim 144 wherein said transmembrane gradient is an ion gradient, a pH gradient, an electrochemical potential gradient, or a solubility gradient.

146. The method of Claim 144 wherein said ion gradient is a gradient of an ion selected from the group of ammonium ion, and a substituted form of ammonium ion comprising at least one C-N bond.

147. The method of Claim 144 wherein said pre-entity is a globally cationic derivative of the entity.

148. The method Claim 143 wherein said compound is a pharmaceutical.

149. The method of Claim 148 wherein said organic compound is a taxane compound

150. The method of Claim 149 wherein said taxane compound is paclitaxel or docetaxel.

151. The method of Claim 149 wherein said pre-entity comprises an ester of the hydroxyl group in any of the positions 2' or 7' of the taxane molecule wherein said ester comprises a titratable amine.

152. The method of Claim 151 wherein said pre-entity is 2'-(2-(N,N'-diethylamino)propionyl)-paclitaxel or 2'-(2-(N,N'-diethylamino)propionyl)-docetaxel.

153. The method of Claim 143 wherein said condition is a change in pH.

154. The method of Claim 143 wherein said condition is an enzymatic cleavage of a labile bond.

155. The method of Claim 143 wherein said condition is the process of hydrolysis, photolysis, radiolysis, thiolysis, ammonolysis, reduction, substitution, oxidation, or elimination.

156. A kit for providing a liposomally encapsulated entity, comprising the composition of any of the claims 1 - 22, an instruction for using the composition to encapsulate an entity, and optionally, in a separate container, the entity.

157. A kit of Claim 156 wherein the entity is a globally cationic substance, a therapeutic entity, or a detectable marker.

158. A composition of any of the Claims 1-119 wherein the liposome comprises a substituted ammonium compound having in a aqueous solution at ambient temperature a pKa value of at least 8.0, at least 8.5, at least 9.0, at least 9.5, or at least 10.0.

159. A method of any of the Claims 120-142 wherein the composition contacted with the entity comprises a liposome, said liposome comprises a substituted ammonium compound having in a aqueous solution at ambient temperature a pKa value of at least 8.0, at least 8.5, at least 9.0, at least 9.5, or at least 10.0.