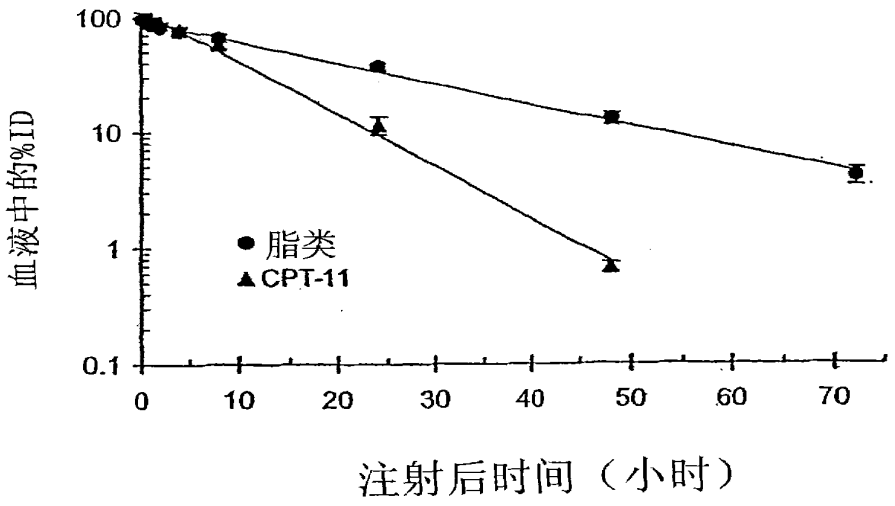


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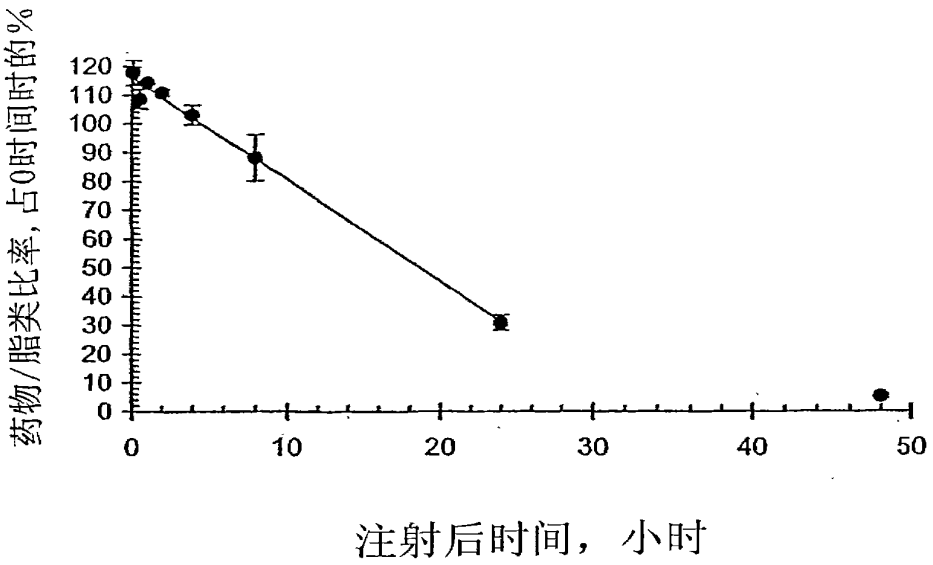


图 2

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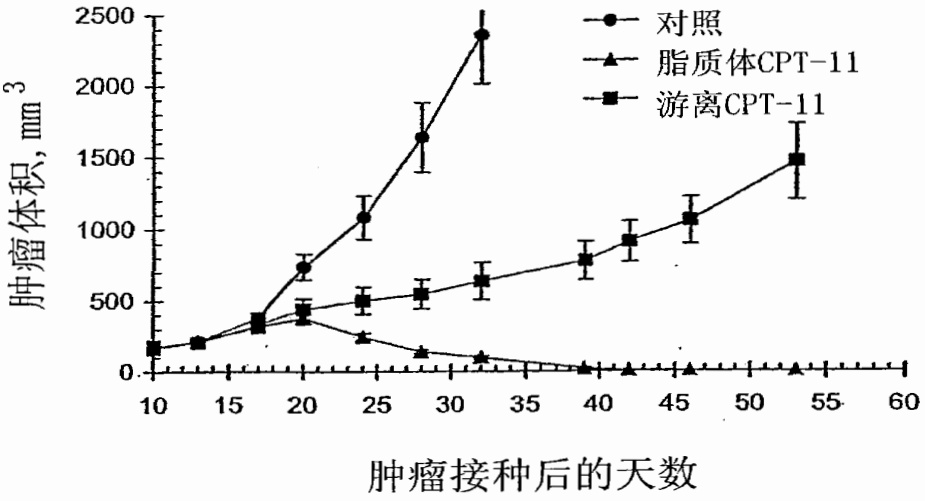


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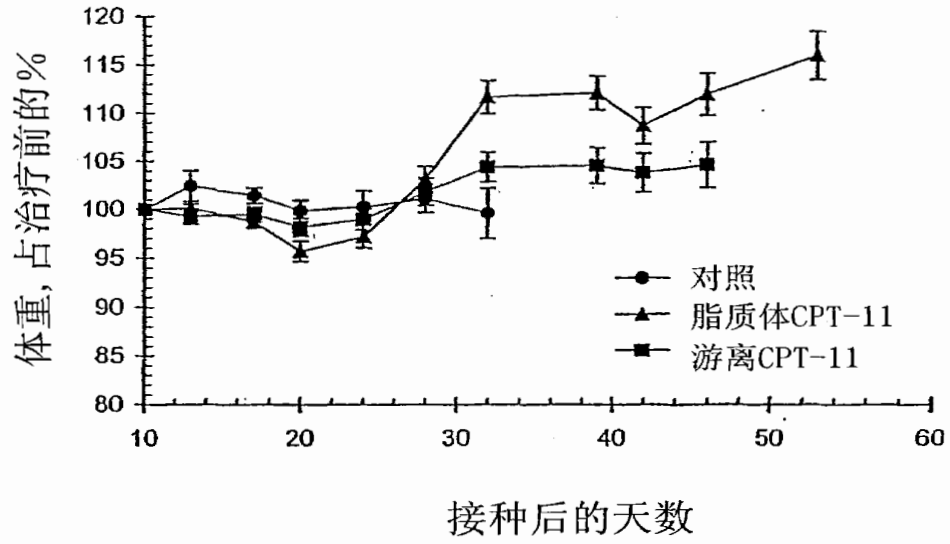


图 4

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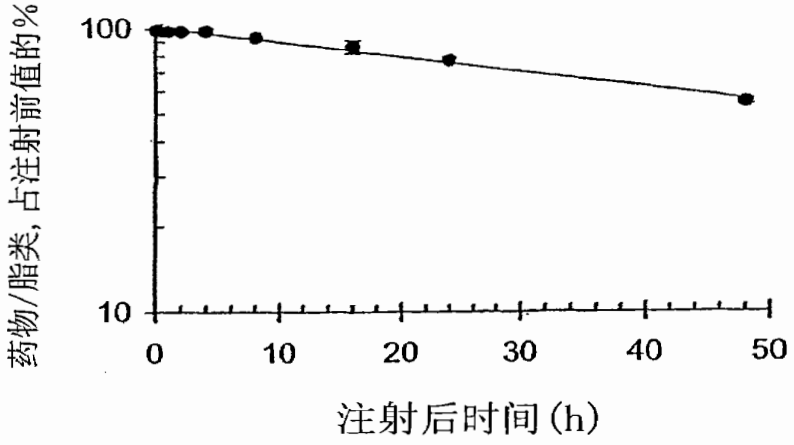


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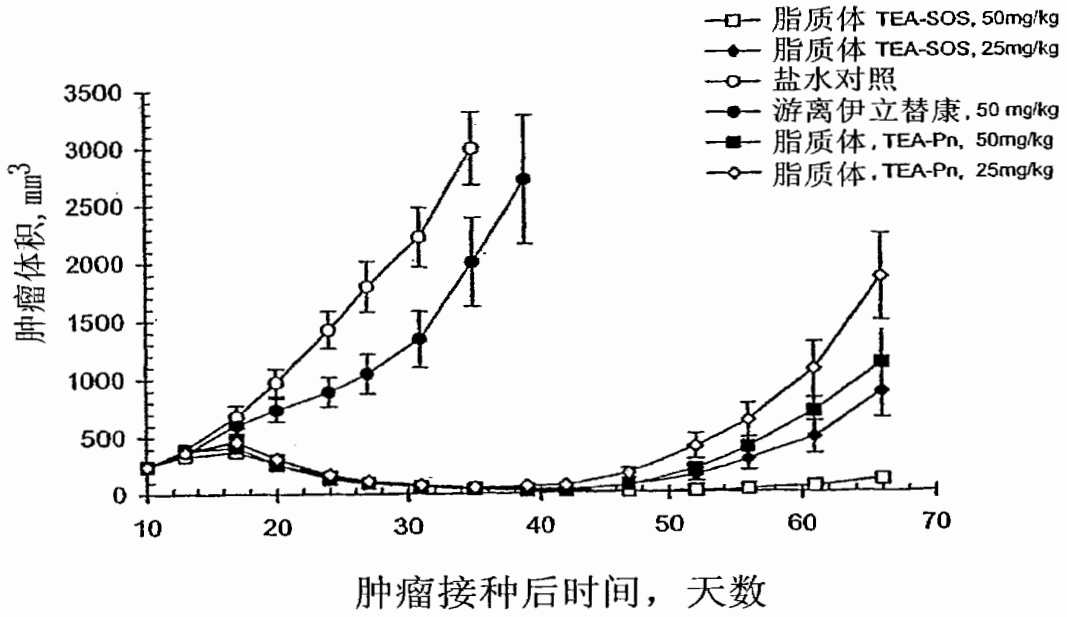


图 6

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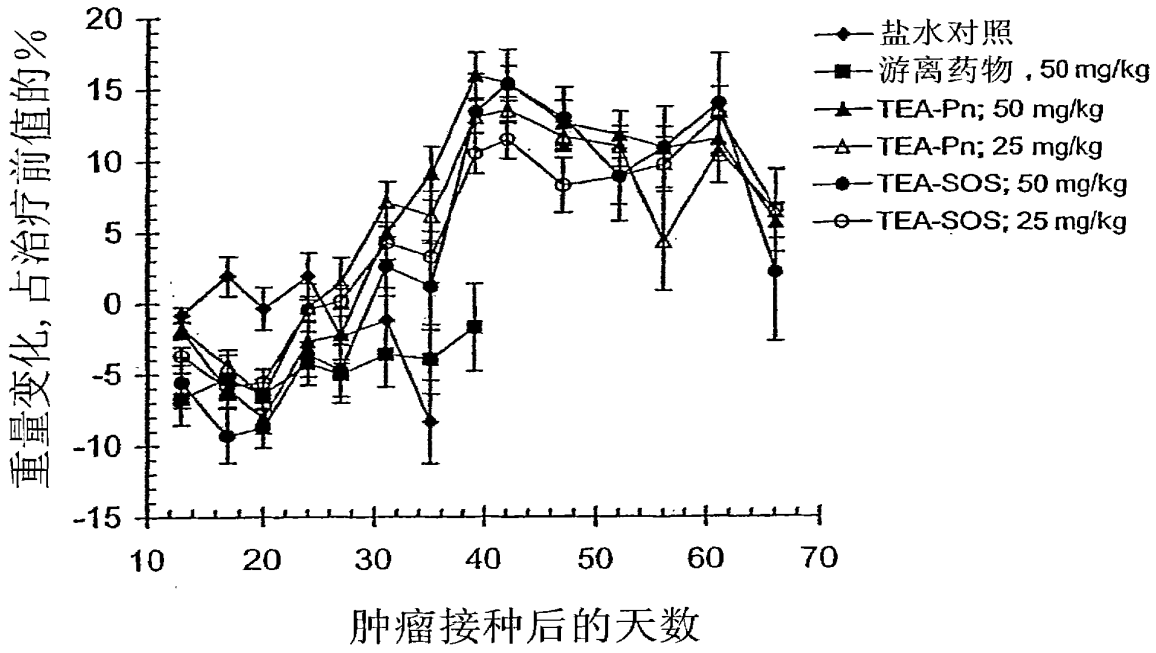


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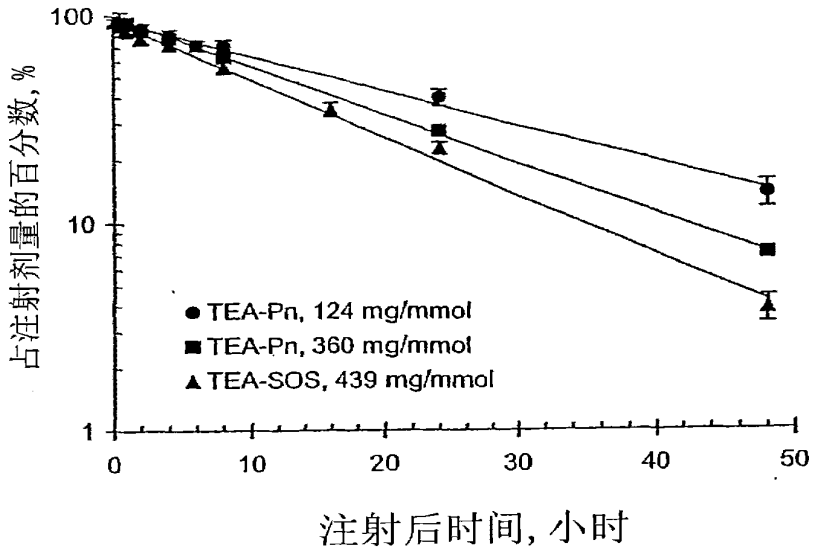


图 8A

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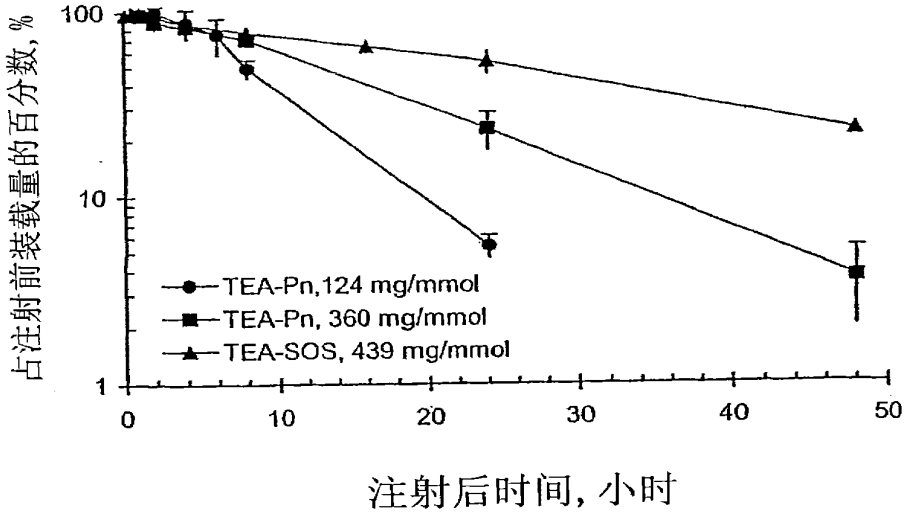


图 8B

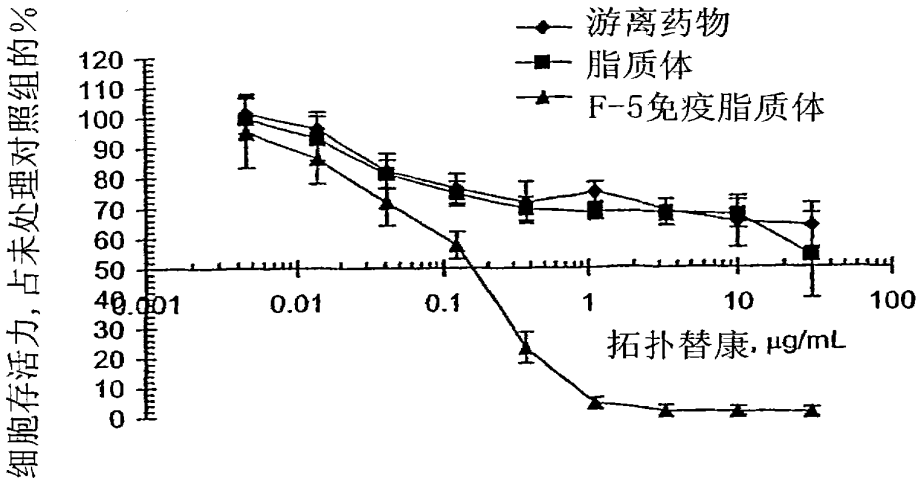


图 9

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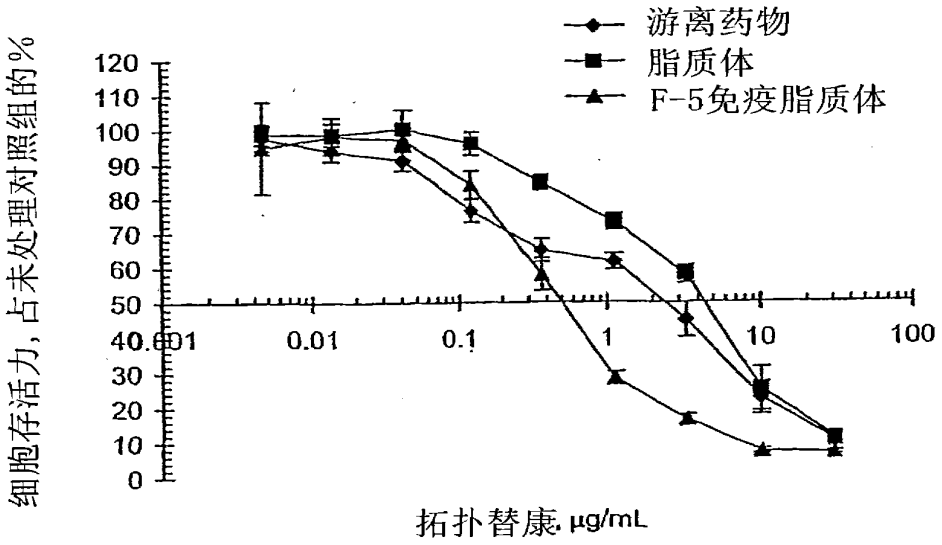


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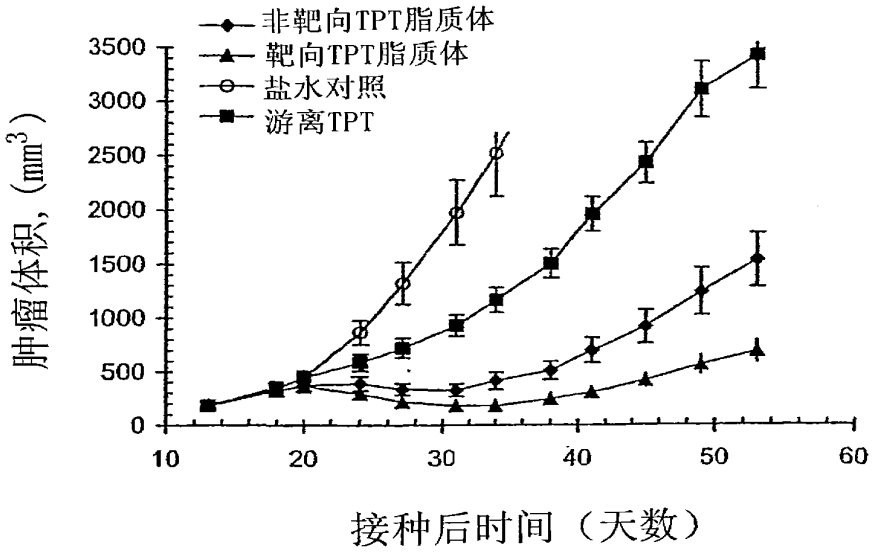


图 11

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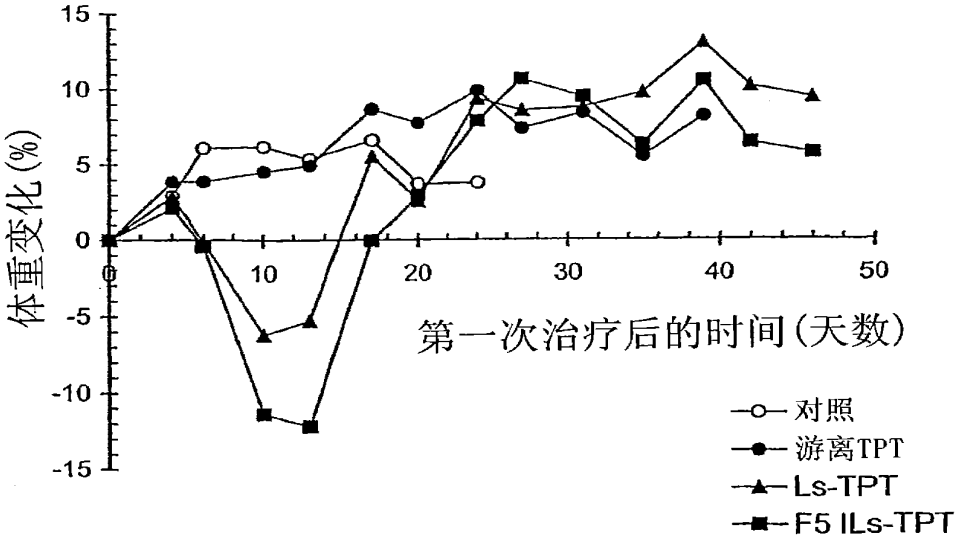


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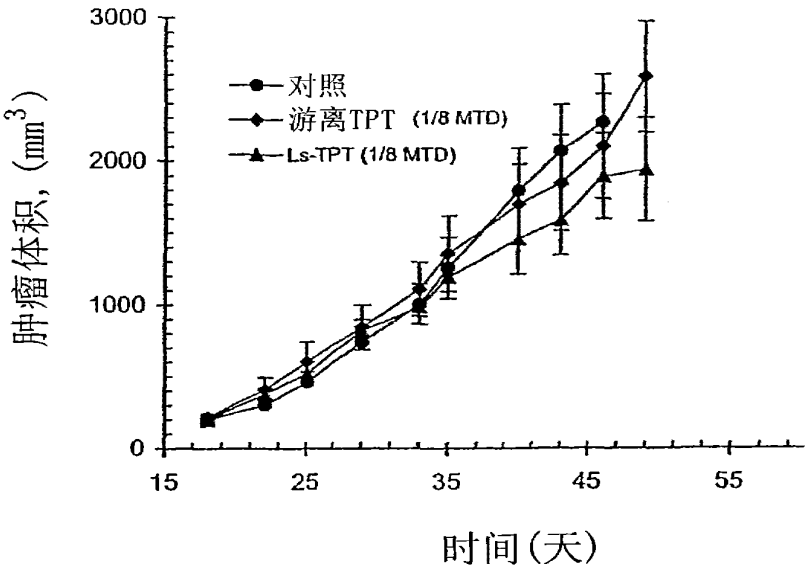


图 13A

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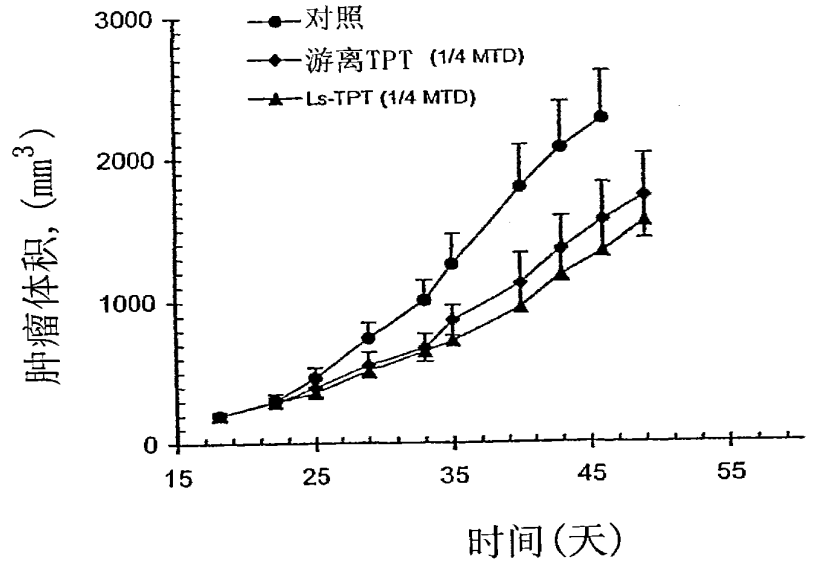


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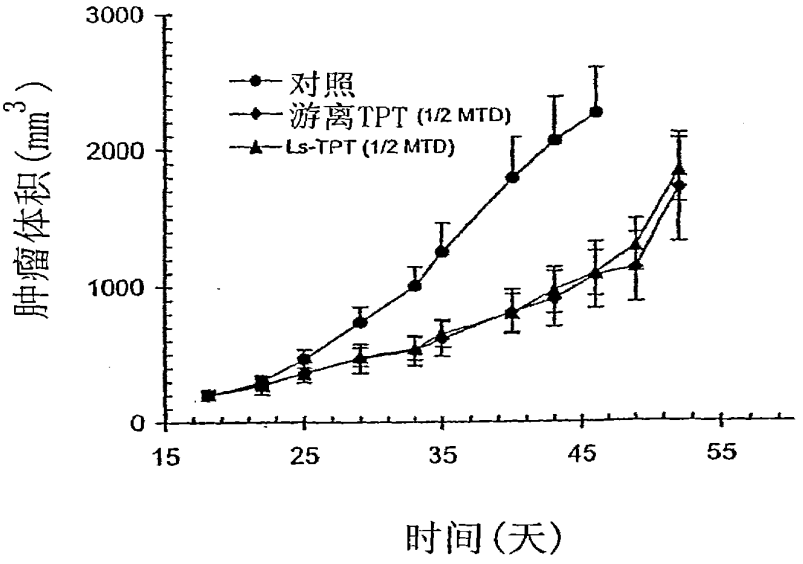


图 13C

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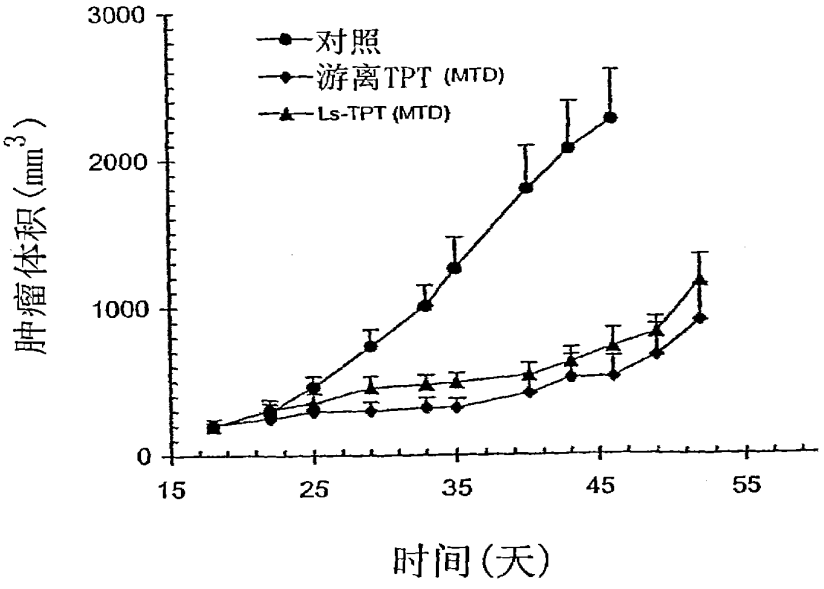


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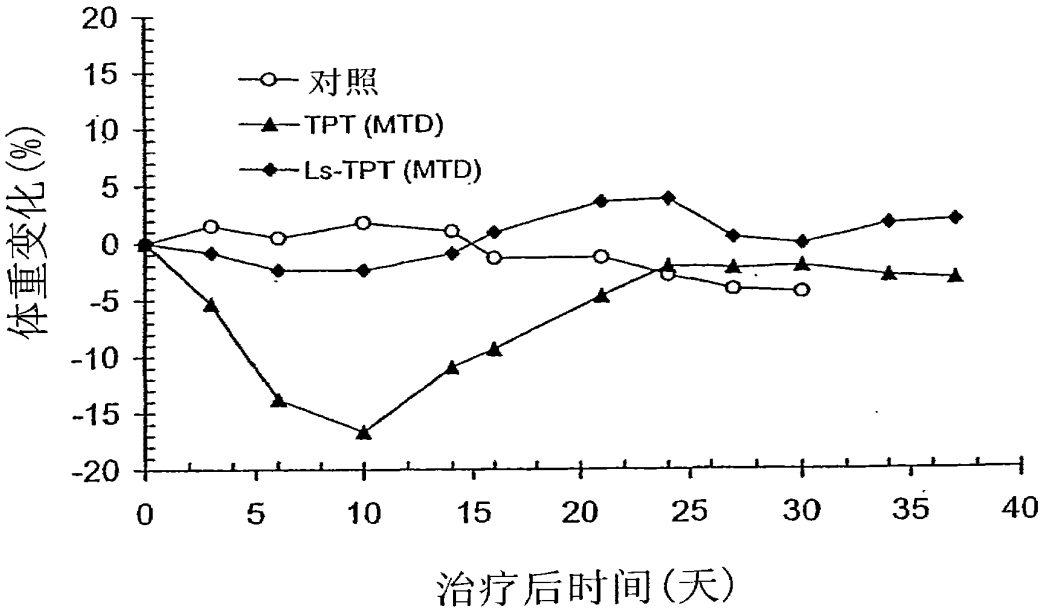


图 14

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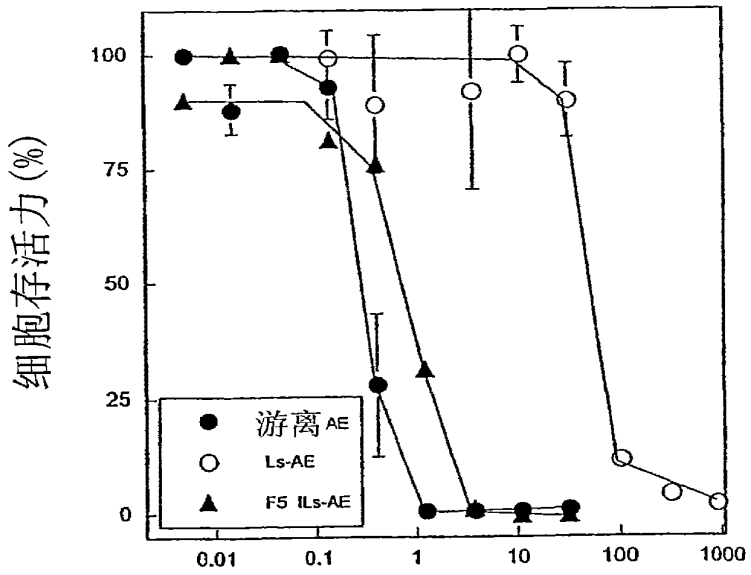


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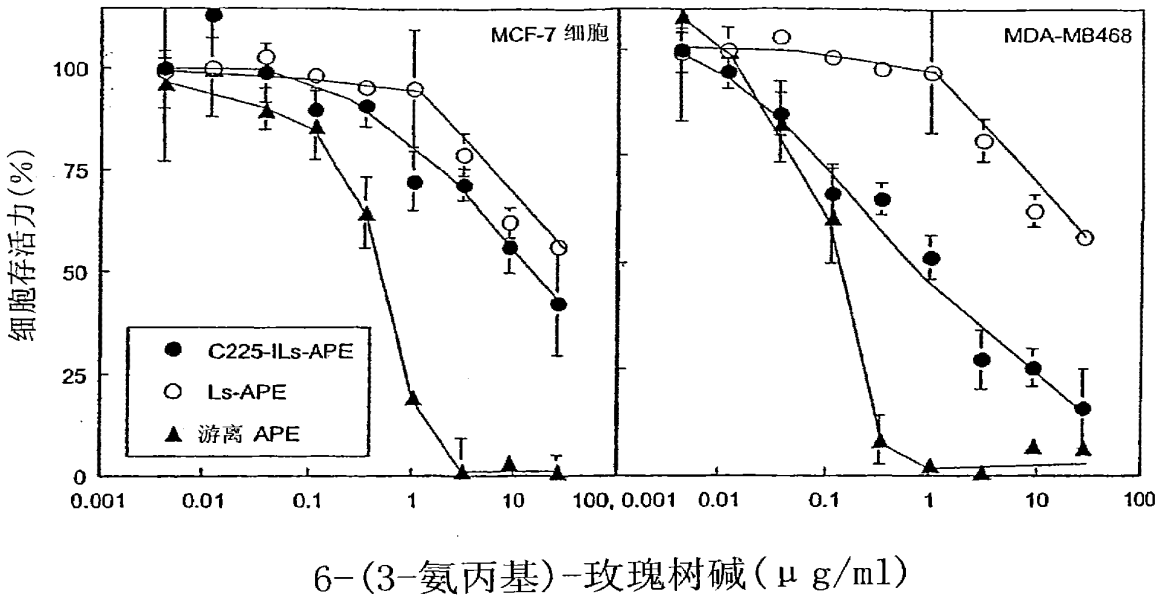


图 16

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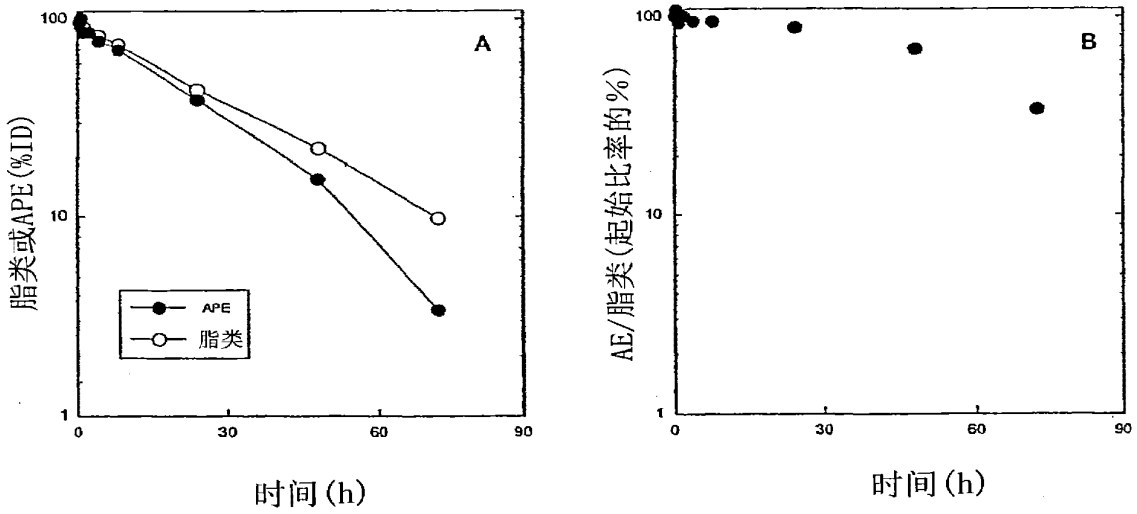


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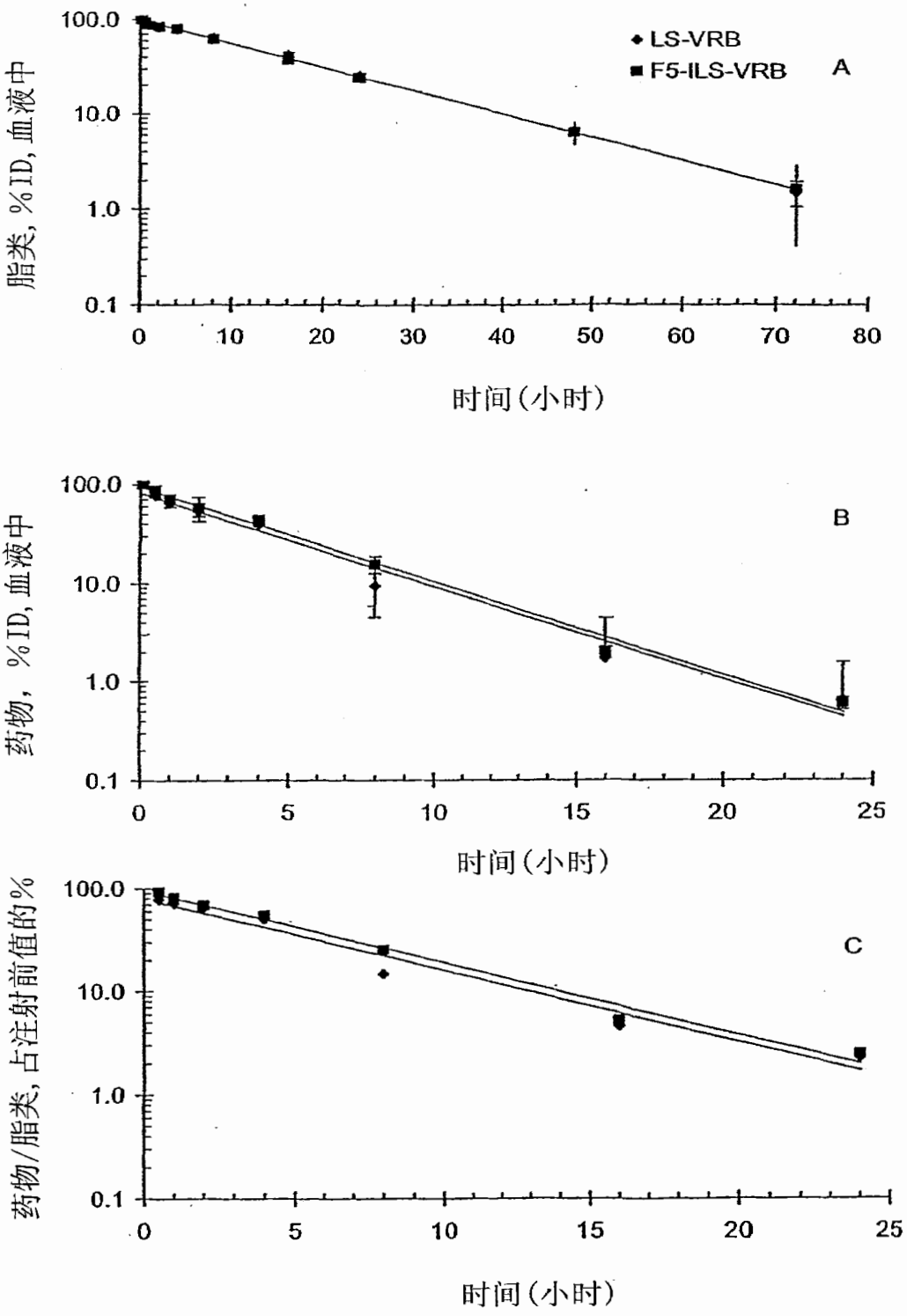


图 18

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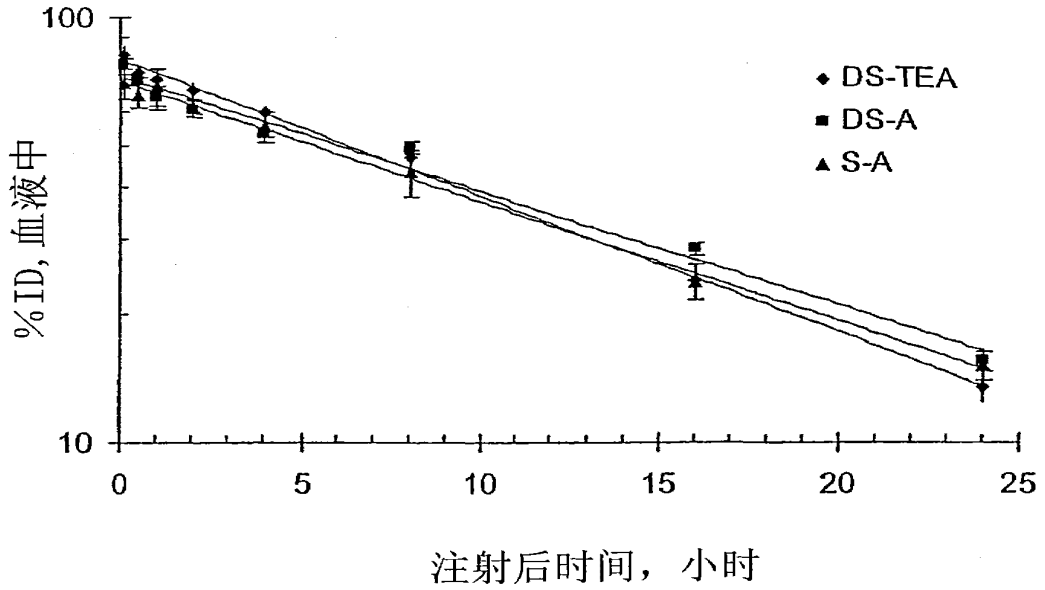


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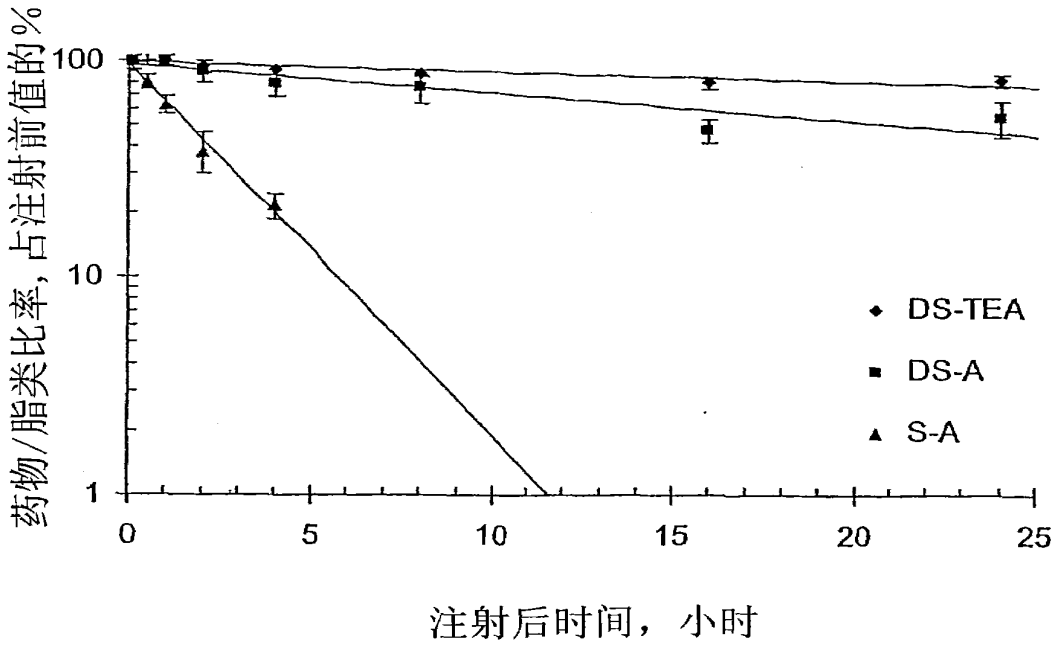


图 20

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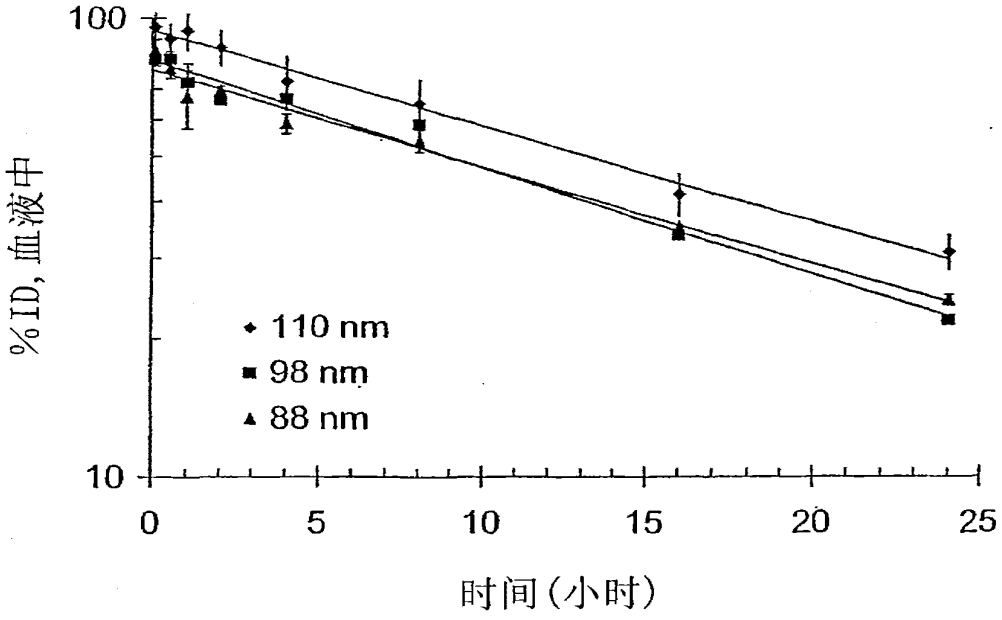


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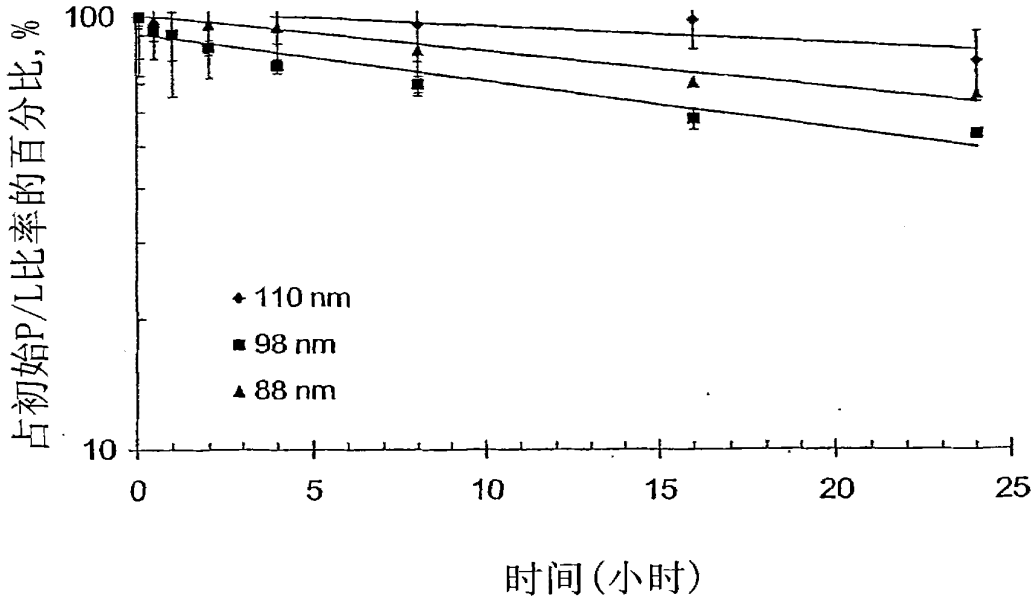


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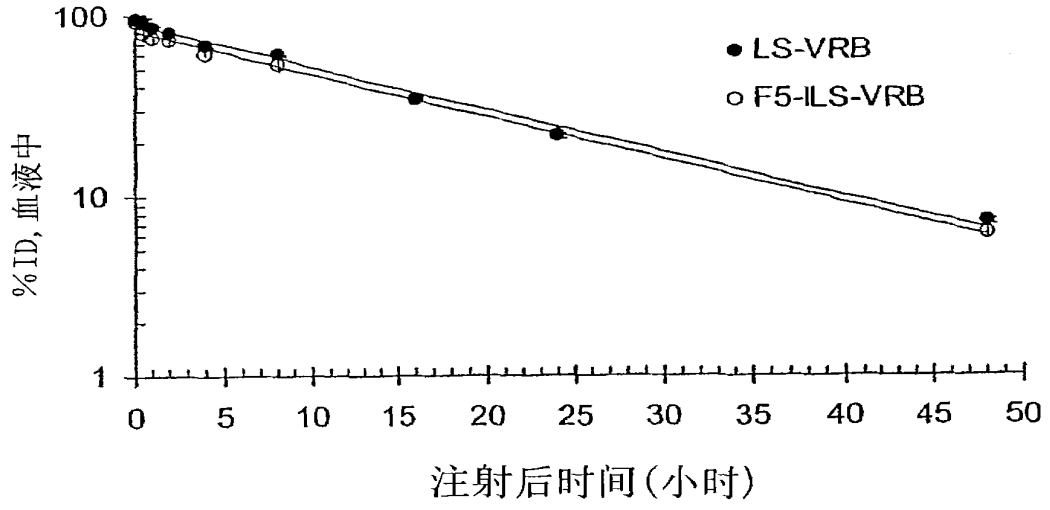


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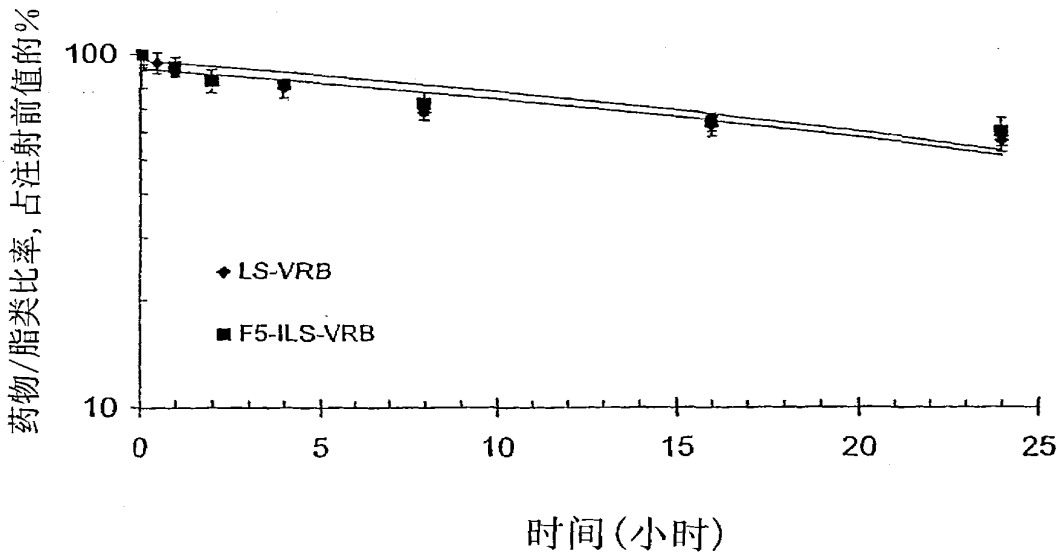


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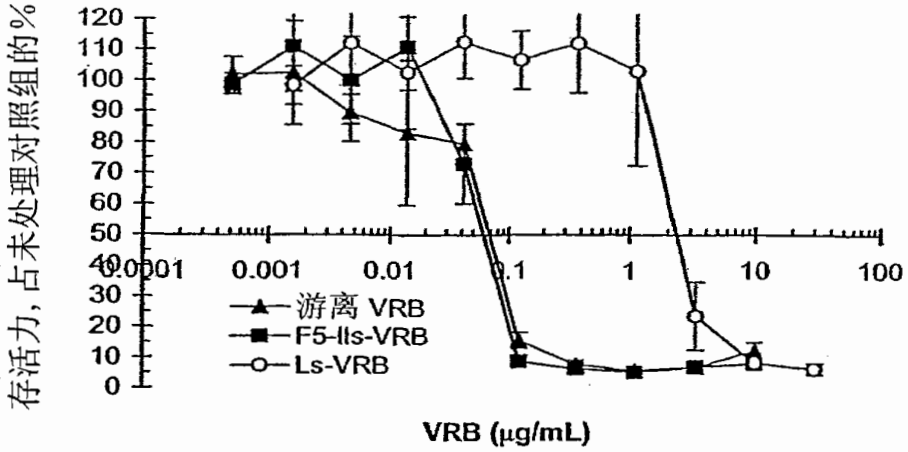


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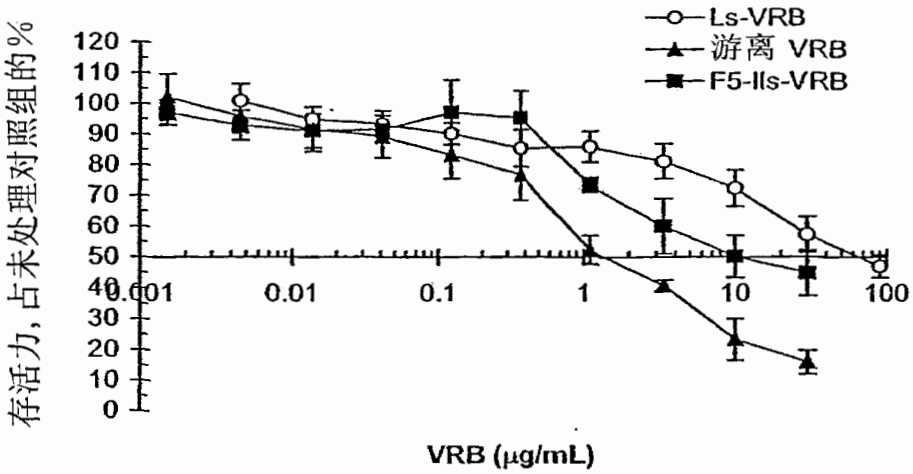


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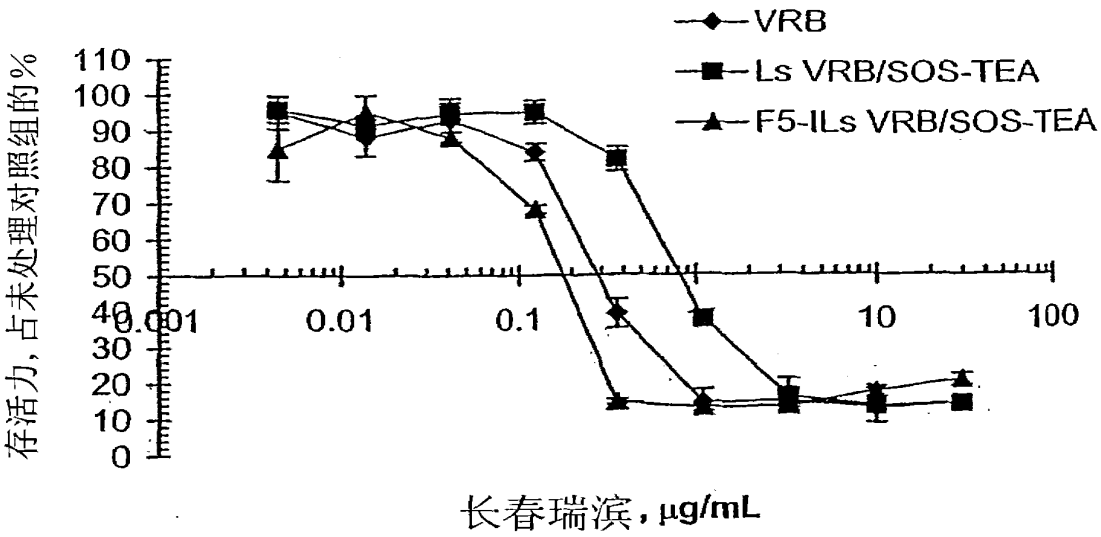


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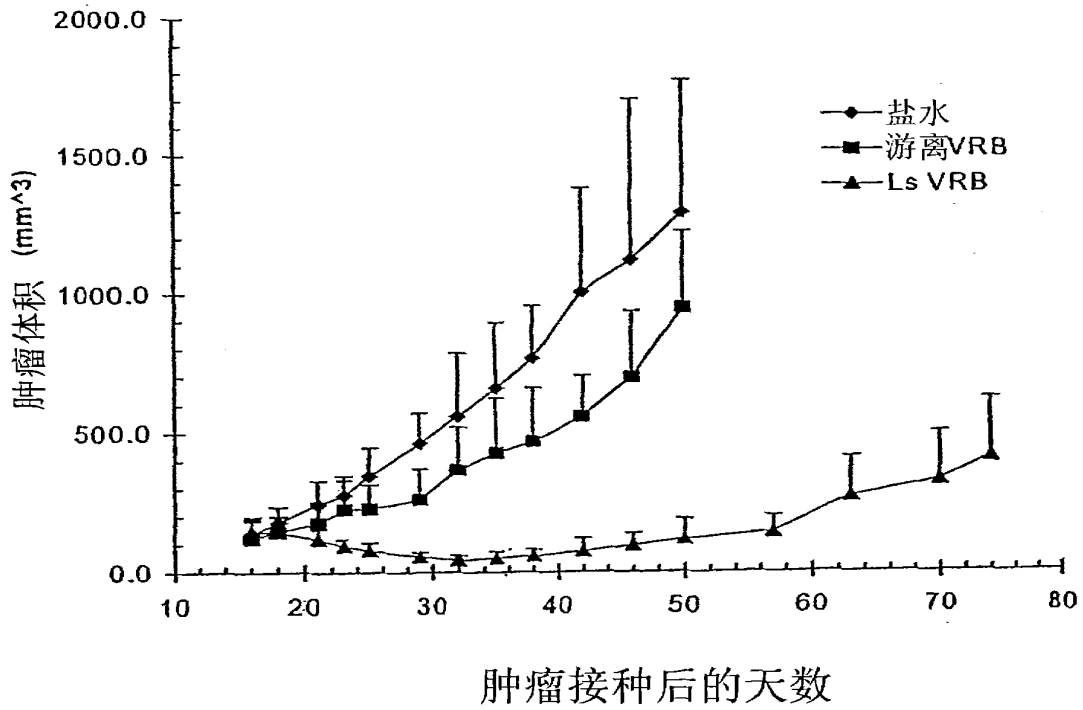


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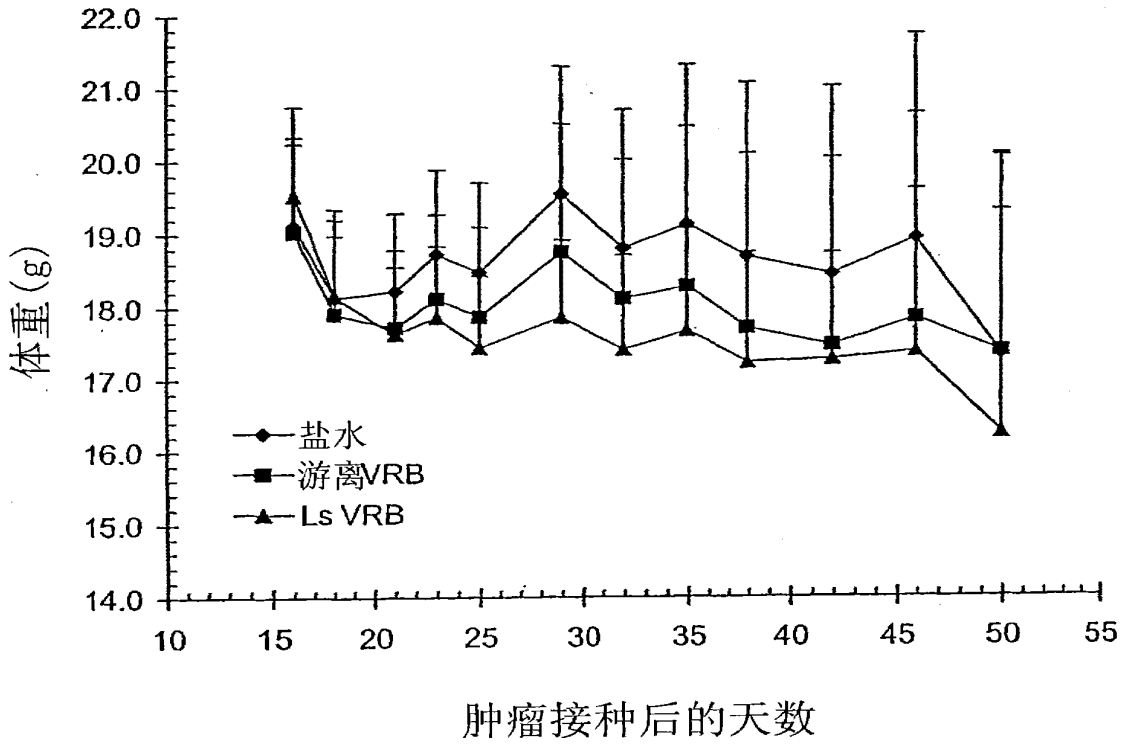


图 29

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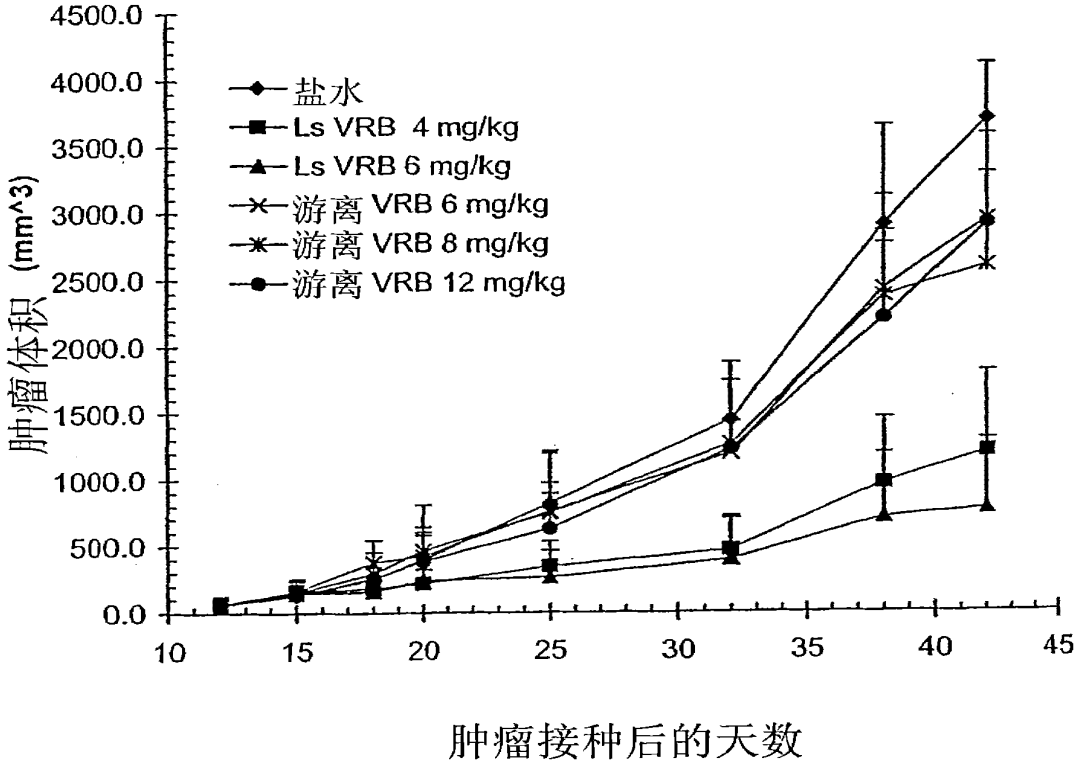


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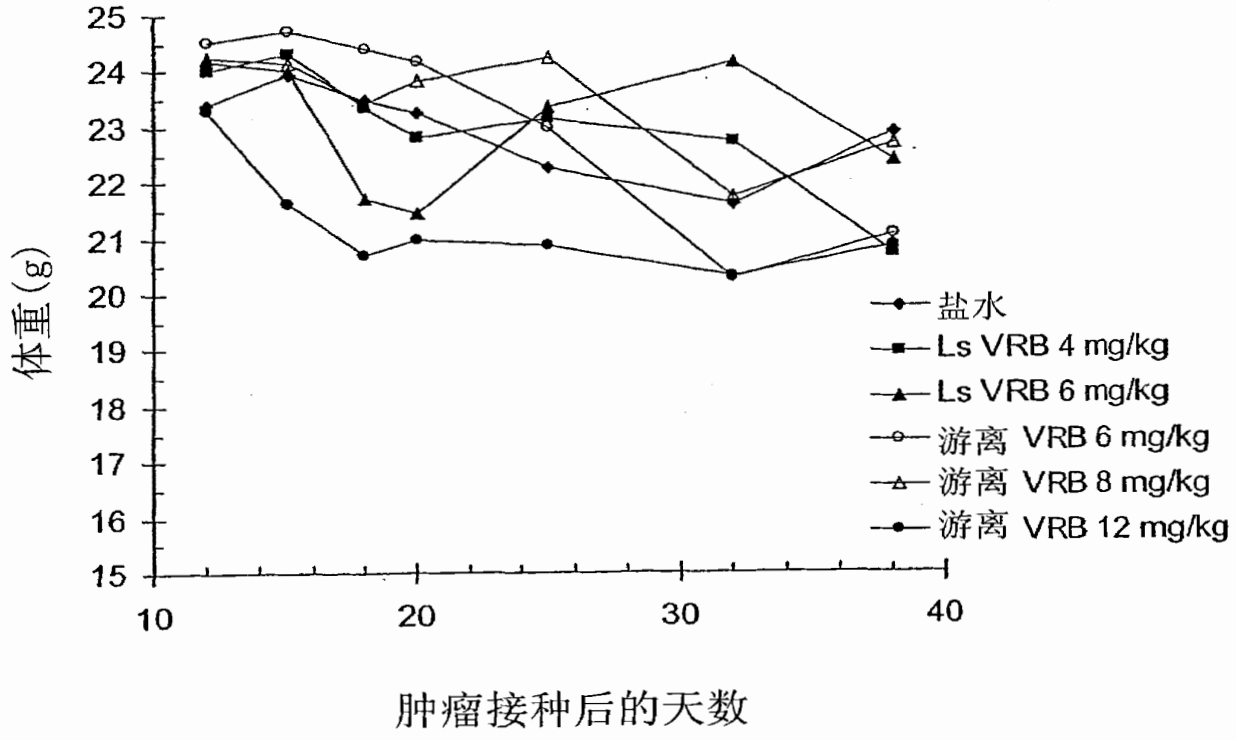


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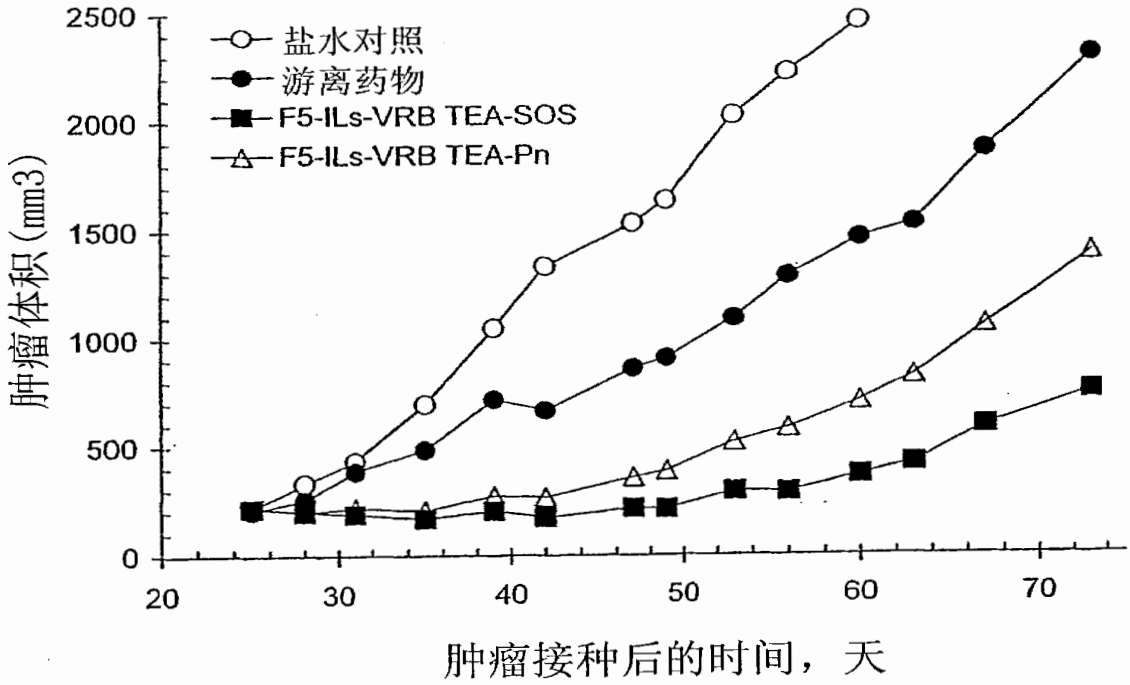


图 32

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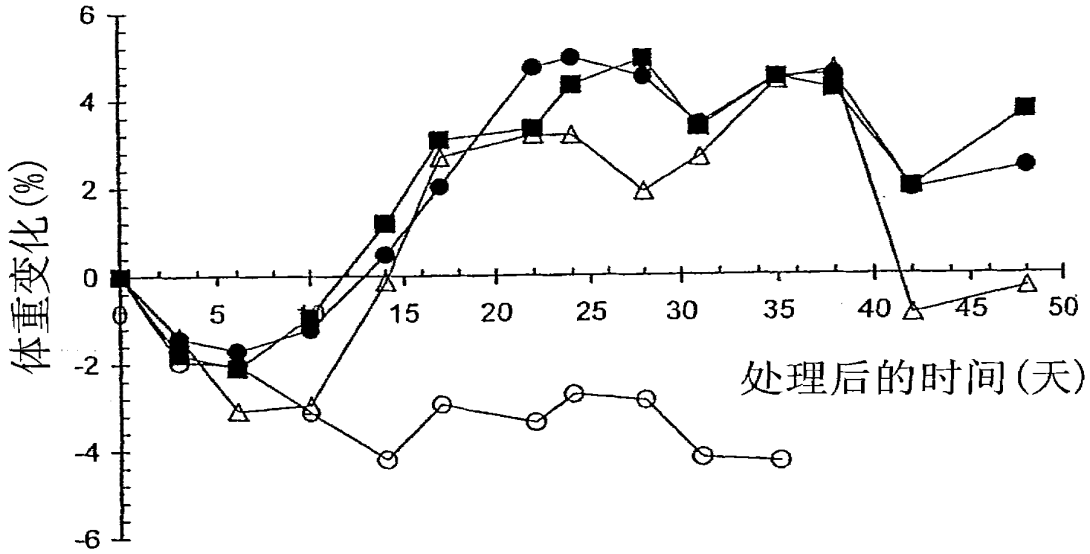


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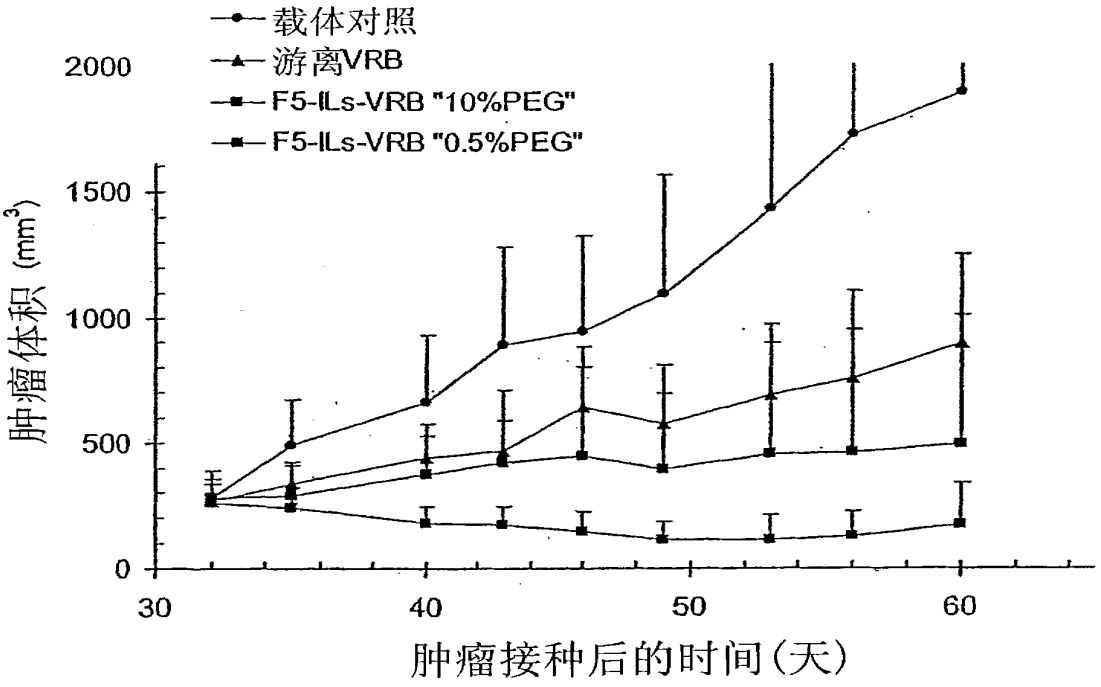


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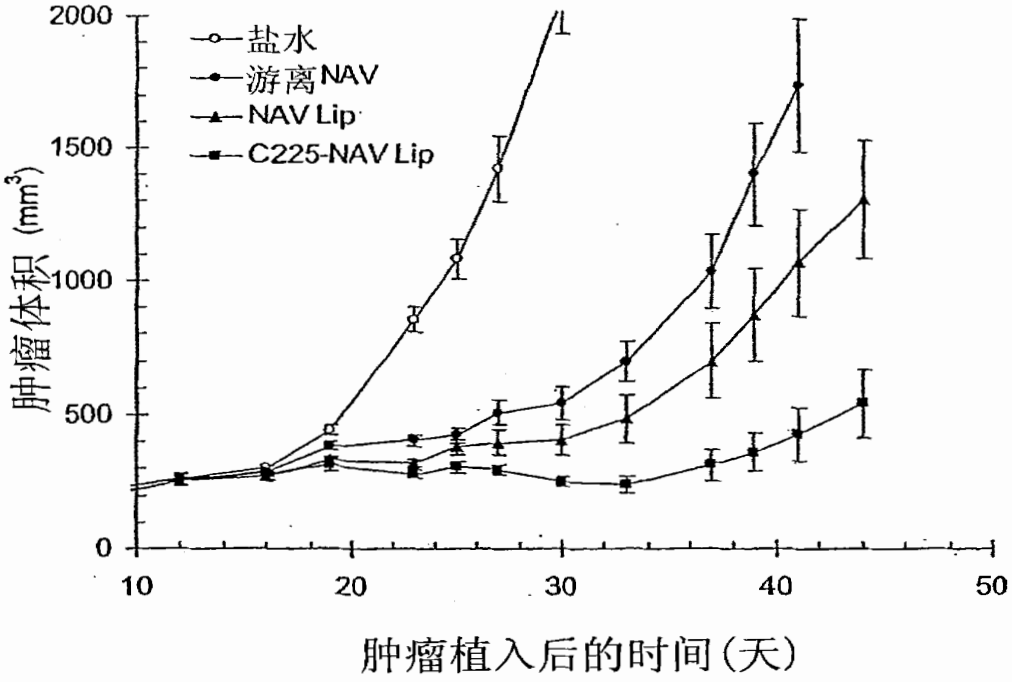


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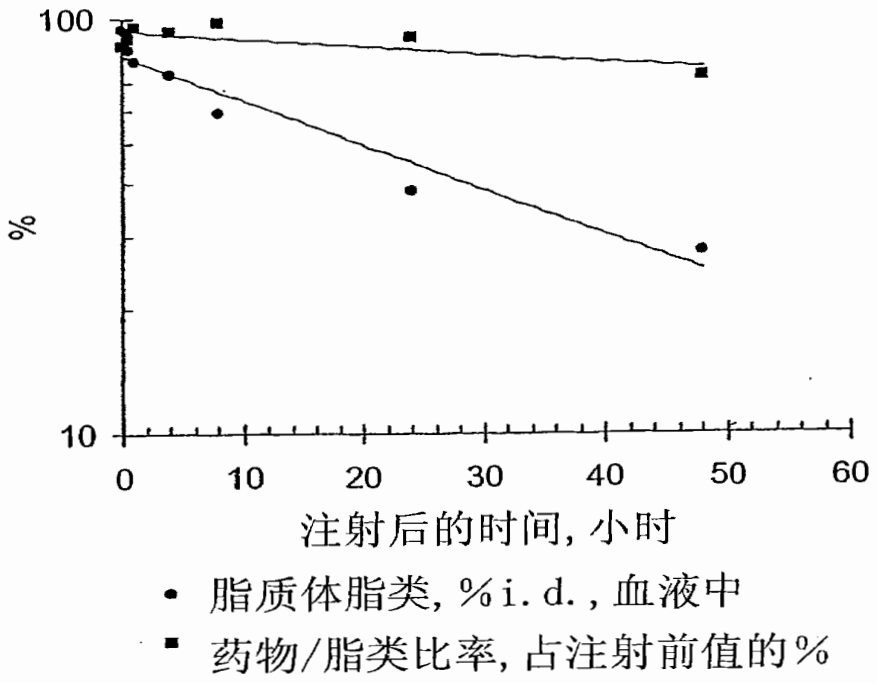


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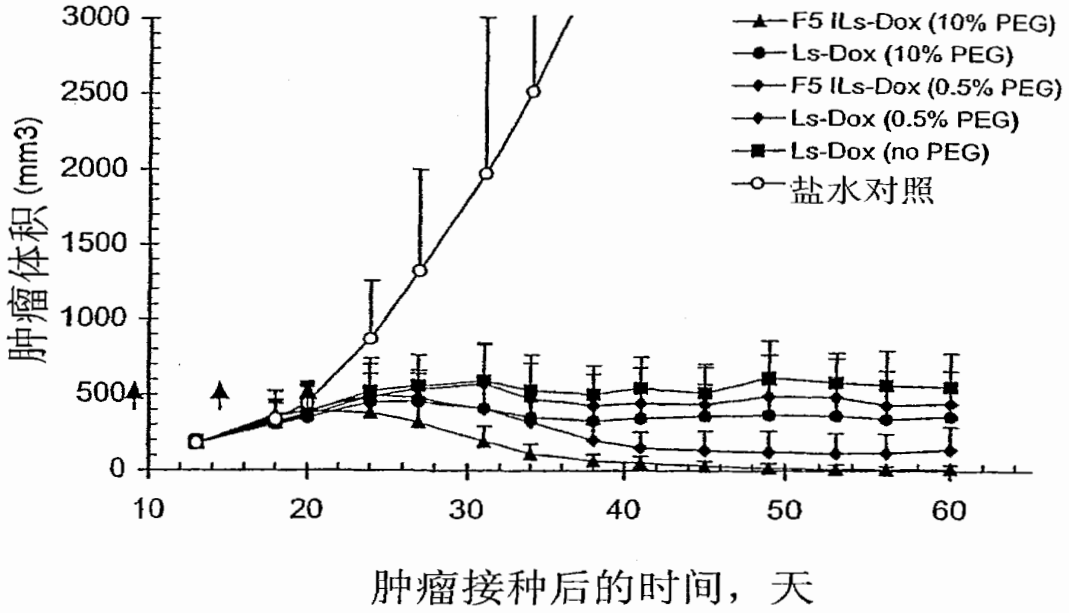


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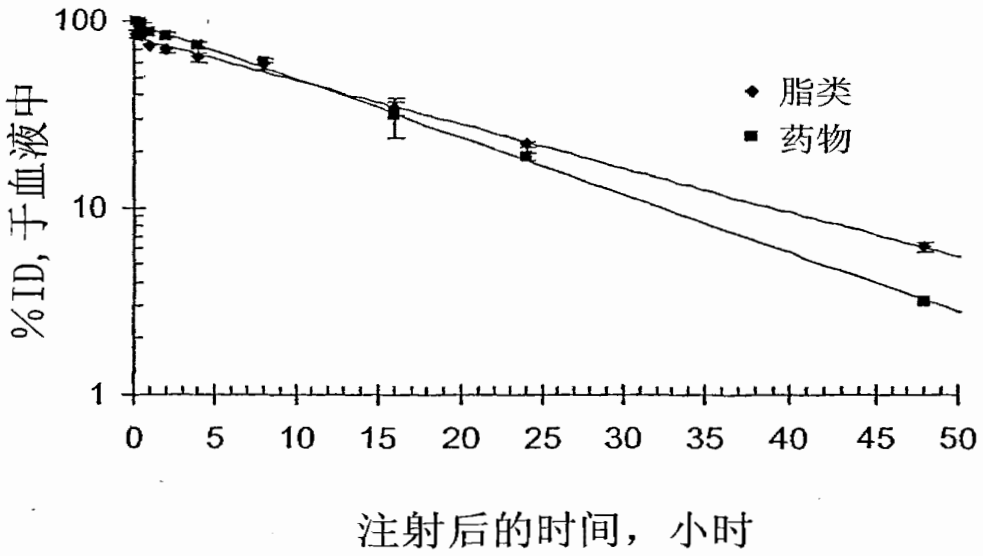


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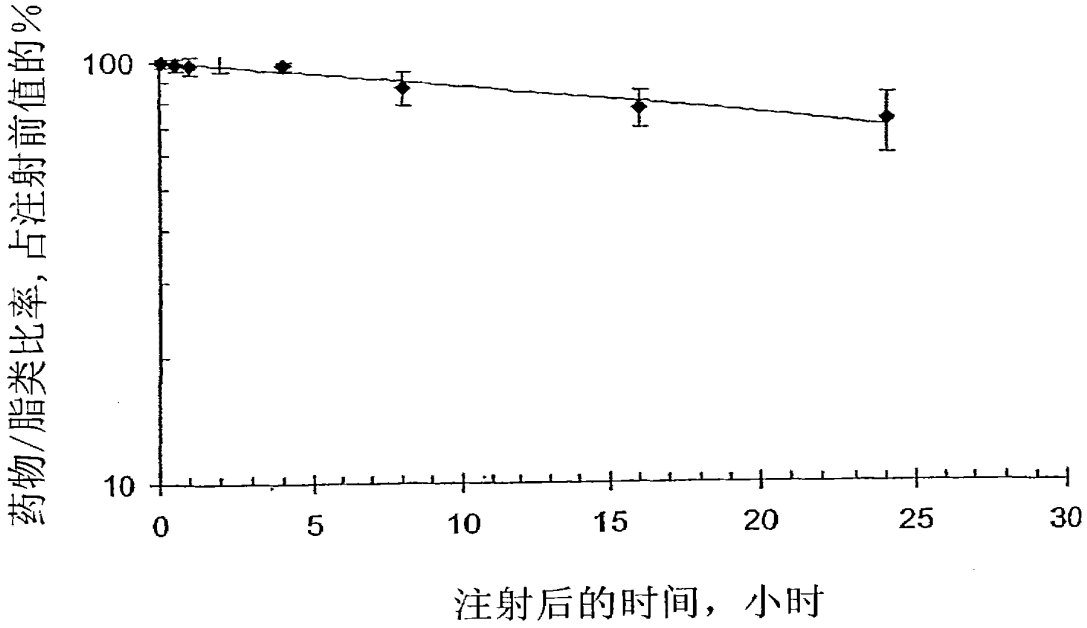


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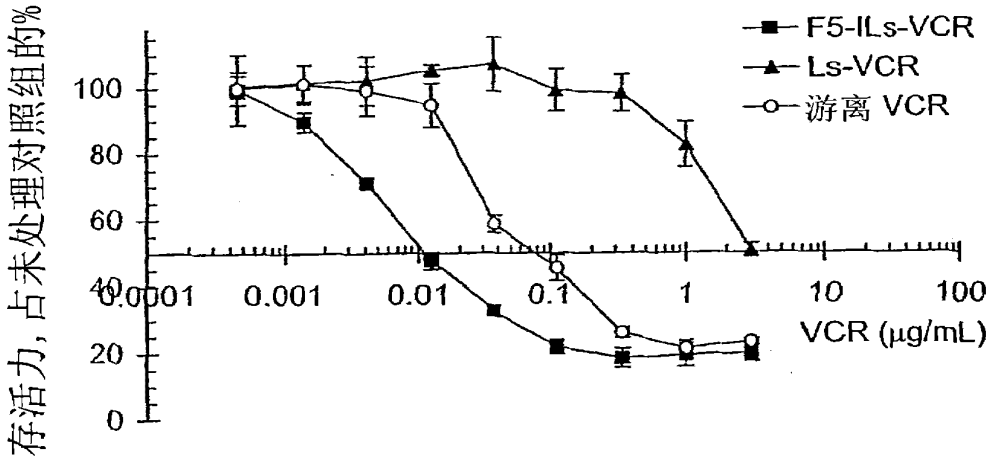


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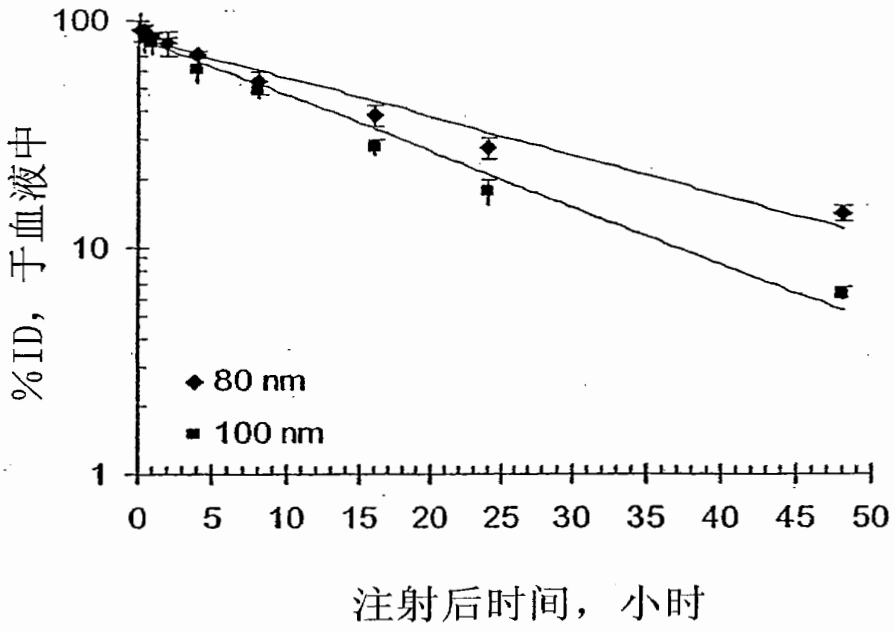


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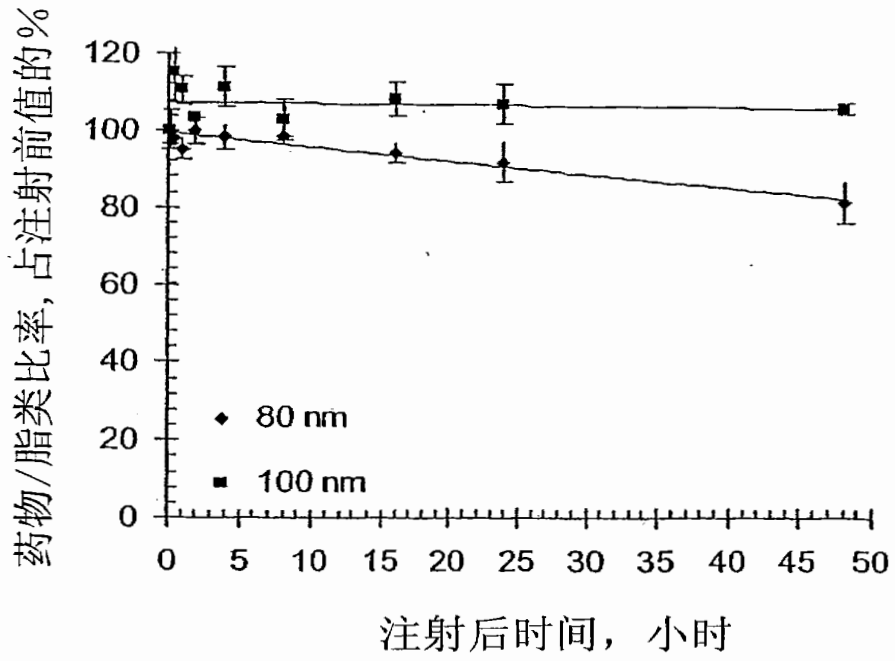


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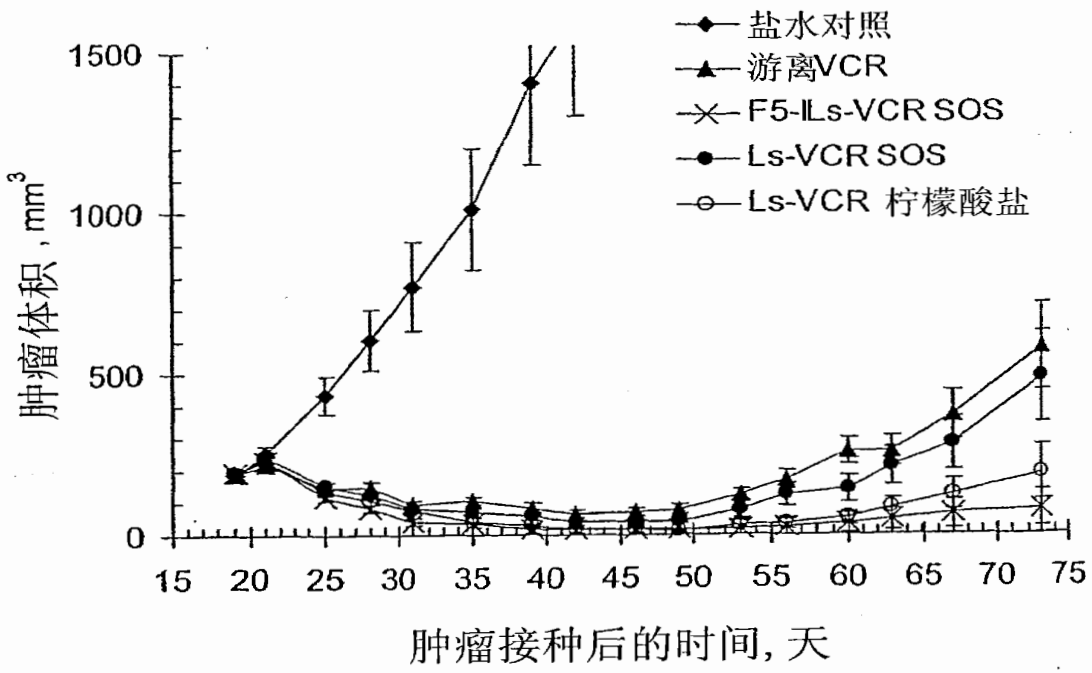


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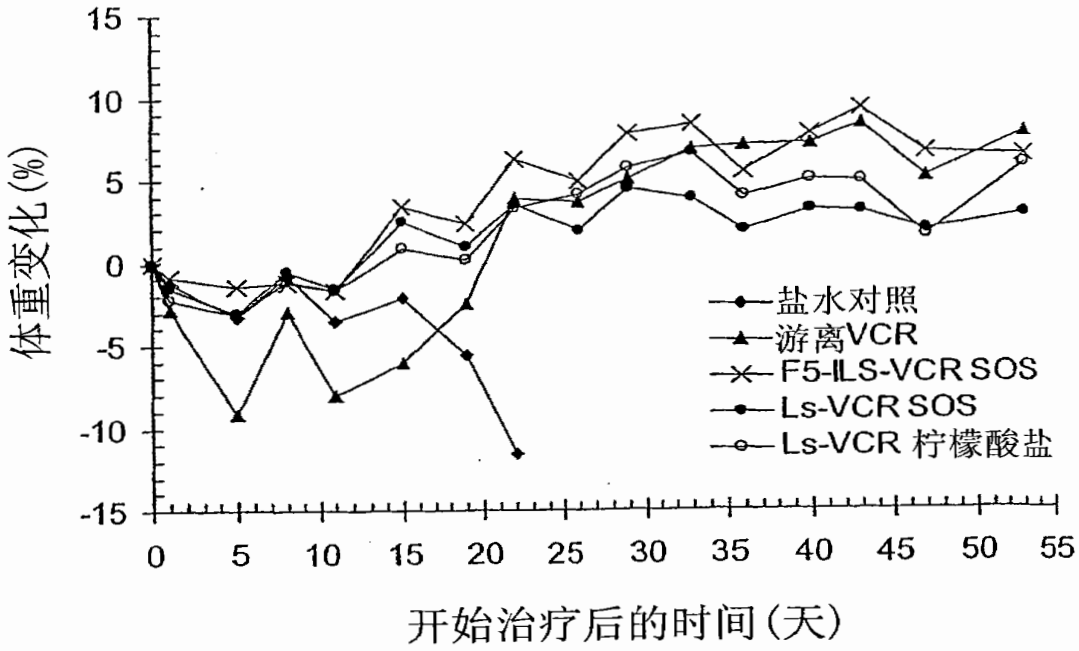


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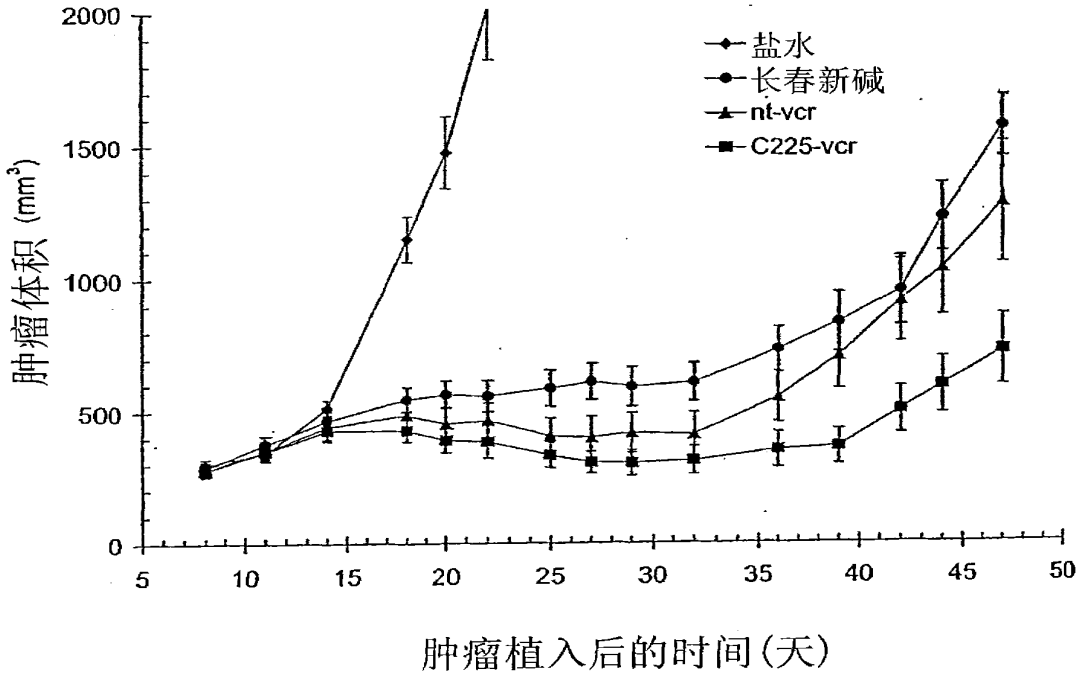


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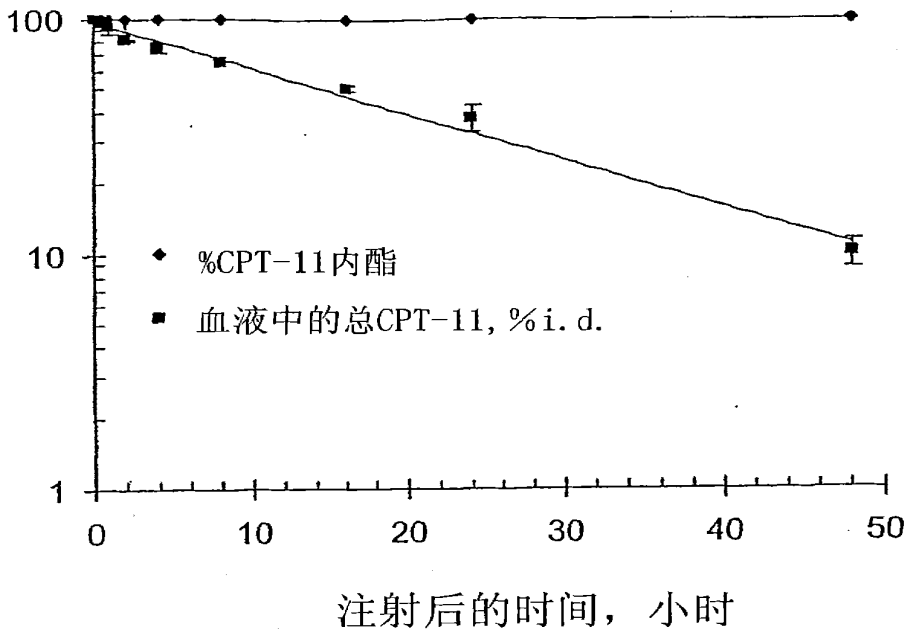


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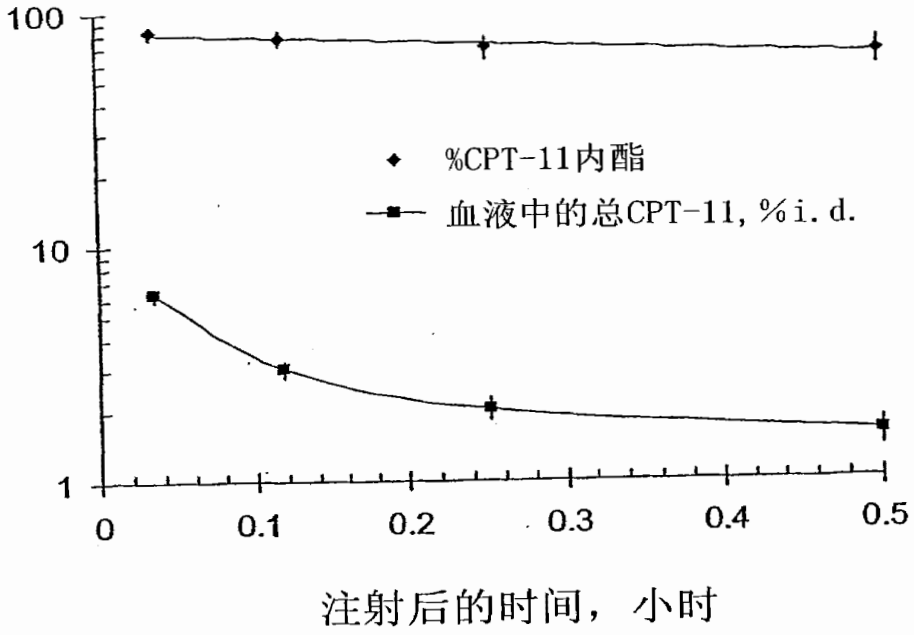


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(71) Applicant: ALZA CORPORATION [US/US]; Alza Plaza, 1900 Charleston Road, Mountain View, CA 94039–7210 (US).

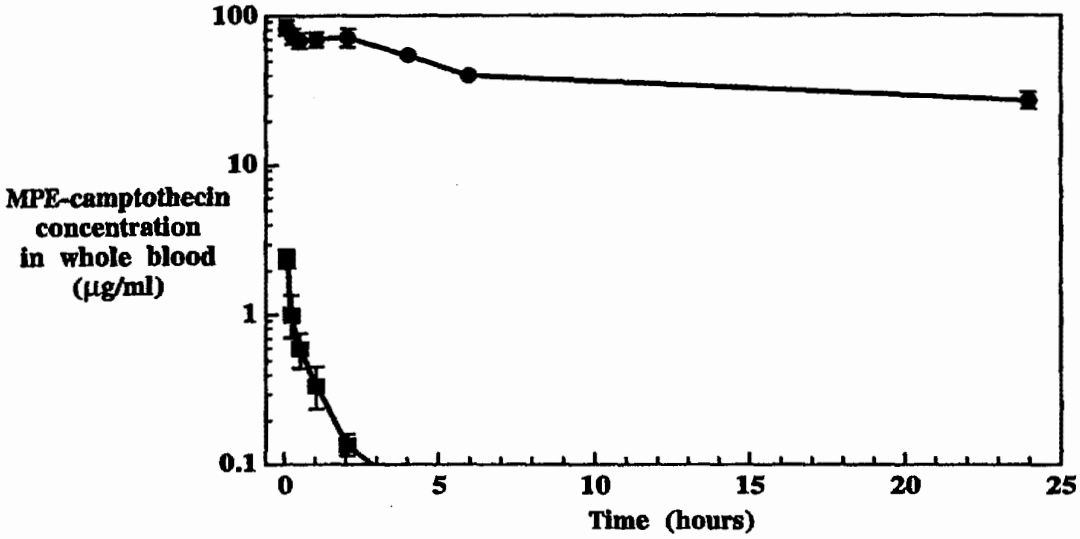
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(54) Title: LIPOSOME–ENTRAPPED TOPOISOMERASE INHIBITORS



(57) Abstract

A composition for administration of a therapeutically effective dose of a topoisomerase inhibitor I or topoisomerase I/II inhibitor is described. The composition includes liposomes having an outer surface and an inner surface defining aqueous liposome compartment, and being composed of a vesicle–forming lipid and of a vesicle–forming lipid derivatized with a hydrophilic polymer to form a coating of hydrophilic polymer chains on both the inner and outer surfaces of the liposomes. Entrapped in the liposomes is the topoisomerase inhibitor at a concentration of at least about 0.10 μmole drug per μmole lipid.

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Liposome-Entrapped Topoisomerase Inhibitors

Field of the Invention

The present invention relates to a liposome composition having an entrapped topoisomerase inhibitor.

Background of the Invention

Next to heart disease, cancer is the major cause of death in the United States, causing over 500,000 fatalities annually (Katzung, B., "Basic and Clinical Pharmacology", 7th Edition, Appleton & Lange, Stamford CT, 1998, p. 882). With present methods of treatment, one-third of patients are cured with local measures, such as surgery or radiation therapy, which are quite effective when the tumor has not metastasized by the time of treatment. Earlier diagnosis might lead to increased cure of patients undergoing such local treatments. However, in many cases, early micrometastasis is a characteristic feature of the neoplasm, indicating that a systemic approach such as chemotherapy may be required, often along with a local treatment method, for effective cancer management.

Cancer chemotherapy can be curative in certain disseminated neoplasms that have undergone either gross or microscopic spread by the time of diagnosis. These include testicular cancer, diffuse large cell lymphoma, Hodgkin's disease and choriocarcinoma as well as childhood tumors such as acute lymphoblastic leukemia. For other forms of disseminated cancer, chemotherapy provides a palliative rather than curative therapy. Effective palliative therapy results in temporary clearing of the symptoms and signs of cancer and prolongation of useful life. Advances in cancer chemotherapy have recently provided evidence that chemical control of neoplasia is possible for a number of cancers.

One category of drugs used for cancer therapy is topoisomerase inhibitors. These compounds inhibit the action of topoisomerase enzymes which play a role in the replication, repair, genetic recombination and transcription of DNA. An example of a topoisomerase inhibitor is camptothecin, a natural compound that interferes with the activity of topoisomerase I, an enzyme involved in DNA replication and RNA transcription. Camptothecin and the camptothecin analogues topotecan and irinotecan are approved for clinical use.

Camptothecin and its analogues are effective in cancer chemotherapy by interfering with the breakage/reunion actions of topoisomerase I. The compounds stabilize and form a reversible enzyme-camptothecin-DNA ternary complex which prevents the reunion step of the breakage/union cycle of the topoisomerase reaction.

One problem with camptothecin is its water insolubility, which hinders the delivery of the drug. Numerous analogues of camptothecin have been prepared to improve the compound's water solubility. Another problem with camptothecin and its analogues is that the compounds are susceptible in aqueous environments to hydrolysis at the α-hydroxy lactone ring. The lactone ring opens to the carboxylate form of the drug, a form that exhibits little activity against topoisomerase I.

Various approaches to improving the stability of camptothecin and its analogues have been described. One approach has been to entrap the compounds in liposomes.

Burke (U.S. Patent No. 5,552,156) describes a liposome composition intended to overcome the instability of camptothecin and its analogues by entrapping the compounds in liposomes having a lipid bilayer membrane which allows the compound to penetrate, or intercalate, into the lipid bilayer. With the compound intercalated into the bilayer membrane, it is removed from the aqueous environment in the core of the liposome and thereby protected from hydrolysis.

One problem with this approach is that the liposomes are quickly removed from the bloodstream by the reticuloendothelial system (RES), preventing delivery, and preferably accumulation, at the tumor site.

Subramanian and Muller (*Oncology Research*, 7(9):461-469 (1995)) describe a liposome formulation of topotecan and report that in liposome-entrapped form, topotecan is stabilized from inactivation by hydrolysis of the lactone ring. However, the biological activity of the liposome-entrapped drug *in vitro* has only 60% of the activity of the free drug.

Lundberg (*Anti-Cancer Drug Design*, 13:453 (1998)) describes two lipophilic, oleic acid ester derivatives of camptothecin analogues which are entrapped in liposomes and intercalated into the bilayer for stabilization of the lactone ring. Daoud (*Anti-Cancer Drugs,* 6:83-93 (1995)) describes a liposome composition including camptothecin, where the drug is also intercalated into the lipid bilayer. The liposomes in both of these references are prepared conventionally, where the drug is passively entrapped in the liposomes to sequester the drug in the lipid bilayer membrane for stabilization. Using

this method of preparation it is difficult to achieve a sufficient drug load in the liposomes for clinical efficacy.

Accordingly, there is still a need in the art for a liposome formulation which (i) includes a topoisomerase inhibitor, such as camptothecin and its analogues; (ii) remains in the bloodstream for a prolonged period of time; (iii) retains antitumor activity; and (iv) includes a sufficient drug load for clinical relevance.

Summary of the Invention

Accordingly, it is an object of the invention to provide a topoisomerase inhibitor composition for improved cancer therapy.

It is another object of the invention to provide a liposome composition for administration of a topoisomerase inhibitor for antitumor therapy.

In one aspect, the invention includes a composition for treating a tumor in a subject, comprising liposomes composed of a vesicle-forming lipid and between about 1-20 mole percent of a vesicle-forming lipid derivatized with a hydrophilic polymer. The liposomes are formed under conditions that distribute the polymer on both sides of the liposomes' bilayer membranes. Entrapped in the liposomes is a topoisomerase I inhibitor or a topoisomerase I/II inhibitor at a concentration of at least about 0.10 μmole drug per μmole lipid. The liposomes have an inside/outside ion gradient sufficient to retain the topoisomerase I inhibitor or topoisomerase I/II inhibitor within the liposomes at the specified concentration.

In one embodiment, the topoisomerase inhibitor is a topoisomerase I inhibitor selected from the group consisting of camptothecin and camptothecin derivatives. For example, the camptothecin derivative can be 9-aminocamptothecin, 7-ethylcamptothecin, 10-hydroxycamptothecin, 9-nitrocamptothecin, 10,11-methlyenedioxycamptothecin, 9-amino-10,11-methylenedioxycamptothecin or 9-chloro-10,11-methylenedioxycamptothecin. In other embodiments, the camptothecin derivative is irinotecan, topotecan, (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin, 7-(4-methylpiperazinomethylene)-10,11-methylenedioxy-20(S)-camptothecin or 7-(2-(N-isopropylamino)ethyl)-(20S)-camptothecin.

In another embodiment, the topoisomerase inhibitor is a topoisomerase I/II inhibitor, such as 6-[[2-(dimethylamino)-ethyl]amino]-3-hydroxy-7H-indeno[2,1-

c]quinolin-7-one dihydrochloride, azotoxin or 3-methoxy-11H-pyrido[3',4'-4,5]pyrrolo[3,2-c]quinoline-1,4-dione.

The hydrophilic polymer included in the liposome composition can be polyvinylpyrrolidone, polyvinylmethylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropylmethacrylamide, polymethacrylamide, polydimethylacrylamide, polyhydroxypropylmethacrylate, polyhydroxyethylacrylate, hydroxymethylcellulose, hydroxyethylcellulose, polyethyleneglycol and polyaspartamide.

In a preferred embodiment, the hydrophilic polymer is polyethyleneglycol having a molecular weight between 500-5,000 daltons.

In still another embodiment, the liposomes further include a vesicle-forming lipid having a phase transition temperature above 37°C.

In yet another embodiment, the vesicle-forming lipid is hydrogenated soy phosphatidylcholine, distearoyl phosphatidylcholine or sphingomyelin. One preferred liposome composition is composed of 20-94 mole percent hydrogenated soy phosphatidylcholine, 1-20 mole percent distearoyl phosphatidylcholine derivatized with polyethyleneglycol and 5-60 mole percent cholesterol.

Another preferred composition is 30-65 mole percent hydrogenated soy phosphatidylcholine, 5-20 mole percent distearoyl phosphatidylcholine derivatized with polyethyleneglycol and 30-50 mole percent cholesterol.

In another aspect, the invention includes a composition for administration of a topoisomerase I inhibitor or a topoisomerase I/II inhibitor, comprising liposomes composed of vesicle-forming lipids and having an inside/outside ion gradient effective to retain the drug within the liposomes. Entrapped in the liposomes is the topoisomerase I inhibitor or the topoisomerase I/II inhibitor at a concentration of at least about 0.20 μmole drug per μmole lipid.

In another aspect, the invention includes a method of treating a tumor in a subject, comprising preparing liposomes composed of vesicle-forming lipids including between 1-20 mole percent of a vesicle-forming lipid derivatized with a hydrophilic polymer chain, the liposomes being formed under conditions that distribute the polymer on both sides of the liposomes' bilayer membrane. The liposomes contain a topoisomerase I inhibitor or a topoisomerase I/II inhibitor entrapped in the liposomes at a concentration of at least about 0.10 mole per μmole lipid, the liposomes having an inside/outside ion gradient sufficient to

retain the topoisomerase I inhibitor or topoisomerase I/II inhibitor within the liposome at the specified concentration. The liposomes are then administered to the subject.

In one embodiment of this aspect, the method further includes entrapping the topoisomerase I inhibitor or topoisomerase I/II inhibitor in the liposomes by remote loading, for example, via an ammonium sulfate gradient.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figs. 1A is a plot of the blood circulation lifetime of liposome-entrapped MPE-camptothecin (solid circles), taken as the percent of injected dose as a function of time, compared to the free form of the drug (solid squares);

Fig. 1B shows the blood concentration of MPE-camptothecin, as a function of time, in hours, after administration of liposome-entrapped MPE-camptothecin (solid circles) and of free (non-liposomal) MPE-camptothecin (solid squares) to rats;

Fig. 2A is a plot showing the body weight of mice, in grams, as a function of days after tumor inoculation with an HT29 colon tumor. The animals were treated on days 10, 16 and 23 after tumor inoculation with liposome-entrapped MPE-camptothecin at dosages of 24 mg/kg (closed circles), 15 mg/kg (closed triangles) and 6 mg/kg (closed squares) and with free MPE-camptothecin at doses of 24 mg/kg (open circles), 15 mg/kg (open triangles) and 6 mg/kg (open squares);

Fig. 2B is a plot showing tumor volume, in mm^3, as a function of days after inoculation with an HT29 colon tumor. The animals were treated on days 10, 16 and 23 after tumor inoculation with liposome-entrapped MPE-camptothecin at dosages of 24 mg/kg (closed circles), 15 mg/kg (closed triangles) and 6 mg/kg (closed squares) and with free drug at doses of 24 mg/kg (open circles), 15 mg/kg (open triangles) and 6 mg/kg (open squares);

Fig. 3A is a plot showing the body weight of mice, in grams, as a function of days after inoculation with an HT29 colon tumor. The animals were treated on days 9, 16 and 23 after tumor inoculation with liposome-entrapped MPE-camptothecin at dosages of 5 mg/kg (open triangles), 3 mg/kg (open inverted triangles), 1 mg/kg (open diamonds), 0.5 mg/kg (open circles) and 0.1 mg/kg (open squares) and with free MPE-camptothecin at a

dose of 20 mg/kg (closed squares);

Fig. 3B is a plot showing tumor volume, in mm^3, as a function of days after inoculation with an HT29 colon tumor. The animals were treated on days 9, 16 and 23 after tumor inoculation with liposome-entrapped MPE-camptothecin at dosages of 5 mg/kg (open triangles), 3 mg/kg (open inverted triangles), 1 mg/kg (open diamonds), 0.5 mg/kg (open circles) and 0.1 mg/kg (open squares) and with free MPE-camptothecin at a dose of 20 mg/kg (closed squares);

Figs. 4A-4B are plots showing the plasma concentration of topotecan as a function of time, in hours, after administration of liposome-entrapped topotecan (solid triangles) and of free (non-liposomal) topotecan (solid squares) to rats at dosages of 2 mg/kg (Fig. 4A) and 5 mg/kg (Fig. 4B);

Fig. 5A is a plot showing the body weight of mice, in grams, as a function of days after inoculation with an HT29 colon tumor. The animals were treated on days 9, 16 and 23 after tumor inoculation with liposome-entrapped topotecan at dosages of 2 mg/kg (diamonds), 5 mg/kg (circles), 8 mg/kg (open squares); liposome-entrapped MPE-camptothecin at 4 mg/kg (triangles); free topotecan at a dose of 25 mg/kg (inverted triangles) and saline (closed squares);

Fig. 5B is a plot showing tumor volume, in mm^3, as a function of days after inoculation with an HT29 colon tumor. The animals were treated on days 9, 16 and 23 after tumor inoculation with liposome-entrapped topotecan at dosages of 2 mg/kg (diamonds), 5 mg/kg (circles), 8 mg/kg (open squares); liposome-entrapped MPE-camptothecin at 4 mg/kg (triangles); free topotecan at a dose of 25 mg/kg (inverted triangles) and saline (closed squares);

Fig. 6 is a plot of plasma concentration of CKD602 as a function of time, in hours, after administration of liposome-entrapped CKD602 (solid circles) and of free (non-liposomal) topotecan (solid squares) to rats at a dosage of 1 mg/kg;

Fig. 7A is a plot showing the body weight of mice, in grams, as a function of days after inoculation with an HT29 colon tumor. The animals were treated on days 9, 16 and 23 after tumor inoculation with liposome-entrapped CKD602 at dosages of 4 mg/kg (diamonds), 2 mg/kg (circles), 1 mg/kg (open squares); liposome-entrapped MPE-camptothecin at 4 mg/kg (triangles); free CKD602 at a dose of 20 mg/kg (inverted triangles) and saline (closed squares); and

Fig. 7B is a plot showing tumor volume, in mm^3, as a function of days after inoculation with an HT29 colon tumor. The animals were treated on days 9, 16 and 23 after tumor inoculation with liposome-entrapped CKD602 at dosages of 4 mg/kg (diamonds), 2 mg/kg (circles), 1 mg/kg (open squares); liposome-entrapped MPE-camptothecin at 4 mg/kg (triangles); free CKD602 at a dose of 20 mg/kg (inverted triangles) and saline (closed squares).

Detailed Description of the Invention

I. Definitions

Unless otherwise indicated, the terms below have the following meaning:

"Effective amount" or "effective dose" refers to the amount necessary or sufficient to inhibit undesirable cell growth, *e.g.*, prevent undesirable cell growth or reduce existing cell growth, such as tumor cell growth. The effective amount can vary depending on factors known to those of skill in the art, such as the type of cell growth, the mode and regimen of administration, the size of the subject, the severity of the cell growth, etc. One of skill in the art would be able to consider such factors and make the determination regarding the effective amount.

"Therapeutically effective antitumor therapy" refers to a therapy which is effective to maintain or decrease the size, *e.g.*, volume, of a primary tumor or metastatic tumor.

"Topoisomerase I inhibitor" refers to any compound that inhibits or reduces the action of topoisomerase I enzyme.

"Topoisomerase I/II inhibitor" refers to any compound that inhibits or reduces the action of both topoisomerase I enzyme and topoisomerase II enzyme.

"Topoisomerase inhibitor" refers to a topoisomerase I inhibitor or a topoisomerase I/II inhibitor.

"MPE-camptothecin" refers to 7-(4-methyl-piperazino-methylene)-10,11-ethylenedioxy-20(S)-camptothecin.

"Topotecan" refers to 9-dimethyl-aminomethyl-10-hydroxycamptothecin.

"CKD-602" refers to 7-(2-(N-isopropylamino)ethyl)-(20S)-camptothecin.

II. Liposome Composition

The present invention is directed to a liposome composition for administration of a topoisomerase I inhibitor or a topoisomerase I/II inhibitor. In studies performed in

support of the invention, three topoisomerase inhibitors were entrapped in liposomes and characterized *in vivo:* topotecan, 7-(4-methyl-piperazino-methylene)-10,11-ethylenedioxy-20(S)-camptothecin (referred to hereing as "MPE-camptothecin") and 7-(2-(N-isopropylamino)ethyl)-(20S)-camptothecin (referred to herein as "CKD-602"). The drugs were entrapped in liposomes by remote loading to achieve a high drug load stably retained in the liposomes, as will be described. *In vivo* studies with the formulations demonstrated that the liposome composition achieves a surprising and unexpected degree of improvement in therapeutic activity when compared to therapy with the topoisomerase inhibitor in free form. More specifically, and as will be described below, the dose of the liposome-entrapped topoisomerase I inhibitor MPE-camptothecin required to achieve therapeutic antitumor therapy is about 20 times lower than the dose required when the drug is administered in free form.

In this section, the liposome composition will be described, including methods for preparing the liposomes.

A. Liposome Components

Liposomes suitable for use in the composition of the present invention include those composed primarily of vesicle-forming lipids. Vesicle-forming lipids can form spontaneously into bilayer vesicles in water, as exemplified by the phospholipids. The liposomes can also include other lipids incorporated into the lipid bilayers, with the hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and the head group moiety oriented toward the exterior, polar surface of the bilayer membrane.

The vesicle-forming lipids are preferably ones having two hydrocarbon chains, typically acyl chains, and a head group, either polar or nonpolar. There are a variety of synthetic vesicle-forming lipids and naturally-occurring vesicle-forming lipids, including the phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol, and sphingomyelin, where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. The above-described lipids and phospholipids whose acyl chains have varying degrees of saturation can be obtained commercially or prepared according to published methods. Other suitable lipids include glycolipids and sterols such as cholesterol.

Cationic lipids are also suitable for use in the liposomes of the invention, where the cationic lipid can be included as a minor component of the lipid composition or as a major or sole component. Such cationic lipids typically have a lipophilic moiety, such as a sterol, an acyl or diacyl chain, and where the lipid has an overall net positive charge. Preferably, the head group of the lipid carries the positive charge. Exemplary cationic lipids include 1,2-dioleyloxy-3-(trimethylamino) propane (DOTAP); N-[1-(2,3,-ditetradecyloxy)propyl]-N,N-dimethyl-N-hydroxyethylammonium bromide (DMRIE); N-[1-(2,3,-dioleyloxy)propyl]-N,N-dimethyl-N-hydroxy ethylammonium bromide (DORIE); N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA); 3 [N-(N',N'-dimethylaminoethane) carbamoly] cholesterol (DC-Chol); and dimethyldioctadecylammonium (DDAB).

The cationic vesicle-forming lipid may also be a neutral lipid, such as dioleoylphosphatidyl ethanolamine (DOPE) or an amphipathic lipid, such as a phospholipid, derivatized with a cationic lipid, such as polylysine or other polyamine lipids. For example, the neutral lipid (DOPE) can be derivatized with polylysine to form a cationic lipid.

In another embodiment, the vesicle-forming lipid is selected to achieve a specified degree of fluidity or rigidity, to control the stability of the liposome in serum and to control the rate of release of the entrapped agent in the liposome.

Liposomes having a more rigid lipid bilayer, or a liquid crystalline bilayer, are achieved by incorporation of a relatively rigid lipid, *e.g.*, a lipid having a relatively high phase transition temperature, *e.g.*, above room temperature, more preferably above body temperature and up to 80°C. Rigid, *i.e.*, saturated, lipids contribute to greater membrane rigidity in the lipid bilayer. Other lipid components, such as cholesterol, are also known to contribute to membrane rigidity in lipid bilayer structures.

On the other hand, lipid fluidity is achieved by incorporation of a relatively fluid lipid, typically one having a lipid phase with a relatively low liquid to liquid-crystalline phase transition temperature, *e.g.*, at or below room temperature, more preferably, at or below body temperature.

Vesicle-forming lipids having a main phase transition temperatures from approximately 2°C-80°C are suitable for use as the primary liposome component of the present composition. In a preferred embodiment of the invention, a vesicle-forming lipid having a main phase transition temperature above about 37°C is used as the primary lipid

component of the liposomes. In another preferred embodiment, a lipid having a phase transition temperature between about 37-70°C is used. By way of example, the lipid distearoyl phosphatidylcholine (DSPC) has a main phase transition temperature of 55.1°C and the lipid hydrogenated soy phosphatidylcholine (HSPC) has a phase transition temperature of 58°C. Phase transition temperatures of many lipids are tabulated in a variety of sources, such as Avanti Polar Lipids catalogue and Lipid Thermotropic Phase Transition Database (LIPIDAT, NIST Standard Reference Database 34).

The liposomes also include a vesicle-forming lipid derivatized with a hydrophilic polymer. As has been described, for example in U.S. Patent No. 5,013,556 and in WO 98/07409, which are hereby incorporated by reference, such a hydrophilic polymer provides a surface coating of hydrophilic polymer chains on both the inner and outer surfaces of the liposome lipid bilayer membranes. The outermost surface coating of hydrophilic polymer chains is effective to provide a liposome with a long blood circulation lifetime *in vivo*. The inner coating of hydrophilic polymer chains extends into the aqueous compartments in the liposomes, *i.e.,* between the lipid bilayers and into the central core compartment, and is in contact with the entrapped compound after the compound is loaded via remote loading. As will be illustrated below, the liposome formulation having a surface coating of hydrophilic polymer chains distributed on the inner and outer liposome surfaces provides for a topoisomerase I inhibitor or topoisomerase I/II inhibitor composition where the compound is retained in the liposomes for improved therapeutic activity.

Vesicle-forming lipids suitable for derivatization with a hydrophilic polymer include any of those lipids listed above, and, in particular phospholipids, such as distearoyl phosphatidylethanolamine (DSPE).

Hydrophilic polymers suitable for derivatization with a vesicle-forming lipid include polyvinylpyrrolidone, polyvinylmethylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropylmethacrylamide, polymethacrylamide, polydimethylacrylamide, polyhydroxypropylmethacrylate, polyhydroxyethylacrylate, hydroxymethylcellulose, hydroxyethylcellulose, polyethyleneglycol, and polyaspartamide. The polymers may be employed as homopolymers or as block or random copolymers.

A preferred hydrophilic polymer chain is polyethyleneglycol (PEG), preferably as a PEG chain having a molecular weight between 500-10,000 daltons, more preferably

between 500-5,000 daltons, most preferably between 1,000-2,000 daltons. Methoxy or ethoxy-capped analogues of PEG are also preferred hydrophilic polymers, commercially available in a variety of polymer sizes, *e.g.*, 120-20,000 daltons.

Preparation of vesicle-forming lipids derivatized with hydrophilic polymers has been described, for example in U.S. Patent No. 5,395,619. Preparation of liposomes including such derivatized lipids has also been described, where typically, between 1-20 mole percent of such a derivatized lipid is included in the liposome formulation. It will be appreciated that the hydrophilic polymer may be stably coupled to the lipid, or coupled through an unstable linkage which allows the coated liposomes to shed the coating of polymer chains as they circulate in the bloodstream or in response to a stimulus.

B. Topoisomerase Inhibitor

The liposomes of the invention include a topoisomerase inhibitor entrapped in the liposome. Entrapped is intended to include encapsulation of an agent in the aqueous core and aqueous spaces of liposomes. It will be appreciated that for compounds having some hydrophobicity, entrapment in the lipid bilayer(s) of the liposomes may also occur.

Topoisomerases catalyze the introduction and relaxation of superhelicity in DNA. Several types of enzymes with varying specifities are known to be important in the replication of DNA, as well as in the repair, genetic recombination and transcription of DNA. The simplest topoisomerases, designated topoisomerase I, relax superhelical DNA, a process that is energetically spontaneous. The gyrases, which are known as topoisomerase II, catalyze the energy-requiring and ATP-dependent introduction of negative superhelical twists into DNA. In DNA replication, topoisomerases I and II have the function of relaxing the positive superhelicity that is introduced ahead of the replicating forks by the action of helicases. In addition, gyrases introduce negative twists into segments of DNA that allow single-strand regions to appear.

Topoisomerase inhibitors, then, are compounds that inhibit topoiosmerase activity. Compounds known as topoisomerase I inhibitors have activity against topoisomerase I, and the topoiosmerase II inhibitors have activity against topoisomerase II. Some compounds have activity against both topoisomerase I and topoisomerase II and are known as topoisomerase I/II inhibitors.

Preferred topoisomerase I inhibitors for use in the present invention are camptothecin

and analogs of camptothecin. Camptothecin is a pentacyclic alkaloid initially isolated from the wood and bark of *Camptotheca acuminata*, a tree indigenous to China (Wall, M.E. *et al., J. Am. Chem. Soc.*, 94:388 (1966)). Camptothecin exerts its pharmacological effects by irreversibly inhibiting topoisomerase I. Methods for the synthesis of camptothecin and camptothecin analogs or derivatives are known, and are summarized and set forth in U.S. Patent No. 5,244,903, which is herein incorporated by reference in its entirety.

Analogues of camptothecin include SN-38 ((+)-(4S)-4,11-diethyl-4,9-dihydroxy-1H-pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-3,14(4H,12H)-dione); 9-aminocamptothecin; topotecan (hycamtin; 9-dimethyl-aminomethyl-10-hydroxycamptothecin); irinotecan (CPT-11; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-camptothecin), which is hydrolyzed *in vivo* to SN-38); 7-ethylcamptothecin and its derivatives (Sawada, S. *et al., Chem. Pharm. Bull.*, 41(2):310-313 (1993)); 7-chloromethyl-10,11-methylene-dioxy-camptothecin; and others (SN-22, Kunimoto, T. *et al., J. Pharmacobiodyn.*, 10(3):148-151 (1987); N-formylamino-12,13,dihydro-1,11-dihydroxy-13-(beta-D-glucopyranosyl)-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione (NB-506, Kanzawa, F *et al., Cancer Res.*, 55(13):2806-2813 (1995); DX-8951f and lurtotecan (GG-211 or 7-(4-methylpiperazino-methylene)-10,11-ethylenedioxy-20(S)-camptothecin) (Rothenberg, M.L., *Ann. Oncol.*, 8(9):837-855 (1997)) and 7-(2-(N-isopropylamino)ethyl)-(20S)-camptothecin (CKD602, Chong Kun Dang Corporation, Seoul Korea).

Topoisomerase inhibitors having activity against both topoisomerase I and topoisomerase II include 6-[[2-(dimethylamino)-ethyl]amino]-3-hydroxy-7H-indeno[2,1-c]quinolin-7-one dihydrochloride, (TAS-103, Utsugi, T., *et al., Jpn. J. Cancer Res.*, 88(10):992-1002 (1997)) and 3-methoxy-11H-pyrido[3',4'-4,5]pyrrolo[3,2-c]quinoline-1,4-dione (AzalQD, Riou, J.F., *et al., Mol. Pharmacol.*, 40(5):699-706 (1991)).

In one embodiment of the invention, the topoisomerase I inhibitor administered is the pharmacologically active enantiomer of a camptothecin analogue having a chiral center. The enantiomer can be resolved from the racemic mixture using techniques known to those of skill in the art.

C. Method of Preparing the Liposome Composition

The liposomes may be prepared by a variety of techniques, such as those detailed in Szoka, F., Jr., *et al., Ann. Rev. Biophys. Bioeng.* 9:467 (1980), and specific examples of liposomes prepared in support of the present invention will be described below. Typically,

the liposomes are multilamellar vesicles (MLVs), which can be formed by simple lipid-film hydration techniques. In this procedure, a mixture of liposome-forming lipids and including a vesicle-forming lipid derivatized with a hydrophilic polymer are dissolved in a suitable organic solvent which is evaporated in a vessel to form a dried thin film. The film is then covered by an aqueous medium to form MLVs, typically with sizes between about 0.1 to 10 microns. Exemplary methods of preparing derivatized lipids and of forming polymer-coated liposomes have been described in co-owned U.S. Patents Nos. 5,013,556, 5,631,018 and 5,395,619, which are incorporated herein by reference.

The therapeutic agent of choice can be incorporated into liposomes by standard methods, including (i) passive entrapment of a water-soluble compound by hydrating a lipid film with an aqueous solution of the agent, (ii) passive entrapment of a lipophilic compound by hydrating a lipid film containing the agent, and (iii) loading an ionizable drug against an inside/outside liposome ion gradient, termed remote loading. Other methods, such as reverse evaporation phase liposome preparation, are also suitable.

In the present invention, a preferred method of preparing the liposomes is by remote loading. In the studies performed in support of the invention, three exemplary topoisomerase I inhibitors were loaded into pre-formed liposomes by remote loading against an ion concentration gradient, as has been described in the art (U.S. Patent No. 5,192,549) and as described in Example 1. In a remote loading procedure, a drug is accumulated in the liposomes' central compartment at concentration levels much greater than can be achieved with other loading methods. In a preferred embodiment of the invention, the topoisomerase I inhibitor or topoisomerase I/II inhibitor is loaded into the liposomes to a concentration of at least about 0.10 μmole drug per μmole lipid, more preferably of at least about 0.15 μmole drug per μmole lipid, most preferably of at least about 0.20 μmole drug per μmole lipid. The liposomes prepared in support of the invention contained MPE-camptothecin, topotecan or CKD602. As set forth in Example 1, these compounds were loaded into the liposomes by remote loading, discussed below, to a drug concentration level of greater than 0.20 μmole drug per μmole lipid (see the table in Example 1).

Liposomes having an ion gradient across the liposome bilayer for use in remote loading can be prepared by a variety of techniques. A typical procedure is as described above, where a mixture of liposome-forming lipids is dissolved in a suitable organic solvent and evaporated in a vessel to form a thin film. The film is then covered with an aqueous

medium containing the solute species that will form the aqueous phase in the liposome interior spaces.

After liposome formation, the vesicles may be sized to achieve a size distribution of liposomes within a selected range, according to known methods. The liposomes are preferably uniformly sized to a selected size range between 0.04 to 0.25 μm. Small unilamellar vesicles (SUVs), typically in the 0.04 to 0.08 μm range, can be prepared by extensive sonication or homogenization of the liposomes. Homogeneously sized liposomes having sizes in a selected range between about 0.08 to 0.4 microns can be produced, *e.g.*, by extrusion through polycarbonate membranes or other defined pore size membranes having selected uniform pore sizes ranging from 0.03 to 0.5 microns, typically, 0.05, 0.08, 0.1, or 0.2 microns. The pore size of the membrane corresponds roughly to the largest size of liposomes produced by extrusion through that membrane, particularly where the preparation is extruded two or more times through the same membrane. The sizing is preferably carried out in the original lipid-hydrating buffer, so that the liposome interior spaces retain this medium throughout the initial liposome processing steps.

After sizing, the external medium of the liposomes is treated to produce an ion gradient across the liposome membrane, which is typically a lower inside/higher outside concentration gradient. This may be done in a variety of ways, *e.g.*, by (i) diluting the external medium, (ii) dialysis against the desired final medium, (iii) molecular-sieve chromatography, *e.g.*, using Sephadex G-50, against the desired medium, or (iv) high-speed centrifugation and resuspension of pelleted liposomes in the desired final medium. The external medium which is selected will depend on the mechanism of gradient formation and the external pH desired, as will now be considered.

In the simplest approach for generating an ion gradient, the hydrated, sized liposomes have a selected internal-medium pH. The suspension of the liposomes is titrated until a desired final pH is reached, or treated as above to exchange the external phase buffer with one having the desired external pH. For example, the original medium may have a pH of 5.5, in a selected buffer, *e.g.*, glutamate or phosphate buffer, and the final external medium may have a pH of 8.5 in the same or different buffer. The internal and external media are preferably selected to contain about the same osmolarity, *e.g.*, by suitable adjustment of the concentration of buffer, salt, or low molecular weight solute, such as sucrose.

In another general approach, the gradient is produced by including in the liposomes, a selected ionophore. To illustrate, liposomes prepared to contain valinomycin in the

liposome bilayer are prepared in a potassium buffer, sized, then exchanged with a sodium buffer, creating a potassium inside/sodium outside gradient. Movement of potassium ions in an inside-to-outside direction in turn generates a lower inside/higher outside pH gradient, presumably due to movement of protons into the liposomes in response to the net electronegative charge across the liposome membranes (Deamer, *et al.*, 1972).

In another more preferred approach, the proton gradient used for drug loading is produced by creating an ammonium ion gradient across the liposome membrane, as described, for example, in U.S. Patent No. 5,192,549. Here the liposomes are prepared in an aqueous buffer containing an ammonium salt, typically 0.1 to 0.3 M ammonium salt, such as ammonium sulfate, at a suitable pH, *e.g.*, 5.5 to 7.5. The gradient can also be produced by using sulfated polymers, such as dextran ammonium sulfate or heparin sulfate. After liposome formation and sizing, the external medium is exchanged for one lacking ammonium ions, *e.g.*, the same buffer but one in which ammonium sulfate is replaced by NaCl or a sugar that gives the same osmolarity inside and outside of the liposomes.

After liposome formation, the ammonium ions inside the liposomes are in equilibrium with ammonia and protons. Ammonia is able to penetrate the liposome bilayer and escape from the liposome interior. Escape of ammonia continuously shifts the equilibrium within the liposome toward the right, to production of protons.

The topoisomerase inhibitor is loaded into the liposomes by adding the drug to a suspension of the ion gradient liposomes, and the suspension is treated under conditions effective to allow passage of the compound from the external medium into the liposomes. Incubation conditions suitable for drug loading are those which (i) allow diffusion of the derivatized compound, with such in an uncharged form, into the liposomes, and (ii) preferably lead to high drug loading concentration, *e.g.*, 5-500 mM drug encapsulated, more preferably between 20-200 mM, most preferably between 50-300 mM.

The loading is preferably carried out at a temperature above the phase transition temperature of the liposome lipids. Thus, for liposomes formed predominantly of saturated phospholipids, the loading temperature may be as high as 60 C or more. The loading period is typically between 15-120 minutes, depending on permeability of the drug to the liposome bilayer membrane, temperature, and the relative concentrations of liposome lipid and drug.

With proper selection of liposome concentration, external concentration of added compound, and the ion gradient, essentially all of the compound may be loaded into the

liposomes. For example, with a pH gradient of 3 units (or the potential of such a gradient employing an ammonium ion gradient), the final internal:external concentration of drug will be about 1000:1. Knowing the calculated internal liposome volume, and the maximum concentration of loaded drug, one can then select an amount of drug in the external medium which leads to substantially complete loading into the liposomes.

Alternatively, if drug loading is not effective to substantially deplete the external medium of free drug, the liposome suspension may be treated, following drug loading, to remove non-encapsulated drug. Free drug can be removed, for example, by molecular sieve chromatography, dialysis, or centrifugation.

In another embodiment of the invention, the topoisomerase inhibitor is loaded into preformed liposomes which include in the liposome interior a trapping agent effective to complex with the topoisomerase inhibitor and enhance retention of the compound. In a preferred embodiment, the trapping agent is a polyanionic polymer, *e.g.*, a molecule consisting of repetitive units of preferably similar chemical structure and having ionizable groups, that is, chemical functional groups capable of electrolytic dissociation resulting in the formation of ionic charge, and preferably an anionic charge. Polymers having a molecular weight over a broad range are suitable, from 400-2,000,000 Daltons.

The polyanionic polymer is entrapped in the liposomes during lipid vesicle formation. Upon loading of a drug into the pre-formed liposomes, the polymer serves to trap or retain the drug within the liposomes. In the studies described herein, dextran sulfate was used as an exemplary polyanionic polymer. Dextran sulfate is a polymer of anhydroglucose with approximately 2.3 sulfate groups per glucosoyl residue. It is composed of approximately 95% alpha-D-(1-6) linkages and the remaining (1-3) linkages account for the branching of dextran. The polymer is readily available in molecular weights ranging from 5,000 to 500,000 Daltons. However, other polymer are suitable including sulfated, sulfonated, carboxylated or phosphated hydrophilic polymers. For example, sulfated proteoglycans, such as sulfated heparin, sulfated polysaccharids, such as sulfated cellulose or cellulose derivatives, carrageenin, mucin, sulfated polypeptides, such as polylysine with sulfated amine groups, glycopeptides with sulfonate-derivatized saccharide or peptide subunits, and hyaluronic acid. Chondroitin sulfates A, B and C, keratin sulfates, dermatan sulfates are also contemplated. The polymer can also be a neutral polymer modified to include an anionic functional group. For example, amylose, pectin, amylopectin, celluloses, and dextran can be modified to include an anionic subunit.

Polymers bearing a sulfo group such as polyvinylsulfate, polyvinylsulfonate polystyrenesulfonate and sulfated rosin gum are also suitable.

Preparation of liposomes which include such a trapping agent is described with respect to Example 4. In this example, the polyanionic polymer dextran sulfate is entrapped in the liposomes by adding the liposome lipids, which are first dissolved in ethanol, to a solution of dextran sulfate ammonium salt and mixed to form liposomes having dextran sulfate ammonium salt entrapped within the liposomes. The external media was exchanged to establish an ammonium ion gradient across the liposomes for remote loading of drug.

III. *In vivo* Administration of the Composition

Liposomes were prepared in support of the invention as described in Example 1. The topoisomerase I inhibitors (7-(4-methylpiperazino)-methylene)-10,11-ethylenedioxy-20(S)-camptothecin), referred to herein as "MPE-camptothecin"; topotecan; and 7-(2-(N-isopropylamino)ethyl)-(20S)-camptothecin, referred to herein as "CKD-602", were loaded into liposomes under an ammonium sulfate ion concentration gradient. The liposomes were composed of hydrogenated soy phosphatidylcholine, cholesterol and polyethylene glycol derivatized to distearoyl phosphatidylethanolamine (PEG-DSPE) in a molar ratio 55.4:39:5.6. The table in Example 1 summarizes the drug to lipid ratios for the liposome formulations prepared. The calculated liposomal drug concentrations for the three compounds, based on an extruded liposome captured volume of 0.9 μl/μmole lipid, are 284 mM for MPE-camptothecin, 264 mM for topotecan and 298 for CKD-602. Based on an extruded liposome captured volume of 1.5 μl/μmole lipid, the calculated liposomal drug concentrations are 189 mM for MPE-camptothecin, 174 mM for topotecan and 198 for CKD-602. The *in vivo* studies performed with each drug will now be described.

1. *In vivo* Administration of MPE-Camptothecin

The long-circulating, PEG-coated liposomes containing MPE-camptothecin were administered to rats to determine the blood circulation lifetime of the drugs in liposome-entrapped form. The pharmacokinetic profile of the liposome-entrapped drug and of the free drug are shown in Fig. 1A as the percent of injected dose as a function of time. As can be seen, the blood circulation time of the topoisomerase I inhibitor in liposome-entrapped form (solid circles) is significantly longer than the free form of the drug (solid

squares). For MPE-camptothecin, the blood circulation half-life of the liposome-entrapped drug was 14 hours, compared to about 50 minutes for the free drug. The blood clearance of the liposome-entrapped drug in rats was approximately 35-fold lower and the area under the curve was approximately 1250-fold higher than that of the free drug. Analytical results indicate that essentially all the drug remains entrapped in the liposomes in the bloodstream.

Fig. 1B shows the concentration of MPE-camptothecin in whole blood after administration of the liposome formulation (solid circles) and of the free drug to rats. The longer circulation lifetime results in a higher concentration of the drug in the blood.

The anti-tumor efficacy of the MPE-camptothecin liposome formulation was determined in xenograft tumor models, where homozygous nude mice were inoculated with human tumor cells of colon, HT29 origin. Surprisingly, these toxicity and antitumor efficacy studies showed that liposomal MPE-camptothecin was significantly more toxic than the free form of the drug at equivalent doses. These studies and the results will now be described.

Liposomes were prepared as set forth in Example 1 to include entrapped MPE-camptothecin. Nude mice with HT-29 colon xenografts were treated with liposome-entrapped MPE-camptothecin at dosages of 24 mg/kg, 15 mg/kg and 6 mg/kg or with free MPE-camptothecin at the same dosages. Treatment began 10 days after tumor inoculation and doses were administered at days 10, 16 and 23. The tumor volume in each animal was assessed during and following treatment as described in Example 2.

The body weight of each test animal and the tumor volume of each animal are shown, respectively in Figs. 2A and 2B, where animals were treated with liposomal entrapped MPE-camptothecin at dosages of 24 mg/kg (closed circles), 15 mg/kg (closed triangles) and 6 mg/kg (closed squares) and with free MPE-camptothecin at doses of 24 mg/kg (open circles), 15 mg/kg (open triangles) and 6 mg/kg (open squares).

With respect to the animals treated with the liposome-entrapped MPE-camptothecin, all of the animals dosed with 15 mg/kg and 24 mg/kg died after two doses due to drug-related toxicity, with most deaths on day five after the first dose. All of the animals treated with 6 mg/kg liposome-entrapped MPE-camptothecin survived until administration of the third dose on day 23, after which five of the ten animals died within a few days. The toxicity of the liposome-entrapped MPE-camptothecin is reflected in the greater body weight losses, as seen in Fig. 2A.

In contrast, all of the animals treated with the free form of the drug survived the study, with the exception of one animal in the 24 mg/kg dosing group that died a few days after the third dose on day 23.

Table 1

Treatment	Dose mg/kg	Number of Test Animals	Number of Surviving Animals		
			after dose 1 (day 9)	after dose 2 (day 16)	after dose 3 (day 23)
Saline	na	20	20	20	20
free MPE-camptothecin	24	10	10	10	9
free MPE-camptothecin	15	10	10	10	10
free MPE-camptothecin	6	10	10	10	10
liposome-entrapped	24	10	1	0	0
liposome-entrapped	15	10	5	0	0
liposome-entrapped	6	10	10	10	5

With respect to antitumor activity of the formulations, the liposome-entrapped MPE-camptothecin was more effective than the free form of the drug in inhibiting tumor growth, despite its greater toxicity. This can be seen in Fig. 2B, where the 6 mg/kg dose of liposome-entrapped MPE-camptothecin was significantly more effective in inhibiting tumor growth (log growth rate of -0.026) than even the highest dose level of free MPE-camptothecin (24 mg/kg, log growth rate 0.0048).

The complete and partial remission of the tumors in the test animals was monitored and is presented in Table 2. Complete remission of a tumor is defined as the elimination of tumor mass until the end of the experiment. A partial remission is defined as a tumor volume of less than 50% of the peak tumor volume for an individual animal.

Table 2

Treatment	Dose mg/kg	Complete Remission[1]	Partial Remission[2]
Saline		0/20	0/20
free MPE-camptothecin	24	3/10	1/10
free MPE-camptothecin	15	2/10	0/10
free MPE-camptothecin	6	0/10	0/10
liposome-entrapped	24	--[3]	--[3]
liposome-entrapped	15	--[3]	--[3]
liposome-entrapped	6	10/10	na[4]

[1] complete remission defined as elimination of tumor mass until experiment termination.
[2] partial remission defined as a tumor volume of less than 50% of the peak tumor volume for an individual animal.
[3] all 10 animals in test groups died after the second dose on day 16.
[4] na = not applicable

As can be seen in Table 2, the liposome-entrapped MPE-camptothecin at a dose of 6 mg/kg was effective to cause a complete remission of tumors in all 10 test animals. This effect was observed within five days after the second treatment on day 16. As noted above, five of the test animals in the 6 mg/kg liposome-entrapped test group died shortly after the third dose. In the surviving five animals, the tumors did not recur by the end of the study, approximately 30 days after the final treatment on day 23. Data is unavailable for the animals treated with 15 mg/kg and 24 mg/kg liposome-entrapped MPE-camptothecin, since all of the animals in these test groups died due to drug-related toxicity, as noted above.

Administration of MPE-camptothecin in free form at a dose of 24 mg/kg resulted in 3 animals with complete tumor remission and 1 animal with partial tumor remission, as seen in Table 2.

Comparison of the results observed for the drug administered in free form and in liposome-entrapped form indicate that the drug is more potent when administered in liposome-entrapped form. In fact, the liposome-entrapped drug is at least four times more potent than the free form of the drug, as can be seen by comparing the results obtained for a 6 mg/kg of liposome-entrapped MPE-camptothecin dosage to a 24 mg/kg free MPE-camptothecin dosage (Fig. 2B, Table 2). It is clear from these results that the dose of liposome-entrapped MPE-camptothecin required for therapeutically effective anti-tumor therapy is four times lower than the dose required when the drug is administered

in free form.

Example 2 describes the details of a second study to determine the maximum tolerated dose and the lowest effective dose of the liposome-entrapped MPE-camptothecin. In this study, liposomes were prepared as described in Example 1 and the liposome formulation was administered to test animals at drug dosages of 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, 3 mg/kg and 5 mg/kg. The free drug was administered at 20 mg/kg as a comparison.

Table 3 summarizes the number of test animals in each group, specifying the number of animals surviving at each dosing phase of the study. As seen in the table, all of the control, saline treated animals and all of the animals treated with free MPE-camptothecin survived for the duration of the study. Of the ten animals treated with 5 mg/kg liposome-entrapped MPE-camptothecin, four of the animals died of drug-related toxicity and one additional animal died of apparently nonspecific causes after the third dose. One of the ten animals in the test group receiving 3 mg/kg liposome-entrapped MPE-camptothecin died after the second dose, but the death was not considered due to drug treatment because of the absence of any correlating signs of toxicity. All other animals treated with liposome-entrapped MPE-camptothecin survived the entire study duration.

Table 3

Treatment	Dose mg/kg	Number of Test Animals	Number of Surviving Animals		
			after dose 1 (day 9)	after dose 2 (day 16)	after dose 3 (day 23)
Saline		20	20	20	20
free MPE-camptothecin	20	10	10	10	10
liposome-entrapped	5	10	10	10	5
liposome-entrapped	3	10	10	9	9
liposome-entrapped	1	10	10	10	10
liposome-entrapped	0.5	10	10	10	10
liposome-entrapped	0.1	10	10	10	10

The results of the study are shown in Figs. 3A-3B, where Fig. 3A shows the body weight of mice, in grams, as a function of days after inoculation with the HT-29 colon tumor. The animals were treated on days 9, 16 and 23 after tumor inoculation with

liposomal entrapped topoisomerase I inhibitor at dosages of 5 mg/kg (open triangles), 3 mg/kg (open inverted triangles), 1 mg/kg (open diamonds), 0.5 mg/kg (open circles) and 0.1 mg/kg (open squares) and with free drug at a dose of 20 mg/kg (closed squares). As can be seen in Fig. 3A, body weight changes were dose-related and, these changes were correlated with other observations of toxicity.

Fig. 3B is a similar plot showing tumor volume, in mm^3, as a function of days after tumor inoculation, where the dosages are represented by the same symbols as in Fig. 3A. Fig. 3B shows that both the 5 mg/kg and 3 mg/kg dose levels of liposome-entrapped MPE-camptothecin were more therapeutically effective in inhibiting tumor growth than the 20 mg/kg dose of the free drug. Treatment with 20 mg/kg of free MPE-camptothecin (log growth rate of 0.011) was approximately equivalent in antitumor activity to the 1 mg/kg dosage level of the drug in liposome-entrapped form (log growth rate of 0.017).

Table 4 summarizes the complete and partial tumor remission in the test animals.

Table 4

Treatment	Dose mg/kg	Complete Remission[1]	Partial Remission[2]
Saline		0/20	0/20
free MPE-camptothecin	20	0/10	1/10
liposome-entrapped MPE-camptothecin	5	10/10	na[3]
liposome-entrapped MPE-camptothecin	3	7/10	1/10
liposome-entrapped MPE-camptothecin	1	0/10	0/10
liposome-entrapped MPE-camptothecin	0.5	0/10	1/10
liposome-entrapped MPE-camptothecin	0.1	0/10	0/10

[1]Complete remission defined as elimination of tumor mass until experiment termination.
[2]Partial remission defined as a tumor volume of less than 50% of the peak tumor volume for an individual animal.
[3]na = not applicable

There were no complete tumor remissions in the animals treated with 20 mg/kg of free MPE-camptothecin. In contrast, all ten of the animals treated with liposome-entrapped MPE-camptothecin at the 5 mg/kg dosage level had complete remissions. At the 3 mg/kg dosage, seven of the animals had complete remission of their tumor.

The results from the study of Example 3 shows that antitumor activity of the liposome-entrapped topoisomerase inhibitor MPE-camptothecin is significantly better

when compared to the free form of the drug, indicating that the liposome-entrapped form was about 20-fold more potent since the antitumor activity of the free drug at a dose of 20 mg/kg was most comparable to the activity of a 1 mg/kg dose of the liposome-entrapped form of the drug. That the 3 mg/kg and 5 mg/kg liposome-entrapped MPE-camptothecin dosages were significantly more effective in antitumor therapy than the 20 mg/kg dose of the drug in free form indicates that the therapeutic index of the drug entrapped in liposomes is approximately four-fold to five-fold higher than the drug in free form.

2. *In vivo* Adminstration of topotecan

In another study performed in support of the invention, topotecan was entrapped in liposomes composed of DSPC and mPEG-DSPE in a 95:5 molar ratio, as described in Example 4. Early studies, not reported here, indicated that topotecan was not readily retained in the liposomes. The lipid bilayer was selected to use a single component phospholipid having an acyl chain length close to DSPE in the mPEG-DSPE component. Such a bilayer has minimal packing defects which arise from imperfections in nearest neighbor interactions in a solid phase bilayer, which have reduced lateral and rotational mobility relative to fluid bilayers. In addition, a dextran-sulfate loading battery was used in order to achieve precipitation of the topotecan in the liposome interior. Other polymers, in particular polyanionic polymers, are suitable for this purpose, such as chondroitin sulfate A, polyvinylsulfuric acid, and polyphosphoric acid.

The pre-formed liposomes containing dextran ammounim sulfate in the central compartment were loaded with topotecan as described in Example 4. After loading, unentrapped drug was removed by diafiltration and the liposomes were characterized. The liposomes were loaded to a drug:lipid ratio of 0.238 and the liposomes had an average particle diameter of 87 nm.

The liposomes containing topotecan were administered intraveneously to rats to determine the blood circulation lifetime. Figs. 4A-4B show the plasma concentration of topotecan as a function of time after administration to rats. Fig. 4A compares the concentration of liposome-entrapped topotecan administered at 2 mg/kg (solid triangles) to the concentration of free topotecan administered at the same dosage (solid squares). Fig. 4B compares the two forms of the drug at a dosage of 5 mg/kg. The calculated pharmacokinetic parameters are given in Table 5.

Table 5

Parameter	Dosage = 2 mg/kg		Dosage = 5 mg/kg	
	Free Topotecan	Liposome-Entrapped	Free Topotecan	Liposome-Entrapped
lasma Cmax (μg/mL)	2.89	54.5	8.23	119.3
AUC (μg/mL h)	0.57	523	1.57	1140
T ½ (h)	0.20	7.2	0.30	9.8
CL (mL/h)	887	0.96	820	1.10
Vol. Dist. (mL)	173	9.2	278	17.5
elimination rate constant (1/h)	3.45	0.096	2.33	0.071

The data in Table 5 shows that the liposome-entrapped drug has a significantly longer circulation time than the free form of the drug.

The efficacy of the liposomes was determined in another study. As described in Example 4, the liposomes were administered to mice bearing a subcutaneous xenograft tumor. Tumor-bearing mice were randomized into six treatment groups of 12 mice for treatment with one of the following: saline, liposome-entrapped MPE-camptothecin 4 mg/kg; free topotecan 25 mg/kg; liposome entrapped topotecan at drug dosages of 2 mg/kg, 5 mg/kg or 8 mg/kg. All treatments were administered as intravenous bolus injections given weekly for 3 treatments, specifically on days 9, 16 and 23.

The tumor size in each animal was measured twice weekly during the study to evaluate therapeutic efficacy. Body weight of each animal was monitored twice weekly to assess toxicity of the formulations. The results are shown in Tables 6 and 7 and in Figs. 5A-5B.

Table 6

Treatment	Dose mg/kg	Complete Remission[1]	Partial Remission[2]	Non-Responsive[3]
Saline		0	0	12
liposome-entrapped MPE-camptothecin	4	8	4	0
free topotecan	25	0	1	11
liposome-entrapped topotecan	2	1	2	9
liposome-entrapped topotecan	5	2	8	2
liposome-entrapped topotecan	8	7	3	2

[1]Complete remission defined as elimination of tumor mass until experiment termination.
[2]Partial remission defined as a tumor volume of less than 50% of the peak tumor volume for an individual animal.
[3]Non-responsive defined as a tumor volume equal to or greater than initial tumor volume.

As can be seen from Figs. 5A and Table 6, left untreated the tumors grew at a rate of 17.8 mm^3 per day for the duration of the study. The animals treated with liposome-entrapped MPE-camptothecin (positive control animals) experienced a tumor growth rate -1.2 mm^3 per day for the duration of the study. Animals treated with nonencapsulated topotecan, which was administered at 25 mg/kg somewhat below the maximum tolerated dosage (MTD) of 40 mg/kg, had tumor growth of 14.1 mm^3 per day. Animals treated with liposome-entrapped topotecan had tumor growth of 0.9 mm^3 per day for a dosage of 2 mg/kg, -1.9 mm^3 per day for a dosage of 5 mg/kg and -0.8 mm^3 per day for a dosage of 8 mg/kg. The negative growth rate indicates regression of tumor size below the starting tumor volume.

The size of treated tumors as a function of the size of control tumors (%T/C) was examined for all treatment groups and is summarized in Table 6. The National Cancer Institute defines significant anti-tumor activity as a %T/C less than 42.

Table 7

Treatment	Dose mg/kg	%T/C[1] Day 29	%T/C Day 33	%T/C Day 36
liposome-entrapped MPE-camptothecin	4	1.8	0.6	1.9
free topotecan	25	82.8	79.0	85.9
liposome-entrapped topotecan	2	19.5	12.9	16.3
liposome-entrapped topotecan	5	10.5	5.6	5.6
liposome-entrapped topotecan	8	2.0	2.2	2.2

[1] %T/C defined as the average tumor volume at day indicated over the average tumor volume of the control, saline treated animals.

3. *In vivo* Adminstration of CKD-602

Example 5 describes another study conducted in support of the invention using the topoisomerase inhibitor CKD-602. The drug was remotely loaded into liposomes against an ammonium-sulfate gradient with dextran as a trapping agent. The liposome lipid composition was identical to that used for the study using topotecan - HSPC and mPEG-DSPE in a 95/5 mole ratio.

Fig. 6 is a plot showing the plasma concentration of CKD-602 as a function of time after administration to rats at a dosage of 1 mg/kg. The liposome-entrapped form of the drug (solid circles) had a calculated half-life of 9.8 hours and an AUC of 274 μg/mL/hr.

The free form of the drug had a calculated half-life of 0.2 hours and an AUC of 0.37 μg/mL/hr.

Therapeutic efficacy of the CKD-602 formulation was evaulated using mice hearing a HT-20 colon cancer xenograft. Seventy-two mice were inoculated with HT-29 tumor cells and nine days later were randomized into six treatment groups. The animals in each group were treated with one of the following formulations: saline, liposome-entrapped MPE-camptothecin 4 mg/kg; free CKD-602 20 mg/kg; liposome entrapped CKD-602 at drug dosages of 1 mg/kg, 2 mg/kg or 4 mg/kg. All treatments were administered as intravenous bolus injections given weekly for 3 treatments, specifically on days 11, 18 and 25.

The tumor size in each animal was measured twice weekly during the study to evaluate therapeutic efficacy. Body weight of each animal was monitored twice weekly to assess toxicity of the formulations. The results are shown in Tables 8 and 9 and in Figs. 7A-7B.

Table 8

Treatment	Dose mg/kg	Complete Remission[1]	Partial Remission[2]	Non-Responsive[3]
Saline		0/10	0/10	10/10
liposome-entrapped MPE-camptothecin	4	6/10	0/10	4/10
free CKD602	20	0/6	0/6	6/6
liposome-entrapped CKD602	1	2/10	7/10	1/10
liposome-entrapped CKD602	2	6/10	2/10	2/10
liposome-entrapped CKD602	4	4/4	0/4	0/4

[1]Complete remission defined as elimination of tumor mass until experiment termination.
[2]Partial remission defined as a tumor volume of less than 50% of the peak tumor volume for an individual animal.
[3]Non-responsive defined as a tumor volume equal to or greater than initial tumor volume.

As can be seen in Table 8 and in Fig. 7B, the animals treated with saline experienced continuous tumor growth, at a rate of 15.45 mm^3 per day for the duration of the study. The animals treated with the liposome-entrapped MPE-camptothecin (positive control animals)had a tumor growth rate of -0.63 mm^3 per day for the duration of the study. Animals treated with free, unentrapped CKD602 had tumor growth of 15.21 mm^3 per day. Animals treated with liposomal CKD602 had tumor growth of –2.21 mm^3 per

day for animals treated with a dose of 1 mg/kg, -0.96 mm^3 per day for a dose of 2 mg/kg and -2.37 mm^3 per day for a dose of 4 mg/kg. The negative growth rate indicates regression of tumor size below the starting tumor volume.

The size of treated tumors as a function of the size of control tumors (%T/C) was examined for all treatment groups and is summarized in Table 9. The National Cancer Institute defines significant anti-tumor activity as a %T/C less than 42.

Table 9

Treatment	Dose mg/kg	%T/C[1] Day 29	%T/C Day 33	%T/C Day 36
liposome-entrapped MPE-camptothecin	4	2.9	2.3	1.6
free CKD602	20	129.1	120.1	99.9
liposome-entrapped CKD602	1	11.4	7.7	4.4
liposome-entrapped CKD602	2	4.8	2.8	1.6
liposome-entrapped CKD602	4	1.0	1.3	0.9

[1]%T/C defined as the average tumor volume at day indicated over the average tumor volume of the control, saline treated animals.

IV. EXAMPLES

The following examples illustrate methods of preparing, characterizing, and using the composition of the present invention. The examples are in no way intended to limit the scope of the invention.

Materials

The topoisomerase inhibitor (7-(4-methyl-piperazino-methylene)-10,11-ethylenedioxy-20(S)-camptothecin trifluoroacetate (GI147211) (MPE-camptothecin), was provided by Glaxo Research Institute, Research Triangle Park, NC. CKD602 (7-(2-(N-isopropylamino)ethyl)-(20S)-camptothecin) was provided by Chong Kun Dang Corporation, Seoul Korea. Topotecan (Hycamtin®) was purchased commercially.

Materials for preparation of the liposomes and all other reagents were from commercially available sources.

Methods

Animal Studies: Homozygous nude mice were obtained from Taconic Farms (Germantown, NY) and allowed to acclimate for 7 days prior to initiation of the experiment. Animals were housed in appropriate isolated caging with ad lib sterile rodent food and acidified water and a 12:12 light:dark cycle. Animals were randomized into treatment groups prior to tumor inoculation based on body weight. Randomization was confirmed based on tumor size immediately prior to initiation of treatment.

Tumors: Tumors were inoculated by trochar placement of fragments from rapidly growing tumors on donor animals. The human colon cancer cell line, HT-29, was used to initiate subcutaneous xenograft tumors. Cultured cells were trypsinized, washed, counted and resuspended at 50 million cells per mL normal growth media. Tumors were inoculated by injection of 0.1mL (5 million cells) at the back of the neck. Tumors were allowed to grow to an average size of 100 mm^3 prior to initiation of treatment.

Monitoring: All animals were observed daily for general well-being throughout the experiments. Animals were weighed prior to tumor inoculation and weekly thereafter. Tumors were measured twice weekly throughout the experiment, beginning 5-10 days after tumor inoculation. Any animal observed to have 15% or greater weight loss from the initial starting weight and any animal observed to have greater than 4,000 mm^3 tumor volume were excluded from the study.

Example 1

Preparation of Liposomes with Entrapped Topoisomerase Inhibitor

Liposomes were prepared and loaded with a selected topoisomerase inhibitor as follows.

A. Liposome Preparation

The lipids hydrogenated soy phosphaticylcholine (HSPC), cholesterol (Chol) and mPEG-DSPE (at a ratio of 56.4:38.3:5.3 mol/mol) were dissolved in ethanol at 65°C in a 250 mL round bottom. The lipids were agitated continuously for at least 30 minutes at 65°C. The total lipid concentration in ethanol solution was 3.7 g total lipid per 10 mL ethanol.

The dissolved lipid solution was transferred to another 250 mL round bottom flask containing 100 mL of 250 mM ammonium sulfate solution equilibrated to 65°C. The

ethanol:lipid:ammonium sulfate hydration mixture was mixed continuously for at least one hour while maintaining the temperature using a 65°C water bath to form oligolamellar ethanol hydration liposomes.

The oligolamellar liposomes were size reduced using a Lipex thermobarrel extruder to pass the hydration mixture through polycarbonate membranes with known pore size dimensions. The mixture was passed 5 times through a 0.20 μm pore diameter membrane, followed by 10 passes through a 0.10 μm pore diameter membrane. The extruded liposomes contained ammonium sulfate within the interior aqueous compartment(s) of the liposomes, as well as in the exterior aqueous bulk phase medium in which they are suspended. The sized liposomes were stored in the refrigerator until diafiltration preceding the remote loading procedure.

100 mg of a selected topoisomerase inhibitor, MPE-camptothecin, CKD-602 or topotecan, was dissolved in 40 mL 10% sucrose solution to yield a concentration of 2.5 mg/mL. After dissolution, the solution was passed through a 0.20 μm filter to remove insoluble particulates.

B. Remote Loading of Liposomes

Ammonium sulfate and ethanol were removed from the external bulk aqueous phase immediately prior to remote loading by hollow fiber tangential flow diafiltration with a 100KDa nominal molecular weight cutoff cartridge. Constant feed volume was maintained, and at least seven exchange volumes were used resulting in liposomes suspended in an exterior aqueous phase comprised of 10% sucrose.

After diafiltration, the liposomes were mixed with a selected drug solution at a ratio (drug solution:liposomes) of 1:4 (vol/vol) and rapidly warmed to 65°C using a pre-equilibrated jacketed vessel containing water. The temperature of the mixture was maintained at 65°C for 40 to 60 minutes, after which the mixture was rapidly cooled in an ice-water bath. After remote loading, a sample of the liposomes was taken to check for the presence of crystals, to determine percent encapsulation and to measure the mean particle diameter.

Unencapsulated drug was removed from the bulk phase medium by hollow fiber tangential flow diafiltration using a 100 kDa nominal molecular weight cutoff cartridge. At least eight exchange volumes were used, resulting in liposomally encapsulated drug suspended in an external aqueous phase comprised of 10% sucrose 10 millimolar

Histidine pH 6.5.

The final liposome preparation was sterile filtered using a 0.22 μm cellulose acetate syringe filter and stored refrigerated and protected from light until use.

C. Characterization of Liposomes

Percent encapsulation was determined using size exclusion chromatography to compare the percent drug in the void volume (liposomal encapsulated) to the total drug (void volume plus included volume). Drug concentration in the column fractions was determined by absorbance. Mean particle diameter was determined using quasielectric laser light scattering (QELS). The total lipid concentration was assayed at the post-sterile filtration stage in order to determine the drug to lipid ratio. Liposomes loaded with MPE-camptothecin, topotecan and 7-(2-(N-isopropylamino)ethyl)-(20S)-camptothecin (CKD-602) were prepared and characterized. The results are shown in the table below.

Parameter	liposome-entrapped MPE-camptothecin	liposome-entrapped Topotecan	liposome-entrapped CKD-602
lot no.	221AZ43A	221AZ43B	221AZ53
Total Lipid concentration	17.81 μmol/mL	15.97 μmol/mL	14.079 μmol/mL
Drug concentration	2.69 mg/mL (4.55 μmol/mL)	1.72 mg/mL (3.76 μmol/mL)	1.77 mg/mL (3.77 μmol/mL)
drug:lipid ratio (mol/mol)	0.26 (1 : 3.92)	0.24 (1 : 4.25)	0.27 (1 : 3.73)
Mean Particle diameter	99 nm	95.4 nm	96.7 nm
Percent Encapsulation	96.4%	99.9%	95.3%

Example 2

In vivo Efficacy of Liposome-entrapped MPE-Camptothecin

Liposomes containing entrapped MPE-camptothecin were prepared as described in Example 1. The liposome entrapped drug and the free drug were diluted in 5% dextrose in water as required to achieve the desired concentrations.

Nude mice were inoculated with the human colon cancer cell line HT-29 as described above in the methods section. Seventy mice were randomized to one of seven treatment groups as follows: free drug at 24 mg/kg, 15 mg/kg or 6 mg/kg; liposome entrapped drug at 24 mg/kg, 15 mg/kg or 6 mg/kg; saline. Treatment was initiated when average tumor volume was approximately 75 mm^3 on day 10 post-tumor inoculation. All treatments were administered as intravenous bolus injections given weekly for 3

treatments, specifically on days 10, 16 and 23.

Tumor size during and following each experiment was used as the primary evaluation of therapeutic efficacy. Body weight was evaluated to assess toxicity. All tumor bearing animals were observed following cessation of treatment, until euthanized based on criteria above. Experiments were concluded when a majority of control tumors achieved the maximal allowed volume (4,000 mm^3).

Tumor size in each animal was measured repeatedly at various time points, thus these measurements were regarded as correlated information. Since the tumor sizes over time after treatment were of interest, repeated measurement analyses was done for each data set. By examining the data, a log transformation seemed reasonable. Let Y denote the original tumor measurement, let $Z = \log(Y+1)$. After transforming data, repeated measurement analyses was done for the transformed data Z. The SAS procedure PROC MIXED was used. The log growth rate for each treatment group was calculated and used to compare the different treatment groups. Statistical significance was declared at the 0.05 level, but due to multiple comparisons, adjustment to the type I error were done and a P-value of <0.0033 indicated a statistically significant difference in any designated comparison.

The results are summarized in Tables 1 and 2 and in Figs. 2A-2B.

Example 3
Dose Finding Study for Liposome-entrapped MPE-Camptothecin

Liposomes containing entrapped MPE-camptothecin were prepared as described in Example 1. The liposome entrapped drug and the free drug were diluted in 5% dextrose in water as required to achieve the desired concentrations.

Nude mice were inoculated with the human colon cancer cell line HT-29 as described above in the methods section. Seventy mice were randomized to one of seven treatment groups as follows: liposome entrapped drug at 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, 3 mg/kg, 5 mg/kg or 20 mg/kg; and saline. Treatment was initiated when average tumor volume was approximately 75 mm^3 on day 9 post-tumor inoculation. All treatments were administered as intravenous bolus injections given weekly for 3 treatments, specifically on days 9, 16 and 23.

The tumor size was evaluated and analyzed as described in Example 2, and the results are shown in Tables 3 and 4 and in Figs. 3A-3B.

Example 4
In vivo Efficacy of Liposome-Entrapped Topotecan

A. Liposome Preparation

Liposomes containing topotecan were prepared as follows.

The lipids distearoylphospatidylcholine (DSPC) and (N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine) (mPEG-DSPE) were combined at a molar ratio of 95:5 and dissolved in ethanol at 70°C using continuous agitation. The lipid concentration in the ethanol solution was 8.9 grams per 10 mL ethanol.

Dextran sulfate-ammonium salt was prepared by ion exchange chromatography using dextran sulfate sodium salt as the starting material. A 100 mg/mL solution of dextran sulfate ammonium salt was prepared by dissolving dextran sulfate sodium salt in water and adjusting the solution pH to 5 using ammonium hydroxide.

100 mL of dextran sulfate solution was heated to 70°C and combined with the ethanol solution of lipid while mixing to form oligolamellar liposomes. The temperature of the oligolamellar ethanol hydration liposome dispersion was maintained at 70°C for one hour with continuous mixing.

The post hydration mixture was heated to 70 degrees and size reduced using a Lipex thermobarrel extruder through a series of polycarbonate membranes to arrive at a particle size near 100 nm mean particle diameter. Typically, the sequence involved 5 passes through an 0.2 μm pore diameter membrane, followed by 10 passes through an 0.1 μm pore diameter membrane.

Unentrapped dextran sulfate polymer and remaining ethanol were removed from the external bulk aqueous phase immediately prior to the active drug loading step with eight volume exchanges using 350 mM sodium chloride solution, followed by eight volume exchanges using a 10% sucrose solution. The diafiltration cartridge employed had a specified nominal molecular weight cutoff of 100,000 Daltons.

A solution of topotecan was prepared at a concentration of 2.5 mg/mL in 10% sucrose. The drug solution and diafiltered liposomes were combined at a volume ratio of 4:1, and the temperature of the resulting mixture was raised to 70°C and maintained with constant stirring for one hour. Active drug loading was terminated by rapidly cooling the post-loading mixture using an ice water bath.

Unentrapped drug was removed by diafiltration employing a cartridge having nominal molecular weight cutoff of 100,000 Daltons. Typically, 8-10 volume exchanges were employed using 10% sucrose 10 mM Histidine pH 6.5 as the exchange buffer.

Drug concentration was adjusted to the final value by assaying for potency with a uv-vis absorbance measurement and diluting accordingly.

The final process step involved sterile grade filtration employing a 0.22 μm filter prior to filling vials.

B. Liposome Characterization

Percent encapsulation was determined using size exclusion chromatography to determine the percent drug in the void volume ("liposomal drug") to the total amount recovered in both the included and void volume fractions. Drug concentration was monitored using uv-vis absorbance spectrophotometry. Mean particle diameter was determined using quasielastic laser light scattering. Total lipid was determined using phosphorous assay. The results are summarized in the table below.

Parameter	Liposome-entrapped Topotecan
total lipid	17.2 mg/mL
total drug	2.1 mg/mL
drug:lipid ratio (mol:mol)	0.238
mean particle diameter	87.3 nm
percent encapsulation	98.8

C. *In vivo* Pharmacokinetics and Efficacy

Seventy two mice were inoculated with HT-29 cancer cells as described above in the methods section. Nine days after tumor inoculation, the animals were treated weekly with one of the following intravenous treatments: saline; liposome-entrapped MPE-camptothecin 4 mg/kg; free topotecan 25 mg/kg; liposome entrapped topotecan at drug dosages of 2 mg/kg, 5 mg/kg or 8 mg/kg. All treatments were administered as intravenous bolus injections given weekly for 3 treatments, specifically on days 9, 16 and 23.

The tumor size was evaluated and analyzed as described in Example 2, and the results are shown in Tables 6 and 7 and in Figs. 5A-5B.

Example 5
In vivo Efficacy of Liposome-Entrapped CKD-602

A. Liposome Preparation and Characterization

Liposomes containing CKD-602 were prepared as described in Example 4, except using a drug solution of CKD-602. The liposomes were characterized as described in Example 4 and the results are summarized below.

Parameter	Liposome entrapped CKD-602
total lipid	12.5 mg/mL
total drug	2.07 mg/mL
drug:lipid ratio (mol:mol)	0.315
mean particle diameter	92.8 nm
percent encapsulation	94.7

B. *In vivo* Pharmacokinetics and Efficacy

Seventy two mice were inoculated with HT-29 cancer cells as described above in the methods section. Eleven days after tumor inoculation, the animals were treated weekly with one of the following intravenous treatments: saline, liposome-entrapped MPE-camptothecin 4 mg/kg; free CKD602 20 mg/kg; liposome entrapped CKD602 at drug dosages of 1 mg/kg, 2 mg/kg or 4 mg/kg. All treatments were administered as intravenous bolus injections given weekly for 3 treatments, specifically on days 11, 18 and 25.

The tumor size in each animal was measured twice weekly during the study to evaluate therapeutic efficacy. Body weight of each animal was monitored twice weekly to assess toxicity of the formulations. The results are shown in Tables 8 and 9 and in Figs. 7A-7B.

Although the invention has been described with respect to particular embodiments, it will be apparent to those skilled in the art that various changes and modifications can be made without departing from the invention.

IT IS CLAIMED:

1. A composition for treating a tumor in a subject, comprising

liposomes composed of a vesicle-forming lipid and between about 1-20 mole percent of a vesicle-forming lipid derivatized with a hydrophilic polymer, said liposomes being formed under conditions that distribute the polymer on both sides of the liposomes' bilayer membranes; and

entrapped in the liposomes, a topoisomerase inhibitor at a concentration of at least about 0.10 μmole drug per μmole lipid, said liposomes having an inside/outside ion gradient sufficient to retain the topoisomerase inhibitor within the liposomes at the specified concentration.

2. The composition of claim 1, where the topoisomerase inhibitor is a topoisomerase I inhibitor selected from the group consisting of camptothecin and camptothecin derivatives.

3. The composition of claim 2, wherein the camptothecin derivative is selected from the group consisting of 9-aminocamptothecin, 7-ethylcamptothecin, 10-hydroxycamptothecin, 9-nitrocamptothecin, 10,11-methlyenedioxycamptothecin, 9-amino-10,11-methylenedioxycamptothecin, 9-chloro-10,11-methylenedioxycamptothecin, irinotecan, topotecan, (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin, 7-(4-methylpiperazinomethylene)-10,11-methylenedioxy-20(S)-camptothecin and 7-(2-N-isopropylamino)ethyl)-(20S)-camptothecin.

4. The composition of claim 1, wherein the topoisomerase inhibitor is a topoisomerase I/II inhibitor selected from the group consisting of 6-[[2-(dimethylamino)-ethyl]amino]-3-hydroxy-7H-indeno[2,1-c]quinolin-7-one dihydrochloride, azotoxin and 3-methoxy-11H-pyrido[3',4'-4,5]pyrrolo[3,2-c]quinoline-1,4-dione.

5. The composition of claim 1, wherein the hydrophilic polymer is polyethyleneglycol having a molecular weight between 500-5,000 daltons.

6. The composition according to any of the preceding claims, wherein the liposomes include a vesicle-forming lipid having a phase transition temperature above 37°C.

7. The composition of claim 6, wherein the vesicle-forming lipid is selected from the group consisting of hydrogenated soy phosphatidylcholine, distearoylphosphatidylcholine and sphingomyelin.

8. The composition of claim 6, wherein the liposomes are composed of 20-94 mole percent hydrogenated soy phosphatidylcholine and 1-20 mole percent distearoylphosphatidylethanolamine derivatized with polyethyleneglycol and 5-60 mole percent cholesterol.

9. The composition of claim 6, wherein the liposomes are composed of 30-65 mole percent hydrogenated soy phosphatidylcholine, 5-20 mole percent distearoylphosphatidylethanolamine derivatized with polyethyleneglycol and 30-50 mole percent cholesterol.

10. The composition of claim 6, wherein the liposomes are composed of 20-94 mole percent distearoylphosphatidycholine and 1-20 mole percent distearoylphosphatidylethanolamine derivatized with polyethyleneglycol.

11. The composition according to any one of claims 1-10, wherein the liposomes include a polyanionic polymer within the liposomes, said polymer capable of forming a complex with said topoisomerase inhibitor.

12. The composition of claim 12, wherein said polyanionic polymer is selected from dextran sulfate, chondroitin sulfate A, polyvinylsulfuric acid, and polyphosphoric acid.

13. A composition for administration of a topoisomerase inhibitor, comprising

liposomes composed of vesicle-forming lipids and having an inside/outside ion gradient effective to retain the drug within the liposomes; and

entrapped in the liposomes, the topoisomerase inhibitor at a concentration of at least about 0.20 μmole drug per μmole lipid.

14. The composition of claim 13, wherein the toposiomerase inhibitor is a topoisomerase I inhibitor selected from MPE-camptothecin, topotecan and (7-(2-N-isopropylamino)ethyl)-(20S)-camptothecin.

15. The composition of claim 13, wherein the toposiomerase inhibitor is a topoisomerase I/II inhibitor selected from the group consisting of 6-[[2-(dimethylamino)-ethyl]amino]-3-hydroxy-7H-indeno[2,1-c]quinolin-7-one dihydrochloride and 3-methoxy-11H-pyrido[3',4'-4,5]pyrrolo[3,2-c]quinoline-1,4-dione.

16. The composition of claim 13, wherein the liposomes further include a polyanionic polymer within the liposomes, said polymer capable of forming a complex with said topoisomerase inhibitor.

17. A composition according to any of the preceding claims for use as a medicament in treating a tumor in a subject.

18. Use of a composition according to any one of claims 1-16 for the manufacture of a medicament for treating a tumor in a subject.

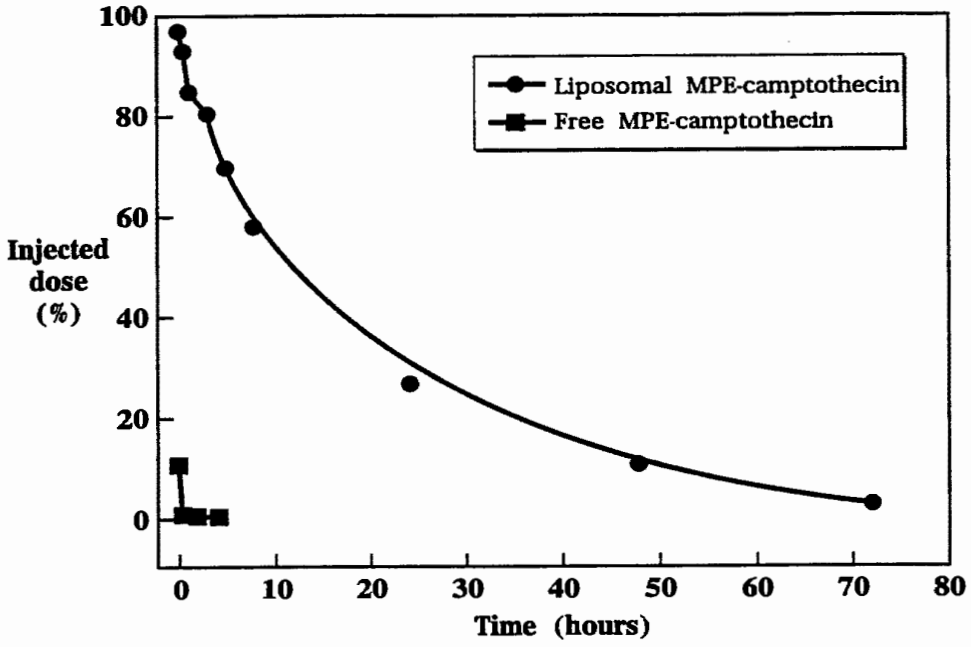

100
80
60
40
20
0
Injected dose (%)
Liposomal MPE-camptothecin
Free MPE-camptothecin
0
10
20
30
40
50
60
70
80
Time (hours)

Fig. 1A

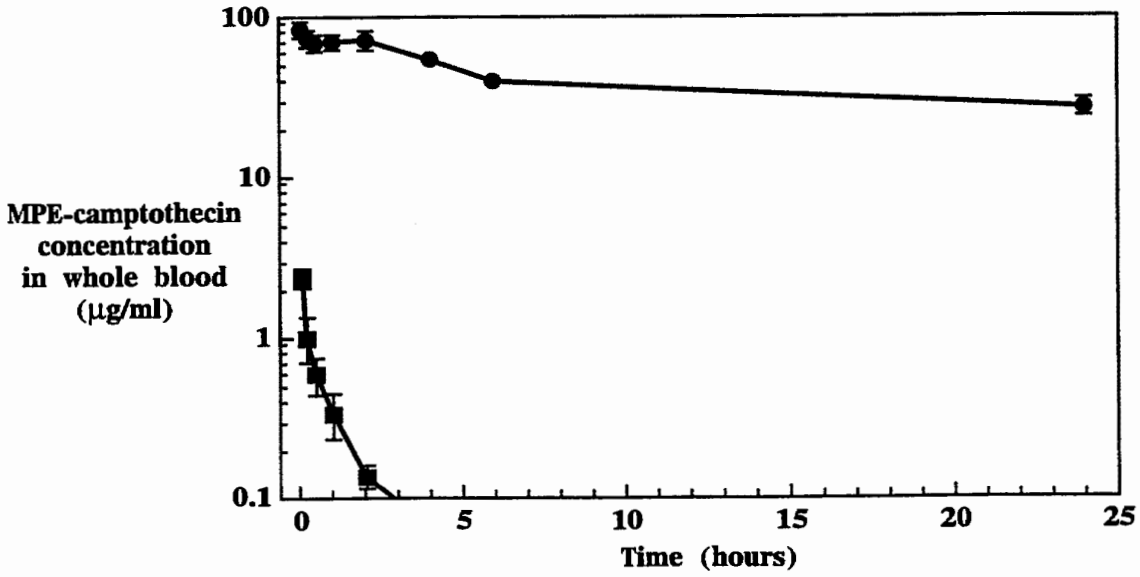

100
10
1
0.1
MPE-camptothecin concentration in whole blood (μg/ml)
0
5
10
15
20
25
Time (hours)

Fig. 1B

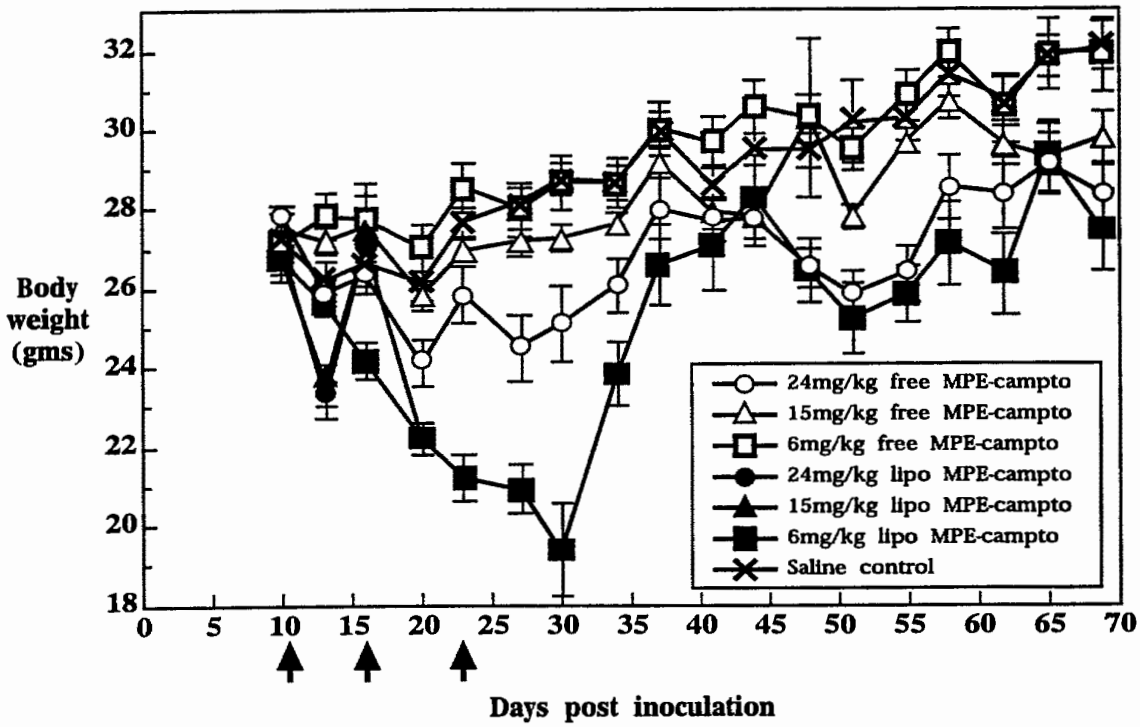

Body weight (gms)
32
30
28
26
24
22
20
18
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Days post inoculation
24mg/kg free MPE-campto
15mg/kg free MPE-campto
6mg/kg free MPE-campto
24mg/kg lipo MPE-campto
15mg/kg lipo MPE-campto
6mg/kg lipo MPE-campto
Saline control

Fig. 2A

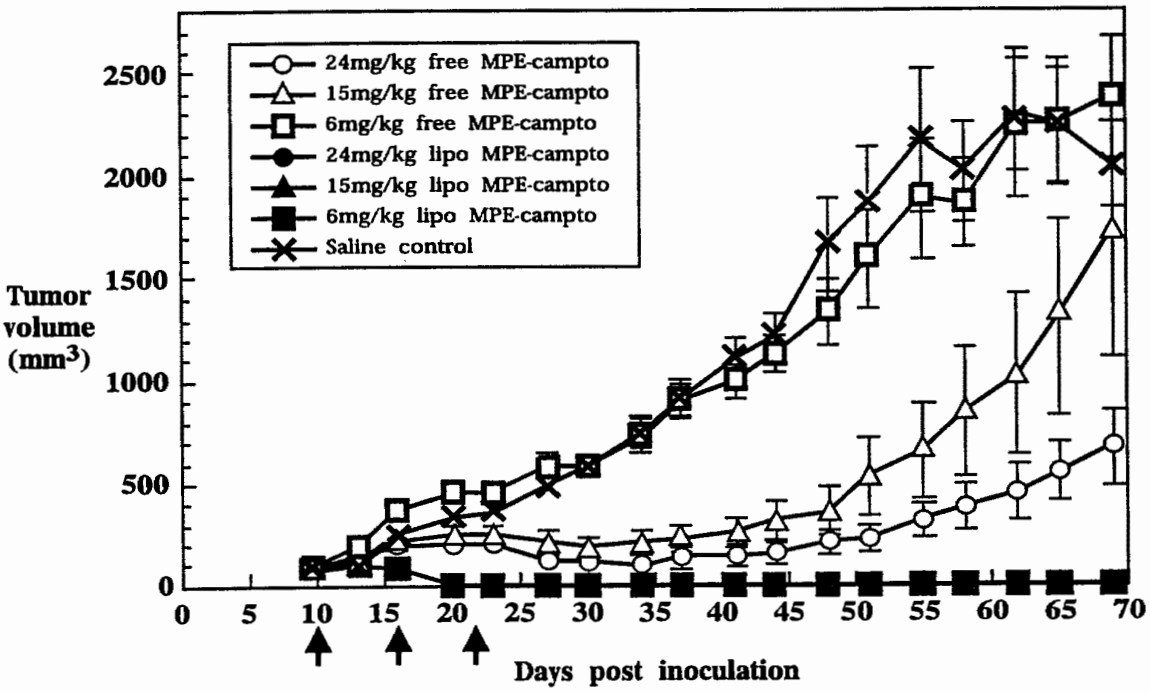

Tumor volume (mm^3)
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Days post inoculation
24mg/kg free MPE-campto
15mg/kg free MPE-campto
6mg/kg free MPE-campto
24mg/kg lipo MPE-campto
15mg/kg lipo MPE-campto
6mg/kg lipo MPE-campto
Saline control

Fig. 2B

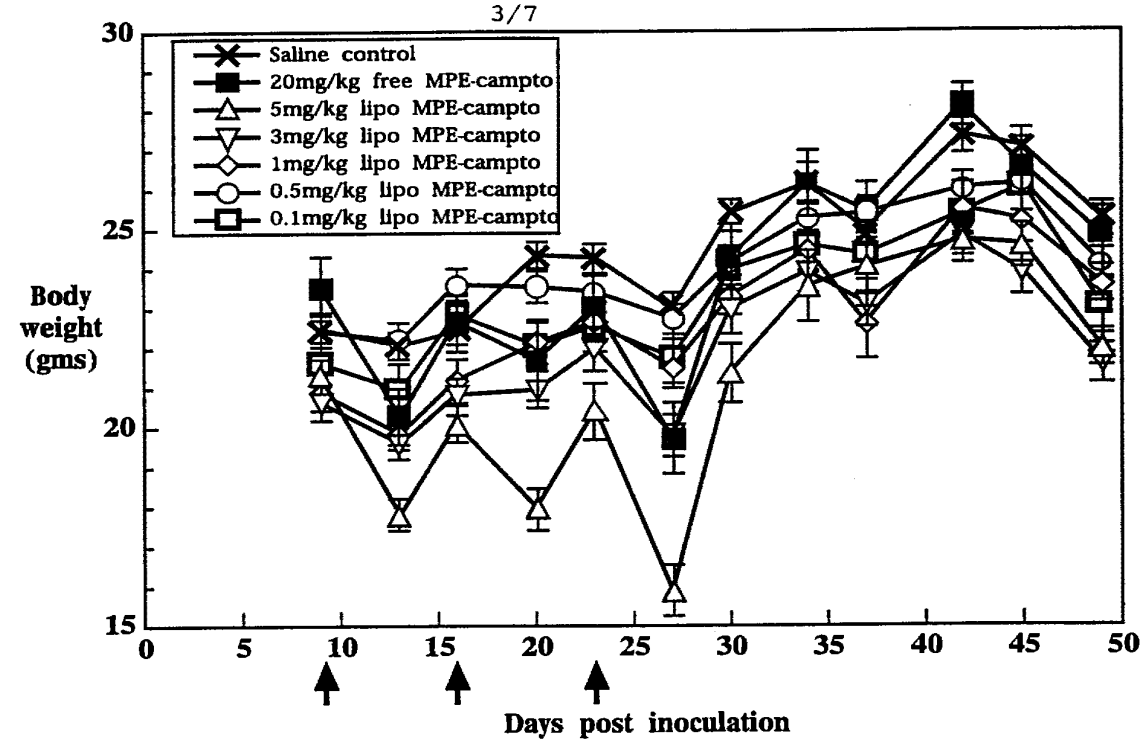

Saline control
20mg/kg free MPE-campto
5mg/kg lipo MPE-campto
3mg/kg lipo MPE-campto
1mg/kg lipo MPE-campto
0.5mg/kg lipo MPE-campto
0.1mg/kg lipo MPE-campto
Body weight (gms)
Days post inoculation

Fig. 3A

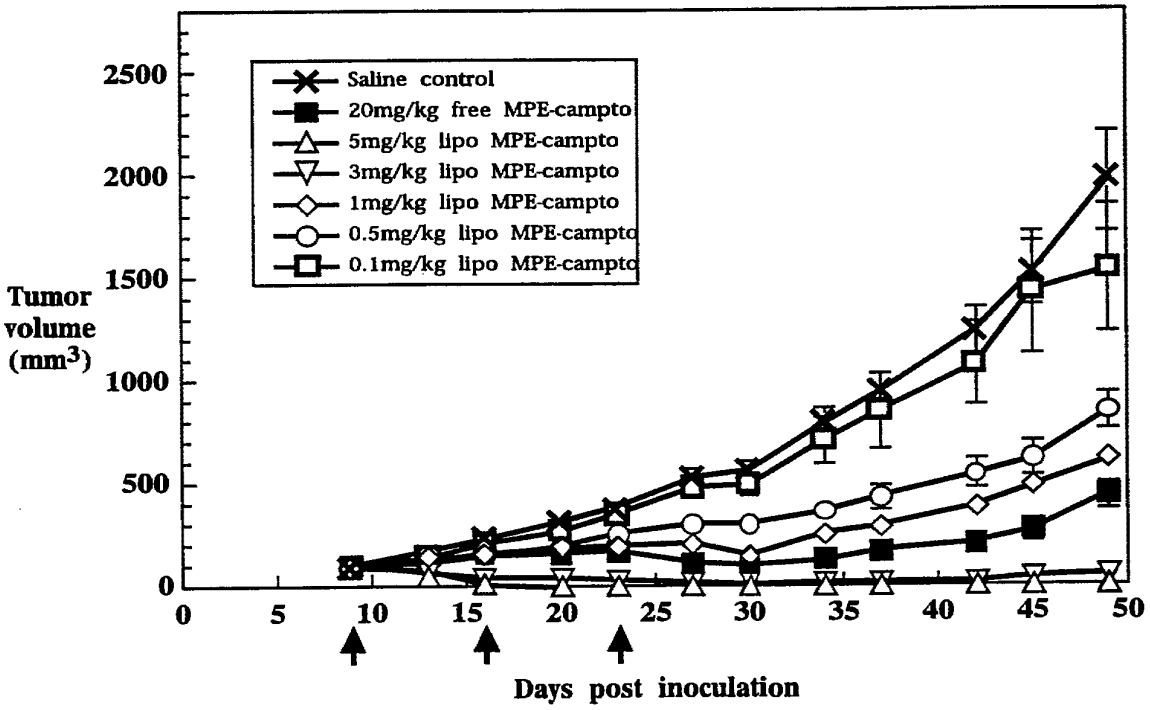

Saline control
20mg/kg free MPE-campto
5mg/kg lipo MPE-campto
3mg/kg lipo MPE-campto
1mg/kg lipo MPE-campto
0.5mg/kg lipo MPE-campto
0.1mg/kg lipo MPE-campto
Tumor volume (mm3)
Days post inoculation

Fig. 3B

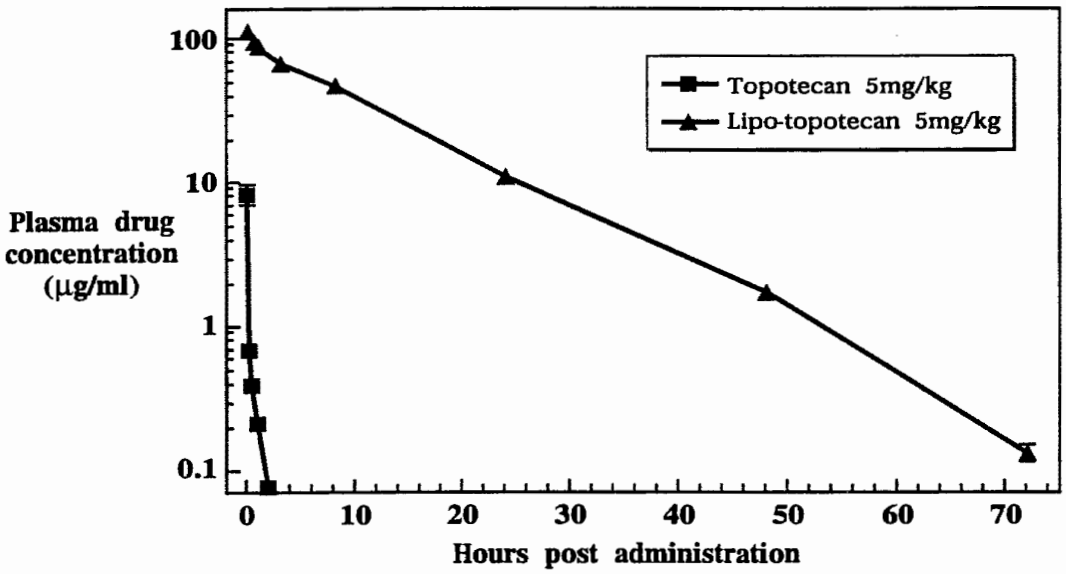

100
10
1
0.1
Plasma drug concentration (μg/ml)
0
10
20
30
40
50
60
70
Hours post administration
Topotecan 5mg/kg
Lipo-topotecan 5mg/kg

Fig. 4A

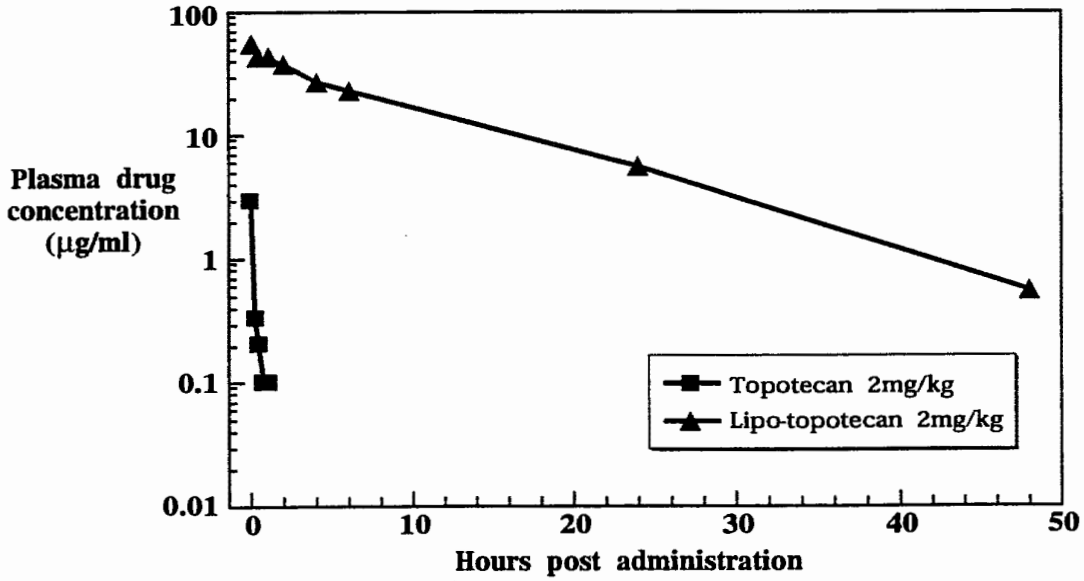

100
10
1
0.1
0.01
Plasma drug concentration (μg/ml)
0
10
20
30
40
50
Hours post administration
Topotecan 2mg/kg
Lipo-topotecan 2mg/kg

Fig. 4B

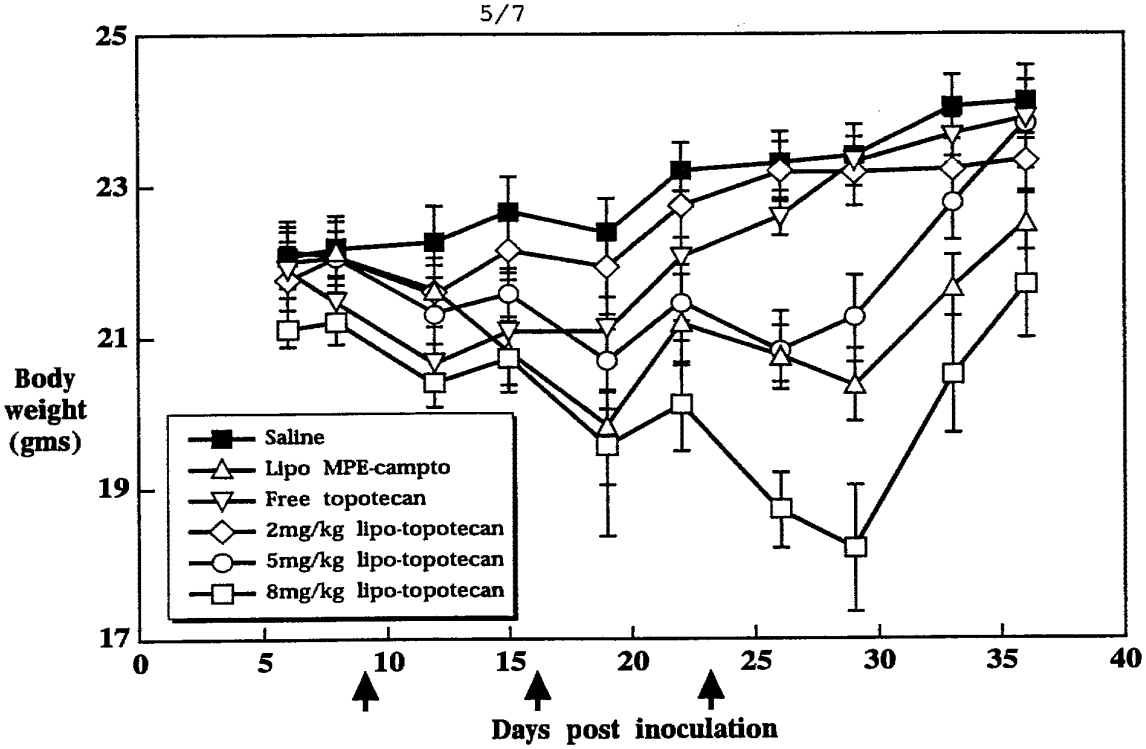

Body weight (gms)
Days post inoculation
Saline
Lipo MPE-campto
Free topotecan
2mg/kg lipo-topotecan
5mg/kg lipo-topotecan
8mg/kg lipo-topotecan

Fig. 5A

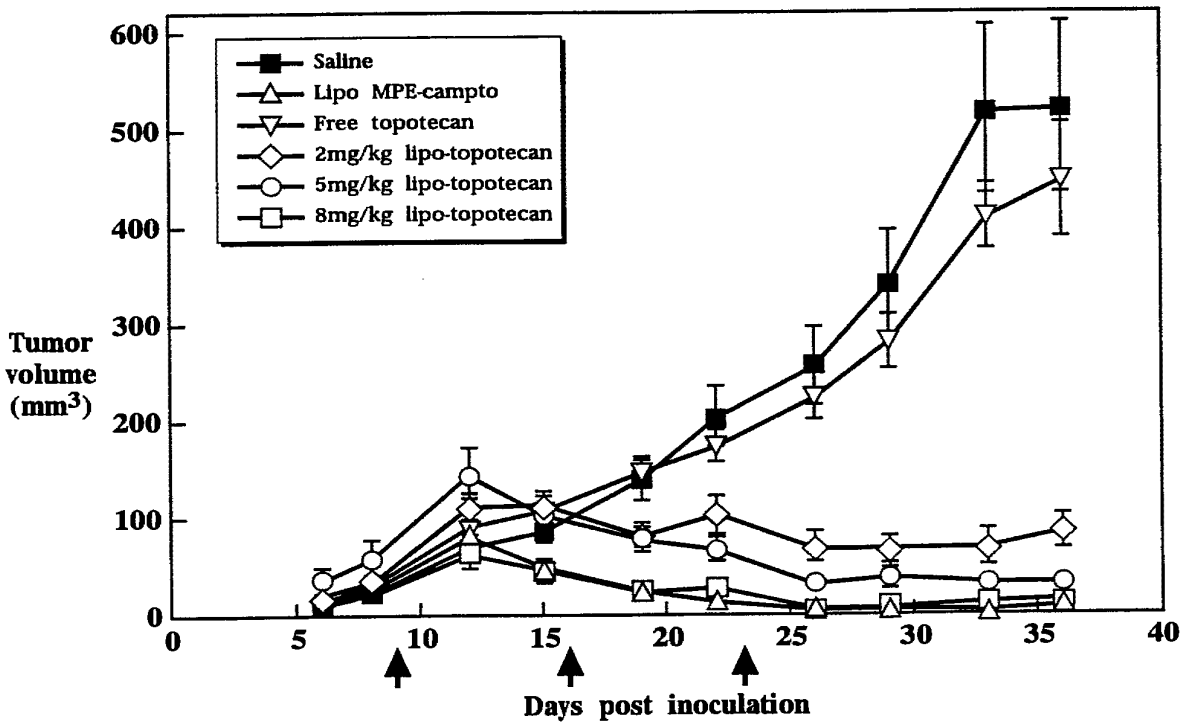

Tumor volume (mm3)
Days post inoculation
Saline
Lipo MPE-campto
Free topotecan
2mg/kg lipo-topotecan
5mg/kg lipo-topotecan
8mg/kg lipo-topotecan

Fig. 5B

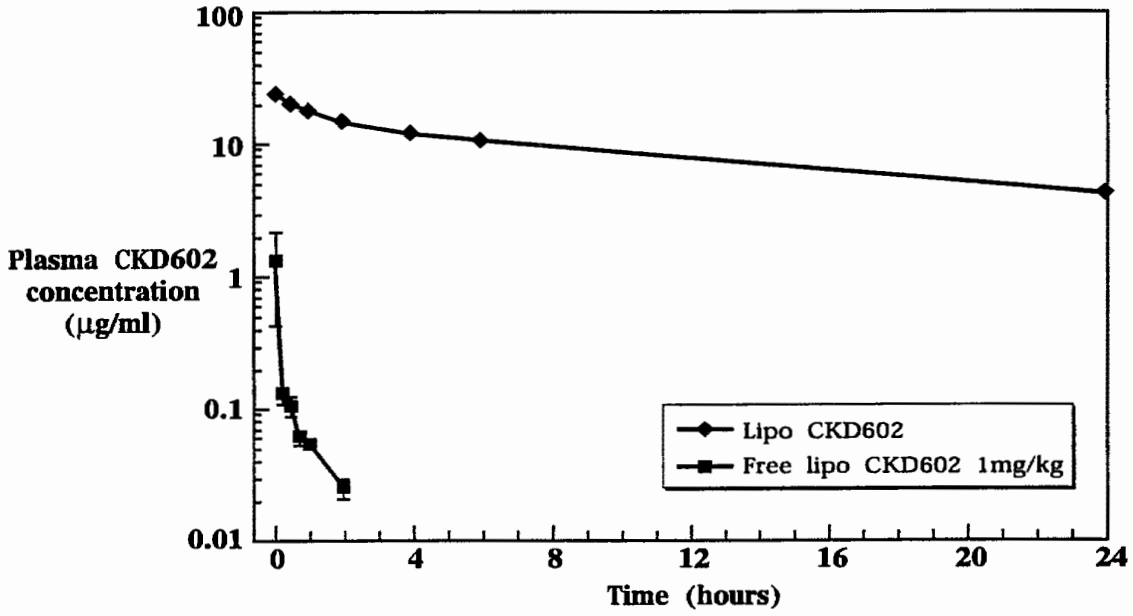

100
10
1
0.1
0.01
Plasma CKD602 concentration (μg/ml)
0
4
8
12
16
20
24
Time (hours)
Lipo CKD602
Free lipo CKD602 1mg/kg

Fig. 6

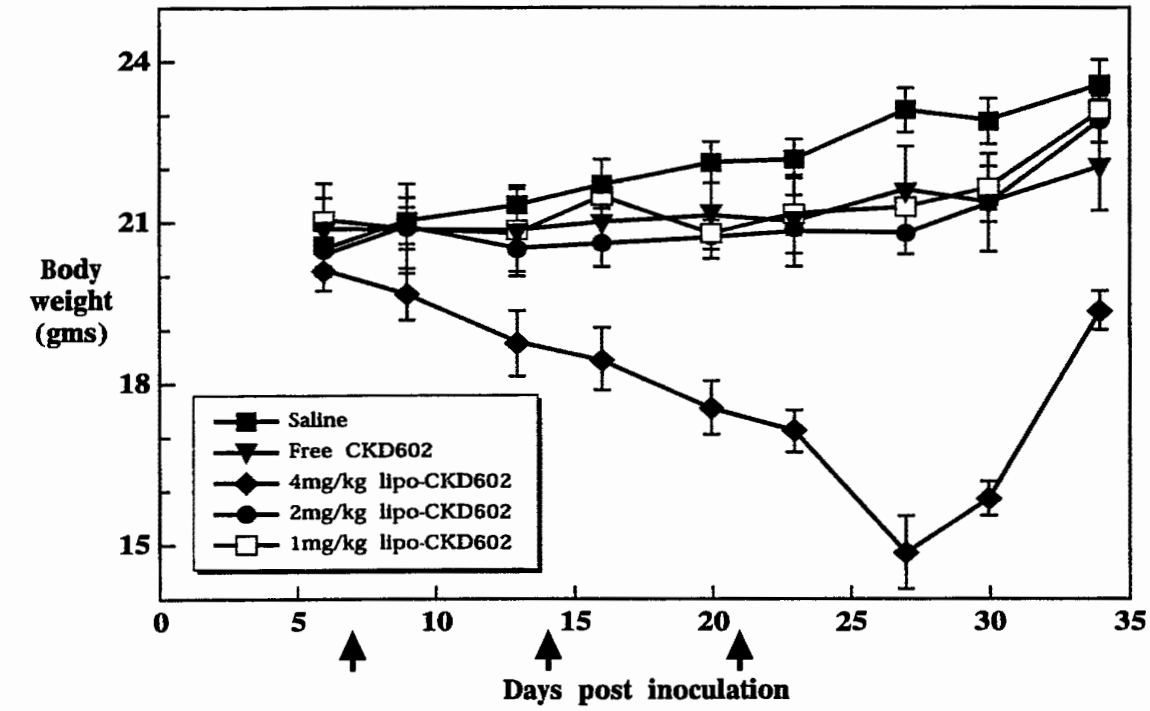

Body weight (gms)
24
21
18
15
0
5
10
15
20
25
30
35
Days post inoculation
Saline
Free CKD602
4mg/kg lipo-CKD602
2mg/kg lipo-CKD602
1mg/kg lipo-CKD602

Fig. 7A

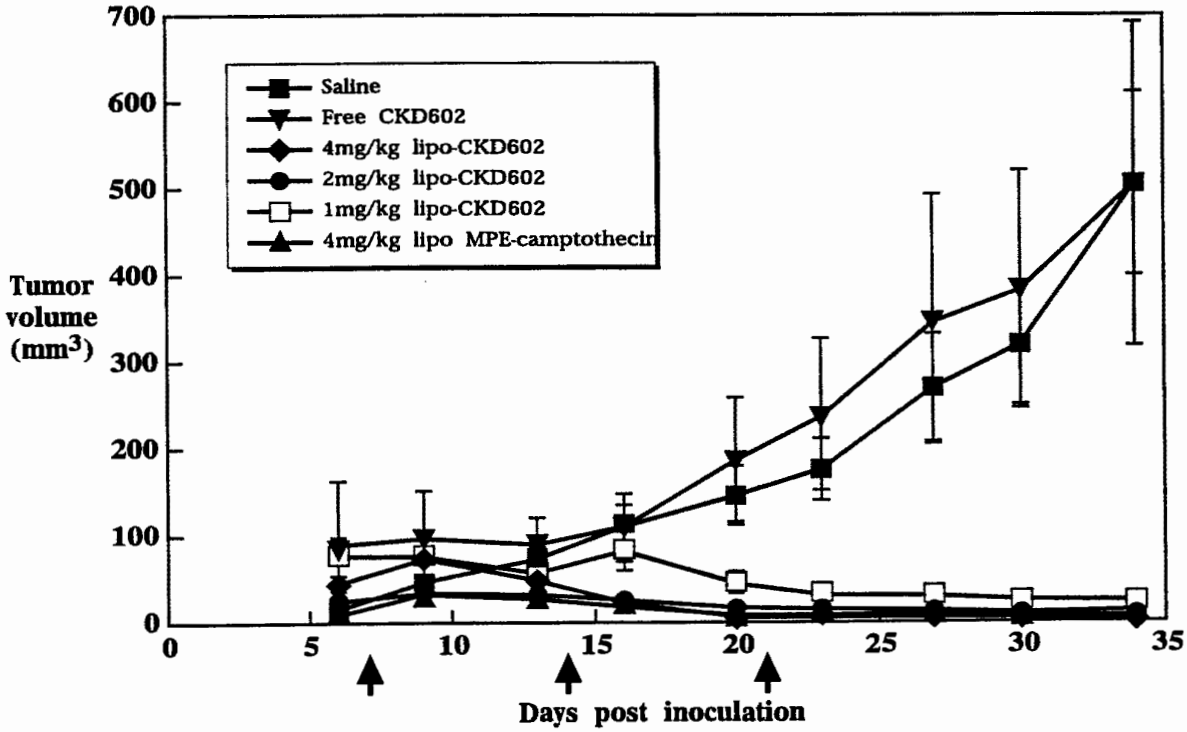

Tumor volume (mm3)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
Days post inoculation
Saline
Free CKD602
4mg/kg lipo-CKD602
2mg/kg lipo-CKD602
1mg/kg lipo-CKD602
4mg/kg lipo MPE-camptothecin

Fig. 7B

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/24228

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 A61K31/4745 A61P35/00

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 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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SADZUKA Y, ET AL.: "Effect of liposomalization on the antitumor activity, side effects and tissue distribution of CPT-11" CANCER LETTERS, vol. 127, no. 1,2, May 1998 (1998-05), pages 99-106, XP000878677 ISSN 0304-3835 paragraphs '02.1!-'02.3! paragraphs '3.2.4!,'03.3!,'03.4!; figures 5-7 —— -/—	1-3,5-7, 17,18

[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
14 February 2000	28/02/2000

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	Epskamp, S

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 08986 A (SMITHKLINE BEECHAM CORP ;CONSTANTINIDES PANAYIOTIS PERI (US)) 6 April 1995 (1995-04-06) page 2, line 15 -page 3, line 9 page 3, line 27 -page 7, line 34 page 9, line 1 -page 10, line 15 examples 1-3,6,7	1-3,5-7, 13,14, 17,18
X	LUNDBERG BB: "Biologically active camptothecin derivatives for incorporation into liposome bilayers and lipid emulsions" ANTI-CANCER DRUG DESIGN, vol. 13, no. 5, July 1998 (1998-07), pages 453-461, XP000878681 ISSN 0266-9536 cited in the application page 454, line 5 -page 455, line 23 page 460; figures 2,3	1,2,5,6, 17,18
A	US 5 552 156 A (BURKE THOMAS G) 3 September 1996 (1996-09-03) cited in the application column 3, line 49 -column 6, line 45 claims; examples 1-7	1-18
P,X	COLBERN GT, ET AL.: "Encapsulation of the topoisomerase inhibitor GL147211C in pegylated (STEALTH) liposomes: pharmacokinetics and antitumor activity in HT29 colon tumor xenografts" CLINICAL CANCER RESEARCH, vol. 4, no. 12, December 1998 (1998-12), pages 3077-3082, XP002130384 ISSN 1078-0432 the whole document in particular: page 3077, right-hand column, line 46 -page 3078, left-hand column, line 12; table 1	1-3,5-9, 13,14, 17,18
P,X	WO 99 13816 A (EMERSON DAVID LLOYD ;NEXSTAR PHARMACEUTICALS INC (US); HU NING (US) 25 March 1999 (1999-03-25) page 6, line 14 -page 7, line 11 page 8, line 17 -page 11, line 15 claims 1-31; examples 1,2; table 4	13,14, 17,18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 24228

Box I Observations where certain claims were found unsearchable (Continuation of item 1 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.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 17 is directed to a method of treatment of 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 II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

CSPC Exhibit 1105

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 99/24228

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9508986 A	06-04-1995	EP 0721328 A	17-07-1996
		JP 9504517 T	06-05-1997
US 5552156 A	03-09-1996	US 5736156 A	07-04-1998
WO 9913816 A	25-03-1999	AU 9387798 A	05-04-1999

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, 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, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
— *without international search report and to be republished upon receipt of that report*

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.

WO 2004/017940 A2

(54) Title: PHARMACEUTICALLY ACTIVE LIPID BASED FORMULATION OF SN38

(57) Abstract: SN38, camptothecin derivatives are poorly water soluble, highly lipophilic camptothecin derivatives and are very active against a variety of human cancers. Because of their very poor water solubility, SN38 has not been used to treat human patients with cancer due to the inability to administer sufficient quantities of dissolved in a pharmaceutical formulation. This invention overcomes these limitations by teaching novel pharmaceutical acceptable SN38 liposome complex formulation for the direct administration of the formulation to human patients with cancer. The claimed invention also describes the methods to prepare liposomal SN38 complexes and antitumor compositions of liposomal SN38 complexes to allow the administration in sufficient amounts to treat various types of cancer and as antiviral agents. This invention is also directed to injectable sterile solutions, antitumor compositions, liposomes. The present invention is for novel compositions and methods for treating diseases caused by cellular proliferation, particularly, for treating cancer in mammals and more particularly in humans. The therapeutic compositions of the present invention include SN38 lipid complexes in which the complexes can contain any of a variety of neutral or charged lipids and, desirably, cardiolipin. The compositions are capable of efficiently incorporating SN38 into complexes and are capable of solubilizing relatively high concentrations of SN38.

PHARMACEUTICALLY ACTIVE LIPID BASED FORMULATION OF SN38

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to United States Provisional Patent Application No. 60/404,668, filed August 20, 2002.

FIELD OF THE INVENTION

This invention pertains to complexes of SN38 with lipids, their methods of manufacture, and their use as antiviral agents and in the treatment of diseases, especially diseases involving eukaryotic cellular proliferation.

DESCRIPTION OF THE BACKGROUND

The compound known as 7-ethyl-10-hydroxycamptothecin (SN38) and more formally as ((+)-(4S)-4,11-diethyl-4,9-dihydroxy-1H-pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-3,14(4H,12H)-dione, first disclosed in U.S. Patent 4,473,692, is an active metabolite of irinotecan, a derivative of camptothecin. It is thought to bind to the enzyme topoisomerase I, the enzyme responsible for relieving torsional strain in DNA by inducing reversible single-strand breaks. The bound SN38 appears to block religation of the single-strand breaks by topoisomerase-I thereby causing cytotoxicity in mammalian cells which, apparently, can not otherwise sufficiently repair the breaks.

The metabolic conversion of irinotecan to SN38 occurs primarily in the liver by carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and a dipiperidino side chain. Subsequently, this derivative undergoes conjugation to form the glucuronide metabolite.

SN38 is approximately 1000 times more potent than irinotecan as an inhibitor of topoisomerase I purified from human and rodent tumor cell lines. In vitro cytotoxicity assays show that SN38 is up to 2,000-fold more potent than irinotecan. Consequently, SN38 has the potential to be a highly effective antineoplastic agent. In addition, SN38 has an advantage over its camptothecin precursors in that it does not require activation by the liver. Therefore, an appropriate formulation could be used in local as well as systemic treatment methods.

SN38 is exceedingly insoluble in aqueous solutions. Despite its lack of solubility in water, it also has a low affinity for lipid membranes from which it tends to precipitate into aqueous phase. These solubility characteristics interfere with the use of SN38 as a therapeutic. Moreover, the effectiveness of SN38 after repeated administrations can be limited by the development of multi-drug resistance which not only reduces its

effectiveness but also reduces the effectiveness of certain other antineoplastic therapeutics. The general toxicity of SN38 also limits its use therapeutically.

Thus, formulations are needed that improve SN38 efficacy such that SN38 can be used effectively in the treatment of diseases associated with cellular proliferation. Such a formulation should have suitable solubility and toxicity characteristics and will be useful as an antiviral agents and in the treatment of certain proliferative diseases such as cancer.

The invention provides such a composition and methods. These and other advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

SUMMARY OF THE INVENTION

The present invention is for novel SN38 compositions, their preparation methods, and their use as antiviral agents and in treating diseases caused by proliferating eukaryotic cells, such as cancer, particularly in mammals, especially humans. The SN38 compositions include SN38 complexed with a lipid wherein more than 40 wt.% of the SN38 is complexed with the lipid. The complexes, include liposomes, and can contain any of a variety of neutral or charged lipid materials and, desirably, cardiolipin. Suitable lipids include any pharmaceutically acceptable lipophilic materials that bind SN38 to provide a stable pharmaceutical formulation and facilitate its administration to mammals. Cardiolipin can be synthetic, derived from natural sources, or be chemically modified. The lipid complexes can carry net negative, or positive charges, or can be neutral. Preferred complexes also contain α-tocopherol. The SN38 complexes can be used advantageously with secondary therapeutic agents other than the SN38 complexes, including antineoplastic (such as cisplatin, taxol, doxorubicin, vinca alkaloids, and temozolomide), antifungal, antibiotic, antiviral, and antimetabolites, or other active agents. Liposome complexes can be multilamellar vesicles, unilamellar vesicles, or their mixtures, as desired. The invention also encompasses methods for preparing such SN38 complexes. The invention is further directed to methods in which a therapeutically effective amount of the SN38 complexes are included in a pharmaceutically acceptable excipient and administered to a mammal, such as a human, as an antiviral agent or to treat proliferative diseases, such as cancer.

This invention also describe the methods to prolong shelf-life of SN38 complexes.

In one particularly preferred method of preparing the SN38 complexes, SN38 is dissolved in an alkaline solution and used to hydrate a lipid film to form liposomes.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions and methods for delivering SN38 to a mammalian host. The compositions and methods are characterized by avoidance of solubility problems of SN38, high SN38 and complex stability, ability to administer SN38 as a bolus or short infusion in a high concentration, reduced SN38 toxicity, increased therapeutic efficacy of SN38, and modulation of multidrug resistance.

The inventive composition is a lipid complex with SN38 in which the complex desirably contains cardiolipin. Suitable complexes are characterized by having SN38 bound with a lipophilic compound that imparts solubility characteristics such that stable pharmaceutical preparations can be generated and used. The complexes include, but are not limited to, liposomes, emulsions, and micelles. In the complexes the SN38 can be bound to the lipid by covalent, hydrophobic, electrostatic, hydrogen, or other bonds and is considered bound even where the SN38 is simply be entrapped within the interior of a liposome. The SN38 compositions include SN38 complexed with a lipid wherein at least about 40% or more, such as at least about 50 wt.% or more of the SN38 is complexed with the lipid, more preferably at least about 70 wt.% or more, even more preferably at least about 80 wt.% or more (e.g., at least about 85% or more), and most preferably at least about 90 wt.% or more (such as at least about 95% or more) of the SN38 is complexed with lipid (e.g., at least a portion of the lipid). Where the compositions are liposomal, desirably, at least about 70 wt.% or more, even more preferably at least about 80 wt.% or more (e.g., at least about 85% or more), and most preferably at least about 90 wt.% or more (such as at least about 95% or more) of the SN38 is entrapped or encapsulated with the liposomes.

Desirably, the SN38 lipid complexes contain cardiolipin. Any suitable cardiolipin can be used. For example, cardiolipin can be purified from natural sources or can be chemically synthesized or chemically modified, such as tetramyristylcardiolipin, by such methods as are known in the art.

SN38 complexes generally contain other complexing agents in addition to cardiolipin. Suitable agents include pharmaceutically acceptable synthetic, semi-synthetic (modified natural) or naturally occurring compounds having a hydrophilic region and a hydrophobic region. Such compounds include amphiphilic molecules which can have net positive, negative, or neutral charges or which are devoid of charge. Suitable complexing agents include compounds, such as phospholipids that can be synthetic or derived from natural sources, such as egg or soy. Suitable phospholipids include compounds such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), sphingomyelin (SPM), and the like, alone or in combination. Phosphatidylglycerols such

as dimyristoylphosphatidylglycerol, dioleoylphosphatidylglycerol, distearoylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, diarachidonoylphosphatidylglycerol, are preferred, as are mixtures thereof. The phospholipids dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), dioleoylphosphatidylglycerol (DOPG), distearoylphosphatidyl choline (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), diarachidonoyl phosphatidylcholine (DAPC), egg phosphatidylcholine, soy phosphatidylcholine, or hydrogenated soy phosphatidylcholine (HSPC) can be used, as can mixtures thereof. Other lipids that can be employed include ganglioside GM1 and polymer modified lipids, such as PEG modified lipids.

The SN38 lipid complexes generally include at least one sterol or steroid component such as cholesterol, polyethylene glycol derivatives of cholesterol (PEG-cholesterols), coprostanol, cholestanol, or cholestane, or α-tocopherol. They may also contain sterol derivatives such as cholesterol hemisuccinate (CHS), cholesterol sulfate, and the like. Organic acid derivatives of tocopherols, such as α-tocopherol hemisuccinate (THS), can also be used. Suitable SN38 complexes can also be formed with glycolipids, or natural or derivatized fatty acids and the like. The preferred SN38 complexing agents include cardiolipin (e.g., natural cardiolipin or synthetic cardiolipin), a phosphatidyl choline, cholesterol, and α-tocopherol which are combined to form of a liposome.

Any suitable amount of SN38 can be used. Suitable amounts of SN38 are those amounts that can be stably incorporated into the complexes of the present invention. The SN38 should preferably be present in the abovementioned compositions at a concentration of about 0.01 mg/ml to about 20 mg/ml, such as between about 0.1 mg/ml and about 20 mg/ml or between about 0.01 mg/ml and about 5 mg/ml, more preferably about 0.1 to about 4 mg/ml, still more preferably about 0.5 to 3 mg/ml, and even more preferably about 0.8 to 2, such as from about 1 or more to about 1.5 mg/ml. Suitable compositions also generally contain from about 1 to about 50 wt.% cardiolipin, or preferably about 2 to about 25 wt.% cardiolipin, or more preferably about 5 wt.% to about 20 wt.% cardiolipin. Such compositions also generally contain about 1 wt.% to about 95 wt.% phosphatidylcholine, or more preferably about 20 wt.% to about 75 wt.% phosphatidylcholine. The preferred compositions also generally contain α-tocopherol in a concentration of about 0.001 wt.% to about 5 wt.%.

The complexing agents can also be considered liposome-forming materials when they are used to generate liposomes by methods such as are known. To generate the desired complexes, they can be dissolved by themselves or with the other lipophilic ingredients, including SN38, in suitable solvents. Suitable solvents are those which provide sufficient solubility and can be evaporated without leaving a pharmaceutically

unacceptable amount of a pharmaceutically unacceptable residue. For example, the cardiolipin can be dissolved in non-polar or slightly polar solvent such as ethanol, methanol, chloroform, methylene chloride, or acetone. SN38 also can be dissolved in an aqueous, typically alkaline, buffer (e.g., sodium carbonate, sodium bicarbonate, sodium hydroxide, sodium phosphate, sodium acetate, sodium citrate, calcium hydroxide, sodium biphosphate, ammonium acetate, Tris (hydroxy-methyl) aminomethane, sodium benzoate, and the like). The aqueous of SN38 can then be added to the lipid film and the resulting mixture vigorously homogenized to produce liposomes, emulsions and micelles, as desired.

The invention further provides a method for forming a lipid composition comprising SN38 or a compound in equilibrium with SN38. SN38 can be said to be stable as long as most of the drug retains its chemical structure or a chemical structure that is in equilibrium with its chemical structure. Chemical structures in equilibrium with SN38 specifically include structures that impart greater solubility at high pH but which are converted to SN38 when the pH is lowered.

Generally, the method involves mixing dissolved lipophilic ingredients together and evaporating or lyophilizing the solvent(s) to form a (preferably homogeneous) lipid phase or lipid film. The lipid phase can be formed, for example, in a suitable organic solvent, such as is commonly employed in the art. The lipid phase then is hydrated with a first aqueous solution including the SN38 (or a compound in equilibrium with SN38) so as to form lipid composition including the compound. Thereafter, the pH of the composition is reduced so as to convert some or all of the compound in equilibrium with SN38 to SN38.

Preferably, the lipid phase is a lipid film, which can be generated by known methods. For example, solvent evaporation can be accomplished by any suitable means that preserves the stability of the components. SN38 complexes, including liposomes or micelles, can then be formed by hydrating the lipid phase, such as by adding a suitable solvent to the dry lipid film mixture. Suitable solvents include pharmaceutically acceptable polar solvents. Generally, solvents are aqueous solutions containing pharmaceutically acceptable salts, buffers, or their mixtures. In one method, a lipid film is hydrated with an aqueous solution of SN38 having an alkaline pH. Suitable pHs range from about 7 to about 11, pHs of about 8 to about 10 are more preferred, and pHs of about 9 to about 10 are most preferred. Aqueous solutions having a suitable pH can be prepared from water having an appropriate amount of NaOH dissolved therein. Alternatively, such solutions can be prepared with buffers, such as Tris·HCl, which have pKs within about 1 pH unit of the desired pH. Other suitable buffers include sodium carbonate, sodium bicarbonate, sodium hydroxide, sodium phosphate, ammonium acetate, sodium citrate,

sodium hydroxide, calcium hydroxide, sodium biphosphate, sodium phosphate, Tris (hydroxy-methyl) aminomethane, sodium benzoate, and the like.

Liposome complexes can be formed (during the hydration step, for example) by dispersing the lipid in the aqueous solution with vigorous mixing. Any method of mixing can be used provided that the chosen method induces sufficient shearing forces between the lipid film and polar solvent to strongly homogenize the mixture and form the desired complexes. For example, mixing can be by vortexing, magnetic stirring, and/or sonicating. Where multilamellar liposomes are desired, they can be formed simply by vortexing the solution. Where unilamellar liposomes are desired, a sonication or filtration step is included in the process.

Liposomal SN38 complexes can be prepared by mixing SN38, cardiolipin, cholesterol, phosphatidyl choline and α-tocopherol in a suitable solvent to form a homogeneous mixture. The mixture is dried to form a lipid film and hydrated into liposomes by the addition of water or an aqueous solution and mixing. Alternatively, SN38 liposomes can be prepared by dissolving the lipophilic ingredients (with the exception of SN38) together and evaporating them to form a lipid film. A solution of SN38 is prepared in an aqueous solution at alkaline pH then is used to hydrate the dry lipid film and form liposomes.

Alternatively, SN38 can be directly dissolved in alkaline aqueous buffer solution, such as previous described. The dissolved SN38 can be added to the liposomes that are prepared by any of the techniques now known or subsequently developed for preparing liposomes. For example, the liposomes can be formed by the conventional technique for preparing multilamellar liposomes (MLVs), that is, by depositing one or more selected lipids on the inside walls of a suitable vessel by dissolving the lipids in chloroform and then evaporating the chloroform, adding the aqueous solution which is to be encapsulated to the vessel, allowing the aqueous solution to hydrate the lipid, and swirling or vortexing the resulting lipid suspension to produce the desired liposomes.

Alternatively, techniques used for producing large unilamellar liposomes (LUVs), such as, reverse-phase evaporation, solvent dilution procedures, infusion procedures, and detergent dilution, can be used to produce the liposomes. A review of these and other methods for producing liposomes can be found in the text Liposomes, Marc J. Ostro, ed., Marcel Dekker, Inc., New York, 1983, Chapter 1.

In general, any suitable method of forming liposomes can be used so long as it generates liposome entrapped SN38. Multilamellar vesicles, stable plurilamellar vesicles, and reverse phase evaporation vesicles can be used. As can be appreciated, the present invention is intended to cover SN38-entrapped liposome compositions, however made.

Suitable liposomes can be neutral, negatively, or positively charged, the charge being a function of the charge of the liposome components and pH of the liposome solution. For example, at neutral pH, positively charged liposomes can be formed from a mixture of phosphatidyl choline, cholesterol and stearyl amine. Negatively charged liposomes can be formed, for example, from phosphatidyl choline, cholesterol, and phosphatidyl serine.

After formation of the lipid composition comprising SN38 or a compound in equilibrium with SN38, the pH of the composition is reduced so as to convert some or all of the compound in equilibrium with SN38 to SN38. Desirably, the pH of the composition is less than about 3.5 (e.g., a pH of from about 1 and 3.5, such as between about 1.5 and about 3), and preferably the pH is about 2.0. The pH can be reduced, in accordance with the inventive method, directly after the hydration stage, e.g., by adding an acidic buffer (such as those described herein), or after a step of dehydration (or drying), storage (if desired), and re-hydration (also termed "resuspension" or "reconstitution"), as described herein. Alternatively, the pH can be reduced during the re-hydration of a dried or lyophilized preparation, for example, where an acidic buffer is employed to reconstitute dried liposomes containing SN38.

Targeting agents can be bound to the SN38 complexes such that the complexes can be targeted to particular tissues or organs. The agents can be bound through covalent, electrostatic, or hydrophobic bonds with the complexes. Suitable targeting agents include carbohydrates and proteins (e.g., antibodies, antibody fragments, peptides, peptide hormones, receptor ligands, and mixtures thereof) or other agents as are known to target desired tissues or organs. For example, U.S. Patent 6,056,973, which is herein incorporated by reference, discloses a number of targeting agents and target cells. (See col. 11, l. 1-41). Methods of preparing suitable conjugates are also disclosed. (See Col. 11, l 55 – col. 14, l. 20).

SN38 complexes can be filtered through suitable filters to control their size distribution. Suitable filters include those that can be used to obtain the desired size range of liposomes from a filtrate. Accordingly, the liposomes produced are preferably treated to reduce their size and to produce a homogeneous population. This may be accomplished by conventional techniques such as extrusion through a filter, preferably of 100 to 800 nm pore size, the filter being either the straight path or tortuous path type. The filter preferably has a pore size of about 5 microns or less, and more preferably about 1 micron or less, such as about 500 nm or less, or even about 200 nm or less or 100 nm or less. Other methods of size reducing the liposomes to a homogenous size distribution are ultrasonic exposure, the French press technique, hydrodynamic shearing, homogenization using, for example, a homogenizer or microfluidization techniques. Alternatively,

filtration can occur after formulation in liquid excipients or diluents, as hereinafter described.

Thus, for example, the liposomes can have a diameter (e.g., average mean diameter) of about 5 microns or less, and more preferably, about 1 micron or less, such as about 500 nm or less, or even about 200 nm or less or 100 nm or less. It is preferred that the liposomes used in the present invention have an average mean diameter from about 20 nm to about 1000 nm and preferably of from about 100 nm to about 800 nm or from about 100 nm to about 400 nm. An average mean diameter of about 160 nm is particularly preferred.

To improve shelf-life, the present invention provides SN38 liposome preparations which can be stored for extended periods of time without substantial leakage from the liposomes of internally encapsulated materials. The present invention provides SN38 liposome preparations which can be dried or dehydrated to form a dried lipid composition, stored for extended periods of time while dehydrated, and then rehydrated when and where they are to be used, without losing a substantial portion of loaded SN38 during the dehydration, storage and rehydration processes. The drying or dehydration can be achieved either after or before the pH of the composition is reduced.

The liposomes are preferably dried or dehydrated to form a dried lipid composition using standard freeze-drying equipment or equivalent apparatus, that is, they are preferably dehydrated under reduced pressure. If desired, the liposomes and their surrounding medium can be frozen in liquid nitrogen before being dehydrated. Alternatively, the liposomes can also be dehydrated without prior freezing, by simply being placed under reduced pressure.

To achieve these and other objects, the invention, in accordance with one of its aspects, provides SN38 liposome preparations that have been dehydrated in the presence of one or more protective sugars. In certain preferred embodiments of the invention, the liposomes are dehydrated with the one or more sugars being present at both the inside and outside surfaces of the liposome membranes. In other preferred embodiments, the sugars are selected from the group consisting of trehalose, maltose, lactose, sucrose, glucose, and dextran, with the most preferred sugars from a performance point of view being trehalose and sucrose. In general, disaccharide sugars have been found to work better than monosaccharide sugars, with the disaccharide sugars trehalose and sucrose being most effective. Other more complicated sugars can also be used. For example, aminoglycosides, including streptomycin and dihydrostreptomycin, have been found to protect liposomes during dehydration. The dehydration is accomplished under vacuum and can take place either with or without prior freezing of the liposome preparation.

It has been found that inventive liposomes having a concentration gradient across their membranes can be dried or dehydrated (preferably in the presence of one or more sugars), subsequently rehydrated, and the concentration gradient then used to create a transmembrane potential which will load SN38 into the liposomes. Alternatively, the concentration gradient can be created after the liposomes have been dehydrated and rehydrated. Accordingly, the invention provides a method of loading liposomes with SN38 or a compound in equilibrium with SN38 involving preparing a preparation which includes liposomes, dehydrating the liposome preparation, rehydrating the dehydrated preparation, replacing the external medium surrounding the liposomes in the rehydrated preparation with a medium (such as an acidic buffer, suitable examples of which are discussed below), which produces an ion concentration gradient capable of generating a transmembrane potential having an orientation which will load SN38 or a compound in equilibrium with SN38 into the liposomes; and admixing SN38 or a compound in equilibrium with SN38 with the liposomes in their replaced external medium.

Dried, dehydrated, or lyophilized SN38 complex liposomes can be resuspended (i.e., reconstituted) into a suitable solution (typically an aqueous solution) by gentle swirling of the solution. The rehydration can be performed at room temperature or at other temperatures appropriate to the composition of the liposomes and their internal contents. When desired, liposomes can be dried such as by evaporation or lyophilization and the liposomes resuspended (i.e., reconstituted) in any desirable polar solvent. Where liposomes are formed as described herein by hydrating lipid films with alkaline, aqueous solvents containing SN38, it is desirable to use a low pH buffer, such as those descried herein, to resuspend (reconstitute) the dehydrated or lyophilized liposomes. Suitable solvents for resuspending (reconstituting) the liposomes include, for example, a buffered solution (typically an aqueous solution) having a pH of less than about 3.5 (e.g., a pH of from about 1 and 3.5, such as between about 1.5 and about 3), and preferably having a pH of about 2.0 (e.g., a lactate buffered solution having a pH of about 2.0). In such embodiments, the resuspension of the dehydrated lipid composition can effect the reduction of pH of the composition.

When the dehydrated or lyophilized liposomes are to be used, rehydration (or reconstitution) can be accomplished by adding an SN38 activating agent to close the lactone ring of SN38. In this sense, the SN38 and compound in equilibrium with SN38 becomes is released as active (pharmaceutically active) SN38. Accordingly, the invention provides a method of making SN38 complexes comprising formulating dehydrated or lyophilized complexes containing liposomes and SN38 or a compound in equilibrium with SN38, dissolving or resuspending the dehydrated or lyophilized complexes in an aqueous solution, and contacting the liposomes with a activating agent such that SN38 becomes

active. The activating agent can be any acidic aqueous buffers, e.g., sodium citrate, citric acid, sodium acetate, acetic acid, ascorbic acid, sodium lactate, lactic acid, sodium tartrate, tartartic acid, sodium succinate, succinic acid, aspartic acid, hydrochloric acid, meleic acid, sodium carbonate, sodium sulfate, sulfuric acid, preferably, sodium lactate, sodium acetate, and the like. In some embodiments, it can be desirable to employ a solubilizing agent to increase the solubility of SN38 during formulation, such as an alkaline buffer, examples of which are discussed herein. Also, it can be desirable for one or more pharmaceutically acceptable excipients to be employed to increase the shelf-life of the composition. Suitable excipients for enhancing shelf life include, for example, protective sugars, as disclosed herein.

The inventive liposomal compositions desirably are stable for at least about 24 hours, and more preferably, they are stable for at least about 48 hours. Most preferably, the liposomal compositions containing SN38, or other lipid complexes of the present invention, are stable for at least about 72 hours. Stability can be assessed either over the time post-formulation or over the period post-reconstitution following drying or lyophilization. In this context, the stability of a liposomal composition of the present invention over time can be assessed, for example, by assaying the change in mean particle size over a 24, 48, or 72 hour period. Typically, stability is assessed after maintaining the composition at room temperature (e.g., about 25 °C) for the desired period of time, but other suitable temperatures can be employed. Desirably, when measured at 25 °C, the mean particle size of the composition after 24, 48, or 72 hours post-formulation or post-reconstitution varies (e.g. is increased or decreased) by less than about 25% (more preferably, the size varies by less than about 20% or 15%, and most preferably by less than about 10% or less than about 5%) of that when the composition is initially formulated or reconstituted. Stability alternatively can be assessed by measuring the pH of the composition over the desired time frame. Desirably, the pH of the composition after 24, 48, or 72 hours post-formulation or post-reconstitution varies (e.g., either is increased or decreased) by at most about 0.5 pH units, and more preferably by at most about 0.4 pH units, from the pH of the composition when the composition is initially formulated or reconstituted. More, preferably, the pH of the composition after 24, 48, or 72 hours post-formulation or post-reconstitution varies by at most about 0.3 pH units from the pH of the composition when initially formulated or reconstituted, and even more preferably by at most about 0.2 pH units from the pH of the composition when initially formulated or reconstituted. Most preferably, the pH of the composition after 24, 48, or 72 hours post-formulation or post-reconstitution varies by at most about 0.1 pH unit from the pH of the composition when initially formulated or reconstituted. Another measurement of stability is the entrapment efficiency of SN38 within the composition, especially a liposomal

composition. Desirably, the entrapment efficiency of SN38 within the composition after 24, 48, or 72 hours post-formulation or post-reconstitution is at least about 80% (more preferably at least about 85%, and most preferably at least about 90% or at least about 95%) of that when the composition is initially formulated or reconstituted. Most preferably, the entrapment efficiency of SN38 within the composition measured 24, 48 or 72 hours post formulation or reconstitution does not appreciably change from that measured when the composition is first formulated or reconstituted.

The invention includes pharmaceutical preparations, which, in addition to non-toxic, inert pharmaceutically suitable excipients, contain the SN38 complex and methods for preparing such compositions. By non-toxic, inert pharmaceutically suitable excipients there are to be understood solid, semi-solid or liquid diluents, fillers and formulation auxiliaries of all kinds.

The invention also includes pharmaceutical preparations in dosage units. This means that the preparations are in the form of individual parts, for example capsules, pills, suppositories and ampoules, of which the content of the SN38 complex corresponds to a fraction or a multiple of an individual dose. The dosage units can contain, for example, 1, 2, 3 or 4 individual doses or 1/2, 1/3 or 1/4 of an individual dose. An individual dose preferably contains the amount of SN38 which is given in one administration and which usually corresponds to a whole, a half, a third, or a quarter of a daily dose.

Tablets, dragees, capsules, pills, granules, suppositories, solutions, suspensions and emulsions, pastes, ointments, gels, creams, lotions, powders and sprays can be suitable pharmaceutical preparations.

For the oral mode of administration, the SN38 complex can be used in the form of tablets, capsules, losenges, powders, syrups, aqueous solutions, suspensions, and the like. Carriers such as lactose, sodium citrate, and salts of phosphoric acid can be used to prepare tablets. Further, disintegrants such as starch, and lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc can be included. Diluents such as lactose and high molecular weight polyethylene glycols can be used in the preparation of dosages in capsule form. The active ingredient can be combined with emulsifying and suspending agents to generate aqueous suspensions for oral use. Flavoring agents such as sweeteners can be added, as desired.

For topical administration and suppositories drug complexes can be provided in the form of such gels, oils, and emulsions as are known by the addition of suitable water-soluble or water-insoluble excipients, for example polyethylene glycols, certain fats, and esters or mixtures of these substances. Suitable excipients are those in which the drug complexes are sufficiently stable to allow for therapeutic use.

The abovementioned pharmaceutical compositions are prepared for administration in the usual manner according to known methods, for example by mixing the complexed SN38 with suitable excipient(s).

The present invention also includes the use of SN38 according to the invention and of pharmaceutical preparations which contain SN38 according to the invention in human and veterinary medicine for the prevention, amelioration and/or cure of diseases, in particular those diseases caused by cellular proliferation, such as cancer, in any mammal, such as a cow, horse, pig, dog or cat. However, it is particularly preferred for use in the treatment of human patients, particularly for cancer and other diseases caused by cellular proliferation. In a preferred embodiment, the inventive method is employed to treat a disease caused by disease caused by proliferating eukaryotic cells in a patient homozygous for the wild-type UGTA1 allele or having at least one copy of a mutant UGTA1 allele (i.e., heterozugous or homozygous), such as, for example, UGTA1*28. Patients having mutations in UGTA1 can exhibit impaired capacity for glucoronidation of SN38; accordingly, employing the inventive compositions, such as the inventive liposomal formulations, can improve efficacy in such patients. The inventive compositions have particular use in treating human cancers and viral infections, in addition to multiple sclerosis. Example of cancers treatable by this invention include, but not limited to lung cancer (such as non-small cell lung cancer); breast cancer; testicular cancer; ovarian cancer; gastro intestinal cancers including colon, rectal, pancreatic, and gastric cancers, hepatocellular carcinoma; head and neck cancers; prostate cancer; renal cell carcinoma; adenocarcinoma; sarcomas; lymphomas; leukemias; and mycosis fugoides; melanoma; high grade glioma, glioblastoma and brain cancers.

The inventive complxes including SN38 (or a compound in equilibrium with SN38) also can be employed to treat viral infections within a patient. In this regard, the invention provides a method of treating viral infections comprising administering to a patient having a viral infection composition comprising a complex comprising SN38 or a chemical in equilibrium with SN38 and a lipid to the patient in an amount sufficient to treat the viral infection within the patient. The application of the inventive method can treat a viral infection by mediating the symptoms of the infection or, in some patients, by killing infected cells or decreasing the viral load within said patient. The method can be employed to treat infections by many viruses, such as adenoviruses, herpes viruses, papillomaviruses, pox viruses, SARS viruses, and immunodeficiency viruses. A preferred viral infection that can be treated in accordance with the inventive method include immunodeficiency viruses, such as SIV, FIV, and, most preferably, HIV.

The active compound or its pharmaceutical preparations can be administered dermally, orally, parenterally, intraperitoneally, intravenously, rectally, or directly to a

tumor (e.g., via intratumoral injection). As SN38 does not require activation by the liver, it is advantageous to employ the present compositions locally, such as by directed injection into an arm or leg, or in the case of a human, a hand or a brain.

In a human of about 70 kg body weight, for example, about 0.1 to 2 mg or about 0.5 to 1 mg SN38 can be administered per kg of body weight can be administered. Preferably, about 0.5 to 2.0 mg of SN38 per kg of body weight is administered. Dosing also can be calculated per body surface area, and, for human patients, it is preferred to administer the inventive composition in amounts of from about 2 mg/m^2 to about 150 mg/m^2 or to deliver a dose of SN38 of such amounts. More preferably, between about 2 or about 2.5 mg/m^2 and about 125 mg/m^2 of the composition, such as between about 2.5 mg/m^2 and about 30 mg/m^2 (e.g., about 2.5 mg/m^2, about 5 mg/m^2, about 10 mg/m^2, about 20 mg/m^2, or about 25 mg/m^2), is administered to a patient, or an amount of the composition is administered to deliver such dosage of SN38 to the patient. Also, dosing of about 30 mg/m^2, about 40 mg/m^2, about 50 mg/m^2, about 60 mg/m^2, about 70 mg/m^2, about 80 mg/m^2, about 90 mg/m^2, or about 100 mg/m^2 also is suitable. However, it can be necessary to deviate from the dosages mentioned and in particular to do so as a function of the nature and body weight of the subject to be treated, the nature and the severity of the illness, the nature of the preparation and if the administration of the medicine or other chemotherapeutic agent, and the time or interval over which the administration takes place. Thus it can suffice in some cases to manage with less that the abovementioned amount of active compound whilst in other cases the abovementioned amount of active compound must be exceeded.

For human patients, a preferred dosing regimen involves administration of the composition over a period of from about 30 or about 180 minutes, such as between about 60 and about 120 minutes, and more preferably for a period of about 90 minutes. Other dosing regimens and the type of administration of the SN38 can be determined by one skilled in the art, by available methods. Suitable amounts are therapeutically effective amounts that do not have excessive toxicity, as determined in empirical studies.

A significant advantage of cardiolipin-containing compositions is that they provide a method of modulating multidrug resistance in cancer cells which are subjected to SN38. In particular, the present compositions reduce the tendency of cancer cells subjected to chemotherapy with SN38 to develop resistance thereto, and reduces the tendency of cancer cells to develop resistance to other therapeutic agents, such as taxol or doxorubicin. Thus, other agents (e.g., secondary therapeutic agents) other than the SN38 complexes (such as the liposomal SN38 compositions) can be advantageously employed with the present treatment in combination with the SN38 complexes. Suitable adjunctive secondary therapeutic agents include, for example, antineoplastic agents (such as cisplatin, taxol,

doxorubicin, vinca alkaloids, and temozolomide), antifungal agents, antibiotic agents, antiviral agents, antimetabolites, imunomodelators, and other secondary active agents. Preferred secondary agents include, for example, Gonadotropin release hormone, antiestrogens, antiandrogens, cisplatin, carboplatin, oxaliplatin, antisense oligonucleotides, paclitaxel, docetexl, vinca alkaloids, such as vincristin, vinblastine, vindestine and vinorelbine, doxorubincine, daunorubicin, epirubicin, mitoxantrone, cytarabine, temozolomide, leuprolide, cyclophosphamide, etoposide, and Tamoxifen, among other secondary agents.

Having described the present invention, reference will now be made to certain examples which are provided solely for purposes of illustration and which are not intended to be limiting.

EXAMPLE 1

SN38 (3 μmoles) can be dissolved in chloroform containing 3 μmoles cardiolipin. To this mixture, 14 μmoles of phosphatidyl choline dissolved in hexane and 10 μmoles cholesterol in chloroform can be added. The mixture can be stirred gently and the solvents can be evaporated under vacuum at below 30° C to form a thin dry film of lipid and drug. Liposomes can then be formed by adding 2.5 ml of saline solution and aggressively mixing the components by vortexing. The flasks can then be vortexed to provide multilamellar liposomes and optionally sonicated in a sonicator to provide small unilamellar liposomes. The efficiency of SN38 encapsulation can be determined by dialyzing an aliquot of the subject liposomes overnight in a suitable aqueous solvent or centrifuging an aliquot of the subject liposomes at 200,000 x g. for 2 hour at 4°C. Thereafter the liposome fraction is dissolved in methanol and analyzed by standard methods using high pressure liquid chromatography (HPLC), such as reverse phase HPLC. Generally the encapsulation efficiency of SN38 in liposomes will be between 80 to 95 % of the initial input dose.

EXAMPLE 2

Similar experimental conditions can be utilized with varying quantities of drug and lipid. For example, concentrations of 6 μM SN38, 6 μM cardiolipin, 28 μM phosphatidyl choline and 20 μM cholesterol can be used by dissolving them in a suitable solvent, evaporating the solvent, and dispersing the dried lipid/drug film in a suitable aqueous solvent such as 5 ml of 7% trehalose-saline solution. Hydration of the liposomes can be facilitated by vortexing and/or sonicating the mixture. The liposomes can then be dialyzed, as desired, and the percent encapsulation of SN38 in liposomes measured, as

described above. Typically, SN38 encapsulation will be greater than about 75% and more generally between about 85 to 95% or more as assayed by HPLC.

EXAMPLE 3

SN38 can be encapsulated in liposomes by using 3 μM of the drug, 15 μM of dipalmitoyl phosphatidyl choline, 1 μM cardiolipin, and 9 μM cholesterol in a volume of 2.5 ml. The drug and lipid mixture can be evaporated under vacuum and resuspended in an equal volume of saline solution. The remainder of the process can be similar to that described above. The SN38 encapsulation efficiency will generally be higher than 75% in this system.

EXAMPLE 4

In this example, liposomes containing 2 μM SN38, 2 μM of phosphatidyl serine, 11 μM phosphatidyl choline, 2 μM cardiolipin, and 7 μM cholesterol prepared by the method described in Example 1 is contemplated with greater than 75% SN38 encapsulation efficiency.

EXAMPLE 5

In this example liposomes containing over 2 mg/ml SN38 in solution are demonstrated.

A lipid film is prepared by adding about 0.2 g of D-α-tocopherol acid succinate to about 1 kg of t-butyl alcohol which is warmed to about 35-40° C. The solution is mixed for about 5 min until the tocopherol is dissolved. About 6.0 g of tetramyristoyl cardiolipin is added to the solution and the solution is mixed for about 5 minutes. About 10 g of cholesterol is added to the solution and the solution is mixed for about 5 more minutes then about 30 g of egg phosphatidyl choline is added and mixed for another 5 min. Approximately 11 grams of the resulting lipid solution is lyophilized to generate a lipid film.

To prepare liposomal SN38, a 4 mg/ml solution of SN38 is prepared by dissolving the drug in an aqueous alkaline solution having a pH of between 8 and 10. Approximately 15 ml of this SN38 solution is added to a vial containing the lipid film. The vial is swirled gently, allowed to hydrate at room temperature for 30 min, vortexed vigorously for 2 min, and sonicated for 10 min in a bath-type sonicator at maximum intensity. The pH of the liposome solution is reduced to acid pH. Using this method more than 90 wt.% of the SN38 is complexed with lipid in the form of liposomes.

EXAMPLE 6

Lipids, DOPC, cholesterol and cardiolipin at the appropriate ratios and tocopheryl acid succinate were dissolved in dichloromethane and subsequently dried under vacuum. The dried lipid film was rehydrated in the SN38 solution in 10% sucrose in 0.1N NaOH (pH>9). The lipid dispersion was extruded under nitrogen through 0.2 μM and 0.1 μM polycarbonate filters and then lyophilized to yield the LE-SN38 cake. The lyophilized cake was hydrated with 10 mM lactate buffer (pH 2.0) in order to convert the SN38 (open-lactone ring, inactive form) to the active form of the drug and allow its migration into the lipid bilayer. Analysis of the batch of reconstituted LE-SN38 showed 99.8% drug entrapment by ultracentrifugation and HPLC methods, stable entrapment upon dilution in normal saline and a mean vesicle size of 150 nm.

EXAMPLE 7

Lipids were dissolved in ethanol. The lipid alcohol mixture was then dispersed in SN38/sucrose solution pH at 8-10. The bulk liposomal SN38 was then extruded through 0.2μM and 0.1μM polycarbonate filters. Following size-reduction, the product was then heated to 40°C under vacuum to evaporate the organic solvent and then sterile filtered through 0.22 μM filters and lyophilized. The drug entrapment efficiency was >95% assay by HPLC method.

EXAMPLE 8

A study was conducted to monitor the physical and chemical stability of LE-SN38 for up to 72 hours post-reconstitution. The objective of this study was to determine SN38 entrapment efficiency, SN38 concentration, liposome particle size, as well as pH at 25°C over 72 hours post-reconstitution.

Table 1 - Stability data for reconstituted liposomal SN38 at 25°C

Time/ Storage Condition		SN38 % of initial	Entrapment Efficiency (%)	Mean Particle Size (nm)	pH
Initial 0 hr	25°C	100	>95	258.8	2.60
8 hr	25°C	101	>95	224.2	2.60
24 hr	25°C	100	>95	226.0	2.59
48 hr	25°C	101	>95	226.8	2.52
72 hr	25°C	102	>95	250.6	2.49

As seen in table 1, there does not appear to be any change in SN38 concentration at 25°C condition over the course of the stability study as the % of initial SN38 concentration is found to be essentially 100% at all time points. In addition, the percent SN38 entrapment remains greater than 95% throughout the 72-hour study at 25°C. The

mean vesicle diameter of reconstituted LE-SN38 at the initial time point is observed to be 258.8 nm. No drastic changes in particle size and pH were observed over 72 hour post-reconstitution. These results demonstrate that the inventive composition is stable over at least about 72 hours.

EXAMPLE 9

A study was conducted to monitor the physical and chemical stability of LE-SN38 for up to 24 hours post-reconstitution and dilution in normal saline. The objective of this study was to determine particle size, SN38 entrapment efficiency, SN38 concentration, as well as pH at 25°C as a function of time.

Table 2

Stability data for 8-fold diluted reconstituted liposomal SN38 at 25°C.

Time/ Storage Condition		SN38 % of initial	SN38 Entrapment (%)	Mean Vesicle Size (nm)	pH
0 hr	25°C	100	>95%	173.5	2.98
8 hr	25°C	99.2	>95%	183.0	2.95
24 hr	25°C	98.7	>95%	193.4	2.96

As seen in table 2, there does not appear to be any significant change in SN38 concentration over the course of the stability study at 25°C. The percent SN38 entrapment remains greater than 95% throughout the 24-hour study at 25°C. No drastic changes in particle size and pH were observed over 24 hour study at 25°C. These results demonstrate that the inventive composition is stable over at least about 24 hours.

EXAMPLE 10

A long-term stability study was conducted to monitor the physical and chemical stability of lyophilized LE-SN38 for up to 12 months. The objective of this study was to determine the visual appearance, particle size, SN38 entrapment efficiency, SN38 concentration, as well as pH at 25°C as a function of time.

Long-term stability data for lyophilized LE-SN38

Table 3 Lot# 1

Time/ Storage Condition		SN38 % of initial	pH	Appearance	Entrapment Efficiency (%)	Mean Particle Size (nm)
Initial	2-8°C	100	2.89	Off-white	>95%	177.4
3 month	2-8°C	102	2.51	Off-white	>95%	180.8
9 month	2-8°C	99.1	2.50	Off-white	>95%	186.8
12 month	2-8°C	102	2.54	Off-white	>95%	181.9

Table 4 Lot# 2

Time/ Storage Condition		SN38 % of initial	pH	Appearance	Entrapment Efficiency (%)
Initial	2-8°C	100	2.73	Off-white	>95%
3 month	2-8°C	100	2.45	Off-white	>95%
10 month	2-8°C	97.0	2.46	Off-white	>95%
12 month	2-8°C	99.6	2.50	Off-white	>95%

As seen in tables 3 and 4, the lyophilized LE-SN38 is stable up to 12 months. There are no significant changes in SN38 concentration, pH, drug entrapment and particle size up to 12 months.

EXAMPLE 11

IN-VITRO CYTOTOXICITY STUDY OF LE-SN38

In vitro cytotoxicity of liposomal SN38 (LE-SN38) and CPT-11 in cancer cell lines was determined using Sulforhodamine B (SRB) assay (Monks, *J Natl Cancer Inst*, 83, 757-766 (1991)). A total of 8 cancer cell lines, including human colon cancer (HT29), human lung cancer (A549), human breast cancer (MX-1), human ovarian cancer (OVCAR-3), human pancreatic cancer (Capan-1), mouse Leukemia (P388), mouse adriamycin resistant leukemia (P388/ADR) and Lewis lung carcinoma (LLC), were included in this study. The GI_{50} value was calculated as the concentration of LE-SN38 or CPT-11 that gives 50% growth inhibition.

Study showed that all eight cell lines studied were sensitive to LE-SN38 with GI_{50} less than 0.1 μM. These results are comparable to previously reported data of free SN38 dissolved in DMSO (Lavelle et al., *Semin Oncol*, 23;1 Suppl 3, 11-20 (1996)); Cavaletti et al., *Toxicol Lett*, 118, 103-107 (2000)) indicating SN38 was released from the liposomes during the period of incubation of LE-SN38 in cell cultures and inhibited cell growth. Results showed that LE-SN38 was approximately 200 to 2000 fold more cytotoxic than CPT-11 against all tumor cell lines.

EXAMPLE 12

MULTIPLE DOSE TOXICITY STUDY OF LE-SN38 IN CD2F1 MICE

CD2F1 mice (Male and Female) were obtained through Harlen Sprague Dawley Laboratories (Indianapolis, IN). The average weight of mice on day 1 of study was 16-22 g for females and 20-27 g for males, and the age was 6-7 weeks. Mice were pre-weighed individually prior to experiment. On days 1-5, animals were injected intravenously via tail vein with LE-SN38 or placebo liposomes at 5, 7.5 and 10 mg/kg dose levels. All animals were observed once daily during the study periods for mortality and clinical signs. Animals showing toxicity as manifested by clinical signs and body weight loss of 25% or more were considered as moribund and euthanized immediately.

The results of the multiple-dose toxicity study of LE-SN38 in CD2F1 mice indicated that the average weight loss ranged from 5.2% for 5 and 7.5 mg/kg dose groups (5 and 7.5 mg/kg X 5 days) and 15.7% for 10 mg/kg dose group (10 mg/kg X 5 days). However, the weight lost was recovered by day 17 post treatment for all LE-SN38 treatment groups. Animals in all groups were acting normal on day 1- 5 post injection of LE-SN38. On day 6-12, animals treated with 5 and 7.5 mg/kg for 5 days were also normal, whereas animals treated with 10 mg/kg for 5 days showed clinical symptoms manifested by hunched posture, rough coat, dehydration and decreased activity. However, on day 14-18 post injection, all animals from all groups recovered. In general, LE-SN38 was well tolerated in mice at all dose levels studied. This could be attributed to the use of non-toxic lipids to form liposomes that buffered the toxicity of SN38. The retention of the drug in the liposomes reduced the tendency of SN38 molecules to directly interact with normal cells, therefore, attenuating the overall toxicity related to free SN38.

EXAMPLE 13

ACUTE DOSE AND MULTIPLE DOSE TOXICITY OF LE-SN38 IN CD2F1 MICE: 30 DAY SURVIVAL

CD2F1 mice (5-8 weeks of age), were obtained from Harlan Sprague Dawley Laboratories (Indianapolis, IN). Animals were housed in cages in temperature and humidity controlled room with 12h light/dark cycles in animal care facility. Mice were offered ad libitum 8656 HT Rodent Diet (Harlan Teklad, Madison, WI). For acute dose toxicity study, LE-SN38 was intravenously (IV) administered to mice (two injections via tail vein/Day within 1hour apart) at doses of 23, 28, 37, 46 and 65 mg/kg. For multiple dose toxicity study, mice were administered LE-SN38 (IV x 5 days, once daily) at doses of 5.0, 7.5 and 10 mg/kg. The animals were observed for clinical signs of toxicity, mortality and body weight changes for up to 30 days.

The acute dose toxicity study suggested 37 and 46 mg/kg maximum tolerated dose (MTD) of LE-SN38 for male and female mice respectively. The MTD of LE-SN38 in a multiple dose toxicity study was found to be 5 and 7.5 mg/kg for male and female mice respectively. No significant loss of body weight was observed in mice at tolerated doses. In addition, no difference in hematological parameters were observed between control and drug treated groups. The results of these experiments are presented in tables 5 and 6.

Table 5

Acute Dose Toxicity of LE-SN38 in CD2F1 Mice: 30 day Survival

Dose of LE-SN38 (mg/kg)	Number of Mice Surviving/Total on Day 30	
	Female	Male
0	5/5	5/5
23	5/5	5/5
28	N/A*	5/5
37	N/A	5/5
46	5/5	0/5
65	4/5	0/5

CD2F1 mice were intravenously administered LE-SN38 (two injections via tail vein/day in 1hour apart). For 0 mg/dose, empty liposomes with a lipid amount of the highest dose group was used. * N/A, not available.

Table 6

Multiple Dose Toxicity of LE-SN38 in CD2F1 Mice: 30 day Survival

Dose of LE-SN38 (mg/kg, once daily x 5 days)	Number of Mice Surviving/Total on Day 30	
	Female	Male
0	10/10	10/10
5	10/10	10/10
7.5	10/10	9/10
10	9/10	7/10

CD2F1 mice were administered LE-SN38 (iv, once daily x 5 days) with doses of 5.0, 7.5 and 10 mg/kg LE-SN38. For 0 mg/dose, empty liposome with a lipid of highest dose group was used.

EXAMPLE 14

THERAPEUTIC EFFICACY OF LE-SN38 AND CPT-11 IN XENOGRAFT MOUSE TUMOR MODELS

Either female CD2F1 (6-8 weeks old) mice or female C.B-17 SCID mice (4-6 weeks old) were obtained from the vendor and maintained as described previously. The

CD2F1 mice were transplanted with P388 murine leukemia tumor cells, whereas the SCID mice were transplanted with HT-29 human colon cancer cells, Capan-1 human pancreatic cancer cells and MX-1 human breast cancer cells. After a suitable waiting period (waiting period varied based on the tumor models), each mouse received intravenous injection via tail vein of placebo liposomes, LE-SN38 or CPT-11 at pre-determined dose levels. For P388 bearing mice, the long term survival for each treatment group was assessed, whereas for solid tumor bearing mice, the tumor growth inhibition of placebo liposomes, LE-SN38 or CPT-11 at different dose levels was measured after 28 day post treatment.

Table 7 summarizes the therapeutic efficacy of LE-SN38 and CPT-11 against different tumors in mice. For the P388 tumor bearing mice administered with CPT-11 at doses of 4, 8 and 16 mg/kg for 5 consecutive days, the median survival time was 16, 20 and 22 days, respectively with no long-term survival. About 22% long-term survival (60 days) was observed for the mice administered with 16 mg/kg CPT-11. In contrast, when the mice were given LE-SN38 at doses of 2.76 mg/kg and 5.52 mg/kg for 5 consecutive days, 60% and 100% long-term survival (60 days) were observed at the respective dose level. There were no clinical signs of toxicity, such as diarrhea, hunched posture, scruffy fur and alopecia or weight loss at these dose levels of LE-SN38. Evidently, LE-SN38 exhibited significantly greater therapeutic efficacy against P388 murine leukemia tumor than the prodrug CPT-11.

When LE-SN38 was given to the mice bearing HT-29 human colon tumor at dose 2, 4 and 8 mg/kg, LE-SN38 inhibited human colon cancer growth by 46, 70 and 88%, respectively at 28 days post treatment. However, when the mice were treated with CPT-11 at the same dose levels, only 36% inhibition was observed at the highest dose level (8 mg/kg). At 2 and 4 mg/kg dose levels, CPT-11 did not show any inhibition against tumor growth. Clearly, LE-SN38 exhibited much greater inhibition against HT-29 induced tumor in mice than the prodrug CPT-11 at the same dose level.

Additionally, LE-SN38 exhibited greater growth inhibition against Capan-1 human pancreatic tumor growth in the animal groups treated with LE-SN38 than those treated with CPT-11 (Table 7). It was demonstrated that the antitumor efficacy of LE- SN38 against human pancreatic tumor in SCID ectopic model was superior to CPT-11. Moreover, it was also found that LE-SN38 induced a dose-dependent tumor regression of MX-1 human breast solid tumor in SCID mice. When the mice were treated with LE-SN38 at 4 and 8 mg/kg dose levels, the tumor regressed by 43.9% and 87.8% respectively. However, when the mice were given CPT-11 at 8 mg/kg dose level, no significant reduction of tumor size was observed.

It is known that intravenous administration of liposomes will lead to their accumulation in extravascular sites that exhibit leaky vasculature, as in the case for the

tumor site. The extent of this accumulation could lead to an increase in tissue specific delivery of SN38 corresponding to several orders of magnitude greater than its precursor, CPT-11. This passive delivery of drug to sites of therapeutic activity may be accounted for the better efficacy of SN38 versus CPT-11. Liposomes also protect SN38 from structural transformation and/or chemical degradation. This protection of the active molecule could also have led to a significant increase in bioavailability, which ultimately enhanced the drug potency and efficacy. In summary, the antitumor efficacy of LE-SN38 was much greater than that of CPT-11 at the same dose levels.

Table 7. The Results of Multiple-Dose Therapeutic Efficacy Studies of LE-SN38 in Comparison with CPT-11

Treatment	Tumor Models			
	P388 Murine Leukemia	HT-29 Human Colon	Capan-1 Human Pancreatic	MX-1 Human Breast
	60-day % Survival (n=10)	% Growth Inhibition [a] (n=5)	% Growth Inhibition [a] (n=5)	% Growth Inhibition [a] (n=5)
Placebo liposomes	0	0	0	0
CPT-11				
2 mg/kg X 5	n/t	0	n/t	n/t
4 mg/kg X 5	0	0	n/t	n/t
8 mg/kg X 5	0	36	47.8	0
16 mg/kg X 5	22	n/t	68.7	n/t
LE-SN38				
2 mg/kg X 5	60 (2.78 mg/kg x5)	46	n/t	n/t
4 mg/kg X 5	100 (5.52 mg/kg x5)	70	60.4	43.9
8 mg/kg X 5	n/t	88	97.8	87.8
12 mg/kg X 5	n/t	n/t	98.0	n/t

n/t: not tested

[a] % Growth Inhibition is defined as the percentage of final tumor volume as compared to the initial tumor volume. It is calculated using the following formula: $\%\ Growth\ \ Inhibition = \frac{V_t}{V_{initial}} \times 100$ The drug treatment was initiated when the tumor reached to a size of 65-120 mm^3. The final tumor was measured on day 28 post treatment. The number of mice used in each treatment for the studies ranged from 5-10.

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EXAMPLE 15

SN38 liposome complexes were prepared using the following procedure: the lipids were mixed with cardiolipin. The mixed powdered lipids were dissolved in chloroform in a round bottomed flask. The clear solution was placed on a Buchi rotary evaporator at 30 °C. for 30 min to make a thin film. The flask containing the thin lipid film was dried under vacuum for 30 min. The film was hydrated in SN38 alkaline solution containing sucrose. The hydrated lipid film was rotated in a 50 °C. The mixture in the flask was votexed and mixed. The mixture was sequentially extruded through decreasing size filters: 800 nm, 400 nm, 200 nm, and 100 nm. The SN38 liposome complexes were then lyophilized under vacuum. The resulting dehydrated complexes can be stored at 2-8 °C for at least 12 months. Prior to administration, the SN38 can be activated by adding acidic buffer.

EXAMPLE 16

This example demonstrates the use of the inventive liposomal SN38 (LE-SN38) formulations in the treatment of patients with advanced cancer.

A study was conducted to assess the maximum tolerated dose and dose limiting toxicity of liposomal SN38, to determine the pharmacokinetics of SN38 after administration of LE-SN38, and to observe antitumor effects of LE-SN38.

The LE-SN38 was prepared by reconstitution with 5mL of 10mM lactate buffer and was stable for up to 8 hours refrigerated at 2-8 °C or at room temperature, 20-25 °C. After dilution with normal saline, LE-SN38 was administered intravenously over 90 minutes on day 1 of a 21 day cycle. The first cycle consisted of a pre-dose, 15 & 45 min after infusion start, end-of-infusion; and a post-infusion at 5, 15 & 30 min; 1, 2, 3, 4, 6, 8, 12 & 24 h; 2, 4, 7, 14 & 21 days.

Patients involved in the study were individuals with advanced solid tumors who had failed conventional therapy. These consisted of three strata according to genotype:

Stratum A: Patients with UGT1A1 wt allele (homozygous)

Stratum B: Patients with UGT1A1*28 allele (heterozygous)

Stratum C: Patients with UGT1A1*28 allele (homozygous)

Dosages of 2.5 mg/m^2, 5 mg/m^2, and 10 mg/m^2 and 20 mg/m^2 were employed in this study. A dose level LE-SN38 had to be tolerated by Stratum A patients before enrollment began at that dose level for Stratum B patients, and a dose level LE-SN38 had to be tolerated by Stratum B patients before enrollment began at that dose level for Stratum C patients. There were between 3 and 6 patients/cohort/strata. Total plasma SN38 concentration and plasma SN38-glucoronide concentration were assessed for each patient.

From this study, it was observed that LE-SN38 was well tolerated when given up to 20 mg/m^2 in Stratum A patients, and LE-SN38 was well tolerated at lower doses when given in the first cohorts to Stratum B Patients. From the study, it was observed that the pharmacokinetics of SN38 is proportional to dose from 2.5 to 10 mg/m^2. Also, no difference was observed in the pharmacokinetics of SN38 of Strata A and Strata B cancer patients given the 2.5 mg/m^2 LE-SN38.

Table 8 presents data concerning the pharmacokinetic parameters of SN38 after IV Infusion of LE-SN38 at 2.5, 5 and 10 mg/m^2 to patients with advanced cancer in Strata A. Table 9 presents data concerning the pharmacokinetic parameters of SN38 after IV Infusion of LE-SN38 at 2.5 mg/m^2 to patients with advanced cancer in Strata B. Table 10 presents data concerning the mean maximum plasma concentration of SN38 (C_{max}) and area under the curve (AUC_{0-inf}) after LE-SN38 administration. Numbers reported for CPT-11 are drawn from published sources. Figure 1 graphically presents values calculated for the mean (SD) plasma concentrations of SN38 after infusion of LE-SN38 at 2.5, 5 and 10 mg/m^2 to patients with advanced cancer in Strata A. Figure 2 graphically presents data concerning the plasma concentrations of SN38 following infusion of LE-SN38 at 2.5 mg/m^2 to advanced cancer patients in Strata A and Strata B through the 4-day timepoint.

Table 8

Dose mg/m^2	No. of Patients	T_{max} h	C_{max} ng/mL	$T_{1/2}$ h	Cl mL/min	AUC_{0-inf} ng*h/mL	Vss L
2.5	3	1 (0.43)	68.0 (45.9)	12.2 (8.19)	790 (480.6)	143 (87.6)	174 (50.1)
5	6	1.06 (0.55)	95.9 (33.9)	13.3 (10.9)	775 (311)	220 (104)	178 (85.7)
10	3	1.25 (0.43)	177 (63.9)	18.9 (14.7)	753 (128)	396 (107)	429 (495)

Table 9

Dose mg/m^2	**No. of Patients**	**T_{max} h** (SD)	**C_{max} ng/mL** (SD)	**$T_{1/2}$ h**	**Cl mL/ min** (SD)	**AUC_{0-inf} ng*h/mL** (SD)	**Vss L** (SD)
2.5	4	1.02 (0.64)	40.6 (19.7)	7.59 (3.20)	869 (328)	90.8 (26.9)	205 (144)

Table 10

Clinical Study (source)	Dose of CPT-11 or LESN38 (mg/m^2)	SN38 C_{max} ng/mL (SD)	SN38 AUC_{0-inf} ng*hr/mL (SD)
DM111 (Camptosar SBA)	50	21.0 (8.84)	173 (92)
DM111 (Camptosar SBA)	100	33.5 (13.3)	581 (473)
DM111 (Camptosar SBA)	165	49.0 (17.6)	667 (484)
DM111 (Camptosar SBA)	250	72.3 (40.9)	876 (672)
DM111 (Camptosar SBA)	350	139	1120
M6475/0027 (Camptosar SBA)	125	39.3 (4.7)	450 (192)
M6475/0001 (Camptosar SBA)	125	34.4 (15.0)	459 (218)
LE-SN38-101 (NeoPharm, Inc.)	10	177 (63.9)	396 (107)

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were

individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

WHAT IS CLAIMED IS:

1. A method of treating viral infections comprising administering to a patient having a viral infection composition comprising a complex comprising SN38 or a chemical in equilibrium with SN38 and a lipid to the patient in an amount sufficient to treat the viral infection within the patient.

2. The method of claim1, wherein the method reduces the viral load within said patient.

3. The method of claim 1, wherein the viral infection is an infection with a virus selected from the group of viruses consisting of adenoviruses, herpes viruses, papillomaviruses, pox viruses, SARS viruses, and immunodeficiency viruses.

4. The method of claim 3, wherein the virus is HIV.

5. A method of treating pancreatic cancer in a patient comprising administering to a patient having pancreatic cancer a complex comprising SN38 or a chemical in equilibrium with SN38 and a lipid to the patient in an amount sufficient to treat the viral infection within the patient.

6. A method of treating a disease caused by proliferating eukaryotic cells in a patient homozygous for the wild-type UGTA1 allele or having at least one copy of the UGTA1*28 allele comprising administering to a patient having a disease caused by proliferating eukaryotic cells a complex comprising SN38 or a chemical in equilibrium with SN38 and a lipid to the patient in an amount sufficient to treat the a disease caused by proliferating eukaryotic cells within the patient, wherein the patient has at least one copy of the UGTA1 allele.

7. The method of claim 6, wherein the disease is cancer.

8. The method of claim 7, wherein the cancer is lung cancer; breast cancer; testicular cancer; ovarian cancer; gastro intestinal cancer including colon, rectal, pancreatic, and gastric cancers, hepatocellular carcinoma; head and neck cancers; prostate cancer; renal cell carcinoma; adenocarcinoma; sarcoma; lymphoma; leukemias and mycosis fugoides; melanoma; high grade glioma, glioblastoma or brain cancers.

9. The method of any of claims 1-8, wherein the host is human.

10. A method of administering a composition comprising a compound selected from SN38 or a chemical in equilibrium with SN38 and a lipid to a human patient, wherein the composition is administered over a period of from about 30 to about 180 minutes.

11. The method of claim 10, wherein the period of infusion is from about 60 to about 120 minutes.

12. The method of claim 10, wherein the period of infusion is about 90 minutes.

13. The method of any of claims 5-12, wherein the medicament is administered directly to a tumor.

14. The method of any of claims 1-12, wherein the medicament is administered dermally.

15. The method of any of claims 1-12, wherein the medicament is administered intravenously.

16. The method of any of claims 1-15, wherein the composition is administered to deliver a dosage of SN38 of from about 0.1 mg/kg to about 2 mg/kg.

17. The method of claim 16, wherein the composition is administered to deliver a dosage of SN38 of about 0.5 mg/kg to about 1 mg/kg.

18. The method of claim 16, wherein the composition is administered to deliver a dosage of SN38 of about 0.5 mg/kg to about 2 mg/kg.

19. The method of any of claims 1-15, wherein the composition is administered to deliver a dosage of SN38 of from about 2 mg/m^2 to about 125 mg/m^2.

20. The method of claim 19, wherein the composition is administered to deliver a dosage of SN38 of from about 2.5 mg/m^2 to about 30 mg/m^2.

21. The method of claim 20, wherein the composition is administered to deliver a dosage of SN-30 of about 2.5 mg/m^2, about 5 mg/m^2, about 10 mg/m^2, about 20 mg/m^2, or about 30 mg/m^2.

22. The method of any of claims 1-21, wherein the composition comprises liposomes.

23. A liposomal composition comprising a compound selected from SN38 or a chemical in equilibrium with SN38, liposomes comprising a lipid, and an acidic buffer, wherein said composition has a pH less than about 3.5.

24. The composition of claim 23, wherein said composition has a pH between about 1.5 and about 3.

25. A liposomal composition comprising a compound selected from SN38 or a chemical in equilibrium with SN38, liposomes comprising a lipid, and an aqueous solution, wherein said composition is stable for about 72 hours.

26. The composition of any of claims 23-25, wherein at least about 80% of the compound is entrapped with the liposomes.

27. The composition of any of claims 23-25, wherein at least about 85% of the compound is entrapped with the liposomes.

28. The composition of any of claims 23-25, wherein at least about 90% of the compound is entrapped with the liposomes.

29. The composition of any of claims 23-25, wherein at least about 95% of the compound is entrapped with the liposomes

30. The composition of any of claims 23-29, wherein the compound is SN38.

31. The composition of any of claims 23-30, wherein the lipid comprises cardiolipin.

32. The composition of claim 31, wherein the cardiolipin is selected from the group consisting of natural cardiolipin, synthetic cardiolipin, and chemically modified cardiolipin.

33. The composition of any of claims 23-32, wherein the lipid comprises at least one of the lipids selected from the group of lipids consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, phosphatidylinositol, sphingomyelin, sterol, tocopherol, fatty acid, cardiolipin, ganglioside GM1 and polymer modified lipids, such as PEG modified lipids, and mixtures thereof.

34. The composition of any of claims 23-32, wherein the lipid comprises a phosphatidylcholine, a sterol, and a tocopherol.

35. The composition of any of claims 23-32, wherein the lipid comprises a phosphatidylglycerol selected from the group consisting of dimyristoylphosphatidylglycerol, dioleoylphosphatidylglycerol, distearoylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, diarachidonoylphosphatidylglycerol, or mixtures thereof.

36. The composition of any of claims 23-32, wherein the lipid comprises a phosphatidylcholine selected from the group consisting of dimyristoylphosphatidylcholine, distearoylphosphatidyl choline, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, diarachidonoyl phosphatidylcholine, egg phosphatidylcholine, soy phosphatidylcholine, hydrogenated soy phosphatidylcholine, and mixtures thereof.

37. The composition of any of claims 23-32, wherein the lipid comprises a sterol selected from the group consisting of cholesterol, polyethylene glycol derivatives of cholesterol, coprostanol, cholestanol, cholestane, cholesterol hemisuccinate, cholesterol sulfate, and mixtures thereof.

38. The composition of any of claims 23-37, wherein about 80 wt.% or more of the compound is in a complex with a portion of the lipid.

39. The composition of any of claims 23-37, wherein about 90 wt.% or more of the compound is in a complex with a portion of the lipid.

40. The composition of any of claims 23-39, wherein the concentration of the compound in the composition is about 0.1 or more to about 20 mg/ml.

41. The composition of any of claims 23-39, wherein the concentration of the compound is about 0.01 or more to about 5 mg/ml.

42. The composition of any of claims 23-39, wherein the concentration of the compound is about 0.1 or more to about 4 mg/ml.

43. The composition of any of claims 23-39, wherein the concentration of the compound is about 0.5 or more to about 3 mg/ml.

44. The composition of any of claims 23-39, wherein the concentration of the compound is about 0.8 or more to about 2 mg/ml.

45. The composition of any of claims 23-39, wherein the concentration of the compound is about 1 or more to about 1.5 mg/ml.

46. The composition of any of claims 23-45, wherein the complex or the liposomes have a diameter of about 1 micron or less.

47. The composition of any of claims 23-45, wherein the complex or the liposomes have a diameter of about 500 nm or less.

48. The composition of any of claims 23-45, wherein the complex or the liposomes have a diameter of about 200 nm or less.

49. The composition of any of claims 23-45, wherein the complex or the liposomes have a diameter of about 100 nm or less.

50. The composition of any of claims 23-49, further including a pharmaceutically acceptably excipient.

51. The composition of any of claims 23-50, further including a targeting agent.

52. The composition of claim 51, wherein the targeting agent is a protein.

53. The composition of claim 52, wherein the protein is selected from the group of proteins consisting of antibodies, antibody fragments, peptides, peptide hormones, receptor ligands, and mixtures thereof.

54. The composition of claim 51, wherein the targeting agent is a carbohydrate.

55. A method of forming a lipid composition comprising a compound selected from SN38 or a chemical in equilibrium with SN38, involving forming a lipid phase and thereafter hydrating the lipid phase with a first aqueous solution including the compound so as to form lipid composition including the compound, and thereafter reducing the pH of the lipid composition including the compound to a pH of less than about 3.5

56. The method of claim 55, wherein the lipid phase is formed in an organic solvent.

57. The method of claim 55 or 56, wherein the first aqueous solution has an alkaline pH.

58. The method of claim 55, wherein the pH of the first aqueous solution is between about 7 and about 11.

59. The method of claim 55, wherein the pH of the first aqueous solution is between about 8 and about 10.

60. The method of any of claims 55-59, wherein the hydration step is performed with vigorous mixing.

61. The method of claims 55-59, wherein the pH of the lipid composition including the compound is reduced to between about 1.5 and about 3.

62. The method of any of claims 55-61, further involving filtering the lipid composition.

63. The method of claim 62, wherein the filtration occurs prior to reducing the pH of the composition.

64. The method of claim 62 or 63, wherein the filtration is through a filter of about 5 microns or less.

65. The method of claim 62 or 63, wherein the filtration is through a filter of about 1 micron or less.

66. The method of claim 62 or 63, wherein the filtration is through a filter of about 500 nm or less.

67. The method of claim 62 or 63, wherein the filtration is through a filter of about 200 nm or less.

68. The method of claim 62 or 63, wherein the filtration is through a filter of about 100 nm or less.

69. The method of any of claims 55-68, further involving dehydrating the lipid composition to form a dried lipid composition.

70. The method of claim 69, wherein the drying occurs prior to reducing the pH of the composition.

71. The method of claim 69 or 70, further involving adding a protective sugar to the lipid composition.

72. The method of claim 71, wherein the sugar is a disaccharide sugar.

73. The method of claim 71, wherein the sugar is trehalose, maltose, sucrose, glucose, lactose, or dextran.

74. The method of claim 71, wherein the sugar is a aminoglycoside.

75. The method of claim 74, wherein the sugar is streptomycin or dihydroxystreptomycin.

76. The method of any of claims 69-75, further involving resuspending the dried lipid composition in a polar solvent.

77. The method of claim 76, wherein the polar solvent is an aqueous solution with an acidic pH less than about 3.5

78. The method of claim 76 or 77, which effects the reduction in pH of the composition.

79. A method of making SN38 complexes comprising formulating lyophilized complexes containing liposomes and SN38 or a compound in equilibrium with SN38, dissolving said lyophilized complexes in an aqueous solution, and contacting the liposomes with a activating agent whereby SN38 becomes active in aqueous solution and releases SN38.

80. The method of claim 79 wherein a solubilizing agent is employed to increase the solubility of SN38.

81. The method of claim 80, wherein the solubilizing agent is an alkaline buffer.

82. The method of claim 81, wherein the alkaline buffer is sodium carbonate, sodium bicarbonate, sodium hydroxide, sodium phosphate, ammonium acetate, sodium citrate, sodium hydroxide, calcium hydroxide, sodium biphosphate, sodium phosphate, Tris (hydroxy-methyl) aminomethane, or sodium benzoate.

83. The method of claim 79 wherein one or more pharmaceutical acceptable excipients are employed to enhance shelf-life of SN38 complex liposomes.

84. The method of claim 83, wherein at least one of said excipients is a protective sugar.

85. A method for loading liposomes with SN38 or a compound in equilibrium with SN38 comprising the steps of:

a. preparing a preparation which includes liposomes;

b. dehydrating the liposome preparation;

c. rehydrating the dehydrated preparation;

d. replacing the external medium surrounding the liposomes in the rehydrated preparation with a medium which produces an ion concentration gradient capable of generating a transmembrane potential having an orientation which will load charged material into the liposomes; and

e. admixing the SN38 with the liposomes in their replaced external medium.

86. The method of claim 85, wherein an activating agent is employed to activate SN38.

87. The method of claim 79 or 86, wherein the activating agent is an acidic buffer.

88. The method of claim 87, wherein the acidic buffer has a pH of about 3.5 or less.

89. The method of claim 87, wherein the acidic buffer has a pH of between about 1.5 and about 3.

90. The method of claim 87 wherein the acidic buffer is citric acid, sodium citrate, sodium lactate, lactic acid, sodium acetate, acetic acid, ascorbic acid, sodium tartrate, tartartic acid, sodium succinate, succinic acid, aspartic acid, hydrochloric acid, meleic acid, sodium carbonate, sulfuric acid, or sodium sulfate.

91. The method of claim 85, wherein the liposome preparation of step (a) includes one or more protective sugars.

92. The method of claim 84 or 91, wherein the sugar is selected from the group consisting of trehalose, maltose, sucrose, glucose, lactose, and dextran.

93. The method of claim 92 wherein the sugar is trehalose.

94. The method of claim 92 wherein the sugar is sucrose.

95. The method of claim 91 wherein the one or more protective sugars are aminoglycosides.

96. The method of claim 95 wherein the sugar is streptomycin.

97. The method of claim 95 wherein the sugar is dihydrostreptomycin.

98. The method of any of claims 79-97, wherein the liposomes comprise cardiolipin.

99. The method of claim 98, wherein the cardiolipin is selected from the group consisting of natural cardiolipin, synthetic cardiolipin, and chemically-modified cardiolipin.

100. The method of any of claims 97-99, wherein the liposomes comprise at least one of the lipids selected from the group of lipids consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, phosphatidylinositol, sphingomyelin, sterol, tocopherol, fatty acid, cardiolipin, and mixtures thereof.

101. The method of any of claims 97-99, wherein the liposomes comprise a phosphatidylcholine, a sterol, and a tocopherol.

102. The method of any of claims 55-63, wherein the lipid phase 97-99, wherein the liposomes comprise group consisting of dimyristoylphosphatidylglycerol, dioleoylphosphatidylglycerol, distearoylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, diarachidonoylphosphatidylglycerol, or mixtures thereof.

103. The method of any of claims 97-99, wherein the liposomes comprise a phosphatidylcholine selected from the group consisting of dimyristoylphosphatidylcholine, distearoylphosphatidyl choline, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, diarachidonoyl phosphatidylcholine, egg phosphatidylcholine, soy phosphatidylcholine, hydrogenated soy phosphatidylcholine, and mixtures thereof.

104. The method of any of claims 97-99, wherein the liposomes comprise a sterol selected from the group consisting of cholesterol, polyethylene glycol derivatives of cholesterol, coprostanol, cholestanol, cholestane, cholesterol hemisuccinate, cholesterol sulfate, and mixtures thereof.

5

Figure 1

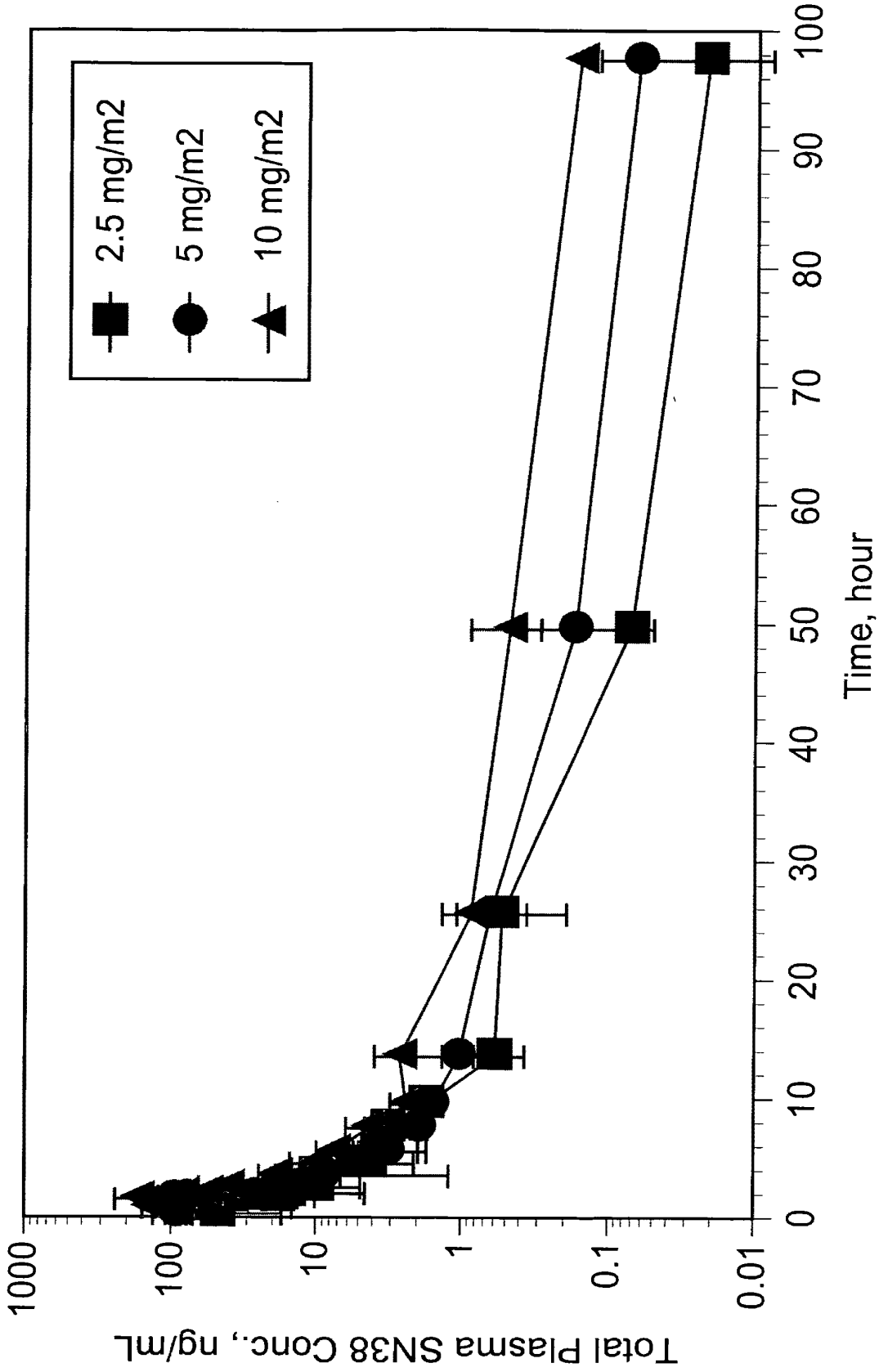
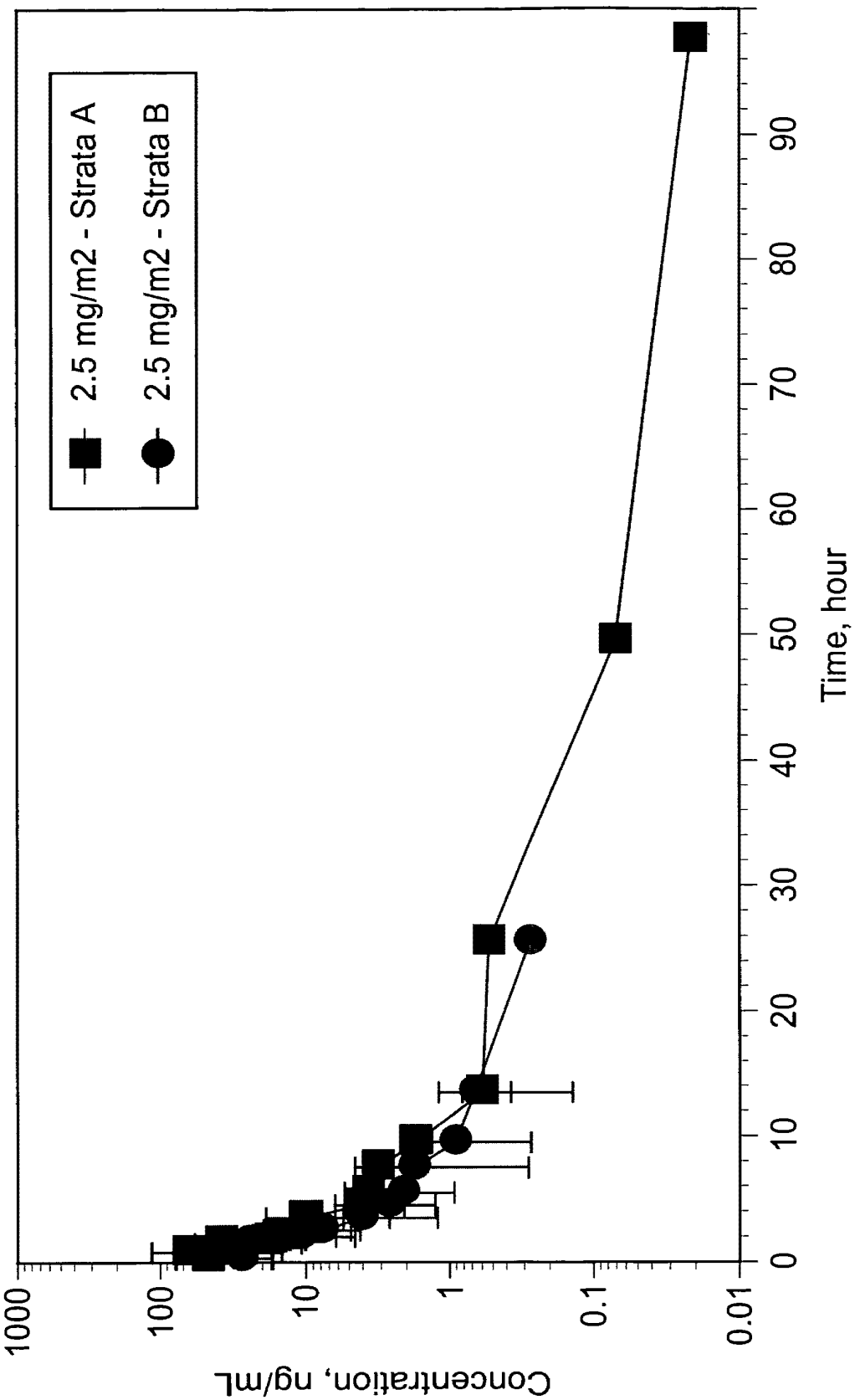

2.5 mg/m2
5 mg/m2
10 mg/m2
Total Plasma SN38 Conc., ng/mL
1000
100
10
1
0.1
0.01
0
10
20
30
40
50
60
70
80
90
100
Time, hour

Figure 2


2.5 mg/m2 - Strata A
2.5 mg/m2 - Strata B
Concentration, ng/mL
1000
100
10
1
0.1
0.01
Time, hour
0
10
20
30
40
50
60
70
80
90

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, 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, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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(54) Title: PHARMACEUTICALLY ACTIVE LIPID BASED FORMULATION OF SN38

(57) Abstract: SN38, camptothecin derivatives are poorly water soluble, highly lipophilic camptothecin derivatives and are very active against a variety of human cancers. Because of their very poor water solubility, SN38 has not been used to treat human patients with cancer due to the inability to administer sufficient quantities of dissolved in a pharmaceutical formulation. This invention overcomes these limitations by teaching novel pharmaceutical acceptable SN38 liposome complex formulation for the direct administration of the formulation to human patients with cancer. The claimed invention also describes the methods to prepare liposomal SN38 complexes and antitumor compositions of liposomal SN38 complexes to allow the administration in sufficient amounts to treat various types of cancer and as antiviral agents. This invention is also directed to injectable sterile solutions, antitumor compositions, liposomes. The present invention is for novel compositions and methods for treating diseases caused by cellular proliferation, particularly, for treating cancer in mammals and more particularly in humans. The therapeutic compositions of the present invention include SN38 lipid complexes in which the complexes can contain any of a variety of neutral or charged lipids and, desirably, cardiolipin. The compositions are capable of efficiently incorporating SN38 into complexes and are capable of solubilizing relatively high concentrations of SN38.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 03/25880

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 A61K31/437 A61P35/00 A61P31/12

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 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, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 03/103596 A (PIGMAN ELIZABETH ANNE ; RAMASWAMI VARADARAJAN (US); ZUTSHI REENA (US);) 18 December 2003 (2003-12-18) abstract paragraphs '0010!, '0 14!, '0018! paragraphs '0056! - '0058! paragraphs '0092! - '0095! examples	1-104
X	WO 02/058622 A (AHMAD IMRAN ; NEOPHARM INC (US); RAHMAN AQUILUR (US); ZHANG JIA-AI ALL) 1 August 2002 (2002-08-01) the whole document	5-104

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[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

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"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
9 March 2004	18/03/2004

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	Villa Riva, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	US 5 834 012 A (PEREZ-SOLER ROMAN ET AL) 10 November 1998 (1998-11-10) column 1, line 42 - line 58 column 2, line 25 - line 38 column 3, lines 9-62 column 6, lines 51-62	5-104
Y	US 6 056 973 A (ALLEN THERESA M ET AL) 2 May 2000 (2000-05-02) column 2, lines 1-21 column 3, lines 1-6 column 5, lines 25-35 column 6, lines 6-55	1-104
Y	EMERSON D L: "Liposomal delivery of camptothecins" PHARMACEUTICAL SCIENCE AND TECHNOLOGY TODAY, ELSEVIER TRENDS JOURNALS, CAMBRIDGE,, GB, vol. 3, no. 6, 1 June 2000 (2000-06-01), pages 205-209, XP002235822 ISSN: 1461-5347 the whole document	1-104
Y	INNOCENTI F ET AL: "PHARMACOGENETICS OF ANTICANCER AGENTS: LESSONS FROM AMONAFIDE AND IRINOTECAN" DRUG METABOLISM AND DISPOSITION, WILLIAMS AND WILKINS., BALTIMORE, MD, US, vol. 29, no. 4, PART 2, April 2001 (2001-04), pages 596-600, XP001008560 ISSN: 0090-9556 the whole document	1-104
P,A	PIZZOLATO J F ET AL: "The camptothecins" LANCET THE, LANCET LIMITED. LONDON, GB, vol. 361, no. 9376, 28 June 2003 (2003-06-28), pages 2235-2242, XP004434302 ISSN: 0140-6736 the whole document	1-104

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 03/25880

Box I Observations where certain claims were found unsearchable (Continuation of item 1 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.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-22 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 II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03103596 A	18-12-2003	US 2003059465 A1	27-03-2003
		WO 03103596 A2	18-12-2003
		US 2004009229 A1	15-01-2004
WO 02058622 A	01-08-2002	CA 2427467 A1	01-08-2002
		CZ 20031515 A3	17-09-2003
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		JP 2001504093 T	27-03-2001
		PT 932391 T	30-06-2003
		TW 520297 B	11-02-2003
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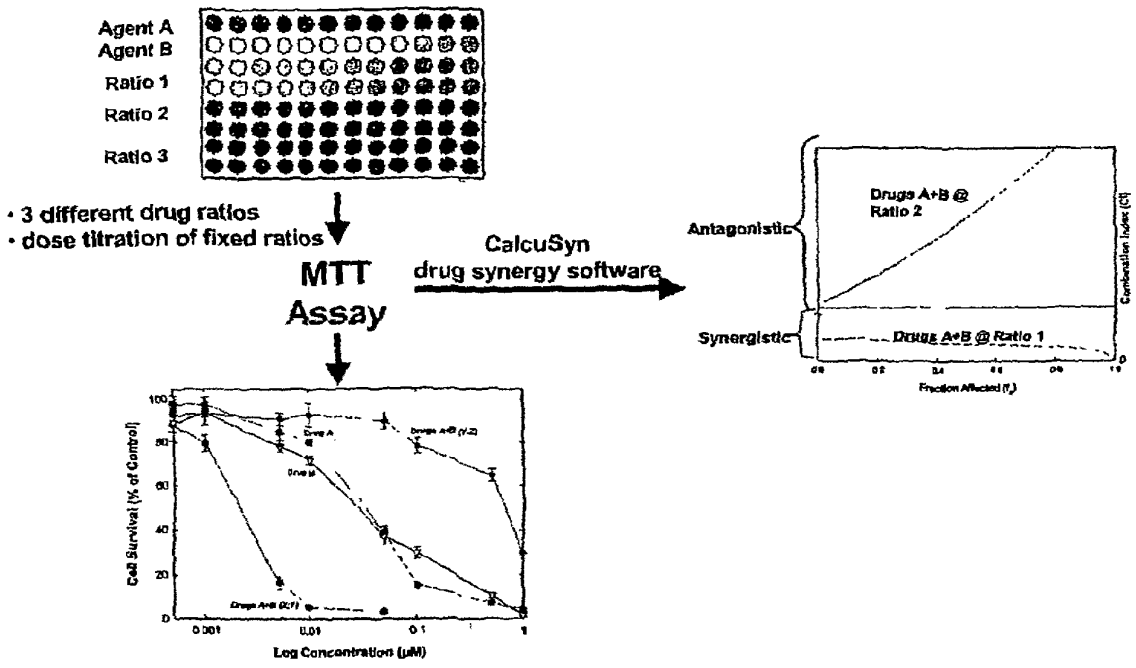
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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, 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.

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[Continued on next page]

(54) Title: COMPOSITIONS FOR DELIVERY OF DRUG COMBINATIONS



(57) Abstract: Compositions which comprise delivery vehicles having stably associated therewith non-antagonistic combinations of two or more agents, such as antineoplastic agents, are useful in achieving non-antagonistic effects when combinations of drugs are administered.

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COMPOSITIONS FOR DELIVERY OF DRUG COMBINATIONS

Cross-Reference to Related Applications

[0001] This application is a continuation-in-part of U.S. Serial No. 10/264,538 filed 3 October 2002, which claims benefit under 35 U.S.C. § 119(e) of provisional applications U.S. Serial No. 60/326,671 filed 3 October 2001; Serial No. 60/341,529 filed 17 December 2001; Serial No. 60/356,759 filed 15 February 2002; Canadian informal application Serial No. CA 2,383,259 filed 23 April 2002; provisional applications U.S. Serial No. 60/401,984 filed 7 August 2002 and U.S. Serial No. 60/408,733 filed 6 September 2002. The contents of these applications are incorporated herein by reference.

Technical Field

[0002] The invention relates to compositions and methods for improved delivery of synergistic or additive combinations of therapeutic agents. More particularly, the invention concerns delivery systems which ensure the maintenance of synergistic or additive ratios when the agents are delivered to an intended target by providing formulations comprising delivery vehicles.

Background Art

[0003] The progression of many life-threatening diseases such as cancer, AIDS, infectious diseases, immune disorders and cardiovascular disorders is influenced by multiple molecular mechanisms. Due to this complexity, achieving cures with a single agent has been met with limited success. Thus, combinations of agents have often been used to combat disease, particularly in the treatment of cancers. It appears that there is a strong correlation between the number of agents administered and cure rates for cancers such as acute lymphocytic leukemia. (Frei, *et al., Clin. Cancer Res.* (1998) 4:2027-2037). Clinical trials utilizing combinations of doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue and cytarabine (ACOMLA) or cyclophosphamide, doxorubicin, vincristine, prednisone and bleomycin (CHOP-b) have been successfully used to treat histiocytic lymphoma (Todd, *et al., J. Clin. Oncol.* (1984) 2:986-993).

[0004] The effects of combinations of drugs are enhanced when the ratio in which they are supplied provides a synergistic effect. Synergistic combinations of agents have also been shown to reduce toxicity due to lower dose requirements, to increase cancer cure rates

(Barriere, *et al., Pharmacotherapy* (1992) 12:397-402; Schimpff, *Support Care Cancer* (1993) 1:5-18), and to reduce the spread of multi-resistant strains of microorganisms (Shlaes, *et al., Clin. Infect. Dis.* (1993) 17:S527-S536). By choosing agents with different mechanisms of action, multiple sites in biochemical pathways can be attacked thus resulting in synergy (Shah and Schwartz, *Clin. Cancer Res.* (2001) 7:2168-2181). Combinations such as L-canavanine and 5-fluorouracil (5-FU) have been reported to exhibit greater antineoplastic activity in rat colon tumor models than the combined effects of either drug alone (Swaffar, *et al., Anti-Cancer Drugs* (1995) 6:586-593). Cisplatin and etoposide display synergy in combating the growth of a human small-cell lung cancer cell line, SBC-3 (Kanzawa, *et al., Int. J. Cancer* (1997) 71(3):311-319).

[0005] Additional reports of synergistic effects are found for:

Vinblastine and recombinant interferon-β (Kuebler, *et al., J. Interferon Res.* (1990) 10:281-291);

Cisplatin and carboplatin (Kobayashi, *et al., Nippon Chiryo Gakkai Shi* (1990) 25:2684-2692);

Ethyl deshydroxy-sparsomycin and cisplatin or cytosine arabinoside (AraC) or methotrexate or 5-FU or vincristine (Hofs, *et al., Anticancer Drugs* (1994) 5:35-42);

All trans retinoic acid and butyric acid or tributyrin (Chen, *et al., Chin. Med. Engl.* (1999) 112:352-355); and

Cisplatin and paclitaxel (Engblom, *et al., Br. J. Cancer* (1999) 79:286-292).

[0006] In the foregoing studies, the importance of the ratio of the components for synergy was recognized. For example, 5-fluorouracil and L-canavanine were found to be synergistic at a mole ratio of 1:1, but antagonistic at a ratio of 5:1; cisplatin and carboplatin showed a synergistic effect at an area under the curve (AUC) ratio of 13:1 but an antagonistic effect at 19:5.

[0007] Other drug combinations have been shown to display synergistic interactions although the dependency of the interaction on the combination ratio was not described. This list is quite extensive and is composed mainly of reports of *in vitro* cultures, although occasionally *in vivo* studies are included.

[0008] In addition to the multiplicity of reports, a number of combinations have been shown to be efficacious in the clinic. These are described in the table below.

REFERENCE	DRUG 1	DRUG 2	DRUG 3
Langer, *et al.* (1999) Drugs 58 Suppl. 3:71-75	Cisplatin or Vindesine	+ UFT (Tegafur/ uracil)	
FDA[a] (Colon or Rectal Cancer)	Leucovorin	+ 5-FU	
FDA (Colon or Rectal Cancer)	Irinotecan	+ Leucovorin	+ 5-FU
FDA (Breast Cancer)	Herceptin	+ Paclitaxel	
FDA (Breast Cancer)	Xeloda (other names: Capecitabine)	+ Docetaxel	
FDA (Ovarian and Lung Cancer)	Paclitaxel	+ Cisplatin	
FDA (Lung Cancer)	Etoposide	+ Other FDA-approved Chemotherapeutic agents	
FDA (Lung Cancer)	Gemcitabine	+ Cisplatin	
FDA (Prostate)	Novantrone (mitoxantrone hydrochloride)	+ Corticosteroids	
FDA (Acute Nonlymphocytic Leukemia)	Novantrone	+ Other FDA-approved drugs	
FDA (Acute Nonlymphocytic Leukemia/Acute Lymphocytic Leukemia)	Daunorubicin (DNR, Cerubidine)	+ Other FDA-approved drugs	
FDA (Chronic Myelogenous Leukemia)	Busulfex (Busulfan; 1,4-butanediol, dimethanesulfonate; BU, Myleran)	+ Cyclophosphamide (Cytoxan)	

[a]FDA: United States Food and Drug Administration

[0009] In addition, certain other combinations can be postulated from various reports in the literature to have the potential for exhibiting non-antagonistic combination effects or clinical efficacy or accepted as the standard of care by region study groups. These are:

DISEASE	DRUG 1	DRUG 2	DRUG 3
(Colon Cancer)	Oxaloplatin	+ 5-FU (or FUDR)	+ Leucovorin
(Metastatic Breast Cancer)	Taxol	+ Doxorubicin	
	Adriamycin (doxorubicin)	+ Cytoxan (cyclophosphamide)	
	Methotrexate	+ 5-FU (or FUDR)	+ Cytoxan
	Vinblastine	+ Doxorubicin	
(Non-small Cell Lung Cancer)	Carboplatin	+ Taxol	
	Cisplatin	+ Docetaxel (Taxotere®)	
	Vinorelbine	+ Cisplatin	
	Irinotecan	+ Cisplatin	
(Small Cell Lung Cancer)	Carboplatin	+ Taxol	
	Cisplatin	+ Etoposide	
(Prostate Cancer)	Estramustine	+ Taxol	
	Estramustine	+ Mitoxantrone	
	Estramustine	+ Taxotere	
(Hodgkin's Lymphoma)	Bleomycin	+Vinblastine (as part of ABDV: Adriamycin, Bleomycin, DTIC, Vinblastine)	
(Non-Hodgkin's Lymphoma)	Carboplatin	+ Etoposide (as part of ICE :Ifosfamide, Carboplatin, Etoposide)	
(Melanoma)	IL-2	+ Cisplatin	
(Acute Myeloid Leukemia)	Daunorubicin	+ Cytosine Arabinoside	
	Vincristine	+ Doxorubicin	
(Bladder Cancer)	Carboplatin	+ Taxol	
	Carboplatin	+ Gemcitabine	
	Gemcitabine	+ Taxol	
	Vinblastine	+ Doxorubicin (as part of MVAC: Methotrexate, Vinblastine, Adriamycin, Cisplatin)	
(Head and Neck Cancer)	5-FU (or FUDR)	+ Cisplatin	+ Leucovorin
(Pancreatic Cancer)	Gemcitabine	+5-FU (or FUDR)	

Additional Combinations:

Carboplatin	+ 5-FU (or FUDR)
Carboplatin	+ Irinotecan
Irinotecan	+ 5-FU (or FUDR)
Vinorelbine	+ Carboplatin
Methotrexate	+ 5-FU (or FUDR)
Idarubicin	+ AraC
Adriamycin	+ Vinorelbine
Safingol	+ Fenretinide

[0010] Despite the aforementioned advantages associated with the use of synergistic drug combinations, there are various drawbacks that limit their therapeutic use. For instance, synergy often depends on various factors such as the duration of drug exposure and the sequence of administration (Bonner and Kozelsky, *Cancer Chemother. Pharmacol.* (1990) 39:109-112). Studies using ethyl deshydroxy-sparsomycin in combination with cisplatin show that synergy is influenced by the combination ratios, the duration of treatment and the sequence of treatment (Hofs, *et al.*, *supra*).

[0011] It is thus known that in order for synergy to be exhibited by a combination of agents, these agents must be present in amounts which represent defined ratios. Indeed, the same combination of drugs may be antagonistic at some ratios, synergistic at others, and additive at still others. It is desirable to avoid antagonistic effects, so that the drugs are at least additive. The present invention recognizes that the result obtained at an individual ratio is also dependent on concentration. Some ratios are antagonistic at one concentration and non-antagonistic at another. The invention ensures ratios of components in the synergistic or additive range by delivering these agents in formulations that maintain the desired or administered ratio when the target location in the subject are reached and by selecting the ratios to be predominantly non-antagonistic at a desired range of concentrations, since the concentration at the target may be different from that administered.

[0012] PCT publication WO 00/51641 describes administering a combination of antiviral agents which is said to be synergistic. *In vitro* tests were used to determine synergistic ratios. However, there is no teaching of any mode of administration which would maintain this ratio *in vivo*. Indeed, the publication states that the components may be administered sequentially or simultaneously.

[0013] PCT publication WO 01/15733 describes putatively synergistic compositions for treating autoimmune disease. Again, the method of formulation does not ensure maintenance of this ratio after delivery.

[0014] Daoud, *et al., Cancer Chemother. Pharmacol.* (1991) 28:370-376, describe synergistic cytotoxic actions of cisplatin and liposomal valinomycin on human ovarian carcinoma cells. This paper describes an *in vitro* assay in which cisplatin which is free and valinomycin which is encapsulated in liposomes are used to treat cultures of CaOV-3, a human ovarian tumor-derived cell line. The authors determined the concentration ranges over which synergism and antagonism was exhibited. Liposome encapsulation was employed to solubilize the valinomycin. As the experiments are performed *in vitro*, *in vivo* delivery is irrelevant.

[0015] U.S. patent 6,214,821 issued 10 April 2001 to Daoud, describes pharmaceutical compositions containing topoisomerase I inhibitors and a staurosporine. The claims appear to be based on the discovery that staurosporines have the ability to abrogate topoisomerase I inhibitor-induced S-phase arrest and to enhance its cytotoxicity to human breast cancer cells lacking normal p53 function. No particular pharmaceutical formulation is suggested.

[0016] U.S. patent 5,000,958 to Fountain, *et al.,* describes mixtures of antimicrobial agents encapsulated in liposomes which are said to exert an enhanced therapeutic effect *in vivo*. Suitable ratios of antimicrobial agents are determined by a combination effect test which empirically tests for synergy *in vitro*. There is no discussion of assuring a synergistic ratio over a range of concentrations.

[0017] Schiffelers, *et al., J. Pharmacol. Exp. Therapeutic* (2001) 298:369-375, describes the *in vivo* synergistic interaction of liposome co-encapsulated gentamicin and ceftazidime. The desired ratios were determined using a similar combination effect test to that of Fountain (*supra*), but there is no discussion of determination of a ratio wherein synergism is maintained over a range of concentrations.

[0018] The present invention recognizes, first, that it is possible to maintain a determined synergistic or additive ratio of therapeutic agents by controlling the pharmacokinetics of the formulation in which they are administered, and second, that the non-antagonistic ratio must be exhibited over a range of concentrations, since the concentration of components in a drug cocktail which reaches the target tissue may not be the same as that which is administered. The problem of maintaining synergy or additivity is solved by the recognition that when therapeutic agents are encapsulated in (*i.e.*, stably associated with) delivery vehicles, such as liposomes, the delivery vehicles determine the pharmacokinetics and thus agents which are encapsulated will behave in a similar manner, and by selecting ratios which are predominantly synergistic/additive over a range of concentrations.

Disclosure of the Invention

[0019] The invention relates to methods for administering non-antagonistic ratios of therapeutic agents, preferably antitumor drugs, using delivery vehicle compositions that encapsulate two or more agents, wherein the agents are present in the vehicles at ratios synergistic or additive (i.e. non-antagonistic) over a range of concentrations. Prior to encapsulation, the ratios of therapeutic agents in the combination are selected so that the combination exhibits synergy or additivity over a desired concentration range. Encapsulation in delivery vehicles allows two or more agents to be delivered to the disease site in a coordinated fashion, thereby assuring that the agents will be present at the disease site at a non-antagonistic ratio. This result will be achieved whether the agents are co-encapsulated in delivery vehicles, or are separately encapsulated in delivery vehicles administered such that non-antagonistic ratios are maintained at the disease site. The pharmacokinetics (PK) of the composition are controlled by the delivery vehicles themselves such that coordinated delivery is achieved (provided that the PK of the delivery systems are comparable).

[0020] Thus, in one aspect, the invention provides a delivery vehicle composition for parenteral administration comprising two or more agents encapsulated in the vehicle composition at a ratio that is synergistic or additive over a desired concentration range. The delivery vehicle composition is prepared by a process comprising encapsulating the agents in the delivery vehicle composition at these ratios. The non-antagonistic ratio of the agents is determined by assessing the biological activity or effects of the agents on relevant cell culture or cell-free systems over a range of concentrations and, in one embodiment, applying an algorithm to determine a "combination index," (CI). As further described below, using recognized algorithms, a combination index can be calculated at each concentration level. Ratios are selected where the CI represents synergy or additivity over a range of concentrations. Preferably the CI is synergistic over a wide concentration range. Preferred agents are antitumor agents. Any method which results in determination of a ratio of agents which maintains a non-antagonistic effect over a desired range of concentrations may be used.

[0021] More particularly, the invention relates to a composition which comprises delivery vehicles, said delivery vehicles having encapsulated therein at least a first therapeutic agent and a second therapeutic agent in a mole ratio of the first agent to the second agent which exhibits a non-antagonistic biologic effect to relevant cells in culture or cell-free system over at least 5% of such concentration range where greater than 1% of the cells are affected (Fraction affected (f_a) > 0.01) or to a composition which comprises delivery vehicles, said delivery

vehicles having encapsulated therein at least a first therapeutic agent and a second therapeutic agent in a mole ratio of the first agent to the second agent which exhibits a non-antagonistic cytotoxic effect or cytostatic effect to relevant cells wherein said agents are antineoplastic agents. By "relevant" cells, applicants refer to at least one cell culture or cell line which is appropriate for testing the desired biological effect. For example, if the agent is an antineoplastic agent, a "relevant" cell would be a cell line identified by the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI)/National Institutes of Health (NIH) as useful in their anticancer drug discovery program. Currently the DTP screen utilizes 60 different human tumor cell lines. The desired activity on at least one of such cell lines would need to be demonstrated.

[0022] In another aspect, the invention is directed to a method to deliver a synergistic or additive ratio of two or more therapeutic agents to a desired target by administering the compositions of the invention. The administration of such compositions need not be in the form of a single composition, but may also include simultaneous or near simultaneous administration of separate compositions comprising therapeutic agents in delivery vehicles such that the pharmacokinetics of the delivery vehicles is coordinated - *i.e.*, designed in such a way that the ratio of therapeutic agents administered is maintained when target tissues or organs are reached. Thus, separate compositions, each comprising delivery vehicles stably associated with one or more therapeutic agents may be delivered to the subject in a ratio of the therapeutic agents which has been determined to be non-antagonistic as described herein.

[0023] In another aspect, the invention is directed to a method to prepare a therapeutic composition comprising delivery vehicles, said delivery vehicles containing a ratio of at least two therapeutic agents which is non-antagonistic over a range of concentrations which method comprises providing a panel of at least two therapeutic agents wherein the panel comprises at least one, but preferably a multiplicity of ratios of said agents, testing the ability of the members of the panel to exert a biological effect on a relevant cell culture or cell-free system over a range of concentrations, selecting a member of the panel wherein the ratio provides a synergistic or additive effect on said cell culture or cell-free system over a suitable range of concentrations; and encapsulating (i.e. stably associating) the ratio of agents represented by the successful member of the panel into drug delivery vehicles. The ratio resulting from the determination described above, in addition to being used as a guide for preparing a single formulation, may also be used to determine the relative amounts to be administered to a subject of separate compositions, each comprising delivery vehicles stably associated with at least one

therapeutic agent. Thus, the ratios of therapeutic agents herein determined to be additive or synergistic may be supplied to the subject in a single composition or in the correct proportion of separately prepared compositions.

[0024] In another aspect, the invention is directed to kits said kits comprising, in separate containers, a first composition comprising a first therapeutic agent stably associated with delivery vehicles and a second composition comprising delivery vehicles stably associated with the second therapeutic agent. The two containers may be calibrated so that the correct proportion of the two compositions is administered; alternatively, or in addition the kit may simply include instructions with regard to the correct ratio.

[0025] As further described below, in a preferred embodiment, in designing an appropriate combination in accordance with the method described above, the non-antagonistic ratios are selected as those that have a combination index (CI) of ≤ 1.1 over a range of at least 5% of those doses or concentrations that affect greater than 1% or more of the cells ($fa > 0.01$), preferably between 20 and 80% of the cells (f_a=0.2 to 0.8), as defined by relevant cell culture or cell-free assay systems.

Brief Description of the Drawings

[0026] FIGURE 1 is a diagram outlining the method of the invention for determining an appropriate ratio of therapeutic agents to include in formulations.

[0027] FIGURE 2 (A-E) illustrates 5 methods for presenting combination and synergy data.

[0028] FIGURE 3A is a graph of combination index (CI) for irinotecan:5-FU at mole ratios of 1:10 (filled squares) and 1:1 (filled circles) as a function of the fraction of HT29 cells affected (f_a).

[0029] FIGURE 3B is a graph of CI for etoposide:carboplatin at mole ratios of 1:10 (filled diamonds) and 10:1 (filled squares) as a function of the fraction of MCF-7 cells affected (f_a).

[0030] FIGURE 4 is a graph of the CI for cisplatin:edelfosine at mole ratios of 10:1 (filled triangles) and 1:1 (filled circles) as a function of the fraction of H460 cells affected (f_a).

[0031] FIGURE 5A is a graph of the CI maximum as a function of carboplatin:daunorubicin at 10:1, 1:1 and 1:10 mole ratios in H460 cells. The inset is a histogram of the CI for carboplatin:daunorubicin at mole ratios of 10:1 and 1:1 at Effective Dose (ED) values of 50, 75 and 90 in MCF-7 cells.

[0032] FIGURE 5B is a graph of the CI for carboplatin:daunorubicin at mole ratios of 1:10 (filled triangles), 1:1 (filled squares) and 10:1 (filled circles) as a function of the fraction of H460 cells affected (f_a). The inset is a histogram of the CI for carboplatin:daunorubicin at mole ratios of 1:10, 1:1 and 10:1 at ED values of 50, 75 and 90 in H460 cells.

[0033] FIGURE 6 is a graph of the carboplatin (open circles) and daunorubicin (filled circles) concentrations in plasma (nmoles/mL) as a function of time after intravenous administration when the drugs are formulated in a single liposome (DSPC/DSPG, 80:20 mol %) at a non-antagonistic ratio (10:1).

[0034] FIGURE 7A is a graph of the carboplatin:daunorubicin mole ratio as a function of time after intravenous administration at three different ratios when the drugs are formulated in a single liposome (DSPC/DSPG, 80:20 mol %) at 10:1 (filled circles), 5:1 (open circles) and 1:1 (filled triangles).

[0035] FIGURE 7B is a graph of the 1:1 carboplatin:daunorubicin data in Figure 7A re-plotted as a function of time after intravenous administration.

[0036] FIGURE 8 is a graph of carboplatin (filled circles) and daunorubicin (open circles) concentrations in plasma (nmoles/mL) as a function of time after intravenous administration when the drugs are formulated at a non-antagonistic mole ratio (10:1) in a single liposome (DSPC/sphingomyelin/DSPE-PEG2000, 90:5:5 mol %).

[0037] FIGURE 9 is a graph comparing the activity of a cocktail of carboplatin and daunorubicin (filled inverted triangles), carboplatin and daunorubicin formulated in a single liposome (open inverted triangles) or saline control (filled circles) given to mice bearing the human H460 non-small cell lung tumor. Carboplatin and daunorubicin were formulated in DSPC/DSPG (80:20 mol %) liposomes at a 1:1 mole ratio. The arrows indicate the days at which the doses were administered.

[0038] FIGURE 10 is a graph comparing the activity of a cocktail of carboplatin and daunorubicin (filled triangles), carboplatin and daunorubicin formulated in a single liposome (open triangles) or saline control (filled circles) given to mice bearing the human H460 non-small cell lung tumor. Carboplatin and daunorubicin were formulated in DSPC/SM/DSPE-PEG2000 (90:5:5 mol %) liposomes at a 10:1 mole ratio. The arrows along the x-axis indicate the dosing schedule.

[0039] FIGURE 11A is a graph of the CI for cisplatin:daunorubicin at mole ratios of 1:1 (filled squares) and 10:1 (filled circles) as a function of the fraction of H460 cells affected (f_a).

[0040] FIGURE 11B is a graph of the CI maximum as a function of the cisplatin:daunorubicin at 10:1, 1:1 and 1:10 mole ratios against H460 cells.

[0041] FIGURE 12 is a graph of cisplatin (open circles) and daunorubicin (closed circles) concentrations in plasma (μmoles/mL) as a function of time after intravenous administration when the drugs are formulated at a non-antagonistic mole ratio (10:1) in a single liposome (DMPC/Chol, 55:45 mol %).

[0042] FIGURE 13 is a graph of cisplatin (closed circles) and daunorubicin (open circles) concentrations in the plasma (μmoles/mL) as a function of time after intravenous administration when the drugs are formulated at a non-antagonistic mole ratio (10:1) in two separate liposomes (DMPC/Chol, 55:45 mol % for cisplatin and DSPC/DSPE-PEG2000, 95:5 mol % for daunorubicin).

[0043] FIGURE 14 is a graph comparing the activity of a cocktail of cisplatin and daunorubicin (filled inverted triangles), cisplatin and daunorubicin formulated in separate liposomes (open inverted triangles) or saline control (filled circles) given to mice bearing the human H460 non-small cell lung tumor. Cisplatin was formulated in DMPC/Chol (55:45 mol %) liposomes and daunorubicin was formulated in DSPC/DSPE-PEG2000 (95:5 mol %) liposomes and administered at a non-antagonistic mole ratio (10:1). Arrows indicate the days on which the doses were administered.

[0044] FIGURE 15 is a graph showing concentrations of cisplatin (closed circles) and daunorubicin (open circles) remaining in the plasma (nmoles/mL) at various times after intravenous administration when the drugs were formulated in a single liposome (DMPC/Chol, 55:45 mol %) at an antagonistic 1:1 mole ratio. The inset shows the cisplatin:daunorubicin mole ratio at various time points after administration.

[0045] FIGURE 16 is a graph comparing the activity of a cocktail of cisplatin and daunorubicin (filled triangles), cisplatin and daunorubicin formulated in a single liposome (open triangles) or saline control (filled circles) given to mice bearing the human H460 non-small cell lung tumor. The drugs were formulated in DMPC/Chol (55:45 mol %) liposomes at an antagonistic mole ratio (1:1). Arrows indicate the days on which the doses were administered.

[0046] FIGURE 17A is a graph of the CI for cisplatin:topotecan at mole ratios of 1:1 (filled circles) and 10:1 (open circles) as a function of the fraction of H460 cells affected (f_a).

[0047] FIGURE 17B is a graph of the CI maximum as a function of the cisplatin:topotecan mole ratio against H460 cells.

[0048] FIGURE 18 is a graph showing concentrations of cisplatin (closed circles) and topotecan (open circles) remaining in the plasma (μmoles/mL) at various times after intravenous administration when the drugs are formulated in separate liposomes (DMPC/Chol, 55:45 mol % for cisplatin and DSPC/Chol, 55:45 mol % for topotecan). The inset shows the cisplatin to topotecan mole ratio at various time points after administration.

[0049] FIGURE 19 is a graph comparing the activity of a cocktail of cisplatin and topotecan (filled triangles), cisplatin and topotecan formulated in separate liposomes (open triangles) or saline control (filled circles) given to mice bearing the human H460 non-small cell lung tumor. Cisplatin was formulated in DMPC/Chol (55:45 mol %) liposomes and topotecan was formulated in DSPC/Chol (55:45 mol %) liposomes and were administered at a non-antagonistic mole ratio (10:1). Arrows indicate the days on which the doses were administered.

[0050] FIGURE 20A is a graph of the CI for cisplatin:irinotecan at mole ratios of 1:1 (squares), 10:1 (circles), 1:5 (triangles) and 1:10 (diamonds) as a function of the fraction of H460 cells affected (f_a).

[0051] FIGURE 20B is a graph of the CI maximum as a function of the cisplatin:irinotecan mole ratio against H460 cells.

[0052] FIGURE 21 is a graph showing the concentrations of cisplatin (filled circles) and irinotecan (open circles) remaining in the plasma (nmoles/mL) at various time points after intravenous administration when the drugs were co-loaded into a single liposome (DSPC/DSPG, 80:20 mol %).

[0053] FIGURE 22 is a graph showing the concentrations of cisplatin (closed circles) and irinotecan (open circles) remaining in the plasma (nmoles/mL) at various time points after intravenous administration when the drugs are formulated in separate liposomes (DMPC/Chol, 55:45 mol % for cisplatin and DSPC/DSPE-PEG2000, 95:5 mol % for irinotecan).

[0054] FIGURE 23 is a graph comparing the activity of a cocktail of cisplatin and irinotecan (filled squares), cisplatin and irinotecan formulated in separate liposomes and administered at different doses (open symbols) or saline control (filled circles) given to mice bearing the human H460 non-small cell lung tumor. Cisplatin formulated in DMPC/Chol (55:45 mol %) liposomes and irinotecan formulated in DSPC/DSPE-PEG2000 (95:5 mol %) liposomes were administered at a non-antagonistic mole ratio (1:5). Arrows indicate the days on which the doses were administered.

[0055] FIGURE 24 is a graph of CI for vinorelbine in combination with POPS (inverted triangles), DPPS (upward triangles), DLPS (circles), DSPS (diamonds) or DOPS (squares) as a function of the H460 cells affected (f_a) at vinorelbine:PS mole ratios of 1:1.

[0056] FIGURE 25A is a graph of the vinorelbine concentration in plasma as a function of time after intravenous administration to SCID/rag2 mice of free vinorelbine (filled circles) or encapsulated in SM/Chol/DPPS/DSPE-PEG2000, 35:45:10:10 mol % liposomes (open circles) at a vinorelbine:PS mole ratio of 1:1.

[0057] FIGURE 25B is a histogram showing plasma concentration area under the curve (AUC) for free vinorelbine (black bar) or encapsulated in SM/Chol/DPPS/DSPE-PEG2000, 35:45:10:10 mol % (grey bar) after intravenous administration to SCID/rag2 mice, using the data of FIGURE 25A.

[0058] FIGURE 26 is a graph comparing the activity of free vinorelbine (open circles), vinorelbine encapsulated in DSPC/Chol/DPPS/DSPE-PEG2000, 35:45:10:10 mol % liposomes (filled inverted triangles), vinorelbine encapsulated in SM/Chol/DPPS/DSPE-PEG2000, 35:45:10:10 mol % liposomes (open triangles) or saline control (filled circles) given to mice bearing the H460 non-small cell lung tumor. Vinorelbine and phosphatidylserine (DPPS) were formulated at a non-antagonistic mole ratio (1:1). Arrows indicate the days on which the doses were administered.

[0059] FIGURE 27 shows the effect of saline control (filled circles); free vinorelbine (open circles); vinorelbine encapsulated in: SM/Chol/DPPS/DSPE-PEG2000, 35:45:10:10 (filled inverted triangles), DAPC/Chol/DPPS/DSPE-PEG2000, 35:45:10:10 mol % (open triangles), and DSPC/Chol/DSPS/DSPE-PEG2000, 35:45:10:10 mol % (filled squares) liposomes given to mice bearing the H460 non-small cell lung tumor. Vinorelbine and phosphatidylserine (DPPS or DSPS) were formulated at a non-antagonistic mole ratio (1:1). Arrows indicate the days on which the doses were administered.

[0060] FIGURE 28 shows the effect of saline control (open triangles); free vinorelbine (filled circles); and vinorelbine encapsulated in SM/Chol/DPPS/DSPE-PEG2000, 35:45:10:10 mol % liposomes (filled inverted triangles) on percent survival of P388 murine leukemia bearing mice. Vinorelbine and phosphatidylserine were formulated at a non-antagonistic mole ratio (1:1). The arrow along the x-axis indicate the day on which the doses were administered.

[0061] FIGURE 29 shows CI plotted as a function of the fraction of HT-29 cells affected by combinations of FUDR:CPT-11 at various ratios: 10:1 (solid squares); 5:1 (solid circles); 1:1 (solid triangles); 1:5 (solid inverted triangles); and 1:10 (open circles).

[0062] FIGURE 30 is a graph of plasma concentration levels of FUDR (solid circles) and CPT-11 (open circles) as a function of time after intravenous administration.

[0063] FIGURE 31 is a graph of tumor volume *versus* time after tumor cell inoculation for saline controls (solid circles) injection of a cocktail of CPT-11/FUDR (open inverted triangles) and the liposomal formulation of CPT-11/FUDR (solid inverted triangles).

Modes of Carrying Out the Invention[1]

[0064] The method of the invention involves determining a ratio of therapeutic drugs which is non-antagonistic over a desired concentration range *in vitro* and supplying this non-antagonistic ratio in a manner that will ensure that the ratio is maintained at the site of desired activity. The synergistic or additive ratio is determined by applying standard analytical tools to the results obtained when at least one ratio of two or more therapeutic agents is tested *in vitro* over a range of concentrations against relevant cell cultures or cell-free systems. By way of illustration, individual agents and various combinations thereof are tested for their biological effect on cell culture or a cell-free system, for example causing cell death or inhibiting cell growth, at various concentration levels. The concentration levels of the preset ratios are plotted against the percentage cell survival to obtain a correlation which can be manipulated by known and established mathematical techniques to calculate a "combination index" (CI). The

[1] Abbreviations

The following abbreviations are used:

PE: phosphatidylethanolamine; PS: phosphatidylserine; DPPS: dipalmitoylphosphatidylserine; DSPS: distearoylphosphatidylserine DLPS: dilauroylphosphatidylserine; DOPS: dioleoylphosphatidylserine; POPS: palmitoyloleoylphosphatidylserine; PC: phosphatidylcholine; SM: sphingomyelin; PG: phosphatidylglycerol; PI: phosphatidylinositol; PA: phosphatidic acid; DSPC: distearoylphosphatidylcholine; DMPC: dimyristoylphosphatidylcholine; DSPG: distearoylphosphatidylglycerol; DSPE: distearoylphosphatidylethanolamine; Chol: cholesterol; CH or CHE: cholesteryl hexadecyl ether;

PEG: polyethylene glycol; DSPE-PEG: distearoylphosphatidylethanolamine-N-[polyethylene glycol]; when PEG is followed by a number, the number is the molecular weight of PEG in Daltons; DSPE-PEG2000: distearoylphosphatidylethanolamine-N-[polyethylene glycol 2000];

SUV: small unilamellar vesicle; LUV: large unilamellar vesicle; MLV: multilamellar vesicle;

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide; DMSO: dimethylsulfoxide; OD: optical density; OGP: N-octyl beta-D-glucopyranoside; EDTA: ethylenediaminetetraacetic acid; HEPES: N-[2-hydroxylethyl]-piperazine-N-[2-ethanesulfonic acid]; HBS: HEPES buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4); SHE: 300 mM sucrose, 20 mM HEPES, 30 mM EDTA; ED50, ED75 and ED90: effective dose required to affect 50, 75 and 90 % of the cells in culture; LD50: dose required to cause 50 % lethality of the cells in culture; CI: combination index; CI max or CI maximum: CI value taken for a single f_a value (between 0.2 and 0.8) where the greatest difference in CI values for the drugs at different ratios is observed; f_a: fraction affected; TEA: triethanolamine;

FDA: United States Food and Drug Administration; NCI: National Cancer Institute.

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mathematics are such that a CI of 1 (*i.e.*, 0.9-1.1) describes an additive effect of the drugs; a CI > 1 (*i.e.*, > 1.1) represents an antagonist effect; and a CI of < 1 (*i.e.*, < 0.9) represents a synergistic effect.

[0065] One general approach is shown in Figure 1. As shown, agents A and B are tested individually and together at two different ratios for their ability to cause cell death or cell stasis as assessed by the MTT assay described below. Initially, correlations between the concentration of drugs A, B, and the two different combination ratios (Y:Z and X:Y) are plotted against cytotoxicity, calculated as a percentage based on the survival of untreated control cells. As expected, there is a dose-dependent effect on cell survival both for the individual drugs and for the combinations. Once this correlation has been established, the cell survival or fraction affected (f_a) can be used as a surrogate for concentration in calculating the CI.

[0066] The results of the CI calculation are also shown in Figure 1; this index is calculated as a function of the fraction of cells affected according to the procedure of Chou and Talalay, *Advance Enz. Regul.* (1985) 22:27-55. In this hypothetical situation, the first ratio (X:Y) of drugs A plus B is non-antagonistic at all concentrations while the combination in the second ratio (Y:Z) is antagonistic. Thus, it is possible to provide a ratio of drugs A plus B (ratio 1) which will be non-antagonistic regardless of concentration over a wide range. It is this ratio that is desirable to include in the compositions of the invention.

[0067] The present inventors have also devised an alternative illustration of the effect of ratio and concentration on synergy by calculating a "CI maximum" for various ratios of combinations of agents. The "CI maximum" is defined as the CI value taken for a single f_a value (between 0.2 and 0.8) where the greatest difference in CI values for the drugs at different ratios was observed. This is illustrated in Figures 2A and 2B; as shown, when the irinotecan/carboplatin ratio is 1:10, its CI differs most from that of the remaining ratios where the fraction affected value is 0.2. The CI value for this ratio at f_a 0.2 is, as shown, approximately 2.0.

[0068] While the determination *in vitro* of non-antagonistic ratios has been illustrated for a combination of only two drugs, application of the same techniques to combinations of three or more drugs provides a CI value over the concentration range in a similar manner.

[0069] The ratio obtained in this way is maintained in the pharmaceutical composition by encapsulating the agents in the predetermined ratio in liposomes or other particulate forms which assures that the non-antagonistic ratio will be maintained. The compositions, thus,

contain delivery vehicles which are particulate in nature and contain the desired ratio of therapeutic agents.

[0070] While it is preferred to co-encapsulate the agents so that both are contained in the same delivery vehicle, this is not necessary. Since particulate carriers can share similar pharmacokinetics, the active substances experience coordinated delivery from the formulation even if encapsulated separately.

[0071] By "encapsulation", it is meant stable association with the delivery vehicle. Thus, it is not necessary for the vehicle to surround the agent or agents as long as the agent or agents is/are stably associated with the vehicles when administered *in vivo*. Thus, "stably associated with" and "encapsulated in" or "encapsulated with" or "co-encapsulated in or with" are intended to be synonymous terms. They are used interchangeably in this specification. The stable association may be effected by a variety of means, including covalent bonding to the delivery vehicle, preferably with a cleavable linkage, noncovalent bonding, and trapping the agent in the interior of the delivery vehicle and the like. The association must be sufficiently stable so that the agents remain associated with the delivery vehicle at a non-antagonistic ratio until it is delivered to the target site in the treated subject.

[0072] Delivery vehicles may include lipid carriers, liposomes, lipid micelles, lipoprotein micelles, lipid-stabilized emulsions, cyclodextrins, polymer nanoparticles, polymer microparticles, block copolymer micelles, polymer-lipid hybrid systems, derivatized single chain polymers, and the like. Liposomes can be prepared as described in Liposomes: Rational Design (A.S. Janoff ed., Marcel Dekker, Inc., N.Y.), or by additional techniques known to those knowledgeable in the art. Liposomes for use in this invention may be prepared to be of "low-cholesterol." Such liposomes are "cholesterol free," or contain "substantially no cholesterol," or "essentially no cholesterol." The term "cholesterol free" as used herein with reference to a liposome means that a liposome is prepared in the absence of cholesterol. The term "substantially no cholesterol" allows for the presence of an amount of cholesterol that is insufficient to significantly alter the phase transition characteristics of the liposome (typically less than 20 mol % cholesterol). The incorporation of less than 20 mol % cholesterol in liposomes can allow for retention of drugs not optimally retained when liposomes are prepared with greater than 20 mol % cholesterol. Additionally, liposomes prepared with less than 20 mol % cholesterol display narrow phase transition temperatures, a property that may be exploited for the preparation of liposomes that release encapsulated agents due to the application of heat (thermosensitive liposomes). Liposomes of the invention may also contain

therapeutic lipids, which include ether lipids, phosphatidic acid, phosphonates, ceramide and ceramide analogues, sphingosine and sphingosine analogues and serine-containing lipids. Liposomes may also be prepared with surface stabilizing hydrophilic polymer-lipid conjugates such as polyethylene glycol-DSPE, to enhance circulation longevity. The incorporation of negatively charged lipids such as phosphatidylglycerol (PG) and phosphatidylinositol (PI) may also be added to liposome formulations to increase the circulation longevity of the carrier. These lipids may be employed to replace hydrophilic polymer-lipid conjugates as surface stabilizing agents. Embodiments of this invention may make use of cholesterol-free liposomes containing PG or PI to prevent aggregation thereby increasing the blood residence time of the carrier.

[0073] Micelles are self-assembling particles composed of amphipathic lipids or polymeric components that are utilized for the delivery of sparingly soluble agents present in the hydrophobic core. Various means for the preparation of micellar delivery vehicles are available and may be carried out with ease by one skilled in the art. For instance, lipid micelles may be prepared as described in Perkins, *et al., Int. J. Pharm.* (2000) 200(1):27-39 (incorporated herein by reference). Lipoprotein micelles can be prepared from natural or artificial lipoproteins including low and high-density lipoproteins and chylomicrons. Lipid-stabilized emulsions are micelles prepared such that they comprise an oil filled core stabilized by an emulsifying component such as a monolayer or bilayer of lipids. The core may comprise fatty acid esters such as triacylglycerol (corn oil). The monolayer or bilayer may comprise a hydrophilic polymer lipid conjugate such as DSPE-PEG. These delivery vehicles may be prepared by homogenization of the oil in the presence of the polymer lipid conjugate. Agents that are incorporated into lipid-stabilized emulsions are generally poorly water-soluble. Synthetic polymer analogues that display properties similar to lipoproteins such as micelles of stearic acid esters or poly(ethylene oxide) block-poly(hydroxyethyl-L-aspartamide) and poly(ethylene oxide)-block-poly(hydroxyhexyl-L-aspartamide) may also be used in the practice of this invention (Lavasanifar, *et al., J. Biomed. Mater. Res.* (2000) 52:831-835).

[0074] Cyclodextrins comprise cavity-forming, water-soluble, oligosaccharides that can accommodate water-insoluble drugs in their cavities. Agents can be encapsulated into cyclodextrins using procedures known to those skilled in the art. For example, see Atwood, *et al.*, Eds., "Inclusion Compounds," Vols. 2 & 3, Academic Press, NY (1984); Bender, *et al.*, "Cyclodextrin Chemistry," Springer-Verlag, Berlin (1978); Szeitli, *et al.*, "Cyclodextrins and Their Inclusion Complexes," Akademiai Kiado, Budapest, Hungary (1982) and WO 00/40962.

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[0075] Nanoparticles and microparticles may comprise a concentrated core of drug that is surrounded by a polymeric shell (nanocapsules) or as a solid or a liquid dispersed throughout a polymer matrix (nanospheres). General methods of preparing nanoparticles and microparticles are described by Soppimath, *et al.* (*J. Control Release* (2001) 70(1-2):1-20) the reference of which is incorporated herein. Other polymeric delivery vehicles that may be used include block copolymer micelles that comprise a drug containing a hydrophobic core surrounded by a hydrophilic shell; they are generally utilized as carriers for hydrophobic drugs and can be prepared as found in Allen, *et al., Colloids and Surfaces B: Biointerfaces* (1999) Nov 16(1-4):3-27. Polymer-lipid hybrid systems consist of a polymer nanoparticle surrounded by a lipid monolayer. The polymer particle serves as a cargo space for the incorporation of hydrophobic drugs while the lipid monolayer provides a stabilizing interference between the hydrophobic core and the external aqueous environment. Polymers such as polycaprolactone and poly(d,1-lactide) may be used while the lipid monolayer is typically composed of a mixture of lipid. Suitable methods of preparation are similar to those referenced above for polymer nanoparticles. Derivatized single chain polymers are polymers adapted for covalent linkage of a biologically active agent to form a polymer-drug conjugate. Numerous polymers have been proposed for synthesis of polymer-drug conjugates including polyaminoacids, polysaccharides such as dextrin or dextran, and synthetic polymers such as N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer. Suitable methods of preparation are detailed in Veronese and Morpurgo, *IL Farmaco* (1999) 54(8):497-516 and are incorporated by reference herein.

[0076] Delivery vehicles are thus provided such that consistent delivery of the administered ratio of the therapeutic components is accomplished. Thus, the ratio may be maintained by simple co-encapsulation of the agents in the vehicles that comprise the composition or the agents can be encapsulated in separate vehicles if the vehicles control the pharmacokinetics of the composition to maintain non-antagonistic drug ratios in the same manner.

[0077] Preferably, the compositions of the invention are used to deliver compositions of antitumor agents that are not antagonistic. The following detailed description sets forth the manner in which the ratios of therapeutic agents are determined and methods for encapsulating the desired ratios into the delivery systems of the invention.

[0078] Briefly, in one scenario, first, individual agents are screened separately in a variety of *in vitro* or *in vivo* assays to determine their individual activities. Then, pairs of agents are

combined and assayed in the same screening method. In this initial screen, the ratios of the agents are the mole ratios of the concentrations having 50% activity (IC_{50} value) identified previously. Alternatively, other fixed ratios (typically mole ratios of 1:10, 1:1 and 10:1) are chosen based on considerations for formulation purposes. The mean values, calculated based on agent effects on cell survival, and drug doses are entered into the CalcuSyn computer program and the output data is evaluated to define a Combination Index (CI) value as a function of the fraction of cells affected (f_a).

[0079] The CalcuSyn method has been successfully applied to test various agents such as antitumor drugs, immunosuppressants for organ transplant, combined purging of leukemic cells for autologous bone marrow transplantation, insecticides, biological response modifiers, multiple drug resistance inhibitors, anti-microbial agents, anti-HIV agents, anti-herpetic and other anti-viral agents.

[0080] Combinations of agents displaying interaction behavior similar to that of cisplatin:daunorubicin at a mole ratio of 1:1 in Figure 11A, *i.e.*, are antagonistic, and are not pursued. Combinations of compounds having non-antagonistic interactions over substantial ranges (preferably at least about 20 %) of f_a values greater than fa > 0.01 (*i.e.*, irinotecan:carboplatin at mole ratios of 1:1 and 10:1; Figure 2A) are re-evaluated in this *in vitro* screening assay at a variety of different drug/drug ratios to define the optimum ratio(s) to enhance both the strength of the non-antagonistic interaction (*i.e.*, lower CI values) and increase the f_a range over which synergy is observed.

[0081] Optimized non-antagonistic drug combinations thus identified define a composition for formulation in a delivery vehicle as a dual-agent composition and/or can be used as a single pharmaceutical unit to determine synergistic or additive interactions with a third agent.

In Vitro Determination of Non-antagonistic Ratios

[0082] In order to prepare the compositions of the invention, the desired ratio of agents contained in the delivery vehicles must first be determined. Desirably, the ratio will be that wherein synergy or additivity is exhibited by the combination over a range of concentrations. Such ratios can be determined *in vitro* in cell cultures or cell-free systems using various mathematical models.

[0083] Determination of ratios of agents that display synergistic or additive combination effects over concentration ranges may be carried out using various algorithms, based on the types of experimental data described below. These methods include isobologram methods

(Loewe, *et al., Arzneim-Forsch* (1953) 3:285-290; Steel, *et al., Int. J. Radiol. Oncol. Biol. Phys.* (1979) 5:27-55), the fractional product method (Webb, Enzyme and Metabolic Inhibitors (1963) Vol. 1, pp. 1-5. New York: Academic Press), the Monte Carlo simulation method, CombiTool, ComboStat and the Chou-Talalay median-effect method based on an equation described in Chou, *J. Theor. Biol.* (1976) 39:253-76; and Chou, *Mol. Pharmacol.* (1974) 10:235-247). Alternatives include surviving fraction (Zoli, *et al., Int. J. Cancer* (1999) 80:413-416), percentage response to granulocyte/macrophage-colony forming unit compared with controls (Pannacciulli, *et al., Anticancer Res.* (1999) 19:409-412) and others (Berenbaum, *Pharmacol. Rev.* (1989) 41:93-141; Greco, *et al., Pharmacol Rev.* (1995) 47:331-385).

[0084] The Chou-Talalay median-effect method is preferred. The analysis utilizes an equation wherein the dose that causes a particular effect, f_a, is given by:

$$D = D_m[f_a/(1-f_a)]^{1/m}$$

in which D is the dose of the drug used, f_a is the fraction of cells affected by that dose, D_m is the dose for median effect signifying the potency and m is a coefficient representing the shape of the dose-effect curve (m is 1 for first order reactions).

[0085] This equation can be further manipulated to calculate a combination index (CI) on the basis of the multiple drug effect equation as described by Chou and Talalay, *Adv. Enzyme Reg.* (1984) 22:27-55; and by Chou, *et al.*, in: Synergism and Antagonism in Chemotherapy, Chou and Rideout, eds., Academic Press: New York 1991:223-244. A computer program for this calculation (CalcuSyn) is found in Chou, Dose-effect analysis with microcomputers: quantitation of ED50, LD50, synergism, antagonism, low-dose risk, receptor ligand binding and enzyme kinetics (CalcuSyn Manual and Software; Cambridge: Biosoft 1987).

[0086] The combination index equation is based on the multiple drug-effect equation of Chou-Talalay derived from enzyme kinetic models. An equation determines only the additive effect rather than synergism and antagonism. However, according to the CalcuSyn program, synergism is defined as a more than expected additive effect, and antagonism as a less than expected additive effect. Chou and Talalay in 1983 proposed the designation of CI=1 as the additive effect, thus from the multiple drug effect equation of two drugs, we obtain:

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 \quad [\text{Eq. 1}]$$

for mutually exclusive drugs that have the same or similar modes of action, and it is further proposed that

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D_1)(D_2)/(D_x)_1(D_x)_2 \quad [\text{Eq. 2}]$$

for mutually non-exclusive drugs that have totally independent modes of action. CI <1,= 1, and >1 indicates synergism, additive effect, and antagonism, respectively. Equation 1 or equation 2 dictates that drug 1, $(D)_1$, and drug 2, $(D)_2$, (in the numerators) in combination inhibit x % in the actual experiment. Thus, the experimentally observed x % inhibition may not be a round number but most frequently has a decimal fraction. $(D_x)_1$ and $(D_x)_2$ (in the denominators) of equations 1 and 2 are the doses of drug 1 and drug 2 alone, respectively, inhibiting x %.

[0087] For simplicity, mutual exclusivity is usually assumed when more than two drugs are involved in combinations (CalcuSyn Manual and Software; Cambridge: Biosoft 1987).

[0088] The underlying experimental data are generally determined *in vitro* using cells in culture or cell-free systems. Preferably, the combination index (CI) is plotted as a function of the fraction of cells affected (f_a) as shown in Figure 1 which, as explained above, is a surrogate parameter for concentration range. Preferred combinations of agents are those that display synergy or additivity over a substantial range of f_a values. Combinations of agents are selected that display synergy over at least 5% of the concentration range wherein greater than 1% of the cells are affected, *i.e.*, an f_a range greater than 0.01. Preferably, a larger portion of overall concentration exhibits a favorable CI; for example, 5% of an f_a range of 0.2-0.8. More preferably 10% of this range exhibits a favorable CI. Even more preferably, 20 % of the f_a range, preferably over 50 % and most preferably over at least 70 % of the f_a range of 0.2 to 0.8 are utilized in the compositions. Combinations that display synergy over a substantial range of f_a values may be re-evaluated at a variety of agent ratios to define the optimal ratio to enhance the strength of the non-antagonistic interaction and increase the f_a range over which synergy is observed.

[0089] While it would be desirable to have synergy over the entire range of concentrations over which cells are affected, it has been observed that in many instances, the results are considerably more reliable in an f_a range of 0.2-0.8. Thus, although the synergy exhibited by combinations of the invention is set forth to exist within the broad range of 0.01 or greater, it is preferable that the synergy be established in the f_a range of 0.2-0.8.

[0090] The optimal combination ratio may be further used as a single pharmaceutical unit to determine synergistic or additive interactions with a third agent. In addition, a three-agent combination may be used as a unit to determine non-antagonistic interactions with a fourth agent, and so on.

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[0091] As set forth above, the *in vitro* studies on cell cultures will be conducted with "relevant" cells. The choice of cells will depend on the intended therapeutic use of the agent. Only one relevant cell line or cell culture type need exhibit the required non-antagonistic effect in order to provide a basis for the compositions to come within the scope of the invention.

[0092] For example, in one preferred embodiment of the invention, the combination of agents is intended for anticancer therapy. Appropriate choices will then be made of the cells to be tested and the nature of the test. In particular, tumor cell lines are suitable subjects and measurement of cell death or cell stasis is an appropriate end point. As will further be discussed below, in the context of attempting to find suitable non-antagonistic combinations for other indications, other target cells and criteria other than cytotoxicity or cell stasis could be employed.

[0093] For determinations involving antitumor agents, cell lines may be obtained from standard cell line repositories (NCI or ATCC for example), from academic institutions or other organizations including commercial sources. Preferred cell lines would include one or more selected from cell lines identified by the Developmental Therapeutics Program of the NCI/NIH. The tumor cell line screen used by this program currently identifies 60 different tumor cell lines representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate and kidney. The required non-antagonistic effect over a desired concentration range need be shown only on a single cell type; however, it is preferred that at least two cell lines exhibit this effect, more preferably three cell lines, more preferably five cell lines, and more preferably 10 cell lines. The cell lines may be established tumor cell lines or primary cultures obtained from patient samples. The cell lines may be from any species but the preferred source will be mammalian and in particular human. The cell lines may be genetically altered by selection under various laboratory conditions, and/or by the addition or deletion of exogenous genetic material. Cell lines may be transfected by any gene-transfer technique, including but not limited to, viral or plasmid-based transfection methods. The modifications may include the transfer of cDNA encoding the expression of a specific protein or peptide, a regulatory element such as a promoter or enhancer sequence or antisense DNA or RNA. Genetically engineered tissue culture cell lines may include lines with and without tumor suppressor genes, that is, genes such as p53, pTEN and p16; and lines created through the use of dominant negative methods, gene insertion methods and other selection methods. Preferred tissue culture cell lines that may be used to quantify cell viability, *e.g.*, to test antitumor agents,

include, but are not limited to, H460, MCF-7, SF-268, HT29, HCT-116, LS180, B16-F10, A549, Capan pancreatic, CAOV-3, IGROV1, PC-3, MX-1 and MDA-MB-231.

[0094] In one preferred embodiment, the given effect (f_a) refers to cell death or cell stasis after application of a cytotoxic agent to a cell culture. Cell death or viability may be measured, for example, using the following methods:

CYTOTOXICITY ASSAY	REFERENCE
MTT assay	Mosmann, *J. Immunol. Methods* (1983) 65(1-2):55-63.
Trypan blue dye exclusion	Bhuyan, *et al., Experimental Cell Research* (1976) 97:275-280.
Radioactive tritium (^{3}H)-thymidine incorporation or DNA intercalating assay	Senik, *et al., Int. J. Cancer* (1975) 16(6):946-959.
Radioactive chromium-51 release assay	Brunner, *et al., Immunology* (1968) 14:181-196.
Glutamate pyruvate transaminase, creatine phosphokinase and lactate dehydrogenase enzyme leakage	Mitchell, *et al., J. of Tissue Culture Methods* (1980) 6(3&4):113-116.
Neutral red uptake	Borenfreund and Puerner, *Toxicol. Lett.* (1985) 39:119-124.
Alkaline phosphatase activity	Kyle, *et al., J. Toxicol. Environ. Health* (1983) 12:99-117.
Propidium iodide staining	Nieminen, *et al., Toxicol. Appl. Pharmacol.* (1992) 115:147-155.
Bis-carboxyethyl-carboxyfluorescein (BCECF) retention	Kolber, *et al., J. Immunol. Methods* (1988) 108:255-264.
Mitochondrial membrane potential	Johnson, *et al., Proc. Natl. Acad. Sci. USA* (1980) 77:990-994.
Clonogenic Assays	Puck, *et al., J. of Experimental Medicine* (1956) 103:273-283.
LIVE/DEAD Viability/Cytotoxicity assay	Morris, *Biotechniques* (1990) 8:296-308.
Sulforhodamine B (SRB) assays	Rubinstein, *et al., J. Natl. Cancer Instit.* (1990) 82:1113-1118.

[0095] The "MTT assay" is preferred.

[0096] Non-antagonistic ratios of two or more agents can be determined for disease indications other than cancer and this information can be used to prepare therapeutic formulations of two or more drugs for the treatment of these diseases. With respect to *in vitro*

assays, many measurable endpoints can be selected from which to define drug synergy, provided those endpoints are therapeutically relevant for the specific disease.

[0097] Thus, for example, one skilled in the art will be able to define non-antagonistic ratios of two or more agents selected for treatment of inflammatory disorders by measuring, *in vitro*, suppression of proinflammatory cytokines such as IL-1, IL-18, COX-2, TNF or interferon-gamma. Other inflammatory signals include, but are not limited to, inhibition of prostaglandin E2 and thromboxane B2. In particular, endotoxin-mediated macrophage activation provides a suitable *in vitro* assay for measuring the anti-inflammatory effects of an added agent or combinations of agents and is commonly used in the art. In such an assay, macrophages grown in large quantities are activated by the addition of an endotoxin, such as lipopolysaccharide. Upon activation, macrophage secretion of cytokines such as IL-1 and TNF is measurable as well as activation of COX-2. Candidate anti-inflammatory drugs are added and evaluated based on their ability to suppress IL-1, TNF and COX-2. Titration with 1×10^{-7} M dexamethasone is typically used as a positive control. It will be apparent to those skilled in the art that assays involving macrophage activation are suitable for wide-spread screening of drug combinations and that suppression of IL-1, TNF and COX-2 are suitable endpoints for defining synergy. In addition to measuring inflammatory signals, investigators can consider the use of *in vitro* models that measure the effect of two or more agents on leukocyte functions. Functional tests can involve, but are not limited to, inhibition of degranulation, superoxide generation, and leukocyte migration.

[0098] Similar to cancer, proliferation is a key event in the development of arteriosclerosis, restenosis or other cardiovascular diseases with vasculoproliferative attributes. Thus, one skilled in the art can find non-antagonistic ratios of two or more agents by assessing drug synergy by the methods set forth herein, applied to relevant proliferating cell populations of blood vessels. In particular, restenosis, such as coronary and peripheral artery restenosis that typically results following angioplasty, is attributable to smooth muscle and endothelial cell proliferation (Fuster, *Arch Mal Coeur Vaiss* (1997) 90 Spec No 6:41-47). Using standard methods, set forth herein, one skilled in the art can measure whether two or more agents act non-antagonistically to inhibit endothelial cell or smooth muscle cell proliferation. These assays can be undertaken using immortalized cell lines or, preferably, using primary cell lines. These cell lines can be obtained from commercial sources (*e.g.*, Clonetics, California) or from fresh tissue (*e.g.*, umbilical veins, arteries, brain) and must be maintained in appropriate growth factors that promote cell proliferation. Similar to assays measuring synergy of two or more

agents on cancer cells, such assays can include, but are not limited to, endpoints of inhibition of proliferation and migration. Proliferation endpoints can rely on live/dead assays such as the MTT assay described in this application, measurements of proliferation that rely on [^{3}H]-thymidine incorporation, or other similar assays. Also similar to dividing cancer cells, proliferation of endothelial cells and smooth muscle cells is regulated by checkpoints in the cell cycle and assays that measure cell cycle inhibition can be used to define non-antagonistic ratios of two or more agents selected for treatment of vasculoproliferative disorders.

[0099] Non-antagonistic combinations of agents may also be identified for their activity against microbial or viral infections. As a first step in identifying antimicrobial agents, the minimum inhibitory concentration (MIC) for an agent can be determined by the classical microtitre broth dilution or agar dilution antimicrobial assays known to those skilled in the art. These assays are regulated by the National Committee of Laboratory Safety and Standards (NCLSS). The standard broth dilution assays are published in Amsterdam (1996) Susceptibility testing of Antimicrobials in liquid media in "Antibiotics in Laboratory Medicine", Lorian, V. 4th Edition, pages 52-111, Williams and Wilkins, Baltimore. The MIC is defined as the lowest concentration of an antibiotic that will inhibit the *in vitro* growth of an infectious organism. In the above-mentioned assays, the MIC can be determined by plating an inoculum of microbes in a small spot (at, for example, 10^4 colony-forming units [CFU] per spot) on growth medium (for example, agar) having different concentrations of the drug. Alternatively, microbes can be inoculated into a suspension of growth media that contains different concentrations of the drug. In addition, the microbes may be either treated as above or may be resident as intracellular infections in a specific cell population (*i.e.*, a macrophage). In the latter instance, mammalian cells grown in culture by standard methods are given intracellular microbial infections by brief exposure to a low concentration of microbes. After a period of time to allow the intracellular replication of the microbes, the cells and their intracellular microbes are treated with a drug in the same manner as described for cytotoxicity tests with mammalian cells. After an appropriate period of time sufficient for the drug to inhibit microbial growth when given at effective concentrations, the bacterial growth can be determined by a variety of means including: (i) determination of the absence or presence (and size, as appropriate) of the inoculum spot; (ii) plating and serial dilution of known volumes of the suspension of treated bacteria onto agar growth plates to allow calculation of the number of microbes that survived treatment; (iii) macroscopic (by eye) determination; (iv) time-kill curves in which microbes in the logarithmic phase of growth are suspended into a growth

media containing a drug(s) and, at various times after inoculation, known volumes are removed and serial diluted onto growth agar for counting of the surviving microbes; (v) other spectroscopic, analytic, *in vitro* or *in vivo* methods known by those skilled in the art to allow the counting of viable microbes. The efficacy of a drug, or combinations of drugs to kill intracellular-resident infections are typically assessed after the host cells are lysed with detergents (such as 1% Triton X-100 plus 0.1% sodium dodecyl sulfate) to release the microbes, then the lysates are serial diluted onto agar growth plates for counting of the numbers of surviving microbes.

[0100] Combinations of effective agents are assessed for their antagonistic, additive or synergistic activity using the means described above. Specifically, pairs of compounds are applied to the bacteria in fixed ratios that can be equimolar, or the ratio of the MIC values or other fixed ratios, and the bacteria treated at a variety of concentrations of the pair of compounds. Activity is determined as described above. Antagonism, additivity or synergy are determined from a variety of mathematical treatments for example by isobolograms, CI, and the like.

[0101] Extensive screening of agents or combinations of agents with antiviral activity can be performed by a number of *in vitro* assays, typically plaque reduction and cytopathic effects (CPE) inhibition assays, which are well known to those of skill in the art. These assays are able to directly measure the extent to which an antiviral drug or drugs inhibits the effects of viral infection in tissue culture. The plaque reduction assay is preferred for virus and cell line combinations which produce a well-defined plaque. Michaelis, *et al.,* demonstrated the use of plaque reduction assays combined with the Chou-Talalay method for determining non-antagonistic antiviral effects of aphidicolin and its derivatives on a number of viruses at various mole ratios (Michaelis, *et al., Arzneimittelforschung* (2002) 52(5):393-399). If a well-defined plaque is not producible by particular virus and cell line combinations, CPE inhibition assays are preferred. Additional methods for rapid and convenient identification of non-antagonistic combinations of antiviral agents include, but are not limited to, cell viability, virus yield and HIV acute or chronic infection assays. Cell viability is used to measure an antiviral agent's or combination of agent's ability to increase cell viability and can be achieved using quantitative assays such as the MTT assay previously described. Alternatively, the virus yield assay and the acute HIV infection assays evaluate an agent's ability to reduce virus yield allowing for direct measurements of antiviral activity. It will be apparent to those knowledgeable in the art that the aforementioned assays are suitable for screening antiviral

drug combinations for synergistic, additive or antagonistic effects *in vitro* and are therefore included within the scope of the invention.

Preferred Agent Combinations

[0102] Various combinations of therapeutic agents, having been found to satisfy the criteria for non-antagonistic effects set forth above, are then provided in the form of formulations of drug delivery vehicles. A "therapeutic agent" is a compound that alone, or in combination with other compounds, has a desirable effect on a subject affected by an unwanted condition or disease.

[0103] Certain therapeutic agents are favored for use in combination when the target disease or condition is cancer. Examples are:

"Signal transduction inhibitors" which interfere with or prevents signals that cause cancer cells to grow or divide;

"Cytotoxic agents";

"Cell cycle inhibitors" or "cell cycle control inhibitors" which interfere with the progress of a cell through its normal cell cycle, the life span of a cell, from the mitosis that gives it origin to the events following mitosis that divides it into daughter cells;

"Checkpoint inhibitors" which interfere with the normal function of cell cycle checkpoints, *e.g.*, the S/G2 checkpoint, G2/M checkpoint and G1/S checkpoint;

"Topoisomerase inhibitors", such as camptothecins, which interfere with topoisomerase I or II activity, enzymes necessary for DNA replication and transcription;

"Receptor tyrosine kinase inhibitors" which interfere with the activity of growth factor receptors that possess tyrosine kinase activity;

"Apoptosis inducing agents" which promote programmed cell death;

"Antimetabolites," such as Gemcitabine or Hydroxyurea, which closely resemble an essential metabolite and therefore interfere with physiological reactions involving it;

"Telomerase inhibitors" which interfere with the activity of a telomerase, an enzyme that extends telomere length and extends the lifetime of the cell and its replicative capacity;

"Cyclin-dependent kinase inhibitors" which interfere with cyclin-dependent kinases that control the major steps between different phases of the cell cycle through phosphorylation of cell proteins such as histones, cytoskeletal proteins, transcription factors, tumor suppresser genes and the like;

"DNA damaging agents";

"DNA repair inhibitors";

"Anti-angiogenic agents" which interfere with the generation of new blood vessels or growth of existing blood vessels that occurs during tumor growth; and

"Mitochondrial poisons" which directly or indirectly disrupt mitochondrial respiratory chain function.

[0104] Especially preferred combinations for treatment of tumors are the clinically approved combinations set forth hereinabove. As these combinations have already been approved for use in humans, reformulation to assure appropriate delivery is especially important.

[0105] Preferred agents that may be used in combination include DNA damaging agents such as carboplatin, cisplatin, cyclophosphamide, doxorubicin, daunorubicin, epirubicin, mitomycin C, mitoxantrone; DNA repair inhibitors including 5-fluorouracil (5-FU) or FUDR, gemcitabine and methotrexate; topoisomerase I inhibitors such as camptothecin, irinotecan and topotecan; S/G2 or G2/M checkpoint inhibitors such as bleomycin, docetaxel, doxorubicin, etoposide, paclitaxel, vinblastine, vincristine, vindesine and vinorelbine; G1/early-S checkpoint inhibitors; G2/M checkpoint inhibitors; receptor tyrosine kinase inhibitors such as genistein, trastuzumab, ZD1839; cytotoxic agents; apoptosis-inducing agents and cell cycle control inhibitors.

[0106] The mechanism of action of one or more of the agents may not be known or may be incorrectly identified. All synergistic or additive combinations of agents are within the scope of the present invention. Preferably, for the treatment of a neoplasm, combinations that inhibit more than one mechanism that leads to uncontrolled cell proliferation are chosen for use in accordance with this invention. For example, the present invention includes selecting combinations that effect specific points within the cell cycle thereby resulting in non-antagonistic effects. For instance, drugs that cause DNA damage can be paired with those that inhibit DNA repair, such as anti-metabolites. The present invention also includes selecting combinations that block multiple pathways that would otherwise result in cell proliferation.

[0107] Particularly preferred combinations are DNA damaging agents in combination with DNA repair inhibitors, DNA damaging agents in combination with topoisomerase I or topoisomerase II inhibitors, topoisomerase I inhibitors in combination with S/G2 or G2/M checkpoint inhibitors, G1/S checkpoint inhibitors or CDK inhibitors in combination with G2/M checkpoint inhibitors, receptor tyrosine kinase inhibitors in combination with cytotoxic agents, apoptosis-inducing agents in combination with cytotoxic agents, apoptosis-inducing agents in

combination with cell-cycle control inhibitors, G1/S or G2/M checkpoint inhibitors in combination with cytotoxic agents, topoisomerase I or II inhibitors in combination with DNA repair inhibitors, topoisomerase I or II inhibitors or telomerase inhibitors in combination with cell cycle control inhibitors, topoisomerase I inhibitors in combination with topoisomerase II inhibitors, and two cytotoxic agents in combination.

[0108] Specific agents that may be used in combination include cisplatin (or carboplatin) and 5-FU (or FUDR), cisplatin (or carboplatin) and irinotecan, irinotecan and 5-FU (or FUDR), vinorelbine and cisplatin (or carboplatin), methotrexate and 5-FU (or FUDR), idarubicin and araC, cisplatin (or carboplatin) and taxol, cisplatin (or carboplatin) and etoposide, cisplatin (or carboplatin) and topotecan, cisplatin (or carboplatin) and daunorubicin, cisplatin (or carboplatin) and doxorubicin, cisplatin (or carboplatin) and gemcitabine, oxaliplatin and 5-FU (or FUDR), gemcitabine and 5-FU (or FUDR), adriamycin and vinorelbine, taxol and doxorubicin, flavopuridol and doxorubicin, UCN01 and doxorubicin, bleomycin and trichlorperazine, vinorelbine and edelfosine, vinorelbine and sphingosine (and sphingosine analogues), vinorelbine and phosphatidylserine, vinorelbine and camptothecin, cisplatin (or carboplatin) and sphingosine (and sphingosine analogues), sphingosine (and sphingosine analogues) and daunorubicin and sphingosine (and sphingosine analogues) and doxorubicin.

[0109] Preferred combinations in general include those set forth hereinabove as already shown to be efficacious in the clinic as recognized by the FDA and those further suggested based on literature reports. While the candidate agents for use in the method of the invention are not limited to these specific combinations, those set forth hereinabove have been disclosed as suitable combination therapies, and are thus preferred for use in the methods and compositions of the present invention.

[0110] Some lipids are "therapeutic lipids" that are able to exert therapeutic effects such as inducing apoptosis. Included in this definition are lipids such as ether lipids, phosphatidic acid, phosphonates, ceramide and ceramide analogues, dihydroxyceramide, phytoceramide, sphingosine, sphingosine analogues, sphingomyelin, serine-containing lipids and sphinganine The term "serine-containing phospholipid" or "serine-containing lipid" as defined herein is a phospholipid in which the polar head group comprises a phosphate group covalently joined at one end to a serine and at the other end to a three-carbon backbone connected to a hydrophobic portion through an ether, ester or amide linkage. Included in this class are the phospholipids such as phosphatidylserine (PS) that have two hydrocarbon chains in the hydrophobic portion

that are between 5-23 carbon atoms in length and have varying degrees of saturation. The term hydrophobic portion with reference to a serine-containing phospholipid or serine-containing lipid refers to apolar groups such as long saturated or unsaturated aliphatic hydrocarbon chains, optionally substituted by one or more aromatic, alicyclic or heterocyclic group(s).

[0111] Combinations of therapeutic lipids and other agents can also be used to achieve synergistic or additive effects (see Examples 17-21).

High Throughput Screening for Determining Ratios That Display Non-antagonistic Combination Effects

[0112] Chemical libraries of agents may be screened against one another at different ratios to identify novel non-antagonistic drug combinations. Chemical libraries may comprise novel or conventional agents. In addition to screening for two agent combinations, three or four agent combinations may also be screened for non-antagonistic combination effects. Preferably, the data analysis methodology employed to determine drug synergy is the aforementioned Median Effect Analysis. According to this method, libraries of agents are tested individually and in combination at different ratios. Combination indexes are then calculated using the aforementioned method developed by Chou and Talalay. Drug combinations that display non-antagonistic effects at specific ratios are encapsulated in delivery vehicles at a non-antagonistic ratio.

[0113] High throughput screening systems are commercially available (see, *e.g.*, Zymark Corp., Hopkinton, Mass.; Air Technical Industries, Mentor, Ohio; Beckman Instruments, Inc. Fullerton, Calif.; Precision Systems, Inc., Natick, Mass., etc.). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start-up, as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols for the various high throughput screening methods.

Preparation of Non-Antagonistic Compositions

[0114] When the appropriate ratios of the agents have been determined as described above, the agents at the appropriate ratio are placed into one or more delivery vehicle compositions wherein one or more delivery vehicles encapsulates two or more agents. Not all the delivery vehicles in the composition need be identical. The delivery vehicles in the compositions are

particles of sizes that depend on their route of administration, which can be suspended in an aqueous or other solvent and are able to encapsulate the agents of the invention. Such vehicles include, for example, lipid carriers, liposomes, cyclodextrins, polymer nanoparticles and polymer microparticles, including nanocapsules and nanospheres, block copolymer micelles, lipid stabilized emulsions, derivatized single-chain polymers, polymer lipid hybrid systems, lipid micelles, lipoprotein micelles as mentioned previously. For intravenous administration, delivery vehicles are typically about 4-6,000 nm in diameter. Preferred diameters are about 5-500 nm in diameter, more preferably 5-200 nm in diameter. For inhalation, intra-thecal, intra-articular, intra-arterial, intra-peritoneal or subcutaneous administration, delivery vehicles are typically from 4 μm to an excess of 50 μm. Delivery vehicle compositions designed for intra-ocular administration are generally smaller.

[0115] As explained above, the biologically active agents may be formulated into a single composition at the predetermined ratio, or separate compositions comprising delivery vehicles with coordinated pharmacokinetics can be employed along with instructions for administering these compositions in a proportion consistent with the predetermined ratio. Thus, the desired ratio may be achieved by administering the agents in separate compositions simultaneously or sequentially in the proportion described.

[0116] The therapeutic agents are "encapsulated" in the delivery vehicles. "Encapsulation," as previously described, includes covalent or non-covalent association of an agent with the delivery vehicle. For example, this can be by interaction of the agent with the outer layer or layers of the delivery vehicle or entrapment of an agent within the delivery vehicle, equilibrium being achieved between different portions of the delivery vehicle. For example, for liposomes, encapsulation of an agent can be by association of the agent by interaction with the bilayer of the liposomes through covalent or non-covalent interaction with the lipid components or entrapment in the aqueous interior of the liposome, or in equilibrium between the internal aqueous phase and the bilayer. For polymer-based delivery vehicles, encapsulation can refer to covalent linkage of an agent to a linear or non-linear polymer. Further, non-limiting examples include the dispersion of agent throughout a polymer matrix, or the concentration of drug in the core of a nanocapsule, a block copolymer micelle or a polymer-lipid hybrid system. "Loading" refers to the act of encapsulating one or more agents into a delivery vehicle.

[0117] Encapsulation of the desired combination can be achieved either through encapsulation in separate delivery vehicles or within the same delivery vehicle. Where

encapsulation into separate delivery vehicles, such as liposomes, is desired, the lipid composition of each liposome may be quite different to allow for coordinated pharmacokinetics. By altering the vehicle composition, release rates of encapsulated drugs can be matched to allow non-antagonistic ratios of the drugs to be delivered to the tumor site. Means of altering release rates include increasing the acyl-chain length of vesicle forming lipids to improve drug retention, controlling the exchange of surface grafted hydrophilic polymers such as PEG out of the liposome membrane and incorporating membrane-rigidifying agents such as sterols or sphingomyelin into the membrane. It should be apparent to those skilled in the art that if a first and second drug are desired to be administered at a specific drug ratio and if the second drug is retained poorly within the liposome composition of the first drug (*e.g.*, DMPC/Chol), that improved pharmacokinetics may be achieved by encapsulating the second drug in a liposome composition with lipids of increased acyl chain length (*e.g.*, DSPC/Chol). Alternatively, two or more agents may be encapsulated within the same delivery vehicle.

[0118] Techniques for encapsulation are dependent on the nature of the delivery vehicles. For example, therapeutic agents may be loaded into liposomes using both passive and active loading methods.

[0119] Passive methods of encapsulating agents in liposomes involve encapsulating the agent during the preparation of the liposomes. In this method, the drug may be membrane associated or encapsulated within an entrapped aqueous space. This includes a passive entrapment method described by Bangham, *et al.*, *J. Mol. Biol.* (1965) 12:238, where the aqueous phase containing the agent of interest is put into contact with a film of dried vesicle-forming lipids deposited on the walls of a reaction vessel. Upon agitation by mechanical means, swelling of the lipids will occur and multilamellar vesicles (MLV) will form. Using extrusion, the MLVs can be converted to large unilamellar vesicles (LUV) or small unilamellar vesicles (SUV). Another method of passive loading that may be used includes that described by Deamer and Bangham, *Biochim. Biophys. Acta* (1976) 443:629. This method involves dissolving vesicle-forming lipids in ether and, instead of first evaporating the ether to form a thin film on a surface, this film being thereafter put into contact with an aqueous phase to be encapsulated, the ether solution is directly injected into said aqueous phase and the ether is evaporated afterwards, whereby liposomes with encapsulated agents are obtained. A further method that may be employed is the Reverse Phase Evaporation (REV) method described by Szoka and Papahadjopoulos, *P.N.A.S.* (1978) 75:4194, in which a solution of lipids in a water

insoluble organic solvent is emulsified in an aqueous carrier phase and the organic solvent is subsequently removed under reduced pressure.

[0120] Other methods of passive entrapment that may be used include subjecting liposomes to successive dehydration and rehydration treatment, or freezing and thawing. Dehydration is carried out by evaporation or freeze-drying. This technique is disclosed by Kirby, *et al., Biotechnology* (1984) 979-984. Also, Shew and Deamer (*Biochim. et Biophys. Acta* (1985) 816:1-8) describe a method wherein liposomes prepared by sonication are mixed in aqueous solution with the solute to be encapsulated, and the mixture is dried under nitrogen in a rotating flask. Upon rehydration, large liposomes are produced in which a significant fraction of the solute has been encapsulated.

[0121] Passive encapsulation of two or more agents is possible for many drug combinations. This approach is limited by the solubility of the drugs in aqueous buffer solutions and the large percentage of drug that is not trapped within the delivery system. The loading may be improved by co-lyophilizing the drugs with the lipid sample and rehydrating in the minimal volume allowed to solubilize the drugs. The solubility may be improved by varying the pH of the buffer, increasing temperature or addition or removal of salts from the buffer.

[0122] Active methods of encapsulating may also be used. For example, liposomes may be loaded according to a metal-complexation or pH gradient loading technique. With pH gradient loading, liposomes are formed which encapsulate an aqueous phase of a selected pH. Hydrated liposomes are placed in an aqueous environment of a different pH selected to remove or minimize a charge on the drug or other agent to be encapsulated. Once the drug moves inside the liposome, the pH of the interior results in a charged drug state, which prevents the drug from permeating the lipid bilayer, thereby entrapping the drug in the liposome.

[0123] To create a pH gradient, the original external medium can be replaced by a new external medium having a different concentration of protons. The replacement of the external medium can be accomplished by various techniques, such as, by passing the lipid vesicle preparation through a gel filtration column, *e.g.*, a Sephadex G-50 column, which has been equilibrated with the new medium (as set forth in the examples below), or by centrifugation, dialysis, or related techniques. The internal medium may be either acidic or basic with respect to the external medium.

[0124] After establishment of a pH gradient, a pH gradient loadable agent is added to the mixture and encapsulation of the agent in the liposome occurs as described above.

[0125] Loading using a pH gradient may be carried out according to methods described in U.S. patent Nos. 5,616,341, 5,736,155 and 5,785,987 incorporated herein by reference. A preferred method of pH gradient loading is the citrate-based loading method utilizing citrate as the internal buffer at a pH of 2-6 and a neutral external buffer.

[0126] Various methods may be employed to establish and maintain a pH gradient across a liposome all of which are incorporated herein by reference. This may involve the use of ionophores that can insert into the liposome membrane and transport ions across membranes in exchange for protons (see for example U.S. patent No. 5,837,282). Compounds encapsulated in the interior of the liposome that are able to shuttle protons across the liposomal membrane and thus set up a pH gradient (see for example U.S. patent No. 5,837,282) may also be utilized. These compounds comprise an ionizable moiety that is neutral when deprotonated and charged when protonated. The neutral deprotonated form (which is in equilibrium with the protonated form) is able to cross the liposome membrane and thus leave a proton behind in the interior of the liposome and thereby cause an decrease in the pH of the interior. Examples of such compounds include methylammonium chloride, methylammonium sulfate, ethylenediammonium sulfate (see U.S. patent No. 5,785,987) and ammonium sulfate. Internal loading buffers that are able to establish a basic internal pH, can also be utilized. In this case, the neutral form is protonated such that protons are shuttled out of the liposome interior to establish a basic interior. An example of such a compound is calcium acetate (see U.S. patent No. 5,939,096).

[0127] Two or more agents may be loaded into a liposome using the same active loading methods or may involve the use of different active loading methods. For instance, metal complexation loading may be utilized to actively load multiple agents or may be coupled with another active loading technique, such as pH gradient loading. Metal-based active loading typically uses liposomes with passively encapsulated metal ions (with or without passively loaded therapeutic agents). Various salts of metal ions are used, presuming that the salt is pharmaceutically acceptable and soluble in an aqueous solutions. Actively loaded agents are selected based on being capable of forming a complex with a metal ion and thus being retained when so complexed within the liposome, yet capable of loading into a liposome when not complexed to metal ions. Agents that are capable of coordinating with a metal typically comprise coordination sites such as amines, carbonyl groups, ethers, ketones, acyl groups, acetylenes, olefins, thiols, hydroxyl or halide groups or other suitable groups capable of donating electrons to the metal ion thereby forming a complex with the metal ion. Examples

of active agents which bind metals include, but are not limited to, quinolones such as fluoroquinolones; quinolones such as nalidixic acid; anthracyclines such as doxorubicin, daunorubicin and idarubicin; amino glycosides such as kanamycin; and other antibiotics such as bleomycin, mitomycin C and tetracycline; and nitrogen mustards such as cyclophosphamide, thiosemicarbazones, indomethacin and nitroprusside; camptothecins such as topotecan, irinotecan, lurtotecan, 9-aminocamptothecin, 9-nitrocamptothecin and 10-hydroxycamptothecin; and podophyllotoxins such as etoposide. Uptake of an agent may be established by incubation of the mixture at a suitable temperature after addition of the agent to the external medium. Depending on the composition of the liposome, temperature and pH of the internal medium, and chemical nature of the agent, uptake of the agent may occur over a time period of minutes or hours. Methods of determining whether coordination occurs between an agent and a metal within a liposome include spectrophotometric analysis and other conventional techniques well known to those of skill in the art.

[0128] Furthermore, liposome loading efficiency and retention properties using metal-based procedures carried out in the absence of an ionophore in the liposome are dependent on the metal employed and the lipid composition of the liposome. By selecting lipid composition and a metal, loading or retention properties can be tailored to achieve a desired loading or release of a selected agent from a liposome.

[0129] Passive and active loading methods may be combined sequentially in order to load multiple drugs into a delivery vehicle. By way of example, liposomes containing a passively entrapped platinum drug such as cisplatin in the presence of $MnCl_2$ may subsequently be used to actively encapsulate an anthracycline such as doxorubicin into the interior of the liposome. This method is likely to be applicable to numerous drugs that are encapsulated in liposomes through passive encapsulation.

Kits

[0130] The therapeutic agents in the invention compositions may be formulated separately in individual compositions wherein each therapeutic agent is stably associated with appropriate delivery vehicles. These compositions can be administered separately to subjects as long as the pharmacokinetics of the delivery vehicles are coordinated so that the ratio of therapeutic agents administered is maintained at the target for treatment. Thus, it is useful to construct kits which include, in separate containers, a first composition comprising delivery vehicles stably associated with at least a first therapeutic agent and, in a second container, a second

composition comprising delivery vehicles stably associated with at least one second therapeutic agent. The containers can then be packaged into the kit.

[0131] The kit will also include instructions as to the mode of administration of the compositions to a subject, at least including a description of the ratio of amounts of each composition to be administered. Alternatively, or in addition, the kit is constructed so that the amounts of compositions in each container is pre-measured so that the contents of one container in combination with the contents of the other represent the correct ratio. Alternatively, or in addition, the containers may be marked with a measuring scale permitting dispensation of appropriate amounts according to the scales visible. The containers may themselves be useable in administration; for example, the kit might contain the appropriate amounts of each composition in separate syringes. Formulations which comprise the pre-formulated correct ratio of therapeutic agents may also be packaged in this way so that the formulation is administered directly from a syringe prepackaged in the kit.

Therapeutic Uses of Delivery Vehicle Compositions Encapsulating Multiple Agents

[0132] These delivery vehicle compositions may be used to treat a variety of diseases in warm-blooded animals and in avian species. Thus, suitable subjects for treatment according to the methods and compositions of the invention include humans, mammals such as livestock or domestic animals, domesticated avian subjects such as chickens and ducks, and laboratory animals for research use. Examples of medical uses of the compositions of the present invention include treating cancer, treating cardiovascular diseases such as hypertension, cardiac arrhythmia and restenosis, treating bacterial, viral, fungal or parasitic infections, treating and/or preventing diseases through the use of the compositions of the present inventions as vaccines, treating inflammation or treating autoimmune diseases.

[0133] In one embodiment, delivery vehicle compositions in accordance with this invention are preferably used to treat neoplasms. Delivery of formulated drug to a tumor site is achieved by administration of liposomes or other particulate delivery systems. Preferably liposomes have a diameter of less than 200 nm. Tumor vasculature is generally leakier than normal vasculature due to fenestrations or gaps in the endothelia. This allows the delivery vehicles of 200 nm or less in diameter to penetrate the discontinuous endothelial cell layer and underlying basement membrane surrounding the vessels supplying blood to a tumor. Selective accumulation of the delivery vehicles into tumor sites following extravasation leads to enhanced drug delivery and therapeutic effectiveness. Because carriers extravasate, it can be

assumed that the carrier drug-to-drug ratio determined in the blood will be comparable to the carrier drug-to-drug ratio in the extravascular space.

Administering Delivery Vehicle Compositions

[0134] As mentioned above, the delivery vehicle compositions of the present invention may be administered to warm-blooded animals, including humans as well as to domestic avian species. For treatment of human ailments, a qualified physician will determine how the compositions of the present invention should be utilized with respect to dose, schedule and route of administration using established protocols. Such applications may also utilize dose escalation should agents encapsulated in delivery vehicle compositions of the present invention exhibit reduced toxicity to healthy tissues of the subject.

[0135] Preferably, the pharmaceutical compositions of the present invention are administered parenterally, *i.e.*, intraarterially, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For example, see Rahman, *et al.*, U.S. patent No. 3,993,754; Sears, U.S. patent No. 4,145,410; Papahadjopoulos, *et al.*, U.S. patent No. 4,235,871; Schneider, U.S. patent No. 4,224,179; Lenk, *et al.*, U.S. patent No. 4,522,803; and Fountain, *et al.*, U.S. patent No. 4,588,578.

[0136] In other methods, the pharmaceutical preparations of the present invention can be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical", it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures that include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as

commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

[0137] Pharmaceutical compositions comprising delivery vehicles of the invention are prepared according to standard techniques and may comprise water, buffered water, 0.9% saline, 0.3% glycine, 5% dextrose and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, and the like. These compositions may be sterilized by conventional, well-known sterilization techniques. 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 solution prior to administration. The compositions may contain 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, and the like. Additionally, the delivery vehicle 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.

[0138] The concentration of delivery vehicles in the pharmaceutical formulations can vary widely, such as from less than about 0.05%, usually at or at least about 2-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, and the like, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. Alternatively, delivery vehicles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. For diagnosis, the amount of delivery vehicles administered will depend upon the particular label used, the disease state being diagnosed and the judgment of the clinician.

[0139] Preferably, the pharmaceutical compositions of the present invention are administered intravenously. Dosage for the delivery vehicle formulations will depend on the ratio of drug to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

[0140] In addition to pharmaceutical compositions, suitable formulations for veterinary use may be prepared and administered in a manner suitable to the subject. Preferred veterinary subjects include mammalian species, for example, non-human primates, dogs, cats, cattle,

horses, sheep, and domesticated fowl. Subjects may also include laboratory animals, for example, in particular, rats, rabbits, mice, and guinea pigs.

[0141] In the instance where a single composition containing more than one active agent is included, the above procedures are followed *per se*. Where the agents are administered in separate delivery vehicle compositions, the administration should be timed in such a manner that the desired ratio is maintained. Typically, this can accomplished by simultaneously administering the compositions in the calculated proportions.

Evaluation of Therapeutic Activity *In Vivo*

[0142] Therapeutic activity of delivery vehicle compositions comprising two or more encapsulated agents may be measured after administration into an animal model. Preferably, the animal model comprises a tumor although delivery vehicle compositions may be administered to animal models of other diseases. Rodent species such as mice and rats of either inbred, outbred, or hybrid origin including immunocompetent and immunocompromised, as well as knockout, or transgenic models may be used.

[0143] Models can consist of solid or non–solid tumors implanted as cell suspensions, bries or tumor fragments in either subcutaneous, intravenous, intraperitoneal, intramuscular, intrathecal, or orthotopic regions. Tumors may also be established via the application or administration of tumorigenic/carcinogenic agents or may be allowed to arise spontaneously in appropriate genetically engineered animal models. Tumor types can consist of tumors of ectodermal, mesodermal, or endodermal origin such as carcinomas, sarcomas, melanomas, gliomas, leukemias and lymphomas.

[0144] In a preferred embodiment, mouse models of tumors are employed. Human xenograft solid tumors grown in immune compromised mice may be utilized and selected on the basis of defined genetics and growth attributes. Tumor cells utilized in these experiments can be genetically manipulated or selected to express preferable properties and are injected into mice.

[0145] Once the tumors have grown to a palpable (measurable) size, delivery vehicle compositions can be administered, preferably intravenously, and their effects on tumor growth are monitored. Intended therapeutic treatments can consist of single bolus or push administrations or multiple or continuous administrations over several days or weeks and by any appropriate route such as by the oral, nasal, subcutaneous, intravenous, intraperitoneal, intrathecal, intratumoral routes using syringes, tablets, liquids, and pumps (such as osmotic).

Dose and schedule dependency may be evaluated in order to determine the maximum anti-tumor activity that can be achieved.

[0146] Various methods of determining therapeutic activity in animal models comprising a tumor may be utilized. This includes solid tumor model evaluation methods and non-solid tumor model evaluation methods.

[0147] Solid tumor model evaluation methods include measurement of tumor volume (mass), tumor weight inhibition (TWI%), tumor growth delay (T-C), tumor regression, cell kill and clonogenic assays.

[0148] Tumor volume measurements are determined from vernier caliper measurements of perpendicular length and width measurements (height measurements can often be obtained as well). Tumor volume (mL) or mass (g) is calculated from: volume = (length x width^2/2; or volume = π/6 x (length x width x height). Data is plotted with respect to time.

[0149] Tumor weight inhibition (TWI%) is determined by measuring the mean tumor weight of a treated group divided by the mean tumor weight of a control group, minus 1 X 100 at a defined time point.

[0150] Tumor growth delay (T-C) is measured as the median time in days for a treated group (T) to reach an arbitrarily determined tumor size (for example, 300 mg) minus median time in days for the control group to reach the same tumor size.

[0151] Tumor regression as a result of treatment may also be used as a means of evaluating a tumor model. Results are expressed as reductions in tumor size (mass) over time.

[0152] Cell kill methods of solid tumor model evaluation can involve measuring tumors repeatedly by calipers until all exceed a predetermined size (*e.g.*, 200 mg). The tumor growth and tumor doubling time can then be evaluated. Log_{10} cell kill parameters can be calculated by:

$$\log_{10} \text{cell kill / dose} = (T–C)/((3.32)(T_d)(\text{No. of doses}))$$

$$\log_{10} \text{cell kill (total)} = (T–C)/((3.32(T_d))$$

$$\log_{10} \text{cell kill (net)} = ((T–C) – (\text{duration of } R_x))/((3.32(T_d))$$

Where: $(T – C)$ = tumor growth delay
T_d = Tumor doubling time

[0153] Clonogenicity assays express the effectiveness of therapy. These assays include excision assays and characterization of cell suspensions from solid tumors.

[0154] Excision assays, used to assess what fraction of cells, in a suspension prepared from tumors, have unlimited proliferative potential (*i.e.*, are clonogenic). Three types of excision assays are:

i) TD_{50}, or endpoint dilution assays: determines the number of cells required to produce tumor takes from inocula *in vivo*.

ii) *In vivo* colony assay: assesses the ability of individual cells to form nodules (colonies) in, for example, the lung.

iii) *In vitro* colony assay, tests the ability of individual cells to grow into colonies either in liquid media, when colonies form on the plastic or glass surface of culture dishes, or in semisolid media such as agar, in which the colonies form in suspension.

[0155] Characterization of cell suspensions from solid tumors are required for *in vitro* and *in vivo* clonogenic assays, flow-cytometric measurements, and for numerous biochemical and molecular analyses performed on a per cell basis. Preparation is by a number of methods such as enzymatic, mechanical, chemical, combinations thereof, and surface activity agents. Evaluations could include, cell yield, cell morphology, tumor cell clonogenicity, retention of biochemical or molecular characteristics.

[0156] Non-solid tumor model evaluation methods include measurement of increase in life span (ILS%), tumor growth delay (T-C), long-term survivors (cures).

[0157] Increase in life-span (ILS%) measures the percentage increase in life-span of treated groups versus control or untreated groups. Tumor growth delay (T-C) measures median time in days for treated (T) group survival minus median time in days for control (C) group survival. Long-term survivors (cures) measures treatment groups that survive up to and beyond 3-times the survival times of untreated or control groups.

[0158] Methods of determining therapeutic activity in humans afflicted with cancer include measurements of survival and surrogate endpoints. The time at which survival is reasonably evaluated depends on the tumor in question. By way of example, survival rates for patients with low-grade lymphomas may be examined at 5 or 10 years post diagnosis, whereas the survival or patients having aggressive diseases such as advanced non-small cell lung cancer may be best evaluated at 6 or 12 months post diagnosis.

[0159] Methods of determining therapeutic activity using surrogate endpoints includes measuring complete response (CR), partial response (PR), progression-free survival (PFS),

time-to-progression (TTP) or duration of response (DOR), plasma and urine markers, enzyme inhibition and/or receptor status, changes in gene expression and quality of life (QOL).

[0160] A complete response means the disappearance of all known sites of disease without the development of any new disease for a period of time appropriate for the tumor type being treated. Assessments are based on a variety of examinations such as those stated above.

[0161] Partial response is at least a 50% decrease in the sum of the products of the bidimensional measurement of all lesions with no new disease appearing for a period of time appropriate for the tumor type being treated. Assessments are based on a variety of examinations (CT scan, MRI, ultrasound, PET scan, bone scan, physical examination) of patients.

[0162] Progression-free Survival (PFS): Duration from treatment in which a patient survives and there is no growth of existing tumor nor appearance of new tumor masses. PFS may be expressed as either the duration of time or as the proportion of patients who are surviving and progression-free at a given time after diagnosis.

[0163] Time-to-progression (TTP) or duration of response (DOR) refer to the duration of time from treatment to a progression of tumor growth, measured either as an increase in size of existing tumor masses or the appearance of new tumor masses.

[0164] Plasma and urine markers include measuring markers such as, but not limited to, the following markers: prostate specific antigen (PSA) and carcinoembryonic antigen (CEA).

[0165] Enzyme inhibition and/or receptor status. Growth factor receptors such as, but not limited to, tyrosine kinase receptors, EGF receptor, PDGF receptor, Her-1 and Her-2 receptors. Enzymes such as, but not limited to, integrin-linked kinases, protein kinases and the like.

[0166] Changes in gene expression include serial analysis of gene expression (genomics) and changes in protein expression (proteomics).

[0167] Quality of Life (QOL) include methods such as the EORTC QLQ-C30 scoring method that evaluates yields scores for five functional scales (physical, role, cognitive, social, and emotional), three symptom scales (nausea, pain, and fatigue), and a global health and quality of life scale. The measure also yields single-item ratings of additional symptoms commonly reported by cancer patients (dyspnea, appetite loss, sleep disturbance, constipation, and diarrhea) as well as the perceived financial impact of the disease and its treatment.

[0168] The following examples are given for the purpose of illustration and are not by way of limitation on the scope of the invention.

EXAMPLES

[0169] The examples below employ the following methods of determining cytotoxicity and for evaluating non-antagonistic effects.

Cytotoxicity Assay

[0170] In the following examples the standard tetrazolium-based colorimetric MTT cytotoxicity assay protocol (Mosmann, *et al., J. Immunol Methods* (1983) 65(1-2):55-63) was utilized to determine the readout for the fraction of cells affected. Briefly, viable cells reduce the tetrazolium salt, 3-(4,5-diethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan which can be read spectrophotometrically. Cells, such as human H460 non-small-cell lung carcinoma (NSCLC) cells grown in 25 cm^2 flasks are passaged (passage number <20), resuspended in fresh RPMI cell culture medium and added into 96-well cell culture plates at a concentration of 1000 cells per well in 100 μL per well. The cells are then allowed to incubate for 24 hours at 37°C, 5% CO_2. The following day, serial drug dilutions are prepared in 12-well cell culture plates. The agents, previously prepared in various solutions, are diluted in fresh RPMI cell culture media. Agents are administered to the appropriate or specified wells for single agents (20 μL) and at specific fixed ratio dual agent combinations (increments of 20 μL) using a Latin square design or "checkerboard" dilution method. The total well volumes are made up to 200 μL with fresh media. The drug exposure is for a duration of 72 hours.

[0171] Following drug exposure, MTT reagent (1 mg/mL in RPMI) is added to each well at a volume of 50 μL per well and incubated for 3-4 hours. The well contents are then aspirated and 150 μL of dimethylsulfoxide (DMSO) is added to each well to disrupt the cells and to solubilize the formazan precipitate within the cells. The 96-well plates are shaken on a plate shaker, and read on a microplate spectrophotometer set at a wavelength of 570 nm. The optical density (OD) readings are recorded and the OD values of the blank wells (containing media alone) are subtracted from all the wells containing cells. The cell survival following exposure to agents is based as a percentage of the control wells (cells not exposed to drug). All wells are performed in triplicate and mean values are calculated.

Median-Effect Analysis for Drug Combinations

[0172] For the drug combination analysis, the software program CalcuSyn, (Biosoft, Ferguson, MO, USA) based on the median-effect principle by Chou and Talalay, was utilized.

The fixed ratios for the dual-agent combinations are initially derived from the IC_{50}:IC_{50} ratios from single agent cytotoxicity profiles. Subsequently, more relevant fixed ratios (e.g. ranging from 10:1 to 1:10; mole ratios) are chosen based upon considerations for formulation purposes. From the mean values calculated based on agent effects on cell survival, doses and respective fractional effect values are entered into the CalcuSyn computer program. The software then determines whether the drug combinations are synergistic, additive or antagonistic based on combination index (CI) values.

Example 1

Multiple Representation of Dose-Effect Analysis

[0173] Quantitative analysis of the relationship between an amount (dose or concentration) of drug and its biological effect as well as the joint effect of drug combinations can be measured and reported in a number of ways. Figure 2 illustrates 5 such methods using, as an example, a combination of irinotecan and carboplatin.

[0174] Based on Chou and Talalay's theory of dose-effect analysis, a "median-effect equation" has been used to calculate a number of biochemical equations that are extensively used in the art. Derivations of this equation have given rise to higher order equations such as those used to calculate Combination Index (CI). As mentioned previously, CI can be used to determine if combinations of more than one drug and various ratios of each combination are antagonistic, additive or synergistic. CI plots are typically illustrated with CI representing the y-axis versus the proportion of cells affected, or fraction affected (f_a), on the x-axis. Figure 2A demonstrates that a 1:10 mole ratio of irinotecan/carboplatin is antagonistic ($CI > 1.1$), while 1:1 and 10:1 have a synergistic effect ($CI < 0.9$).

[0175] The present applicants have also designed an alternative method of representing the dependency of CI on the drug ratios used. The maximum CI value is plotted against each ratio to better illustrate trends in ratio-specific effects for a particular combination as seen in Figure 2B. The CI maximum is the CI value taken at a single f_a value (between 0.2 and 0.8) where the greatest difference in CI values for the drugs at different ratios was observed.

[0176] Because the concentrations of drugs used for an individual ratio play a role in determining the effect (*i.e.*, synergism or antagonism), it can also be important to measure the CI at various concentrations. These concentrations, also referred to as "Effective Doses" (ED) by Chou-Talalay, are the concentration of drug required to affect a designated percent of the cells in an *in vitro* assay, *i.e.*, ED_{50} is the concentration of drug required to affect 50% of the

cells relative to a control or untreated cell population. As shown in Figure 2C, trends in concentration-effect are readily distinguished between the various ratios. The error bars shown represent one standard deviation around the mean and is determined directly through the CalcuSyn program.

[0177] A synergistic interaction between two or more drugs has the benefit that it can lower the amount of each drug required in order to result in a positive effect, otherwise known as "dose-reduction." Chou and Talalay's "dose-reduction index" (DRI) is a measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses for each drug alone. DRI has been important in clinical situations, where dose-reduction leads to reduced toxicity for the host while maintaining therapeutic efficacy. The plot in Figure 2D shows that the concentrations of irinotecan and carboplatin required to achieve a 90% cell kill on their own is significantly higher than their individual concentrations required when they are combined at a non-antagonistic ratio.

[0178] Furthermore the aforementioned data can be represented in a classical isobologram (Figure 2E). Isobolograms have the benefit that they can be generated at different ED values; however, they become more difficult to read as more effect levels are selected for interpretation. For this reason, the data in the examples below are generally presented in accordance with the types of plots shown in Figures 2A and 2B.

Example 2

CI is Dependent upon Concentrations

[0179] Drug combinations of irinotecan and 5-Fluorouracil (5-FU) at mole ratios of 1:1 and 1:10 and etoposide and carboplatin at mole ratios of 10:1 and 1:10 were tested for additive, synergistic or antagonistic effects using the standard tetrazolium-based colorimetric MTT cytotoxicity assay and the median-effect analysis as described in the previous example sections. HT29 or MCF-7 cells were exposed to single agents as well as agents in combination at defined ratios. Eight drug concentrations were utilized for single agents and combinations. Optical density values were obtained from the MTT assay, converted into a percentage of the control, averaged and then converted into fraction affected values. Dose and fraction affected values were entered into CalcuSyn which yielded the CI versus f_a graph, shown in Figure 3.

[0180] Figure 3A shows that irinotecan and 5-FU at a mole ratio of 1:1 were non-antagonistic over the entire range of concentrations as measured by the fraction-affected dose. In contrast, at a mole ratio of 1:10, the same two drugs were non-antagonistic at low

concentrations, yet antagonistic at higher concentrations. As seen in Figure 3B, etoposide and carboplatin were antagonistic at a mole ratio of 10:1 over the entire concentration range. In contrast, at a 1:10 mole ratio, etoposide and carboplatin were antagonistic at low concentrations while non-antagonistic at higher concentrations.

[0181] Cisplatin and edelfosine at mole ratios of 10:1 and 1:1 were also shown to exhibit distinct combination effects in H460 cells as summarized by plotting CI versus f_a. As shown in Figure 4, the combination at a 10:1 mole ratio was non-antagonistic for approximately 50 % of the fraction affected range at low concentrations and antagonistic at higher concentrations, while a 1:1 mole ratio demonstrated synergy over the entire concentration range.

[0182] These results thus demonstrate that synergy is highly dependent on not only the ratio of the agents to one another but also their concentrations.

Example 3

Determination of CI for Various Two-drug Combinations

[0183] Various drug combinations presented in the table below were tested for additive, synergistic or antagonistic effects using the MTT cytotoxicity assay protocol and the median-effect analysis procedure described above. Results from the CI versus f_a graphs are tabulated below. The approximate percentage of the f_a range that exhibited a non-antagonistic effect is reported in brackets following the ratio. Measurements were taken between f_a values of 0.2 and 0.8 and the percent of that f_a range exhibiting a synergistic or additive effect (non-antagonistic) was calculated by determining the percentage of the curve falling below a CI value of 1.1. Data is derived from at least one experiment performed in triplicate.

DRUG COMBINATION	CELL LINE	MOLE RATIO [% Synergistic or Additive[a]]
Irinotecan:5-FU	H460	1:10 [83%], 1:1 [17%], 10:1 [100%]
Irinotecan:5-FU	MCF-7	1:10 [48% additive[b]], 1:1 [58%], 10:1 [90%]
Irinotecan:5-FU	HT29	1:10 [75%], 1:1 [100%]
FUDR:Irinotecan	HCT-116	1:10 [0%], 1:5 [92%], 1:1 [100%], 5:1 [100%], 10:1 [100%]
FUDR:Irinotecan	HT29	1:10 [40%], 1:5 [73%], 1:1 [100%], 5:1 [100%], 10:1 [95%]

DRUG COMBINATION	CELL LINE	MOLE RATIO [% Synergistic or Additive[a]]
5-FU:Carboplatin	H460	1:10 [48%], 1:1 [100%], 10:1 [100%]
FUDR:Carboplatin	H460	1:10 [37%], 1:5 [100%], 1:1 [100%], 5:1 [100% additive[b]], 10:1 [100% additive[b]]
Irinotecan:Carboplatin	H460	1:10 [0%], 1:1 [13%], 10:1 [100% additive[b]]
Irinotecan:Carboplatin	A549	1:10 [0%], 1:1 [100%], 10:1 [100%]
Cisplatin:Irinotecan	H460	1:10 [100%], 1:1 [56%], 10:1 [100% additive[b]]
Cisplatin:Irinotecan	MCF-7	1:10 [100%], 1:1 [92%], 10:1 [50%]
Etoposide:Carboplatin	H460	1:10 [55%], 1:1 [76% additive[b]], 10:1 [0%]
Etoposide:Carboplatin	MCF-7	1:10 [65%], 1:1 [30%], 10:1 [0%]
Carboplatin:Taxol	H460	1:10 [100%], 1:1 [100%], 1:100 [0%]
Carboplatin:Taxol	MCF-7	1:10 [100%], 1:1 [43%], 1:100 [0%]
Taxol:Doxorubicin	H460	1:5 [52%], 1:1 [37% additive[b]], 1:10 [22%]
Taxol:Doxorubicin	MCF-7	1:5 [70%], 1:1 [100%], 1:10 [63%]
Camptothecin:Taxol	H460	1:1 [0%], 1:10 [100%]
Doxorubicin:Vinorelbine	H460	20:1 [0%], 1:1 [100%]
Cisplatin:Etoposide	H460	50:1 [0%], 1:1 [100%]
Cisplatin:Etoposide	MCF-7	25:1 [0%], 1:1 [100%]
Suramin:Vinorelbine	H460	10:1 [0%], 20,000:1 [72%]
Cisplatin:Edelfosine	H460	10:1 [72%], 1:1 [100%]
Cisplatin:Safingol	H460	1:1 [0%], 0.1:1 [100%]
Cisplatin:Safingol	MCF-7	1:1 [58%], 0.1:1 [100%]
Cisplatin:β-sitosterol	H460	10:1 [0%], 0.1:1 [100%]
Cisplatin:β-sitosterol	MCF-7	10:1 [34%], 0.1:1 [100%]

DRUG COMBINATION	CELL LINE	MOLE RATIO [% Synergistic or Additive[a]]
Cisplatin:Suramin	H460	1:100 [37%], 1:40 [0%]
Vinorelbine:Cisplatin	H460	1:500 [0%], 1:200 [8% additive[b]]
Vinorelbine:Edelfosine	H460	1:10 [0%], 1:1 [0%]
Doxorubicin:Cytosine Arabinoside	H460	1:0.45 [0%]
Doxorubicin:Methotrexate	H460	1:0.36 [0%]

[a] "% Synergistic or Additive" is calculated as the percent of the f_a range that does not fall in the antagonistic range (CI values > 1.1 are antagonistic) on a CI vs. fraction affected (f_a) plot, based on the Chou-Talalay Method, between f_a values of 0.2 to 0.8. CI was measured by entering dose and f_a values into CalcuSyn.
[b] The data set for this ratio was in the "additive" range (CI between 0.9 and 1.1).

Example 4

Synergism of Carboplatin and Daunorubicin

[0184] The procedure set forth above for measuring additive, synergistic or antagonistic effects was repeated using carboplatin/daunorubicin at 10:1, 1:1 and 1:10 mole ratios in H460 cells and at 10:1 and 1:1 ratios in MCF-7 cells. A combination index was determined for each dose by producing CI versus f_a curves as described above and then determining the CI at f_a values of 0.50, 0.75 and 0.90 (to yield CI values at ED50, ED75 and ED90, respectively). Standard deviations were calculated by the CalcuSyn program. As shown in the inset of Figure 5A, carboplatin and daunorubicin at a mole ratio of 10:1 displays a synergistic interaction at ED50, ED75 and ED90 values in MCF-7 cells. As further shown in the inset of Figure 5A, carboplatin and daunorubicin at a 1:1 mole ratio is synergistic, as judged by the mean CI values at ED75 and ED90 while being additive at ED50. In H460 cells, a plot of the CI maximum versus mole ratio of carboplatin/daunorubicin reveals that at a mole ratio of 10:1, the drugs are synergistic while at a mole ratio of 1:1, a slightly antagonistic effect is observed. In contrast, a strongly antagonistic effect is exhibited at a ratio of 1:10 (Figure 5A). Data have also been plotted in Figure 5B as CI versus the fraction of H460 cells affected to better illustrate the effect of concentration on synergy. A 1:1 mole ratio of carboplatin/daunorubicin is non-antagonistic at fraction affected values up to 0.42. At a ratio of 10:1, synergy is observed over a substantial range of f_a values (greater than 0.2) and a 1:10 ratio is antagonistic at all f_a values. The inset of Figure 5B shows that at a 10:1 ratio in H460 cells, synergy (as

judged by the mean CI values) is observed at ED50, 75 and 90 and at a 1:1 ratio, additivity is indicated at the ED50. At a 1:10 ratio, carboplatin/daunorubicin is strongly antagonistic at ED50, 75 and 90 values. Based on these results, carboplatin and daunorubicin at a 1:10 mole ratio would therefore not be selected for further formulation and *in vivo* studies, as antagonism is observed at all ED values measured and over the full f_a range in the CI versus f_a plots. Mole ratios of 10:1 and 1:1 carboplatin:daunorubicin are selected for formulation and efficacy studies as at each of these ratios, the drugs demonstrate synergistic effects over at least 5 % of the f_a range (where greater than 1 % of the cells are affected).

Example 5

Maintaining Synergism of Carboplatin and Daunorubicin *In Vivo*

[0185] Carboplatin and daunorubicin were co-loaded into a single cholesterol-free liposome at mole ratios of 10:1, 5:1 and 1:1 (carboplatin/daunorubicin). DSPC was dissolved in chloroform and DSPG was dissolved in chloroform/methanol/water (50:10:1 vol/vol) with trace amounts of ^{14}C-CHE. The solutions were combined at a mole ratio of 80:20 (DSPC/DSPG). Solvent was removed under a stream of N_2 gas while maintaining the temperature at greater than 60°C. The lipid film was then placed in a vacuum pump for 2 minutes and subsequently redissolved in chloroform only. The chloroform was then removed as above. The resulting lipid films were left under vacuum overnight to remove any residual solvent followed by rehydration in 150 mM $CuSO_4$, pH 7.4 (pH adjusted with triethanolamine) containing 80 mg/mL carboplatin with 4 % (v/v) DMSO to increase carboplatin solubility. The resulting multilamellar vesicles (MLVs) were extruded at 70°C through two stacked 80 and 100 nm pore size filters for a total of ten passes. The samples were exchanged into saline and then into 300 mM sucrose, 20 mM HEPES, 30 mM EDTA, pH 7.4 (SHE) using tangential flow dialysis. Daunorubicin (with trace amounts of ^{3}H-daunorubicin) was loaded into the liposomes by incubation at 60°C for 5 minutes at drug to lipid ratios to achieve carboplatin/daunorubicin mole ratios of 10:1, 5:1 and 1:1. Subsequently, each sample was buffer exchanged into saline by tangential flow. To determine the extent of drug loading at various times, during preparation of the co-loaded formulation, daunorubicin and lipid levels were measured by liquid scintillation counting. Carboplatin concentrations were measured by atomic absorption spectrometry. Balb/c mice were intravenously administered 8 mg/kg carboplatin and daunorubicin was dosed at 1.2 mg/kg, 6 mg/kg and 12 mg/kg for mole ratios of 10:1, 5:1 and 1:1 carboplatin/daunorubicin, respectively in the co-loaded formulation. At the

indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Liquid scintillation counting was used to quantitate plasma daunorubicin and lipid levels; plasma carboplatin levels were determined by atomic absorption spectrometry. For quantitation by atomic absorption spectrometry, samples were diluted in 0.1 % nitric acid to fall within the linear range of a standard curve.

[0186] Results in Figure 6, where the mean plasma drug concentration (+/- standard deviation, SD) is plotted at the specified times, indicate that the co-loaded liposomal formulations containing carboplatin and daunorubicin at a 10:1 mole ratio maintained the ratio of the drugs after intravenous administration as the mole plasma concentrations of carboplatin were present at ten times that of daunorubicin. Results in Figures 7A and 7B demonstrate that 10:1, 5:1 and 1:1 mole ratios of carboplatin to daunorubicin formulated in DSPC/DSPG liposomes were maintained in the blood compartment over the 24 hour time course (3 mice per time point) after intravenous administration of formulations prepared at these ratios (Figure 7B more clearly highlights the results obtained following administration of the 1:1 carboplatin/daunorubicin formulation). These results thus demonstrate that coordinated release kinetics of two drugs at a variety of mole ratios can be achieved.

[0187] Carboplatin and daunorubicin were also co-formulated into DSPC/SM/DSPE-PEG2000 (90:5:5 mol %) liposomes in order to determine whether coordinated release of the drugs *in vivo* could be achieved using this formulation as well. A mole ratio of 10:1 was selected that was determined to be synergistic in Example 4.

[0188] Lipid films (with trace amounts of ^{14}C-CHE) were prepared as described above by solubilizing the lipids in chloroform, removing the chloroform under N_2 gas and placing the samples in a vacuum pump overnight. The resulting lipid films were hydrated in 150 mM $CuSO_4$, 20 mM histidine, pH 7.4 (pH adjusted with triethanolamine) containing 40 mg/mL carboplatin. MLVs were extruded at 70°C through two stacked filters of 100 nm pore sizes for a total of ten passes. Samples were then exchanged into 300 mM sucrose, 20 mM HEPES, pH 7.4 by tangential flow dialysis to remove unencapsulated metal solution (or carboplatin). Daunorubicin loading (with trace levels of ^{3}H-daunorubicin) was carried out at 60°C for 5 minutes at a drug concentration to achieve a 10:1 mole ratio of carboplatin/daunorubicin. To determine the extent of drug loading, daunorubicin and lipid levels were measured by liquid scintillation counting; carboplatin levels were determined by atomic absorption spectrometry. Male SCID/rag2 mice were administered 2.25 mg/kg daunorubicin and 15 mg/kg carboplatin

intravenously of the combination co-loaded in DSPC/SM/DSPE-PEG2000 liposomes. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Plasma carboplatin and daunorubicin levels were determined by atomic absorption spectrometry and liquid scintillation counting, respectively.

[0189] The results set forth in Figure 8, where the mean plasma drug concentration (+/- standard deviation, SD) is plotted at the indicated times, reveal that carboplatin and daunorubicin were eliminated from the plasma compartment at the same rate following intravenous administration when formulated in DSPC/SM/DSPE-PEG2000 liposomes. Carboplatin and daunorubicin were thus maintained at a 10:1 mole ratio, as the plasma concentration of carboplatin (nmoles/mL) was present at roughly ten times that of daunorubicin (nmoles/mL) during the time course. These results illustrate that a variety of formulations can be utilized to coordinate the pharmacokinetics of two drugs co-encapsulated in a single liposome such that similar pharmacokinetic release profiles are achieved.

Example 6

Efficacy of Liposomal Carboplatin and Daunorubicin

[0190] DSPC/DSPG liposomes (80:20 mol %) co-encapsulated with daunorubicin and carboplatin at a mole ratio of 1:1 (that was selected for formulation in Example 4) were prepared as described in Example 5 except lipid films were hydrated in a 150 mM $CuSO_4$, pH 7.4 (pH adjusted with triethanolamine), solution containing 25 mg/mL of carboplatin. As well, the lipid films were re-dissolved after being dried down in chloroform to remove methanol or water and then solvent was removed as described previously.

[0191] As in the method of Example 26, efficacy studies were carried out by first inoculating H460 cells (1 x 10^6 cells) subcutaneously into the flank of female SCID/rag2 mice. Tumors were allowed to grow until about 50 mg (0.05 cm^3) in size at which time (day 12) the formulations were injected via the tail vein. Animals (4 mice per group) were treated with three injections, with injections being given every fourth day (q4d schedule; on days 12, 16 and 20). Tumor growth was determined by direct caliper measurements. Mice were treated with saline, free drug cocktail at a 1:1 mole ratio or a liposomal formulation of carboplatin/daunorubicin at a 1:1 mole ratio. For both the free and liposome-formulated treatments, the doses were 6.6 mg/kg carboplatin and 10 mg/kg daunorubicin. Lipid doses were 260 mg/kg lipid for liposome formulated samples.

[0192] Results presented in Figure 9 (points represent mean tumor size +/- standard error of the mean (SEM) determined on the specified day) show that administration of liposomal carboplatin and daunorubicin at a 1:1 mole ratio increased efficacy in relation to free drug cocktail and saline controls.

[0193] Efficacy was also examined in sphingomyelin containing liposomes co-loaded with carboplatin and daunorubicin at a 10:1 mole ratio (determined to be synergistic in Example 4) to examine if the large improvements in efficacy observed for DSPC/DSPG liposomes could be achieved using this formulation as well. Carboplatin and daunorubicin were co-formulated into DSPC/SM/DSPE-PEG2000 (90:5:5 mol %) liposomes according to the procedure outlined in Example 5 except liposomes were extruded through an 80 nm and a 100 nm pore size filter ten times. As well, the samples were buffer exchanged into SHE buffer prior to loading of daunorubicin by fixed volume dialysis rather than tangential flow dialysis. As detailed in Example 26, H460 tumor bearing female SCID/rag2 mice (4 mice per group) were administered 15 mg/kg carboplatin and 2.25 mg/kg daunorubicin for liposome formulated drug and free drug cocktail on days 14, 18 and 22. Liposomal drug was administered at a lipid dose of 375 mg/kg.

[0194] Results presented in Figure 10 (points represent mean tumor size +/- SEM determined on the specified day) show that liposomal carboplatin and daunorubicin encapsulated at a 10:1 non-antagonistic mole ratio in sphingomyelin-containing liposomes exhibit substantially increased efficacy in relation to controls consisting of free drug and saline

Example 7

Synergism of Cisplatin and Daunorubicin

[0195] Cisplatin/daunorubicin combinations were tested for additive, synergistic or antagonistic effects using the methods described above. The results are summarized in Figure 11. As shown in Figure 11A, synergy was observed at a cisplatin/daunorubicin mole ratio of 10:1 over the entire f_a range while the 1:1 mole ratios displayed antagonism over the complete f_a range. Figure 11B, a plot of CI maximum (CI max) vs. cisplatin-to-daunorubicin ratio, further illustrates the dependence of the combination ratio of two agents on the combination index. These results show that at a 10:1 mole ratio, the CI max value is synergistic while at 1:1 and 1:10 mole ratios the CI max value is antagonistic.

Example 8

Maintaining Synergism of Cisplatin and Daunorubicin *In Vivo*

[0196] Cisplatin and daunorubicin were co-loaded into DMPC/Chol (55:45 mol%) liposomes at a 10:1 mole ratio identified in Example 7 as being non-antagonistic.

[0197] Cisplatin was passively entrapped in liposomes by first solubilizing the drug (at 40 mg/mL) in a solution consisting of 150 mM $CuCl_2$, 20 mM histidine (pH 7.4, pH adjusted with triethanolamine) plus 4 % (v/v) DMSO and heating the resulting solution to 80°C to enhance the solubility of cisplatin. The cisplatin solution was then added at 80°C to a lipid film composed of DMPC and cholesterol with trace levels of ^{14}C-CHE. The hydrated lipid films were extruded at 80°C through two 100 nm filters and the liposomes cooled to room temperature. Upon cooling, the samples were centrifuged in a bench top centrifuge at 2000 x g for 5 minutes to pellet any unencapsulated cisplatin, and the supernatant collected. Removal of excess metal ions was carried out by passage through a Sephadex G-50 gel filtration column and collection of the liposome fraction.

[0198] The cisplatin-loaded liposomes were further loaded with daunorubicin (labeled with trace levels of ^{3}H-daunorubicin) at a 10:1 cisplatin/daunorubicin mole ratio by incubation of the liposomes with the drug at 60°C for 15 minutes. In order to determine the extent of drug loading, cisplatin levels were measured by atomic absorption spectrometry and ^{3}H-daunorubicin and lipid levels were measured by liquid scintillation counting.

[0199] In order to determine whether coordinated release was achieved by this formulation, the loaded liposomes were injected into the tail vein of male SCID/rag2 mice at 5.0 mg/kg cisplatin and 1.0 mg/kg daunorubicin per mouse. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Liposomal lipid and daunorubicin levels in the plasma were both determined by liquid scintillation counting and cisplatin levels were measured by atomic absorption spectrometry.

[0200] Results depicted in Figure 12 (points represent mean drug concentration in plasma +/- SD determined at the specified time) indicate that coordinated release of daunorubicin and cisplatin was achieved as the concentrations in the plasma (μmoles/mL) were maintained at a mole ratio of 10:1 at the time points measured.

[0201] Although liposomes may be co-loaded with cisplatin and daunorubicin by the method described above, other techniques may be employed to load the drugs into a single

liposome. An alternative method employs the use of a pH gradient to load daunorubicin after passively entrapping cisplatin along with citrate, pH 4.0, and imposing a pH gradient across the membrane by buffer exchange. This technique may be carried out as follows:

[0202] Lipid films consisting of DSPC/Chol (55:45 mol %) are prepared as described above along with trace amounts of ^{3}H-CHE. A cisplatin solution is prepared by dissolving cisplatin powder into 150 mM NaCl and 150 mM citrate (pH 4). To maximize the solubility of cisplatin in the buffer, the solution is heated to 65°C and added to the lipid films. The resulting MLVs are extruded at 65°C through two 100 nm pore size filters for a total of ten passes. Unencapsulated cisplatin is then removed from the formulation by centrifuging the solution at 2000 x g for 10 minutes. The resulting supernatant containing liposomal cisplatin is passed down a Sephadex G-50 column that is pre-equilibrated in 150 mM NaCl and 20 mM HEPES (pH 7.4) to remove any residual unentrapped cisplatin and to establish a pH gradient across the bilayer.

[0203] Daunorubicin is subsequently loaded into the liposomes by first incubating the liposomes at 60°C for 5 minutes to achieve thermal equilibration and then adding daunorubicin to the lipid formulation at a 0.1:1 drug/lipid mole ratio while vortexing. To determine the extent of drug loading at various times, the concentration of daunorubicin is determined by solubilizing the liposomes with OGP and measuring the absorbance of daunorubicin at 480 nm. The cisplatin concentration of the formulation is measured using atomic absorption spectrometry. Lipid concentrations are measured by liquid scintillation counting.

[0204] An alternative means of coordinating the release kinetics of two drugs can be achieved by formulating each drug in separate carriers. This was demonstrated by formulating cisplatin in DMPC/cholesterol liposomes and daunorubicin in DSPC/DSPE-PEG2000 liposomes and administering them intravenously to mice at a 10:1 mole ratio.

[0205] Liposomal cisplatin was prepared by first dissolving cisplatin (8.5 mg/mL) in 150 mM NaCl at 80°C. The solution was next added to a DMPC/cholesterol (55:45 mol %) lipid film containing trace amounts of ^{3}H-CHE and allowed to hydrate. The resulting MLVs were extruded at 80°C through two 100 nm pore size filters and the liposomes were subsequently exchanged into 20 mM HEPES, 150mM NaCl (pH 7.4) (HBS) by tangential flow dialysis to remove excess metal ions. The liposomes were centrifuged to pellet any unencapsulated cisplatin after extrusion. The cisplatin concentration was determined by atomic absorption spectrometry and lipid levels were determined by liquid scintillation counting.

[0206] Liposomal daunorubicin was prepared by hydration of a lipid film composed of DSPC/ DSPE-PEG2000 (95:5 mol %) and trace amounts of ^{14}C-CHE with a solution of 300 mM $CuSO_4$. The resulting MLVs were extruded by ten passes through two stacked 100 nm pore size filters at 70°C. After extrusion, the liposomes were exchanged into HBS (pH 7.4) by tangential flow dialysis. Loading of daunorubicin (with trace levels of ^{3}H-daunorubicin) was initiated by the addition of daunorubicin to a final drug/lipid weight ratio of 0.1 and holding the solution at 60°C for 10 minutes. The extent of drug loading was measured by liquid scintillation counting to measure ^{3}H-daunorubicin and ^{14}C-CHE levels.

[0207] Male SCID/rag 2 mice were injected intravenously with liposomal cisplatin at a drug dose of 2 mg/kg and liposomal daunorubicin at a drug dose of 0.375 mg/kg. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Plasma cisplatin levels were determined by atomic absorption spectrometry and daunorubicin levels were determined by scintillation counting.

[0208] Results shown in Figure 13 (points represent mean drug concentrations determined in plasma +/- SD at the specified time points) reveal that cisplatin and daunorubicin formulated in separate liposomes were maintained at a 10:1 mole ratio at various time points after intravenous administration.

Example 9

Efficacy of Liposomal Cisplatin and Daunorubicin

[0209] The efficacy of cisplatin and daunorubicin formulated in separate liposomes was determined in SCID/rag2 mice (H460 xenograft model) as detailed in Example 26. H460 tumor bearing mice (4 mice per group) were treated with saline or with cisplatin/daunorubicin at a 10:1 mole ratio that was identified *in vitro* in Example 7 as being non-antagonistic. Cisplatin and daunorubicin were formulated in DMPC/Chol (55:45 mol%) and DSPC/DSPE-PEG2000 (95:5 mol %) liposomes respectively as set forth in Example 8, except DMPC/Chol liposomes were dialyzed against HBS after extrusion. Animals treated with the drug combination received the agents as either a cocktail of the free agents (cocktail; 10:1, mole ratio) or by co-administration of liposomal daunorubicin and liposomal cisplatin (liposome formulation; 10:1 mole ratio) on days 14, 17 and 21. For both the free and formulated treatments, the doses were 2.0 mg/kg of cisplatin and 0.375 mg/kg of daunorubicin. Lipid doses were 400 mg/kg for liposomal cisplatin and 3.75 mg/kg for liposomal daunorubicin.

[0210] Figure 14 shows the results, where each data point represents mean tumor size +/- SEM determined on the specified day. The saline control (solid circles) did not inhibit tumor growth; similarly, the free cocktail (solid inverted triangles) showed only a slight effect on tumor growth. In comparison, the liposomal formulation (open triangles) inhibited tumor growth over a period of at least 32 days.

Example 10

Effect of Liposomal Administration of a Drug Combination at an Antagonistic Mole Ratio

[0211] Cisplatin and daunorubicin were co-loaded into DMPC/Chol (55:45 mol %) liposomes at a 1:1 mole ratio that was determined in Example 7 to be antagonistic. Cisplatin was passively entrapped and daunorubicin actively entrapped to achieve a cisplatin/daunorubicin mole ratio of 1:1. The procedure outlined in Example 8 was employed to load the drugs into a single liposome.

[0212] In order to determine whether coordinated release was achieved by formulation in DMPC/Chol liposomes, the loaded liposomes were injected into the tail vein of Balb/c mice at 2 mg/kg cisplatin and 3.75 mg/kg daunorubicin. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Lipid and daunorubicin plasma levels were both determined by liquid scintillation counting and cisplatin levels were measured by atomic absorption spectrometry. Results summarized in Figure 15 (data points represent mean drug concentrations determined in plasma +/- SD at the specified time points) show that daunorubicin and cisplatin were eliminated from the plasma at the same rate, thus the concentrations in the plasma (nmoles/mL) were maintained at a mole ratio of 1:1 (see insert to Figure 15).

[0213] Efficacy studies were carried out as described in Example 26, where H460 tumor bearing female SCID/rag2 mice were dosed at 2.5 mg/kg cisplatin, 4.7 mg/kg daunorubicin in either cocktail or liposomal formulation and 52.83 mg/kg lipid on days 11, 15 and 19.

[0214] Efficacy results in Figure 16 (data points represent mean tumor size +/- SEM determined on the specified day) show that treatment with daunorubicin and cisplatin at an antagonistic ratio is ineffective at reducing tumor growth when compared to results at a non-antagonistic ratio (10:1 mole ratio) of the agents where tumor growth was substantially inhibited (see Figure 14). These results thus highlight the importance of selecting drug combinations at ratios that exhibit non-antagonistic effects over a range of concentrations *in*

vitro. It should be noted that the drug doses used in Figure 16 (2.5 mg/kg cisplatin and 4.7 mg/kg daunorubicin) are actually higher than those used in Figure 14 (2 mg/kg cisplatin, 0.375 mg/kg daunorubicin).

Example 11

Synergism of Cisplatin and Topotecan

[0215] The procedure set forth above (see Example 1) for determining synergistic, additive or antagonistic effects was repeated using cisplatin/topotecan, both at a 10:1 mole ratio and at a 1:1 mole ratio. As shown in Figure 17A, cisplatin/topotecan at a10:1 mole ratio has a non-antagonistic interaction over a wide range of doses that affect 5% to 99% of cells (f_a =0.05 to f_a=0.99). In contrast, cisplatin/topotecan at a 1:1 mole ratio was strongly antagonistic over the same f_a range (Figure 17A).

[0216] This effect of concentration was also evidenced by calculating a CI maximum for various mole ratios of cisplatin/topotecan. As shown in Figure 17B, an antagonist effect appears maximized at a 1:1 mole ratio and non-antagonistic effects are apparent when either drug is in excess.

Example 12

Maintaining Synergism of Cisplatin and Topotecan *In Vivo*

[0217] Cisplatin and topotecan were formulated into DMPC/Chol and DSPC/Chol liposomes, respectively, and injected intravenously into mice at a 10:1 mole ratio identified in Example 11 to be synergistic.

[0218] Liposomal cisplatin was prepared by hydration of a lipid film consisting of DMPC and cholesterol (55:45 mol %) with a solution consisting of 150 mM NaCl and 8.5 mg/mL of cisplatin. The resulting MLVs were extruded at 80°C by ten passes through two stacked 100 nm pore size filters. After extrusion, the sample was cooled and precipitated cisplatin was removed by centrifugation. The remaining soluble cisplatin that was not encapsulated in the liposomes was removed by dialysis against HBS. After the removal of non-encapsulated cisplatin, the concentration of the drug was measured by atomic absorption spectrometry.

[0219] Liposomal topotecan was prepared by hydration of a lipid film composed of DSPC and cholesterol (55:45 mol %) with a solution of 300 mM $MnSO_4$. The resulting MLVs were extruded at 65°C by ten passes through two stacked 100 nm filters. After extrusion, the liposomes were exchanged into SHE buffer (300 mM sucrose, 20 mM HEPES and 30 mM

EDTA, pH 7.4) by gel filtration chromatography. Loading of topotecan was initiated by the addition of 1 μg of A23187/μmol lipid (A23187 is a cationic ionophore that mediates the exchange of a divalent metal ion for two protons across a bilayer) and topotecan to a final topotecan/lipid ratio of 0.08 (w/w), then holding the solution at 65°C for 15 minutes. The extent of topotecan loading was measured by absorbance at 380 nm after separation of encapsulated and non-encapsulated drug using gel filtration chromatography and solubilization in Triton X-100.

[0220] The preparations were injected intravenously via the tail vein into SCID/rag2 female mice. Doses of the liposomal formulations were 5 mg/kg of cisplatin and 0.758 mg/kg of topotecan. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Liquid scintillation counting was used to quantitate radiolabeled lipid. Cisplatin was measured using atomic absorption spectrometry while topotecan was measured by fluorescence spectroscopy (excitation at 380 nm and emission at 518 nm) after disruption of the liposomes with excess detergent.

[0221] Figure 18 (data points represent mean drug concentrations determined in plasma +/- SD at the specified time points) shows that plasma levels of cisplatin and topotecan were maintained at a 10:1 mole ratio as plasma levels of cisplatin were roughly ten times that of topotecan at various time points after intravenous administration when they were delivered in the above-described liposomes. These results demonstrate that the drug retention and liposome elimination characteristics of two encapsulated agents in two different liposomes can be coordinated such that coordinated drug elimination rates are realized. The inset of Figure 18 shows that the plasma cisplatin-to-topotecan mole ratios (+/- SD) present in the plasma after intravenous administration vary little over time.

[0222] Cisplatin and topotecan can also be formulated in a single liposome in order to ensure non-antagonistic ratios are maintained *in vivo*. This may be carried out by passive entrapment of cisplatin followed by ionophore-mediated loading of topotecan. A cisplatin solution is first prepared by dissolving cisplatin powder into a solution of 150 mM $MnCl_2$. To maximize the solubility of cisplatin in the $MnCl_2$ solution, the solution is heated to 65°C. A lipid film composed of DSPC/Chol (55:45 mol %) along with trace amounts of ^{3}H-CHE is hydrated with the cisplatin/$MnCl_2$ solution. The resulting MLVs are extruded at 65°C through two 100 nm filters for a total of ten passes. Insoluble cisplatin is then removed from the formulation by cooling the formulation to room temperature and centrifuging the solution at

2000 x g. The resulting supernatant containing liposomal and soluble but unencapsulated cisplatin is dialyzed against SHE buffer, 300 mM sucrose, 20 mM HEPES, and 30 mM EDTA (pH 7.4) overnight at room temperature.

[0223] Topotecan is subsequently loaded into the liposomes using an ionophore-mediated proton gradient. Drug uptake is performed at a 0.08:1 drug to lipid weight ratio (w/w). The divalent cation ionophore A23187 (1 μg ionophore/μmol lipid) is added to the liposomes, and then the mixture is incubated at 60°C for 15 minutes to facilitate A23187 incorporation into the bilayer. Subsequently, topotecan is added, and the mixture is incubated at 60°C for 60 minutes to facilitate drug uptake. Unencapsulated topotecan and A23187 are removed from the preparation by dialyzing the sample against 300 mM sucrose. The extent of topotecan loading is quantified by measuring absorbance at 380 nm. Cisplatin levels are measured by atomic absorption spectrometry and lipid levels by liquid scintillation counting.

Example 13

Efficacy of Liposomal Cisplatin and Topotecan

[0224] The efficacy of cisplatin and topotecan loaded into separate liposomes was investigated by formulating the two drugs in separate liposomes and administering the formulation at a 10:1 mole ratio identified in Example 11 as being non-antagonistic. Liposomal cisplatin was passively entrapped in DMPC/Chol (55:45 mol %) liposomes as described in the procedures of Example 12. Topotecan was formulated in DSPC/Chol (55:45 mol %) as in Example 12 as well, except loading of topotecan was to a final topotecan/lipid weight ratio of 0.1 (w/w). Following loading, the external buffer was exchanged into HBS.

[0225] Efficacy studies were conducted as detailed in Example 26, where H460 tumor bearing female SCID/rag2 mice (4 mice per group) were treated intravenously (on days 13, 17, 21) with saline (control), free cocktail or a liposomal mixture of cisplatin/topotecan at a 10:1 mole ratio identified as non-antagonistic in Example 11. For both the free and liposome-formulated treatments, the doses were 1.6 mg/kg of cisplatin and 0.25 mg/kg of topotecan. Lipid doses were 250 mg/kg arising from the cisplatin formulation plus 2.5 mg/kg from the topotecan formulations.

[0226] Figure 19 shows the results (data points represent mean tumor size +/- SEM determined on the specified day). The saline control (solid circles) and the cocktail of cisplatin/topotecan 10:1 (solid triangles) did not effectively arrest tumor volume. However, the

liposomal preparation of cisplatin/topotecan 10:1 (open triangles) prevented the increase in tumor volume for a period of at least 35 days.

Example 14

Synergism of Cisplatin and Irinotecan

[0227] Combinations of cisplatin and irinotecan at mole ratios of 1:1, 10:1, 1:5 and 1:10 were tested for synergy, additivity or antagonism according to the methods described above (see Example 1). Results summarized in Figure 20A show that mole ratios of 10:1, 1:5 and 1:10 were non-antagonistic over the complete range of f_a values whereas a 1:1 ratio was antagonistic over a substantial range of f_a values. Figure 20B further illustrates the dependency of the ratio on the nature of the combination effect as summarized by plotting the combination index maximum against the cisplatin to irinotecan mole ratio.

Example 15

Maintaining Synergism of Cisplatin and Irinotecan *In Vivo*

[0228] Cisplatin and irinotecan were co-loaded into DSPC/DSPG (80:20 mol %) liposomes, which were prepared as described in Example 5 except that lipid films were rehydrated in 225 mM copper (75 mM $CuCl_2$, 150 mM $CuSO_4$, triethanolamine (TEA), pH 6.8) containing 6.0 mg/mL of cisplatin. The liposomal cisplatin concentration after extrusion and removal of unencapsulated drug was 0.025 mole cisplatin/mole lipid. The resulting liposomes were dialyzed against SHE, pH 6.8 overnight. Irinotecan was then added to the preparation and the liposomes were incubated at 45°C for 1.5 hours. The liposomes loaded 60% of the added irinotecan as determined by HPLC. The liposomes were then buffer exchanged into 0.9% saline by tangential flow. After tangential flow, the liposomes retained approximately 80% of the original cisplatin and irinotecan. Analysis of cisplatin and irinotecan, as determined by atomic absorption spectrometry and HPLC analysis, respectively, indicated that the final preparation had a cisplatin-to-irinotecan mole ratio of 1:3. SCID/rag2 mice were intravenously administered 2 mg/kg cisplatin and 38.6 mg/kg irinotecan. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Plasma irinotecan and cisplatin levels were determined by HPLC and atomic absorption spectrometry, respectively.

[0229] Results in Figure 21 (data points represent mean drug concentrations determined in plasma +/- SD at the specified time points) show that following intravenous injection of formulations containing cisplatin and irinotecan, co-loaded into DSPC/DSPG liposomes, the rates of drug elimination were comparable and non-antagonistic mole drug ratios could be maintained over the 24-hour time course after administration.

[0230] Coordinated release of liposomal cisplatin and irinotecan *in vivo* was also achieved by formulating the two drugs in separate delivery vehicles and administering the drugs at a 1:5 mole ratio (cisplatin/irinotecan).

[0231] Liposomal cisplatin was prepared according to the passive loading technique described above. Lipid films consisting of DMPC/Chol (55:45 mol %) were hydrated with a solution of 150 mM NaCl containing 8.5 mg/mL cisplatin, then extruded as described above. The liposomes were collected in the supernatant after centrifugation as above then exchanged into HBS by tangential flow dialysis.

[0232] Liposomal irinotecan was prepared by hydrating lipid films consisting of DSPC/DSPE-PEG2000 (95:5 mol %) with a solution consisting of 150 mM $CuCl_2$, 20 mM histidine, pH 6.8 (pH adjusted with TEA). The resulting MLVs were extruded at 65°C through two stacked 100 nm pore size filters and buffer exchanged with HBS by tangential flow. The extruded liposomes were loaded with irinotecan at 60°C for 1 minute at a 0.1:1 drug to lipid weight ratio. The extent of loading of irinotecan was determined by absorbance at 370 nm after solubilization in Triton X-100; lipid levels were measured by liquid scintillation counting.

[0233] Liposomal cisplatin was administered to male SCID/rag2 mice at a drug dose of 2.0 mg/kg and liposomal irinotecan was administered to the mice at 20 mg/kg. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Plasma irinotecan levels were measured by HPLC and cisplatin was measured by atomic absorption spectrometry.

[0234] Cisplatin and irinotecan administered together in these liposomal formulations at this synergistic ratio (1:5 mole ratio) maintain this ratio at 1:5 following intravenous administration as evidenced by the plasma concentrations of irinotecan (nmoles/mL) being roughly five times that of cisplatin (nmoles/mL) at various time points (Figure 22).

Example 16

Efficacy of Liposomal Cisplatin and Irinotecan

[0235] Efficacy studies were carried out on liposomal cisplatin and irinotecan formulated into separate liposomes. Cisplatin was passively entrapped in DMPC/Chol (55:45 mol %) liposomes and irinotecan was loaded into DSPC/DSPE-PEG2000 (95:5 mol %) liposomes as detailed in Example 15. Liposomal cisplatin and irinotecan were co-administered to H460 tumor bearing SCID/rag2 mice according to the methods described in Example 26 at a 1:5 mole ratio determined to be non-antagonistic in Example 14. Liposomal cisplatin and irinotecan were administered (4 mice per group on days 14, 18 and 22) at the non-antagonistic mole ratio of 1:5 with doses of 1 mg/kg cisplatin, 10 mg/kg irinotecan and 130 mg/kg lipid (open squares); 2.5 mg/kg cisplatin, 25 mg/kg irinotecan and 175 mg/kg lipid (open upward triangles); or, 5 mg/kg cisplatin, 50 mg/kg irinotecan and 250 mg/kg lipid (open inverted triangles). Free cisplatin/irinotecan was dosed at 1 mg/kg cisplatin and 10 mg/kg irinotecan which reflects a 1:5 mole ratio (solid squares).

[0236] Figure 23 (data points represent mean tumor size +/- SEM determined on the specified day) illustrates that tumor growth for the liposomal preparations was substantially suppressed in relation to free drug cocktail and saline treated mice.

Example 17

Synergism of Drug and Lipid Combinations

[0237] Combinations comprising vinorelbine at a 1:1 mole ratio with various potentially therapeutic lipids incorporated into the lipid bilayer, such as POPS (inverted triangles), DPPS (upward triangles), DLPS (circles), DSPS (diamonds) or DOPS (squares), were tested for additive, synergistic or antagonistic effects using the method described above (see Example 1).

[0238] Results in Figure 24 show that all combinations of vinorelbine and lipids tested on H460 cells exhibit synergy over a substantial range of f_a values. In particular, the combinations of vinorelbine with DLPS, DSPS and DOPS exhibit synergy at the majority of f_a values, most notably between f_a=0.2 to f_a=0.8.

Example 18

Pharmacokinetics of Liposomal Vinorelbine and Phosphatidylserine

[0239] Liposomes consisting of SM/Chol/DPPS/DSPE-PEG2000 (35:45:10:10 mol %) were prepared and loaded with vinorelbine as follows:

[0240] Lipids were dissolved in chloroform at 100 mg/mL, and then combined in the appropriate amounts. The exception to this is DPPS which was dissolved at 25 mg/mL using $CHCl_3$/methanol/H_20/citrate buffer (20:10.5:1:1 v/v). Trace amounts of the radioactive lipid ^{3}H-CHE was added at this point to follow the lipid throughout the formulation process. The chloroform was removed under a stream of N_2 gas until very little solvent remained. The resulting lipid films were left under vacuum overnight to remove any residual solvent. The lipid films were rehydrated in citrate buffer (300 mM, pH 4.0) and the resulting MLVs were extruded at 65°C through two 100 nm pore size filters for a total of ten passes.

[0241] Vinorelbine was loaded into these formulations using the pH gradient loading method by titrating up the external buffer pH with the use of 0.2 M Na_2HPO_4. A known amount of liposomes were combined with the corresponding amount of vinorelbine (0.1 drug/lipid weight ratio (w/w)) and incubated at 60°C for 15 minutes. In order to establish a pH gradient, 0.2 M Na_2HPO_4 was added at ten times the volume of the citrate buffer. Vinorelbine was loaded into the liposomes to achieve a vinorelbine/phosphatidylserine mole ratio that was identified as non-antagonistic in Example 17.

[0242] The detergent OGP was used to solubilize the vinorelbine-loaded liposomes; drug levels were measured by absorbance at 270 nm and liquid scintillation counting was used to quantify lipid.

[0243] The resulting vinorelbine-loaded liposomes and free vinorelbine were administered intravenously into SCID/rag2 mice at a drug dose of 10 mg/kg. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was carefully transferred to another tube. Blood was analyzed for remaining ^{3}H-CHE liposomal marker using scintillation counting. Plasma levels of vinorelbine were assayed by HPLC.

[0244] Figures 25A and 25B show that SM/Chol/DPPS/DSPE-PEG2000 liposomes encapsulating vinorelbine exhibit substantially increased plasma drug levels in relation to administration of free vinorelbine. The free vinorelbine mean area under the curve (AUC) of

0.112 μg h/mL was increased to 125.3 μg h/mL by formulation in the liposomes, representing a 1120 fold increase in mean AUC.

Example 19

Efficacy of Liposomal Phosphatidylserine and Vinorelbine in the H460 Human Lung Cancer Model

[0245] DSPC/Chol/DSPS/DSPE-PEG2000 (35:45:10:10 mol %), SM/Chol/DPPS/DSPE-PEG2000 (35:45:10:10 mol %) and DAPC/Chol/DPPS/DSPE-PEG2000 (35:45:10:10 mol %) liposomes were prepared and loaded with vinorelbine as described in Example 18. Phosphatidylserine and vinorelbine were present in the liposomes at a non-antagonistic mole ratio (1:1). Efficacy studies were carried out in the H460 human lung cancer model as described in Example 26.

[0246] Figure 26 shows for H460 tumor bearing mice (4 mice per group) given intravenous administration of liposomes consisting of DSPC/Chol/DPPS/DSPE-PEG2000 and SM/Chol/DPPS/DSPE-PEG2000 and encapsulated vinorelbine, that treatment engendered decreased tumor growth rates relative to those observed following treatment with free vinorelbine and saline. Free vinorelbine was administered at 5 mg/kg and liposomal vinorelbine was administered at a dose of 5 mg/kg of the drug and 50 mg/kg lipid at 13, 17 and 21 days post tumor cell inoculation.

[0247] Figure 27 (data points represent mean tumor size +/- SEM determined on the specified day) shows that liposomes consisting of SM/Chol/DPPS/DSPE-PEG2000; DAPC/Chol/DPPS/ DSPE-PEG2000 and DSPC/Chol/DSPS/DSPE-PEG2000 and encapsulating vinorelbine display decreased tumor volume with time relative to free vinorelbine and saline. Tumor-bearing mice (4 per group) were treated at a vinorelbine dose of 5 mg/kg (free and liposomal) and a lipid dose of 50 mg/kg for the liposomal group. Mice were treated intravenously on days 13, 17 and 21.

Example 20

Efficacy of Liposomal Phosphatidylserine and Vinorelbine in the Murine Leukemia Cancer Model

[0248] Liposomes consisting of SM/Chol/DPPS/DSPE-PEG2000 (35:45:10:10 mol %) were prepared and loaded with vinorelbine as described in Example 18, except that liposomes were extruded through a 100 nm pore filter stacked with an 80 nm filter.

[0249] P388/wt cells were inoculated intraperitonealy into BDF-1 mice as described in Example 27. Subsequently, BDF1 female mice were intraperitonealy administered one of the following: saline; free vinorelbine (10 mg/kg) and SM/Chol/DPPS/DSPE-PEG2000 liposomes loaded with vinorelbine (10 mg/kg vinorelbine and 100 mg/kg lipid). Intraperitoneal administration of free and liposomal vinorelbine was carried out on day 1 with 4 mice per treatment group.

[0250] The survival curves shown in Figure 28 demonstrate that administration of vinorelbine encapsulated in liposomes consisting of SM/Chol/DPPS/DSPE-PEG2000 results in substantially increased survival rates in BDF-1 mice relative to free vinorelbine and saline treatment.

Example 21

Co-Formulation of Sphingosine and Doxorubicin

[0251] Other therapeutic lipids besides phosphatidylserine may be incorporated into liposome membranes. For instance, sphingosine and sphingosine analogues are lipids that are amenable to formulation in liposomes and may be co-formulated with a therapeutic agent that is encapsulated in the aqueous interior (for example, doxorubicin). The preparation of such a pharmaceutical composition (sphingosine) may be carried out as follows:

[0252] A typical liposomal formulation of sphingosine is composed of DSPC/Chol/sphingosine (45:45:10 mol %). Lipid films are prepared as detailed in the previous examples. The lipid films are rehydrated in citrate buffer (300 mM, pH 4) and the resulting MLVs are extruded at 65°C through two 100 nm filters for a total of ten passes. Doxorubicin is subsequently loaded into these formulations using the pH gradient loading method by exchanging the external buffer of the liposomes by passage down a Sephadex G-50 column that is equilibrated in HBS (pH 7.4) to establish a pH gradient.

[0253] The liposomes and doxorubicin solution are then incubated together at 60°C to allow loading to occur. To determine the extent of loading at various times, 100 uL of the sample is applied to a 1 mL Sephadex G-50 spun column and then centrifuged. A drug to lipid ratio for the spun column eluent is generated using liquid scintillation counting to quantitate lipid and absorbance at 480 nm to quantitate doxorubicin. To assay for drug, the liposomes are solubilized by incubation in Triton X-100 before absorbance readings are taken.

Example 22

Synergism of Floxuridine (FUDR) and Irinotecan (CPT-11)

[0254] The procedure set forth above for measuring additive, synergistic or antagonistic effects was repeated using FUDR/CPT-11 at 10:1, 5:1, 1:1, 1:5 and 1:10 mole ratios in HT 29 cells. A combination index was determined for each dose by producing CI versus f_a curves as described above. Data in Figure 29, plotted as CI versus the fraction of HT-29 cells affected, clearly illustrates the effect of concentration on synergy. At a ratio of 5:1 or 1:1 synergy is observed over the entire range of fraction affected values (0.2 to 0.8) while a 10:1 ratio is non-antagonistic at f_a values below 0.76 and a 1:5 mole ratio of FUDR/CPT-11 is non-antagonistic at f_a values less than 0.62. A 1:10 ratio is antagonistic over a substantial range of f_a values (more than 50%). Based on these results, a mole ratio of 1:1 FUDR:CPT-11 was selected for formulation and efficacy studies as this ratio demonstrated synergistic effects over a significant range of f_a values (at least 20% where greater than 1 % of the cells are affected). Formulations prepared at the 5:1 and 10:1 ratio would also meet the requirements of a defined non-antagonistic ratio over a substantial range of f_a values.

Example 23

Maintaining Synergism of FUDR and CPT-11 *In Vivo*

[0255] FUDR and CPT-11 were formulated into DSPC/DSPG/Chol (70:20:10 mol %) liposomes at a 1:1 mole ratio identified in Example A to be synergistic. Lipid films were prepared by dissolving DSPC and cholesterol in chloroform and DSPG in chloroform/methanol/water (16/18/1). The solutions were combined together such that the specified mole ratio was achieved and trace quantities of ^{14}C-CHE were added as a liposomal lipid label. Following solvent removal the resulting lipid films were hydrated in a solution consisting of 250 mM $CuSO_4$ and 25 mg/mL of FUDR (with trace amounts of ^{3}H-FUDR) at

70°C. The resulting MLVs were extruded at 70°C by ten passes through two stacked 100 nm pore size filters. Subsequently, the liposomes were buffer exchanged into SHE, pH 7.4, by tangential flow dialysis, thus removing any unencapsulated FUDR and $CuSO_4$.

[0256] CPT-11 was added to these liposomes such that the FUDR to CPT-11 mole ratio would be 1:1. Loading of CPT-11 into the liposomes was facilitated by incubating the samples at 50°C for 5 minutes. After loading, the samples were exchanged into HBS, pH 7.4, by tangential flow dialysis to remove EDTA or unencapsulated drug. The extent of CPT-11 loading was measured using HPLC. FUDR and lipid levels were measured using liquid scintillation.

[0257] The preparations were injected intravenously via the tail vein into Balb/c female mice. Doses of the liposomal formulations were 8.38 mg/kg of FUDR and 20 mg/kg of CPT-11. At the indicated time points (3 mice per time point), blood was collected by cardiac puncture and placed into EDTA coated microtainers. The samples were centrifuged and plasma was transferred to another tube. Liquid scintillation counting was used to quantitate radiolabeled lipid and FUDR in the plasma. CPT-11 plasma levels were quantified with HPLC.

[0258] Figure 30 shows that plasma levels of FUDR and CPT-11 were maintained at a 1:1 mole ratio as plasma levels of FUDR were roughly equal to that of CPT-11 at various time points after intravenous administration when they were delivered in the above-described liposomes. Data points represent mean drug concentrations (nmoles drug/mL plasma) determined in plasma +/- standard deviation at the specified time points.

Example 24

Efficacy of Liposomal FUDR and CPT-11

[0259] DSPC/DSPG/Chol (70:20:10 mol %) liposomes co-encapsulated with FUDR and irinotecan at a mole ratio of 1:1 were prepared as described in Example B except that after drug loading the external liposome buffer was exchanged to 0.9% NaCl.

[0260] Using the methods of Example 26, efficacy studies were carried out in female SCID/rag2 mice that had been inoculated subcutaneously in the flank with 2 x 10^6 HT-29 cells. Tumors were allowed to grow until they measured to be 180 mg (0.18 cm^3) in size, at which time (day 21) the indicated formulations were injected. Tumor growth was determined by direct caliper measurements. Mice were treated with a single dose (arrow) of saline, free drug cocktail at a 1:1 mole ratio or a liposomal formulation of FUDR/CPT-11 at a 1:1 mole ratio.

For both the cocktail and liposome-formulated treatments, the doses were 9.25 mg/kg FUDR and 25 mg/kg CPT-11. Lipid doses were 278 mg/kg lipid for liposome formulated samples.

[0261] Results presented in Figure 31 show that administration of FUDR and CPT-11 encapsulated in a single liposome at a 1:1 mole ratio provided significantly better therapeutic activity when compared to animals injected with either the free drug cocktail or saline. Data points represent mean tumor size +/- standard error of the mean (SEM).

Example 25

Determination of CI for Various Three-Drug Combinations

[0262] Combinations comprising topotecan, cisplatin, HB5-5A (an analog of edelfosine) and sphingosine were tested for additive, synergistic or antagonistic effects using the standard tetrazolium-based colorimetric MTT cytotoxicity assay (see Examples – Cytotoxicity Assay). Combination effects were calculated using the median-effect analysis described in the previous examples. CI versus f_a graphs were created as described in the preceding examples and CI values corresponding to f_a values at 0.50, 0.75 and 0.90 (represented by ED50, 75 and 90) are reported in table below:

AGENT 1	AGENT 2	AGENT 3	FIXED RATIO	COMBINATION INDEX[a]		
				ED_{50}[b]	ED_{75}	ED_{90}
Topotecan	Cisplatin	HB5-5A	1:10:1	0.56	0.34	0.26
Topotecan	Cisplatin	HB5-5A	1:10:10	0.73	0.53	0.43
Topotecan	Cisplatin	HB5-5A	1:10:100	2.22	1.78	1.45
Topotecan	Cisplatin	Sphingosine	1:10:1	0.23	0.12	0.07
Topotecan	Cisplatin	Sphingosine	1:10:10	0.47	0.34	0.29
Topotecan	Cisplatin	Sphingosine	1:10:100	1.22	0.95	0.76

[a]Combination Index (CI) is used to determine synergy (CI < 0.9) or additivity (CI between 0.9 and 1.1) based on the Chou-Talalay theory of dose-effect analysis. Values are calculated using CalcuSyn Software.
[b]ED_{50}, ED_{75}, ED_{90} refer to the dose of the agent(s) affecting 50, 75 or 90% of the measured response, respectively.

Example 26

Preparation of Tumor Models, Cell Preparation and Implantation for a Solid Subcutaneous Tumor Method

[0263] H460 human non-small cell lung carcinoma cells are obtained from the DCTC Tumor Repository of the NCI. The cells are maintained in tissue culture for up to 20 passages. After 20 passages, new cells are expanded from a frozen stock stored in liquid nitrogen. When the cultured cells reached a confluence of 80-90% they are rinsed with Hanks Balanced Salt

Solution and the adherent cells are removed with a 0.25% trypsin solution. Cells are counted on a haemocytometer and diluted with media to a concentration of 20 x 10^6 cells/mL.

[0264] A patch of hair approximately 2 cm x 2 cm is shaved using electric clippers in the lower back region of each mouse. Using a 28g needle, mice are inoculated subcutaneously with 1 x 10^6 tumor cells on day 0 (one inoculum/mouse) in a volume of 50 µL.

[0265] When tumors reach a defined size of approximately 0.50-to-0.100 cm^3, either one-day prior to treatment or on the day of treatment (~day 10-14), all tumors are measured. After selecting the appropriate tumor sizes, excluding tumors too small or large, the tumors are randomly distributed (n=4) and the mean tumor volume of the groups are determined.

[0266] Mice are organized into appropriate treatment groups and consist of control and treatment groups such as, saline control, vehicle control, positive control and various dilutions of test articles.

[0267] Treatment groups are as follows:

GROUP	MICE/GROUP	TREATMENT	DOSE (MG/KG)	SCHEDULE [a]	VOLUME INJECTION
1	4	Saline control	N/A	q4dx3	10 µL/g
2	4	Vehicle control	20	q4dx3	10 µL/g
3	4	Positive control	10	q4dx3	10 µL/g
4	4	Test agent (low dose)	5	q4dx3	10 µL/g
5	4	Test agent (medium dose)	10	q4dx3	10 µL/g
6	4	Test agent (high dose)	20	q4dx3	10 µL/g

[a]Alternative dosing schedules can be considered such as a single dose or 3 doses every 4-7 days

[0268] Mice are injected intravenously with the required volume of sample to administer the prescribed dose (10 µL/g as indicated) to the animals based on individual mouse weights.

[0269] Tumor growth measurements are monitored using vernier calipers beginning on the day of treatment. Tumor length measurements (mm) are made from the longest axis and width measurements (mm) will be perpendicular to this axis. From the length and width measurements tumor volumes (cm^3) are calculated according to the equation (L X W^2/2)/1000. Animal weights are collected at the time of tumor measurement.

[0270] Individual mouse body weights are recorded at various days (generally two days apart such as Monday, Wednesday and Friday) during the efficacy study for a period of 14-days after the last dosing.

[0271] All animals are observed at least once a day, more if deemed necessary, during the pre-treatment and treatment periods for mortality and morbidity. In particular, signs of ill health are based on body weight loss, change in appetite, rough coat, lack of grooming,

behavioral changes such as altered gait, lethargy and gross manifestations of stress. Should signs of severe toxicity or tumor-related illness be seen, the animals are euthanized (CO_2 asphyxiation) and a necropsy is performed to assess other signs of toxicity. Moribund animals must be terminated for humane reasons and the decision to terminate will be at the discretion of the Animal Care Technician and the Study Director/Manager. Any and all of these findings will be recorded as raw data and the time of death will be logged as the following day.

[0272] Data are presented in either tabular or figure form as follows:

1. Plot of individual mouse tumor volumes with respect to each group, prior to treatment start and after grouping.
2. Mean body weights for each group as a function of time.
3. Mean tumor volumes for each group as a function of time.
4. Raw data including figures and tables are generated and include tumor growth vs. time, tumor growth inhibition, and tumor growth delay.
5. Summary of abnormal or remarkable observations.

Example 27

Preparation of Tumor Models, Cell Preparation and Implantation for an Intraperitoneal Tumor Method

[0273] Mice are grouped according to body weight. Animals (n=4) are inoculated (Day = 0) with 1 x10^6 P388 cells implanted in the peritoneum cavity of BDF-1 mice in a volume of 500 µL with a 25 g needle. P388 cells from the ATCC tumor repository are maintained as an ascitic fluid in the BDF-1 mouse, which are passaged to new mice weekly. Mice are euthanized, and the ascitic cells removed through the abdominal wall with a 20 g needle. The cells used for experiment are used within passage 3-20. After 20 passages in the mice, new cells are brought up from the frozen stock in liquid nitrogen, and mice are inoculated. For experiments, cells are rinsed with Hanks Balanced Salt Solution, counted on a haemocytometer and diluted with HBSS to a concentration of 2 x 10^6 cells/mL.

[0274] Study groupings are performed randomly after all mice have been administered tumor cells. The required groupings are similar to what is performed for solid tumor studies (see Example 26).

[0275] Mice are injected intravenously or intraperitonealy with the required volume of sample to administer the prescribed dose (10 μL/g as indicated) to the animals based on individual mouse weights. With intraperitoneal tumors, administrations generally begin 1-day post tumor cell inoculation.

[0276] Animal well-being is closely monitored daily. Signs of ill health and progression of morbidity are closely monitored as described in Example 26. Animals are weighed at the time of examination.

[0277] Upon termination of any mice, gross necropsies are performed to evaluate the extent of tumor burden and/or physiologically observable changes in organ appearances. Findings are recorded.

[0278] Group body weights are recorded Monday through Friday during the efficacy study for a period of 14 days after the last dosing.

[0279] All animals are observed at least once a day, more if deemed necessary, during the pre-treatment and treatment periods for mortality and morbidity. In particular, signs of ill health are based on body weight loss, change in appetite, behavioral changes such as altered gait, lethargy and gross manifestations of stress. Should signs of severe toxicity or tumor-related illness be seen, the animals are terminated (CO_2 asphyxiation) and a necropsy is performed to assess other signs of toxicity. Moribund animals must be terminated for humane reasons and the decision to terminate will be at the discretion of the animal care technician and the study manager. These findings are recorded as raw data and the time of death is logged on the following day.

[0280] Data is presented in tables or figures and includes mean body weights for each group as a function of time and increase in life-span.

Claims

1. A kit for treatment of a subject which kit comprises

in a first container a composition comprising delivery vehicles stably associated with at least one first therapeutic agent;

in a second container a second composition comprising delivery vehicles stably associated with at least a second therapeutic agent;

wherein the delivery vehicles in said first and second compositions are coordinated with respect to pharmacokinetic behavior; and

wherein said kit further contains instructions for administering said first and second composition at ratios of said first and second therapeutic agent that are non-antagonistic and/or wherein the amounts of said first and second compositions in said containers is proportional to a ratio of said first and second therapeutic agent that is non-antagonistic and/or said containers are calibrated to dispense amounts of said first and second composition wherein the ratio of first and second therapeutic agents is non-antagonistic.

2. The kit of claim 1, wherein the containers are syringes.

3. The kit of claim 1 or 2, wherein said agents are antineoplastic agents.

4. The kit of any of claims 1-3, wherein said non-antagonistic effect is exhibited over at least 5% of the concentration range where > 1% of relevant cells are affected ($f_a > 0.01$) in an *in vitro* assay for cytotoxicity.

5. The kit of claim 4, wherein said non-antagonistic effect is exhibited over at least 5% of the concentration range such that 10-90% of the cells are affected ($f_a = 0.1$-0.9) in said *in vitro* assay.

6. The kit of claim 5, wherein said non-antagonistic effect is exhibited over at least 5% of the concentration range such that 20-80% of the cells are affected ($f_a = 0.2$-0.8) in said *in vitro* assay.

7. The kit of claim 6, wherein said non-antagonistic effect is exhibited over at least 20% of the concentration range such that 20-80% of the cells are affected in said *in vitro* assay.

8. The kit of any of claims 1-7, wherein said delivery vehicles have a mean diameter of between 4.5 and 500 nm.

9. The kit of claim 8, wherein said vehicles have a mean diameter of less than 250 nm.

10. The kit of any of claims 1-9, wherein said delivery vehicles comprise
liposomes, and/or
lipid micelles, and/or
block copolymer micelles, and/or
microparticles, and/or
nanoparticles, and/or
polymer lipid hybrid systems, and/or
derivatized single chain polymers.

11. The kit of any of claims 1-10, wherein at least one of the agents is selected from the group consisting of a DNA damaging agent, a DNA repair inhibitor, a topoisomerase I inhibitor, a topoisomerase II inhibitor, a cell checkpoint inhibitor, a CDK inhibitor, a receptor tyrosine kinase inhibitor, a cytotoxic agent, an apoptosis inducing agent, an antimetabolite, a cell cycle control inhibitor, a therapeutic lipid, a telomerase inhibitor, an anti-angiogenic agent, a mitochondrial poison, a signal transduction inhibitor and an immunoagent.

12. The kit of any of claims 1-10, wherein the first agent is a cytotoxic agent and the second agent is a cell-cycle inhibitor, or

wherein the first agent is a DNA damaging agent and the second agent is a DNA repair inhibitor, or

wherein the first agent is a topoisomerase I inhibitor and the second agent is a S/G_2- or a G_2/M-checkpoint inhibitor, or

wherein the first agent is a G_1/S checkpoint inhibitor or a cyclin-dependent kinase inhibitor and the second agent is a G_2/M checkpoint inhibitor, or

wherein the first agent is a receptor kinase inhibitor and the second agent is a cytotoxic agent, or

wherein the first agent is an apoptosis-inducing agent and the second agent is a cytotoxic agent, or

wherein the first agent is an apoptosis-inducing agent and the second agent is a cell-cycle control agent, or

wherein the first agent is a telomerase inhibitor and the second agent is a cell-cycle control inhibitor, or

wherein the first and second agents are antimetabolites, or

wherein the first and second agents are cytotoxic agents, or

wherein the first agent is a therapeutic lipid and the second agent is a cytotoxic agent, or

wherein the first agent is a topoisomerase I inhibitor and the second agent is a DNA repair inhibitor, or

wherein the apoptosis-inducing agent is a serine-containing lipid.

13. The kit of any of claims 1-10, wherein the first agent is irinotecan and the second agent is 5-FU or FUDR, or

wherein the first agent is cisplatin (or carboplatin) and the second agent is 5-FU or FUDR, or

wherein the first agent is idarubicin and the second agent is AraC or FUDR, or

wherein the first agent is oxaliplatin and the second agent is 5-FU or FUDR, or

wherein the first agent is irinotecan and the second agent is cisplatin (or carboplatin), or

wherein the first agent is gemcitabine and the second agent is cisplatin (or carboplatin), or

wherein the first agent is methotrexate and the second agent is 5-FU or FUDR, or

wherein the first agent is paclitaxel and the second agent is cisplatin (or carboplatin), or

wherein the first agent is etoposide and the second agent is cisplatin (or carboplatin), or

wherein the first agent is docetaxel or paclitaxel and the second agent is doxorubicin, or

wherein the first agent is doxorubicin and the second agent is vinorelbine, or

wherein the first agent is carboplatin and the second agent is vinorelbine, or

wherein the first agent is 5-FU or FUDR and the second agent is gemcitabine.

14. A method to treat a disease condition in a subject which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a first

composition comprising delivery vehicles stably associated with at least a first therapeutic agent and a second composition comprising delivery vehicles stably associated with at least a second therapeutic agent,

wherein the delivery vehicles in said first and second composition are coordinated with respect to pharmacokinetics; and

wherein said administering is at a ratio of first therapeutic agent to second therapeutic agent that is non-antagonistic.

15. The method of claim 14, wherein said non-antagonistic effect is exhibited over at least 5% of the concentration range such that 1%-99% of the cells are affected (f_a = 0.01-0.99) in an *in vitro* assay for cytotoxicity or cytostasis.

16. The method of claim 15, wherein said non-antagonistic effect is exhibited over at least 5% of the concentration range such that 10-90% of the cells are affected (f_a = 0.1-0.9) in an *in vitro* assay for cytotoxicity or cytostasis.

17. The method of claim 16, wherein said non-antagonistic effect is exhibited over at least 5% of the concentration range such that 20-80% of the cells are affected (f_a = 0.2-0.8) in an *in vitro* assay for cytotoxicity or cytostasis.

18. The method of claim 17, wherein said synergistic effect is exhibited over at least 20% of the concentration range such that 20-80% of the cells are affected in an *in vitro* assay for cytotoxicity or cytostasis.

19. The method of any of claims 14-18, wherein said delivery vehicles have a mean diameter of between 4.5 and 500 nm.

20. The method of any of claims 14-18, wherein said vehicles have a mean diameter of less than 250 nm.

21. The method of any of claims 14-18, wherein said delivery vehicles comprise

liposomes, and/or

lipid micelles, and/or

block copolymer micelles, and/or

microparticles, and/or

nanoparticles, and/or

polymer lipid hybrid systems, and/or

derivatized single chain polymers.

22. The method of any of claims 14-21, wherein at least one of the agents is selected from the group consisting of a DNA damaging agent, a DNA repair inhibitor, a topoisomerase I inhibitor, a topoisomerase II inhibitor, a cell checkpoint inhibitor, a CDK inhibitor, a receptor tyrosine kinase inhibitor, a cytotoxic agent, an apoptosis inducing agent, an antimetabolite, a cell cycle control inhibitor, a therapeutic lipid, a telomerase inhibitor, an anti-angiogenic agent, a mitochondrial poison, a signal transduction inhibitor and an immunoagent.

23. The method of any of claims 14-21, wherein the first agent is a cytotoxic agent and the second agent is a cell-cycle inhibitor, or

wherein the first agent is a DNA damaging agent and the second agent is a DNA repair inhibitor, or

wherein the first agent is a topoisomerase I inhibitor and the second agent is a S/G_2- or a G_2/M-checkpoint inhibitor, or

wherein the first agent is a G_1/S checkpoint inhibitor or a cyclin-dependent kinase inhibitor and the second agent is a G_2/M checkpoint inhibitor, or

wherein the first agent is a receptor kinase inhibitor and the second agent is a cytotoxic agent, or

wherein the first agent is an apoptosis-inducing agent and the second agent is a cytotoxic agent, or

wherein the first agent is an apoptosis-inducing agent and the second agent is a cell-cycle control agent, or

wherein the first agent is a telomerase inhibitor and the second agent is a cell-cycle control inhibitor, or

wherein the first and second agents are antimetabolites, or

wherein the first and second agents are cytotoxic agents, or

wherein the first agent is a therapeutic lipid and the second agent is a cytotoxic agent, or

wherein the first agent is a topoisomerase I inhibitor and the second agent is a DNA repair inhibitor, or

wherein the apoptosis-inducing agent is a serine-containing lipid.

24. The method of any of claims 14-21, wherein the first agent is irinotecan and the second agent is 5-FU or FUDR, or

wherein the first agent is cisplatin (or carboplatin) and the second agent is 5-FU or FUDR, or

wherein the first agent is idarubicin and the second agent is AraC or FUDR, or

wherein the first agent is oxaliplatin and the second agent is 5-FU or FUDR, or

wherein the first agent is irinotecan and the second agent is cisplatin (or carboplatin), or

wherein the first agent is gemcitabine and the second agent is cisplatin (or carboplatin), or

wherein the first agent is methotrexate and the second agent is 5-FU or FUDR, or

wherein the first agent is paclitaxel and the second agent is cisplatin (or carboplatin), or

wherein the first agent is etoposide and the second agent is cisplatin (or carboplatin), or

wherein the first agent is docetaxel or paclitaxel and the second agent is doxorubicin, or

wherein the first agent is doxorubicin and the second agent is vinorelbine, or

wherein the first agent is carboplatin and the second agent is vinorelbine, or

wherein the first agent is 5-FU or FUDR and the second agent is gemcitabine.

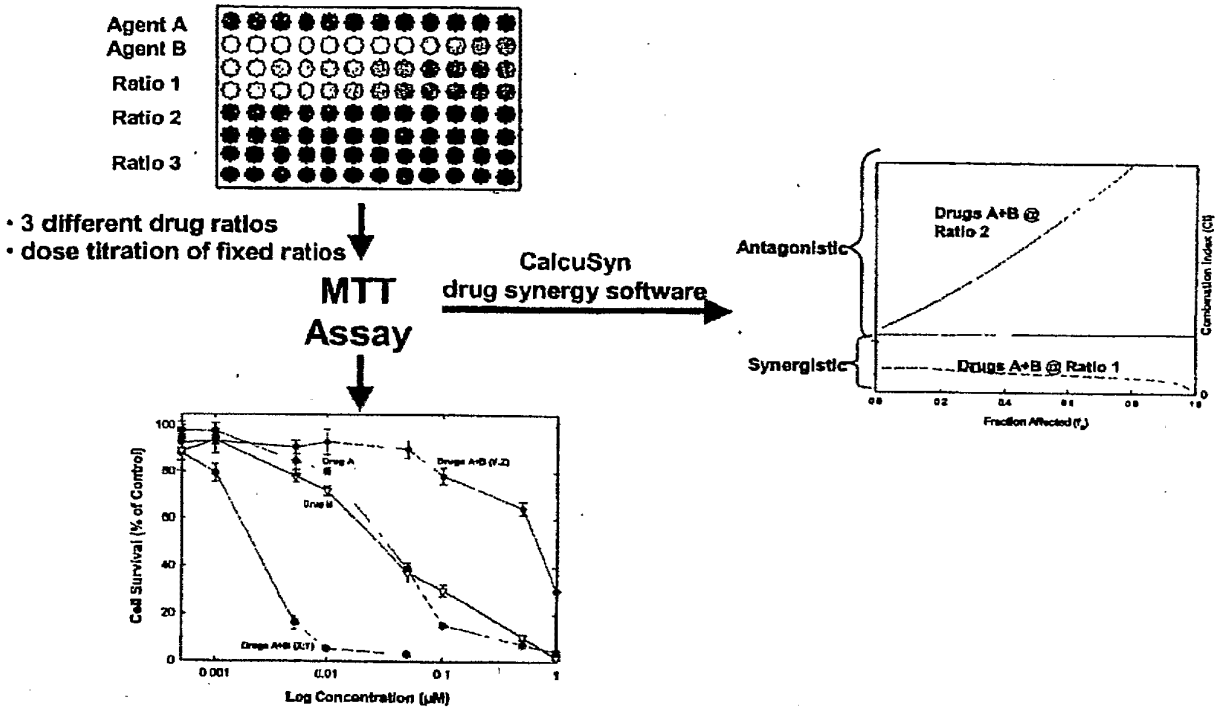

Agent A
Agent B
Ratio 1
Ratio 2
Ratio 3
• 3 different drug ratios
• dose titration of fixed ratios
MTT Assay
CalcuSyn drug synergy software
Antagonistic
Synergistic
Drugs A+B @ Ratio 2
Drugs A+B @ Ratio 1
Combination Index (CI)
Fraction Affected (Fa)
Cell Survival (% of Control)
Log Concentration (μM)
0.001
0.01
0.1
1

FIGURE 1

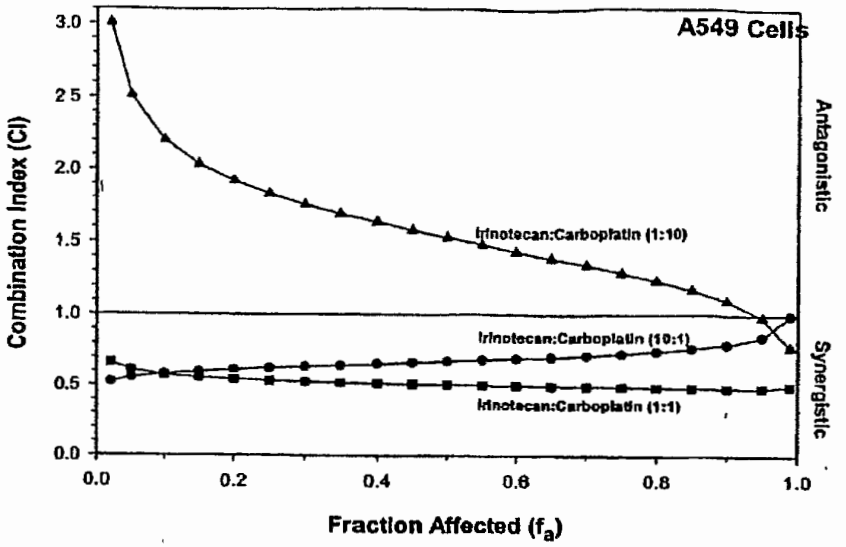


FIGURE 2A

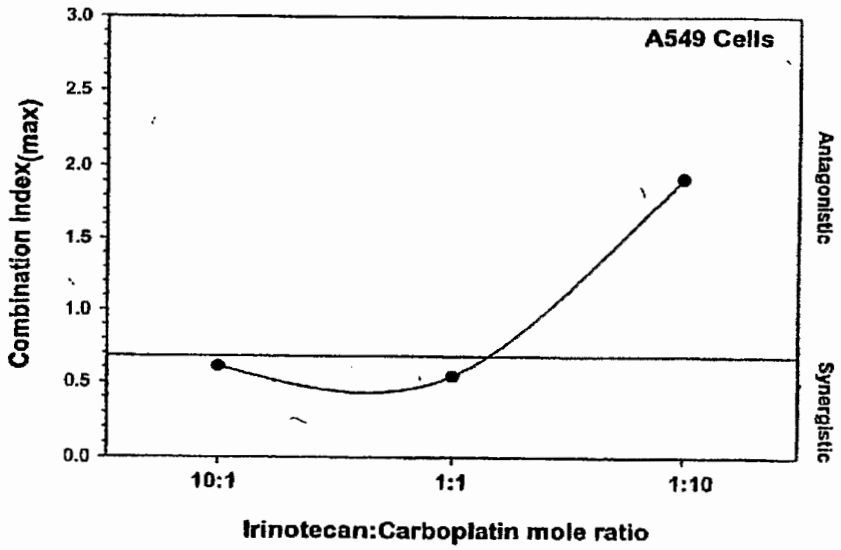


FIGURE 2B

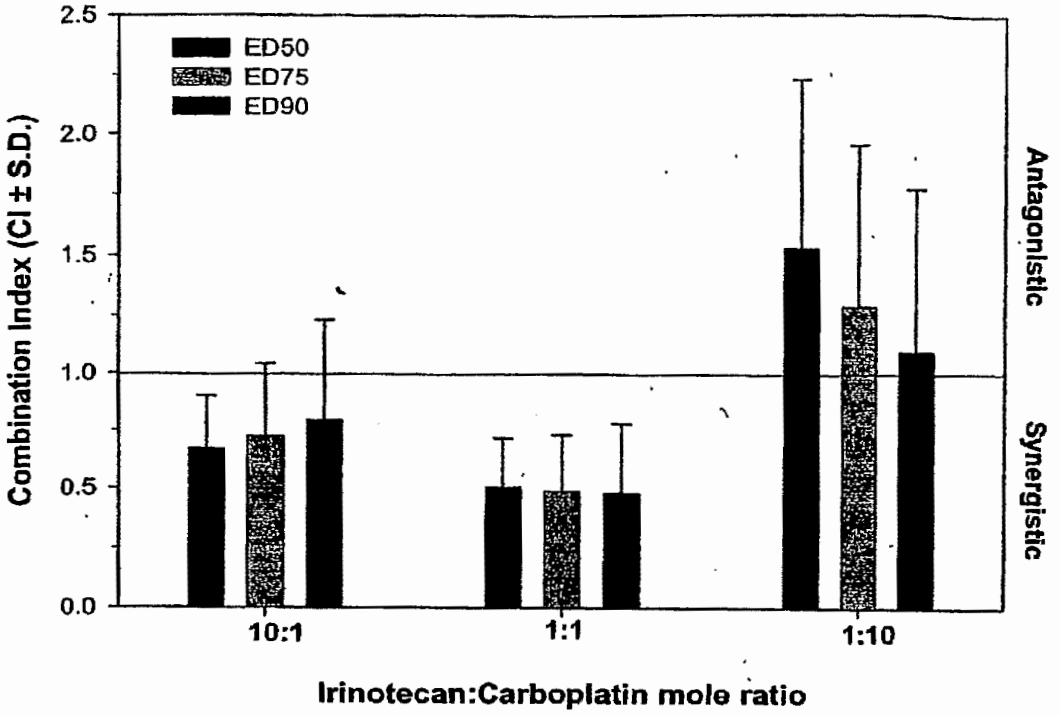

2.5
2.0
1.5
1.0
0.5
0.0
ED50
ED75
ED90
Combination Index (CI ± S.D.)
Antagonistic
Synergistic
10:1
1:1
1:10
Irinotecan:Carboplatin mole ratio

FIGURE 2C

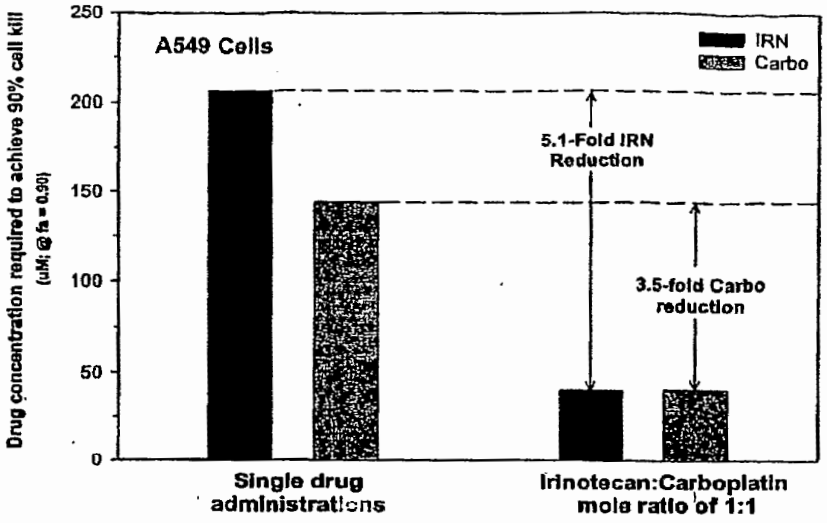


FIGURE 2D

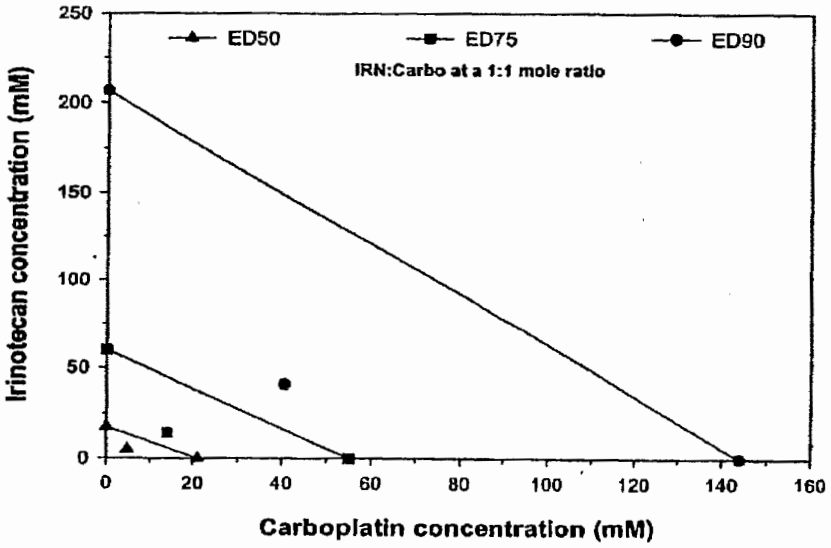


FIGURE 2E

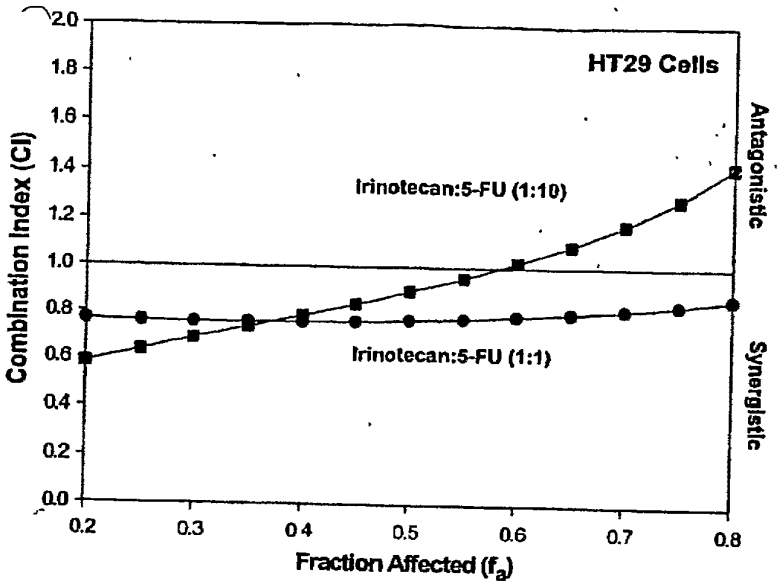


FIGURE 3A

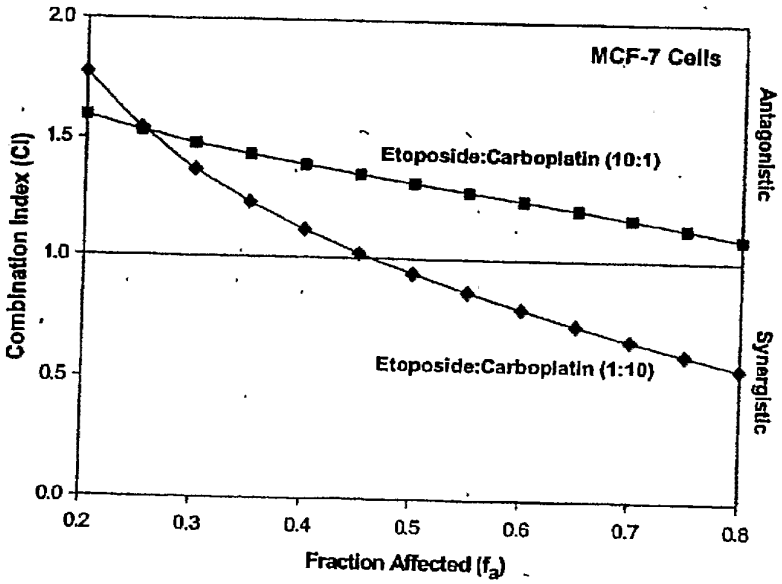


FIGURE 3B

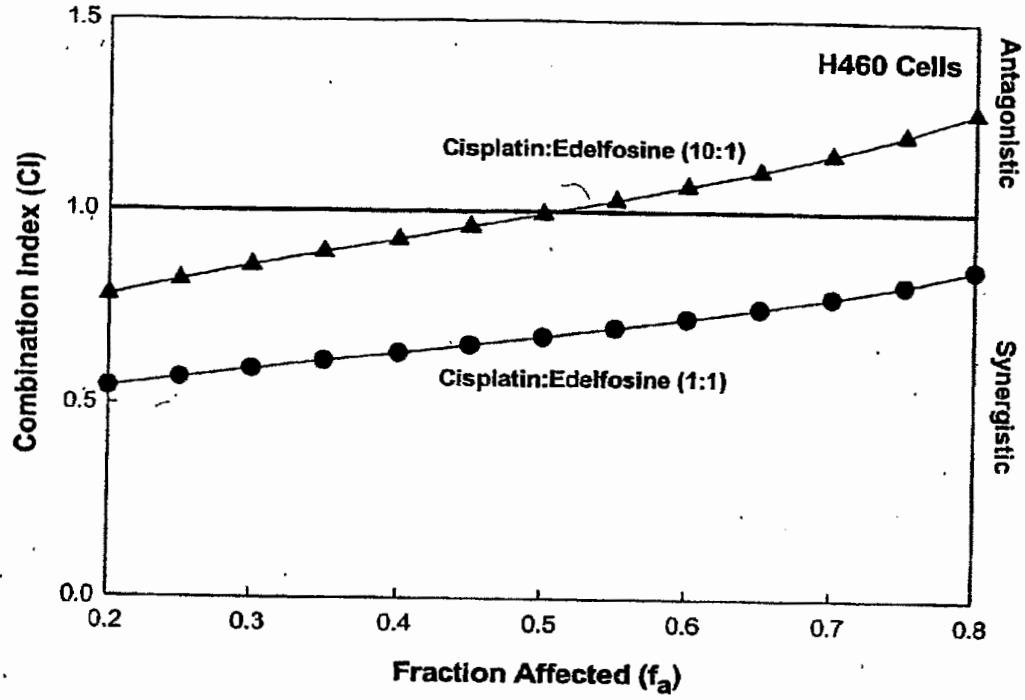

H460 Cells
Cisplatin:Edelfosine (10:1)
Cisplatin:Edelfosine (1:1)
Antagonistic
Synergistic
Combination Index (CI)
Fraction Affected (f_a)
1.5
1.0
0.5
0.0
0.2
0.3
0.4
0.5
0.6
0.7
0.8

FIGURE 4

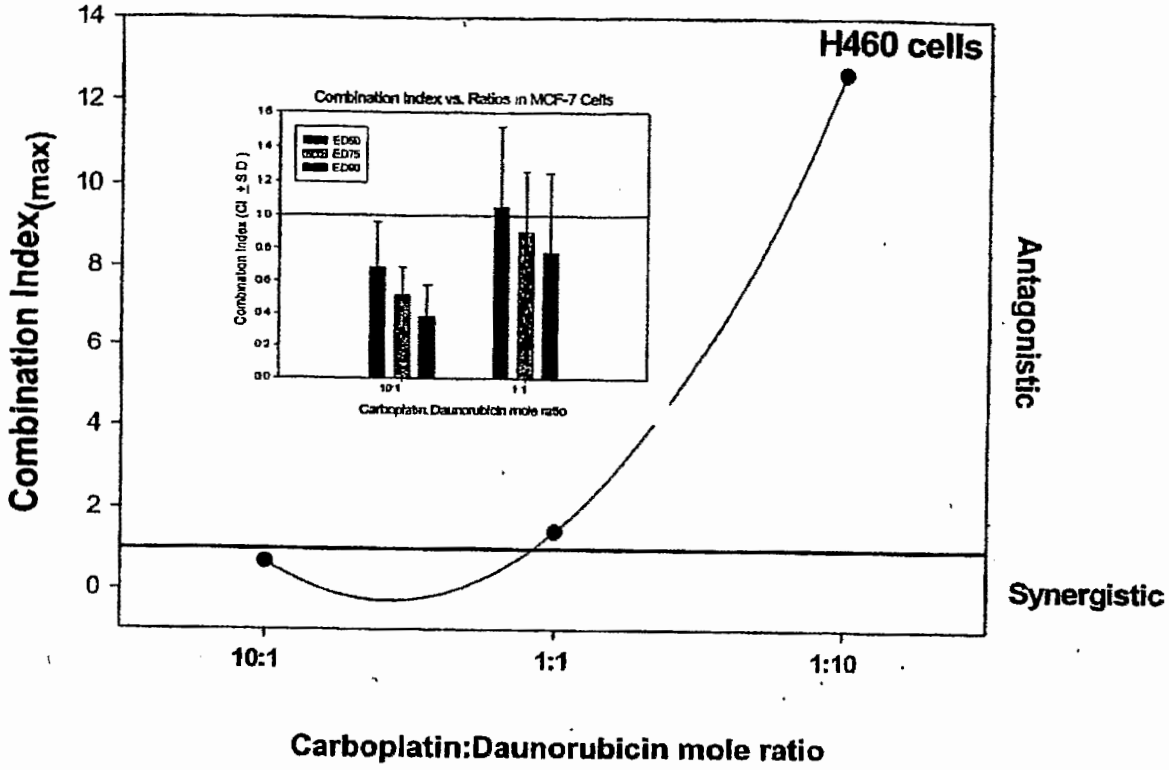

H460 cells
Combination Index(max)
14
12
10
8
6
4
2
0
10:1
1:1
1:10
Antagonistic
Synergistic
Carboplatin:Daunorubicin mole ratio
Combination Index vs. Ratios in MCF-7 Cells
ED50
ED75
ED90
Combination Index (CI ± S.D.)
Carboplatin:Daunorubicin mole ratio

FIGURE 5A

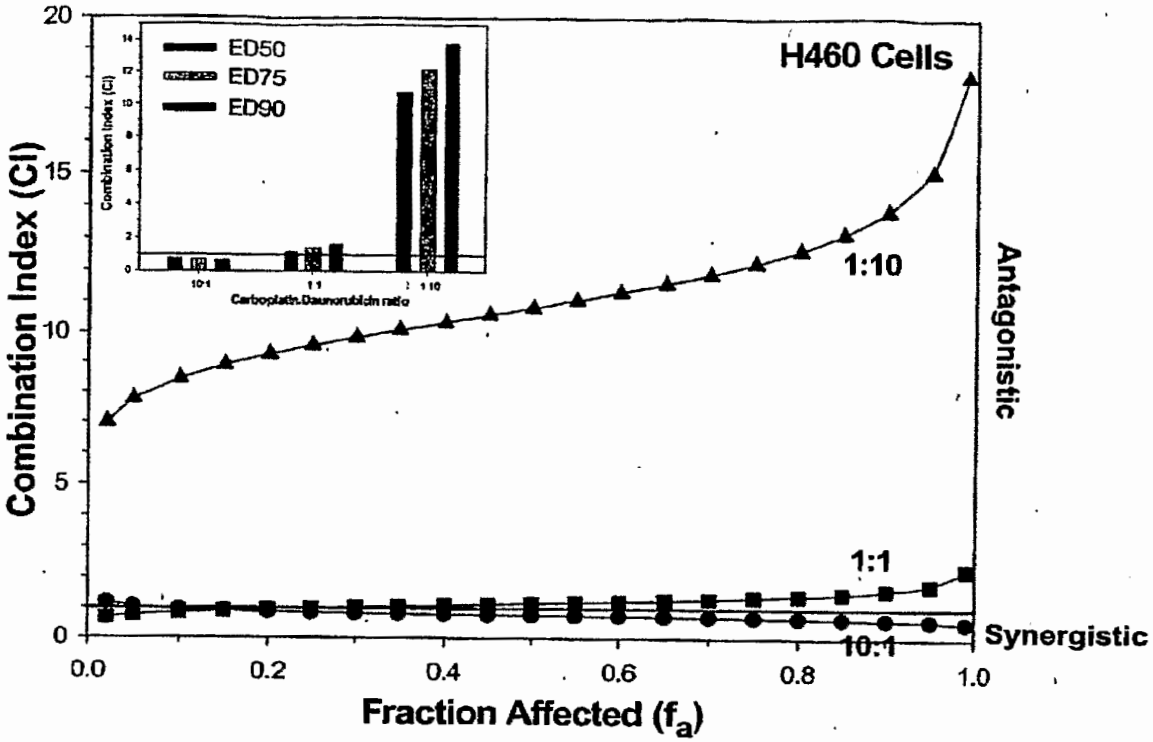

H460 Cells
ED50
ED75
ED90
Combination Index (CI)
Carboplatin:Daunorubicin ratio
1:10
1:1
10:1
Antagonistic
Synergistic
Combination Index (CI)
Fraction Affected (f_a)
0
5
10
15
20
0.0
0.2
0.4
0.6
0.8
1.0

FIGURE 5B

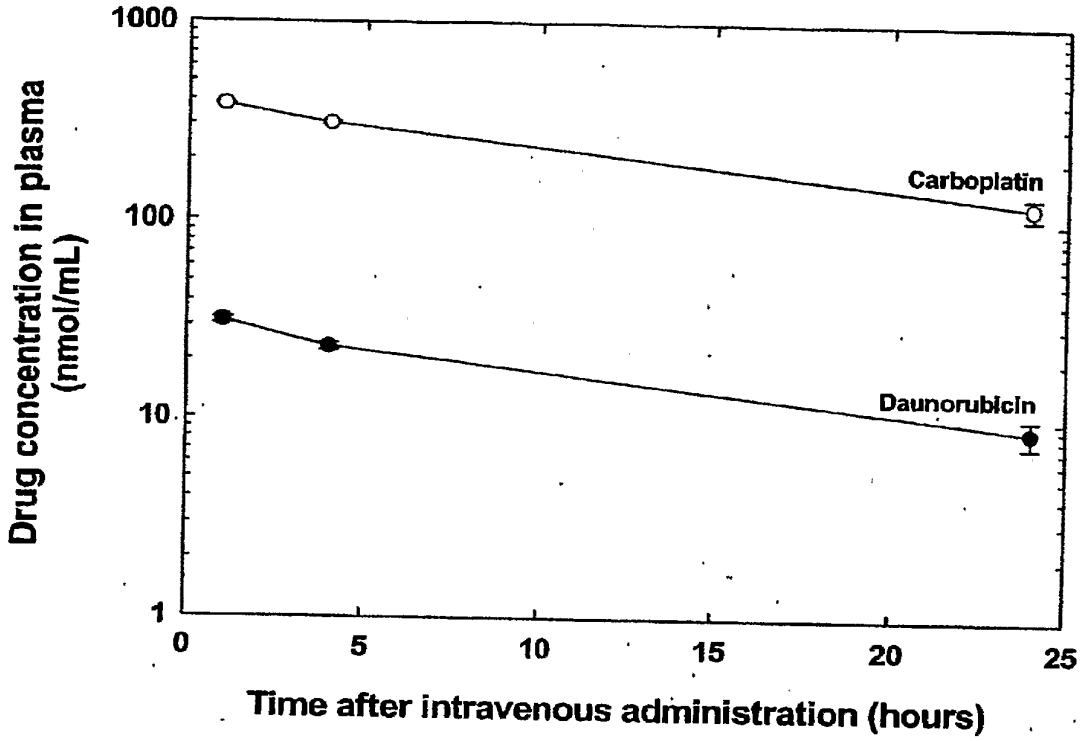

1000
100
10
1
Drug concentration in plasma (nmol/mL)
Carboplatin
Daunorubicin
0
5
10
15
20
25
Time after intravenous administration (hours)

FIGURE 6

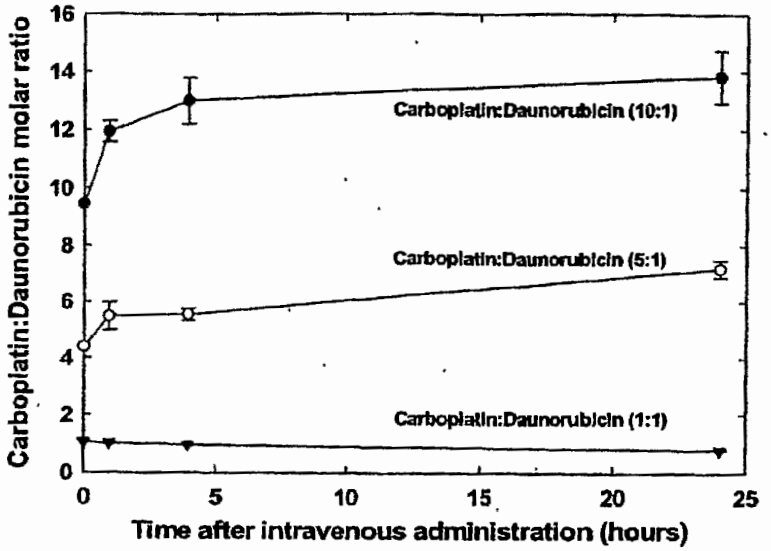


FIGURE 7A

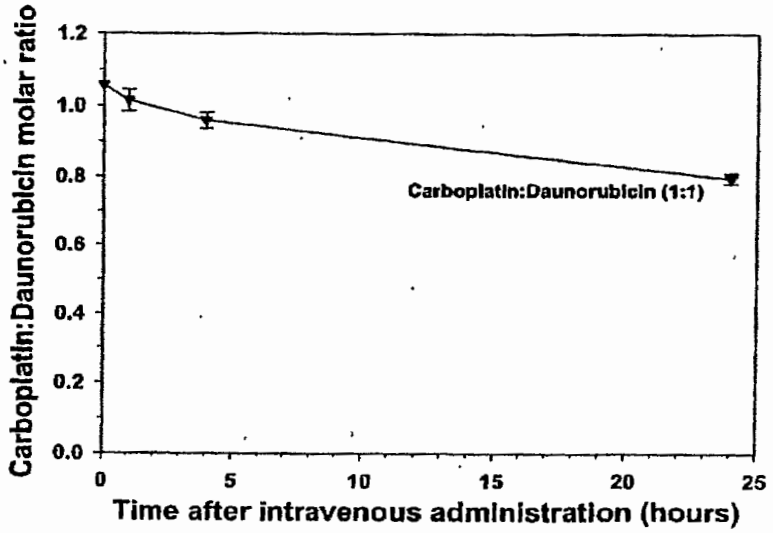


FIGURE 7B

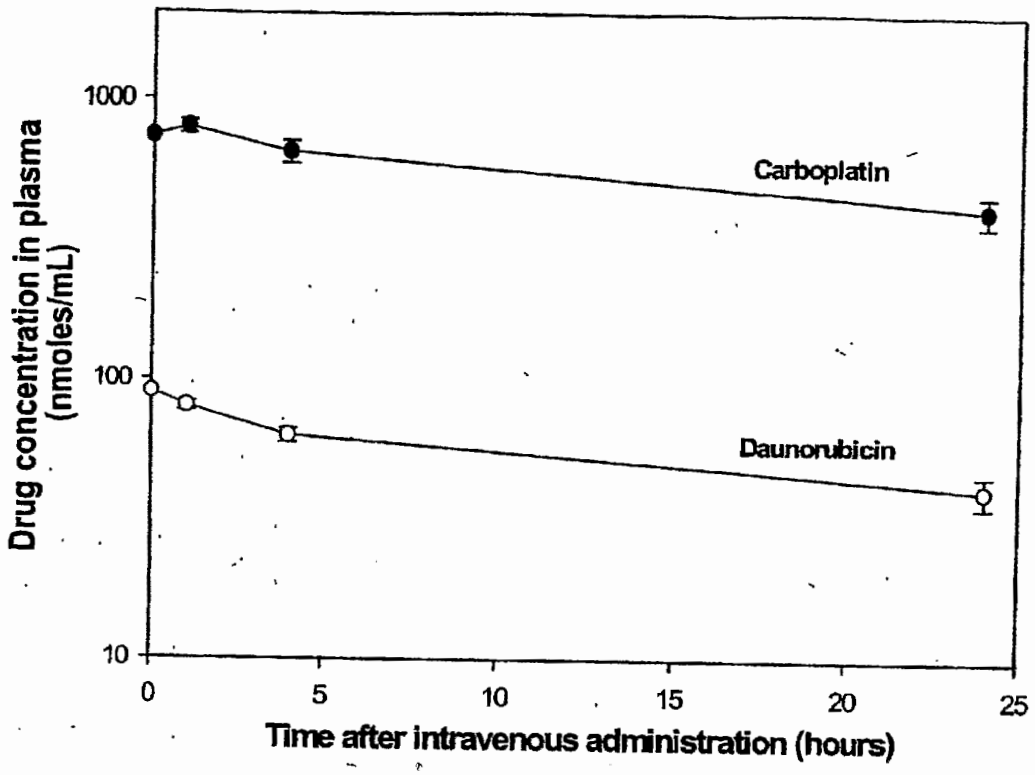

Drug concentration in plasma (nmoles/mL)
1000
100
10
Carboplatin
Daunorubicin
0
5
10
15
20
25
Time after intravenous administration (hours)

FIGURE 8

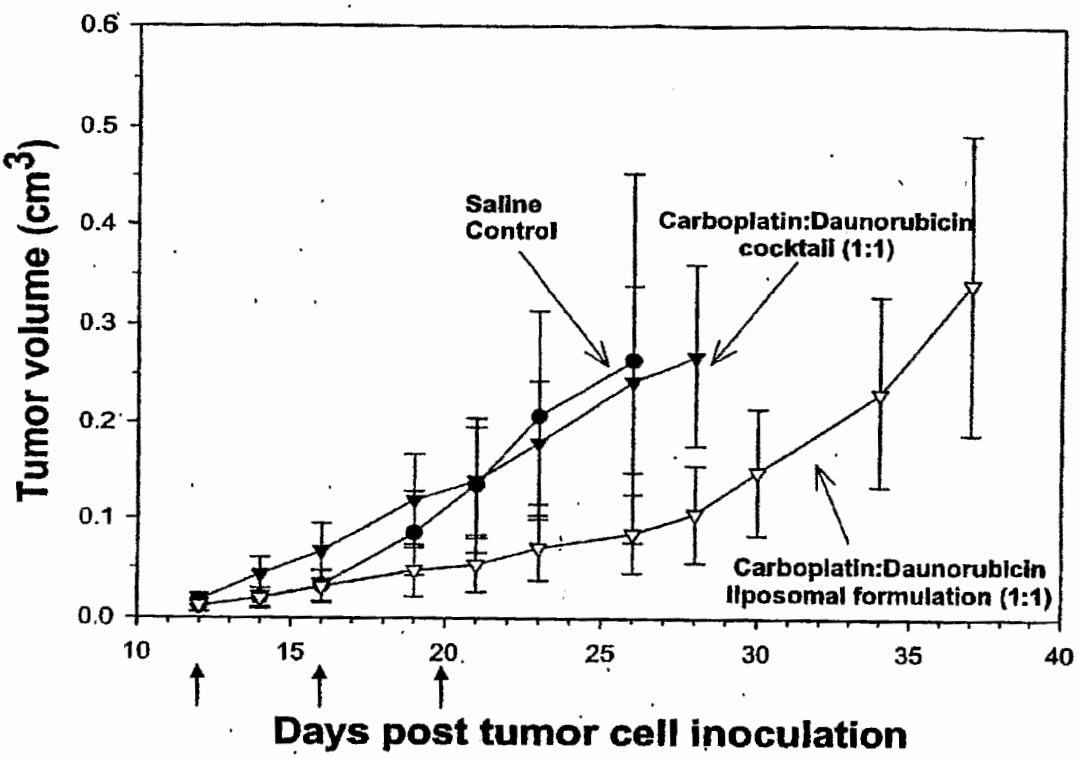

Tumor volume (cm3)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Saline Control
Carboplatin:Daunorubicin cocktail (1:1)
Carboplatin:Daunorubicin liposomal formulation (1:1)
10
15
20
25
30
35
40
Days post tumor cell inoculation

FIGURE 9

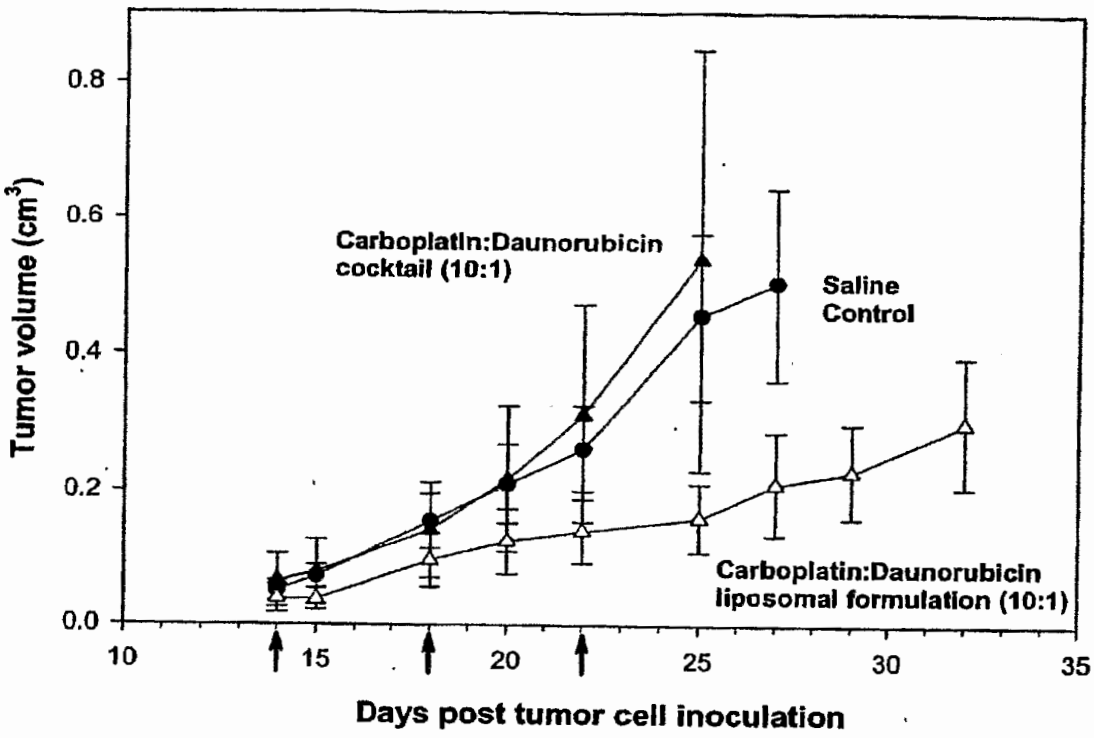

Tumor volume (cm3)
0.0
0.2
0.4
0.6
0.8
10
15
20
25
30
35
Days post tumor cell inoculation
Carboplatin:Daunorubicin cocktail (10:1)
Saline Control
Carboplatin:Daunorubicin liposomal formulation (10:1)

FIGURE 10

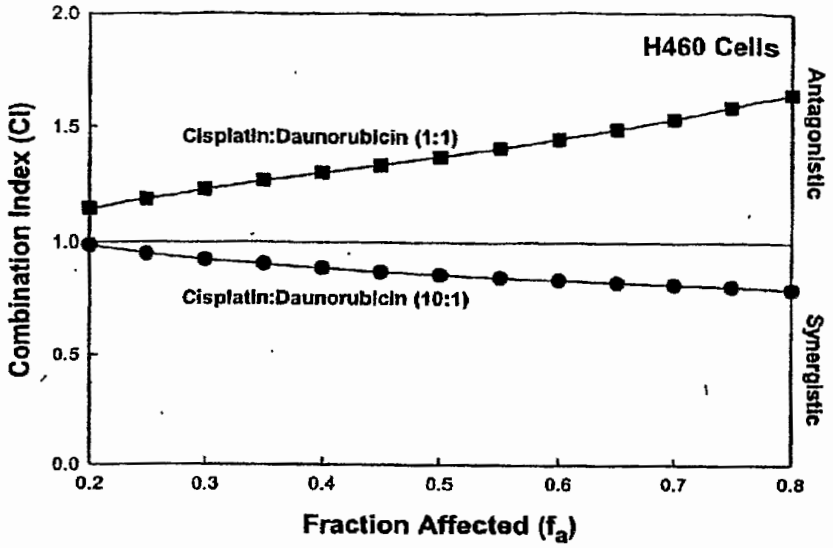


FIGURE 11A

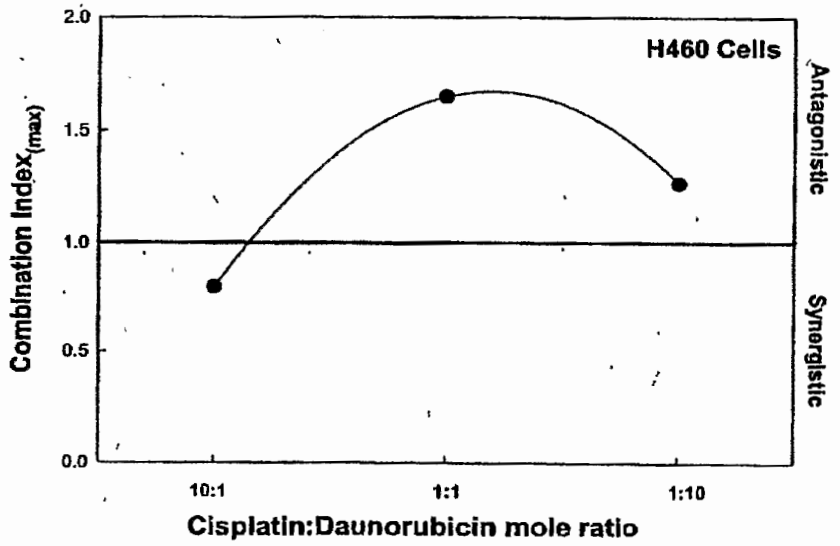


FIGURE 11B

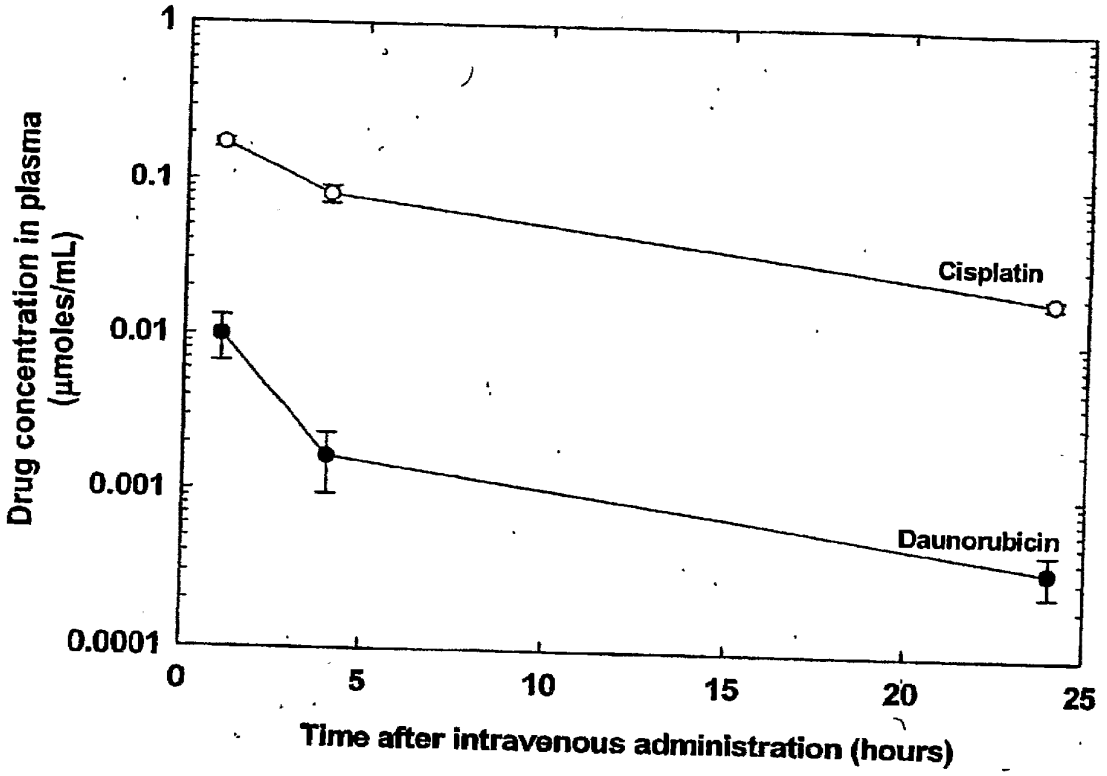

1
0.1
0.01
0.001
0.0001
Drug concentration in plasma (µmoles/mL)
Cisplatin
Daunorubicin
0
5
10
15
20
25
Time after intravenous administration (hours)

FIGURE 12

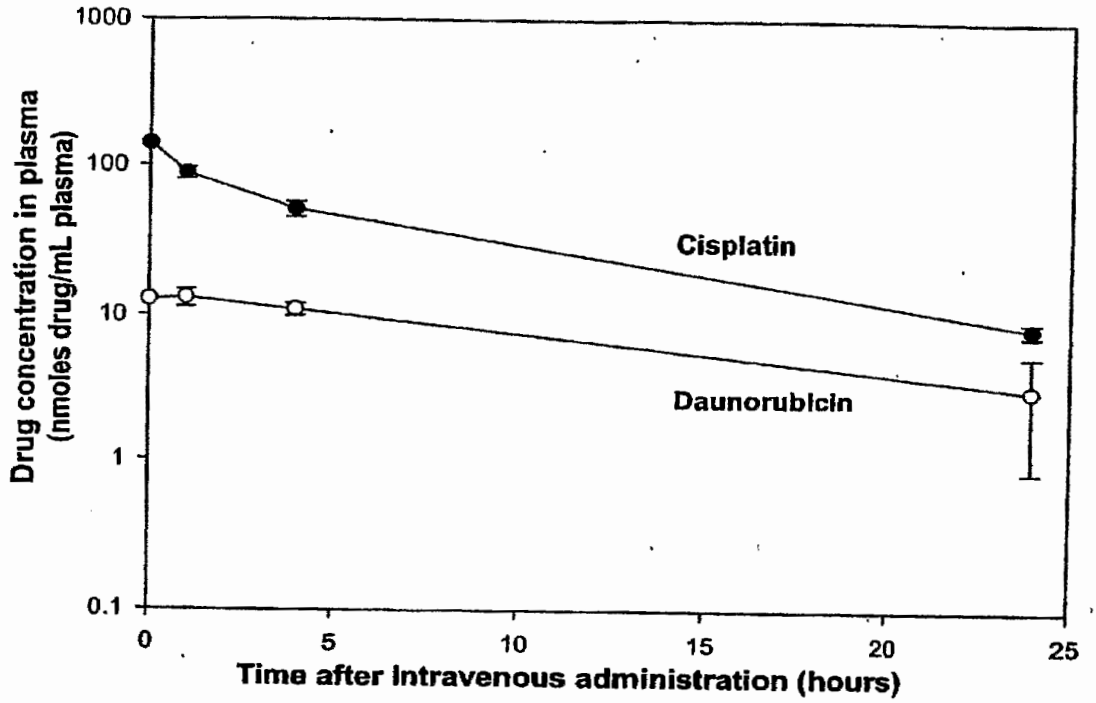

1000
100
10
1
0.1
Drug concentration in plasma
(nmoles drug/mL plasma)
Cisplatin
Daunorubicin
0
5
10
15
20
25
Time after intravenous administration (hours)

FIGURE 13

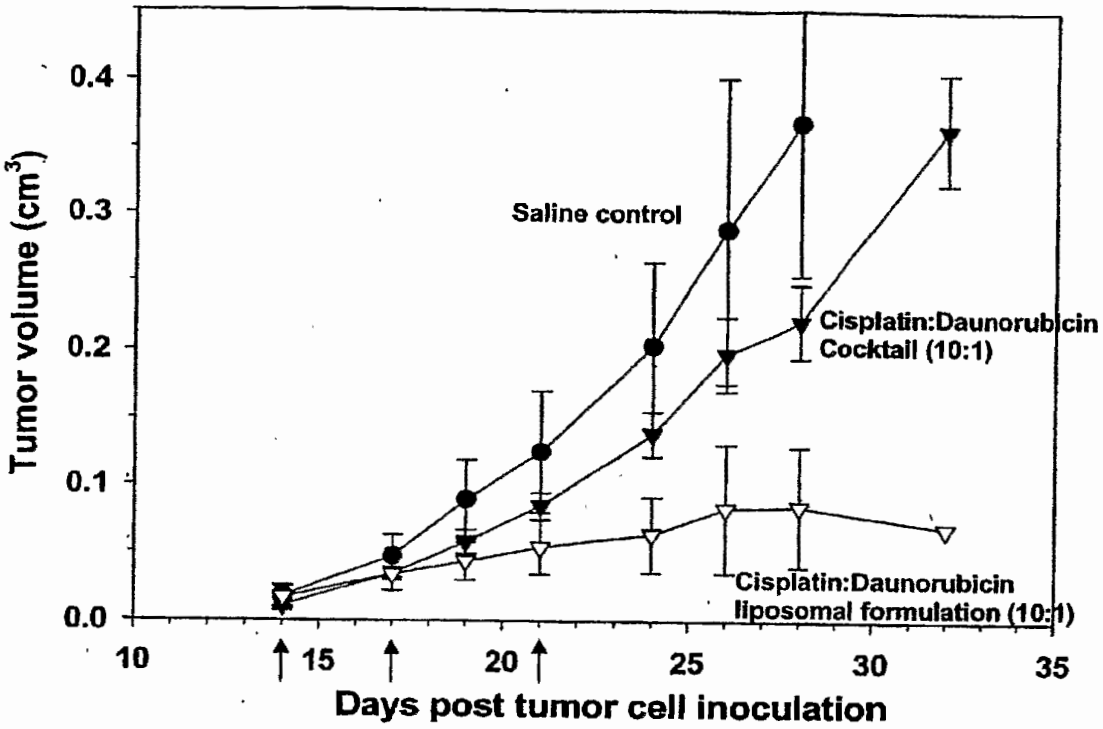

Tumor volume (cm3)
0.4
0.3
0.2
0.1
0.0
Saline control
Cisplatin:Daunorubicin
Cocktail (10:1)
Cisplatin:Daunorubicin
liposomal formulation (10:1)
10
15
20
25
30
35
Days post tumor cell inoculation

FIGURE 14

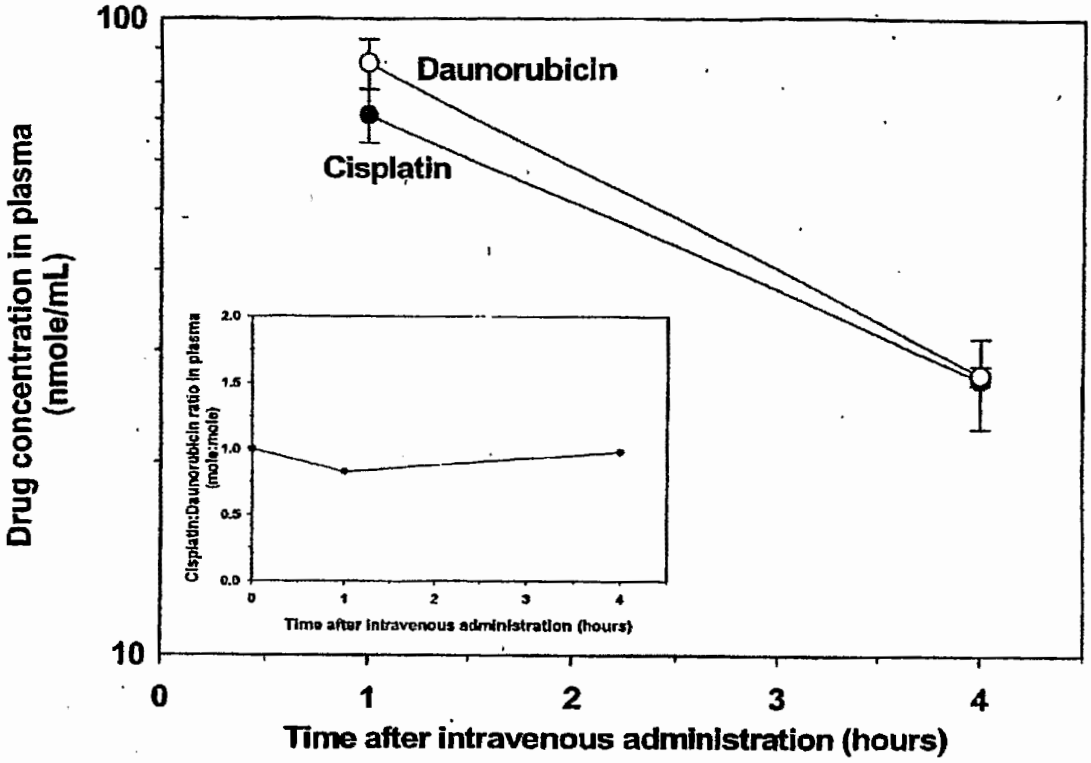

100
10
Drug concentration in plasma (nmole/mL)
Daunorubicin
Cisplatin
0
1
2
3
4
Time after intravenous administration (hours)
Cisplatin:Daunorubicin ratio in plasma (mole:mole)
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
Time after intravenous administration (hours)

FIGURE 15

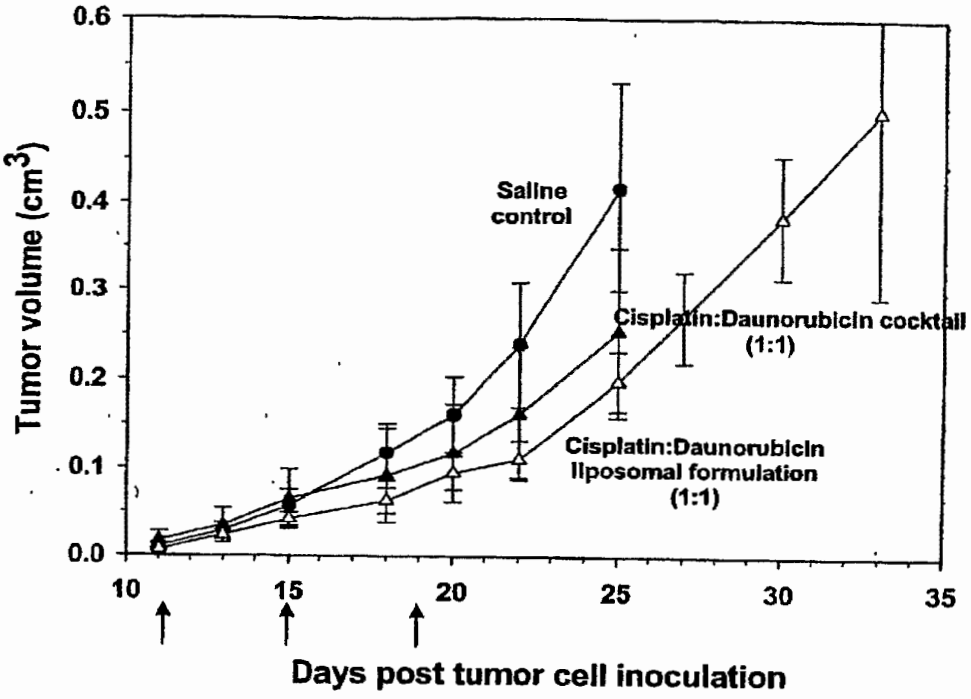

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Tumor volume (cm3)
Saline control
Cisplatin:Daunorubicin cocktail (1:1)
Cisplatin:Daunorubicin liposomal formulation (1:1)
10
15
20
25
30
35
Days post tumor cell inoculation

FIGURE 16

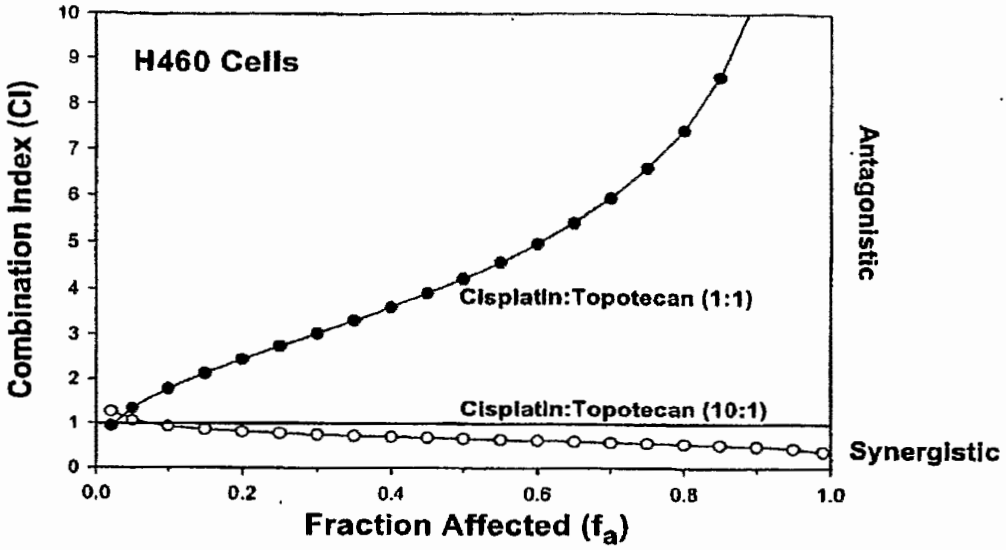


FIGURE 17A

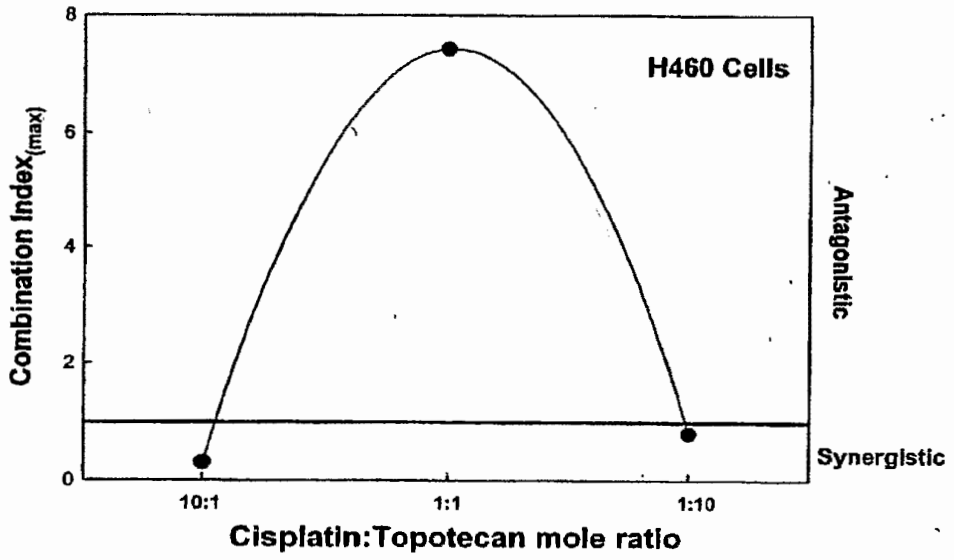


FIGURE 17B

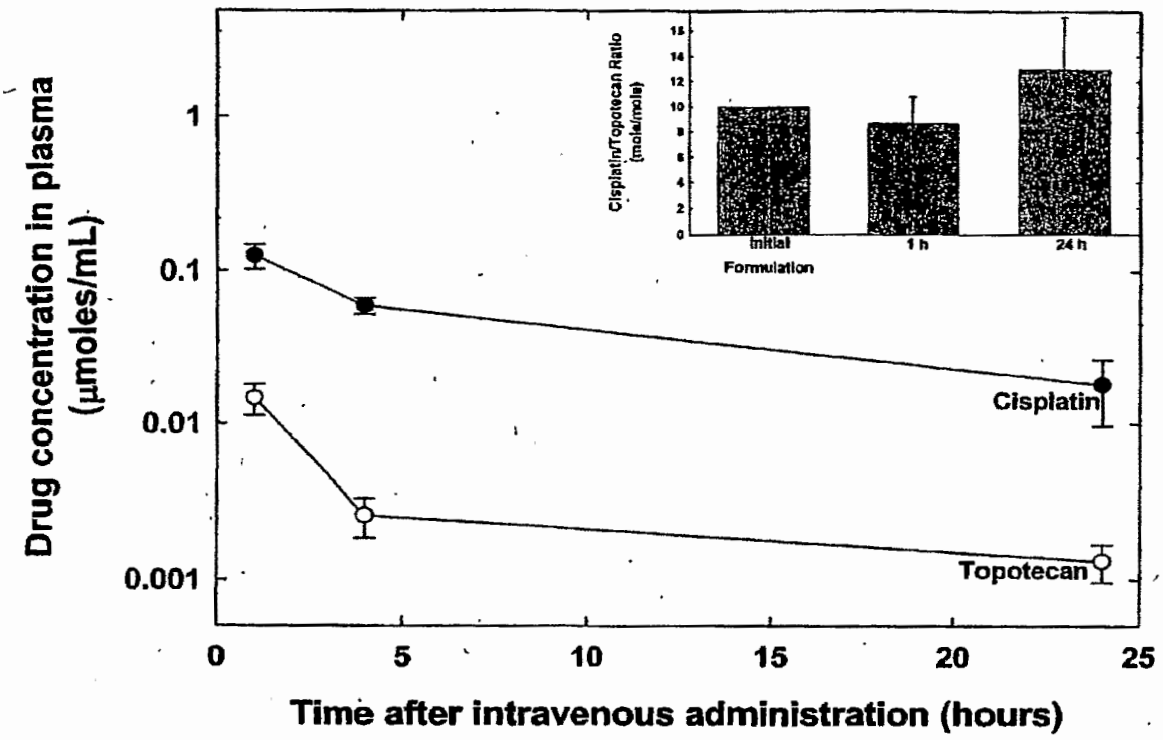

Drug concentration in plasma (µmoles/mL)
1
0.1
0.01
0.001
0
5
10
15
20
25
Time after intravenous administration (hours)
Cisplatin
Topotecan
Cisplatin/Topotecan Ratio (mole/mole)
Initial
1 h
24 h
Formulation

FIGURE 18

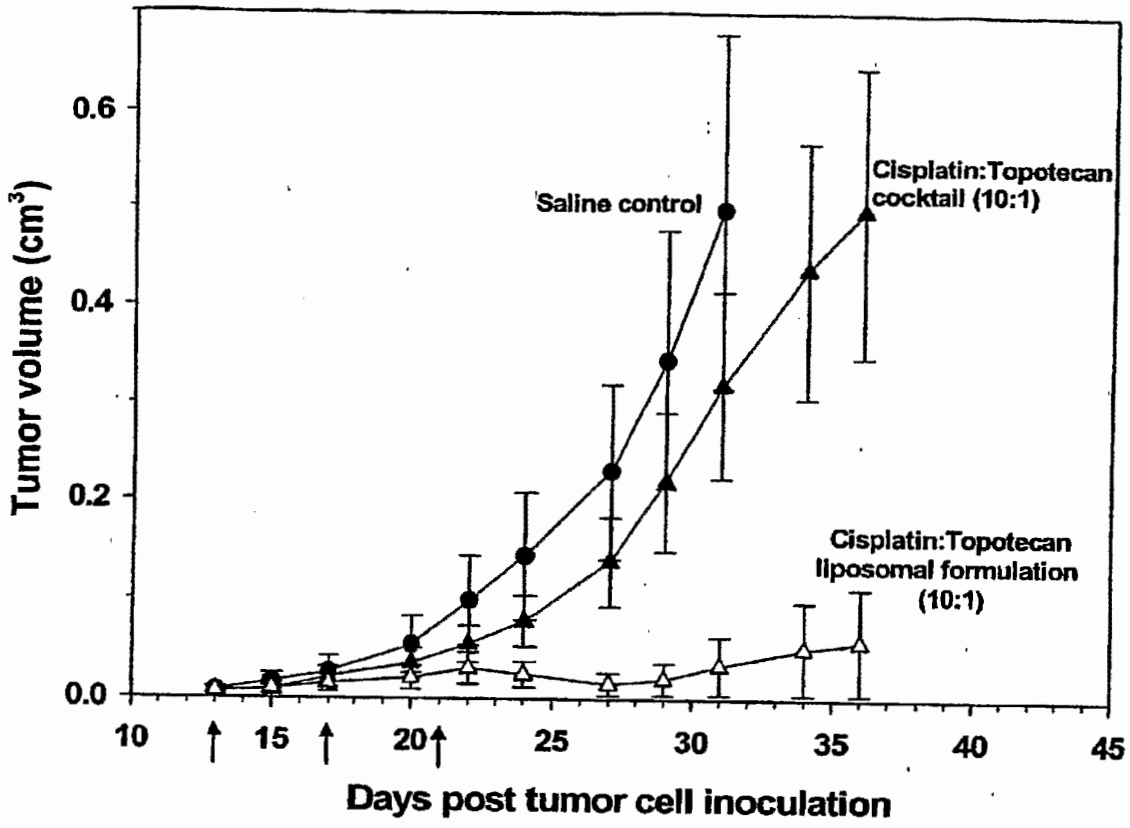

Tumor volume (cm^3)
0.6
0.4
0.2
0.0
Saline control
Cisplatin:Topotecan cocktail (10:1)
Cisplatin:Topotecan liposomal formulation (10:1)
10
15
20
25
30
35
40
45
Days post tumor cell inoculation

FIGURE 19

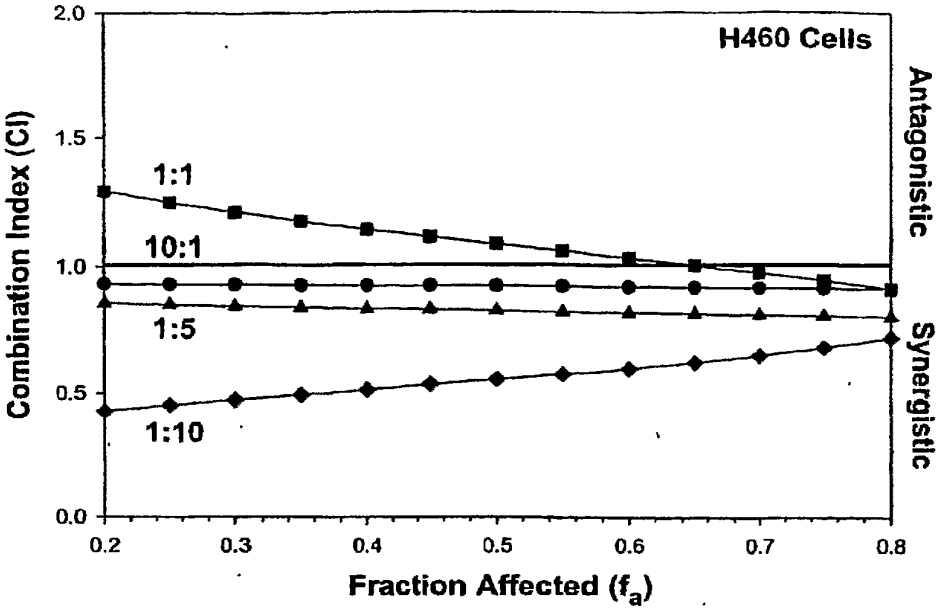


FIGURE 20A

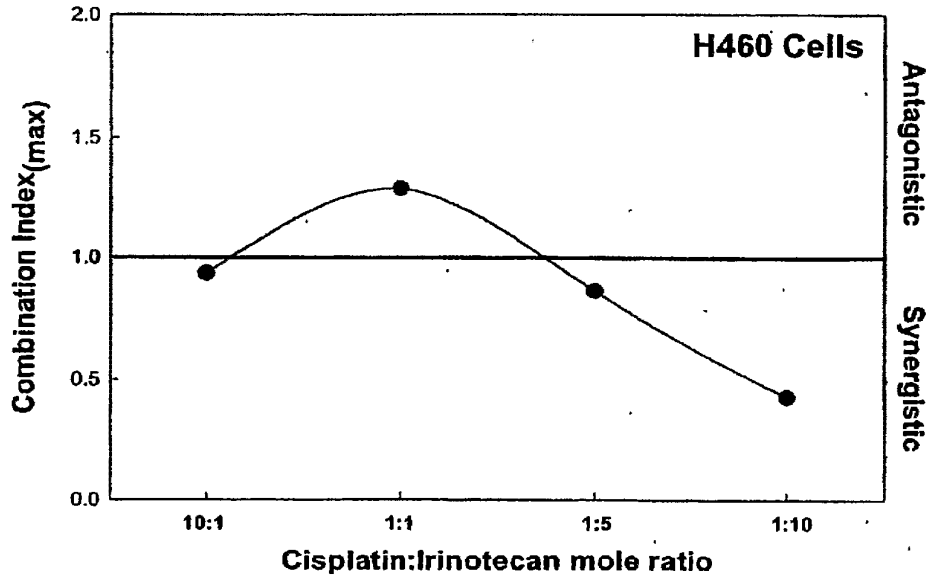


FIGURE 20B

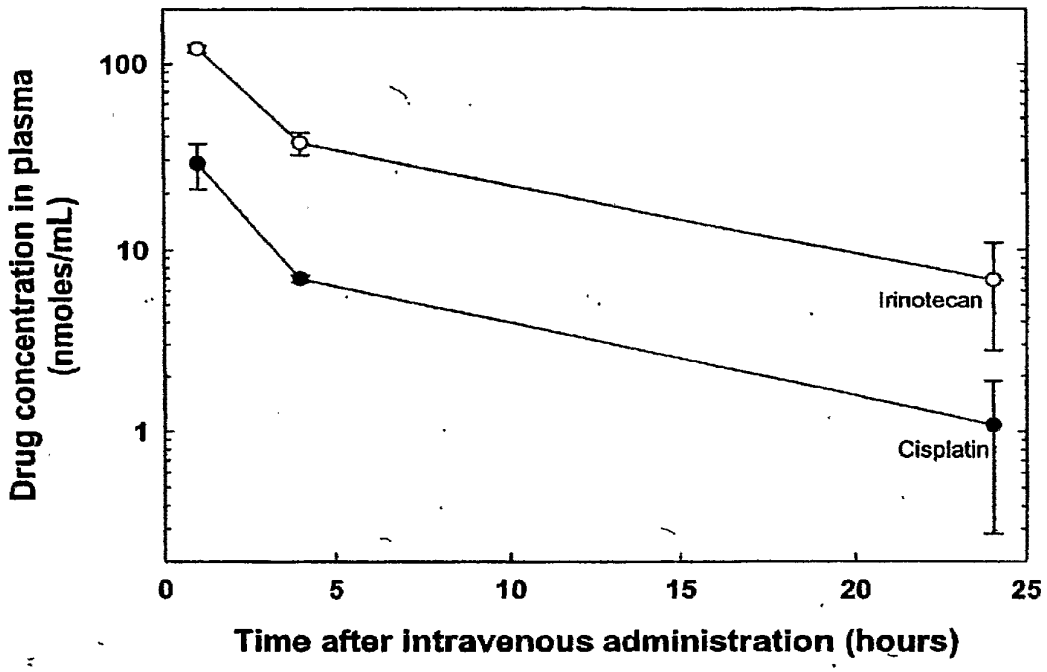

Drug concentration in plasma (nmoles/mL)
100
10
1
0
5
10
15
20
25
Time after intravenous administration (hours)
Irinotecan
Cisplatin

FIGURE 21

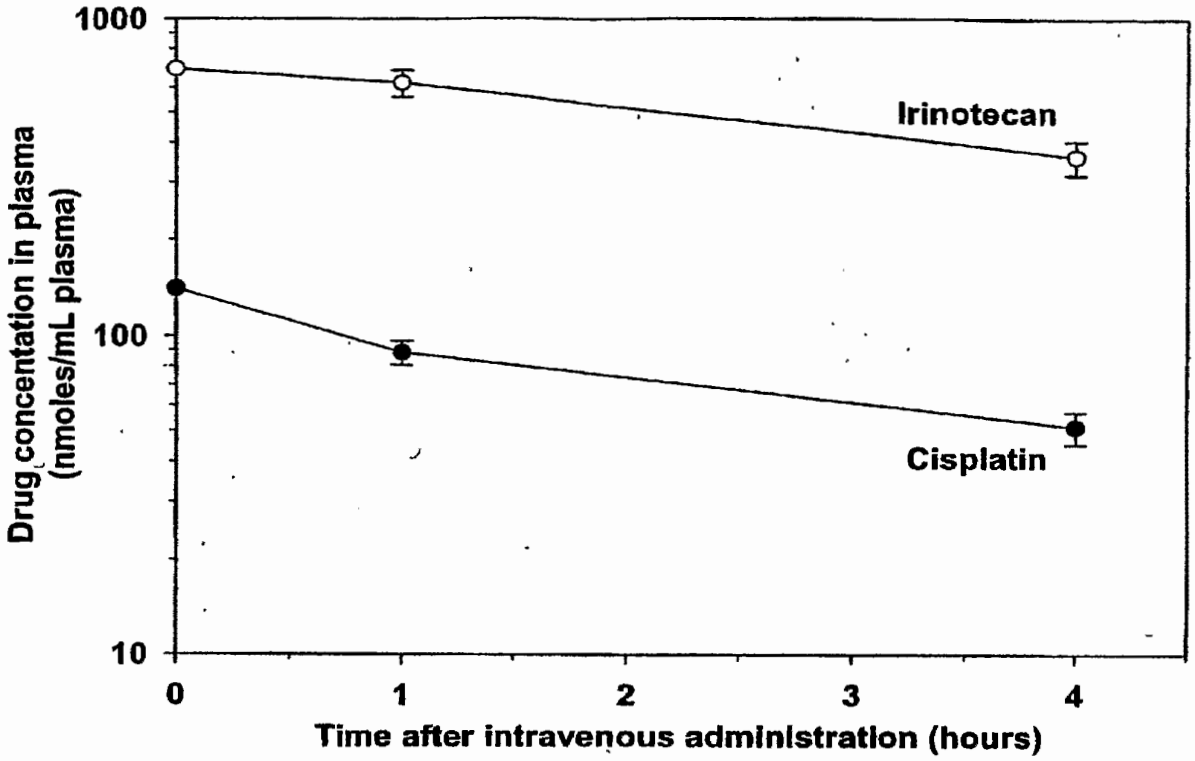

1000
100
10
Drug concentation in plasma (nmoles/mL plasma)
Irinotecan
Cisplatin
0
1
2
3
4
Time after intravenous administration (hours)

FIGURE 22

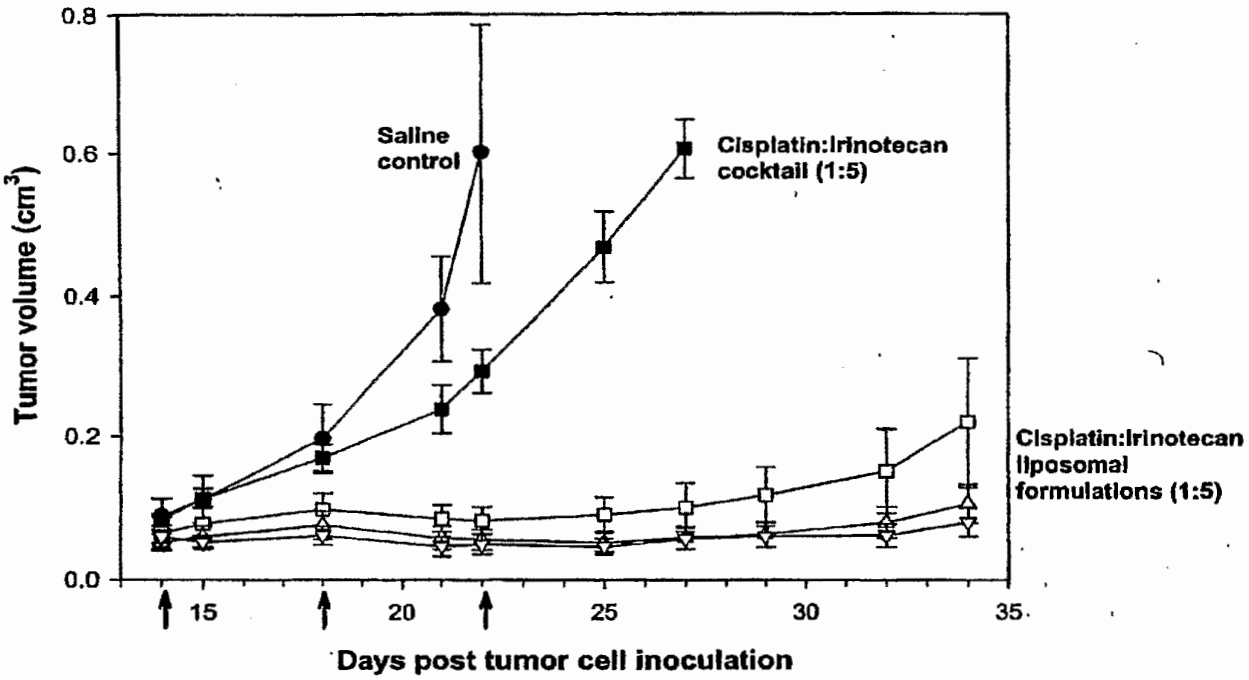

0.8
0.6
0.4
0.2
0.0
Tumor volume (cm3)
Saline control
Cisplatin:Irinotecan cocktail (1:5)
Cisplatin:Irinotecan liposomal formulations (1:5)
15
20
25
30
35
Days post tumor cell inoculation

FIGURE 23

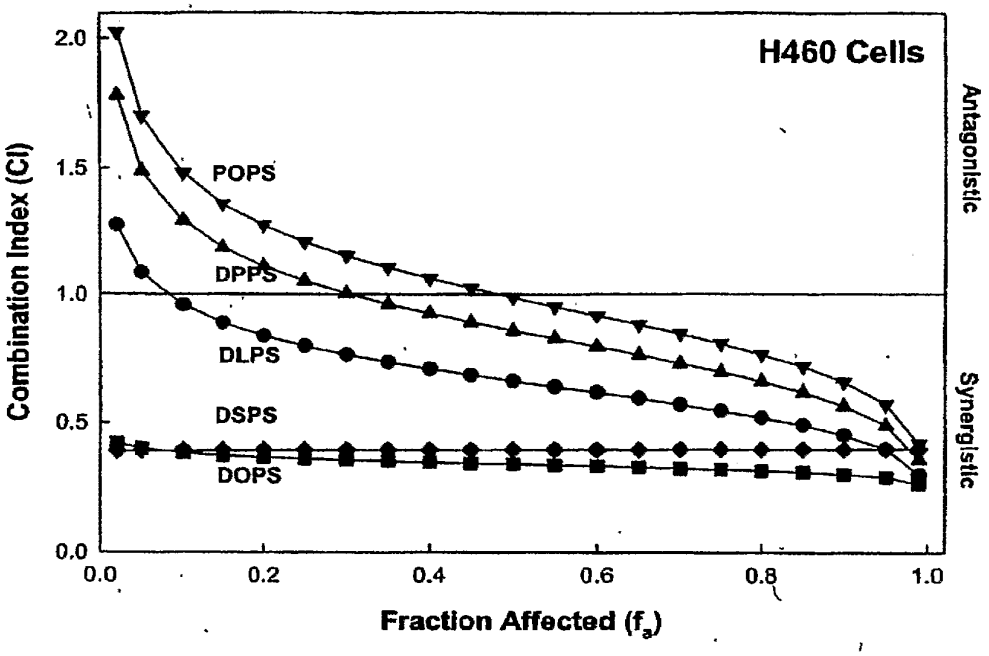

H460 Cells
Combination Index (CI)
Fraction Affected (f_a)
Antagonistic
Synergistic
POPS
DPPS
DLPS
DSPS
DOPS
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0

FIGURE 24

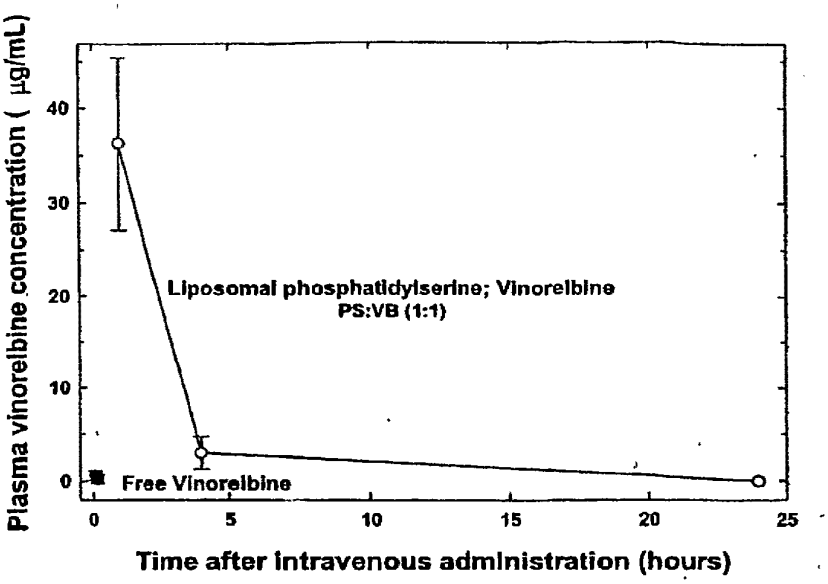


FIGURE 25A

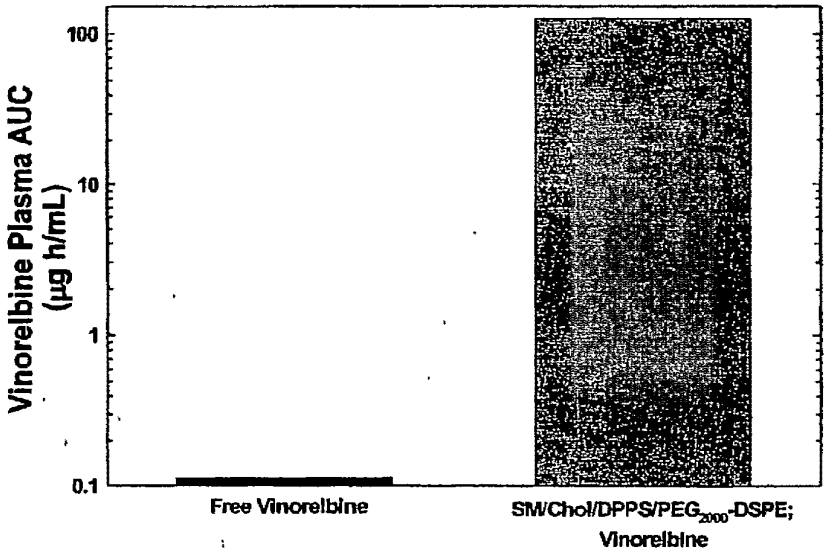


FIGURE 25B

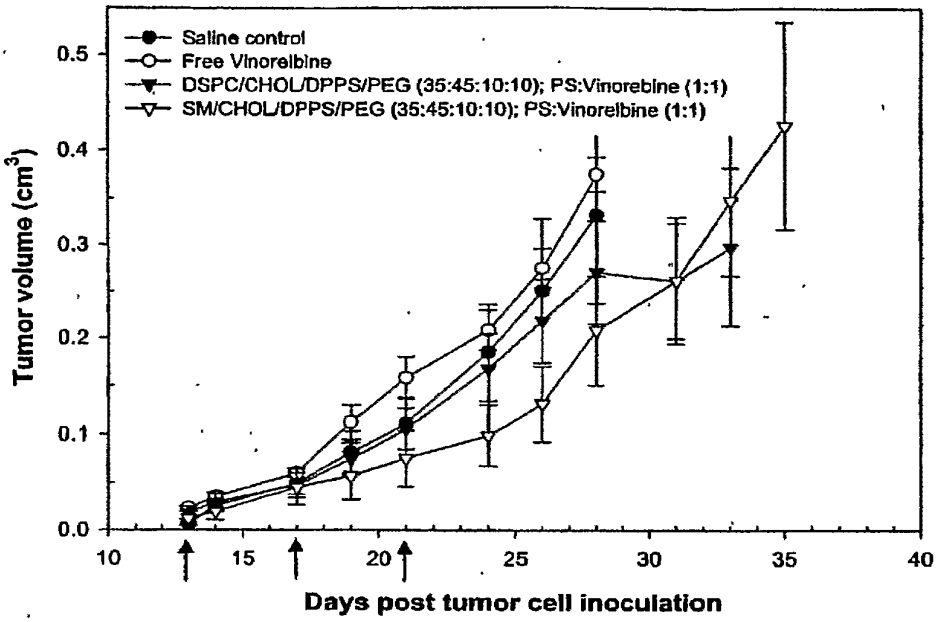

Saline control
Free Vinorelbine
DSPC/CHOL/DPPS/PEG (35:45:10:10); PS:Vinorelbine (1:1)
SM/CHOL/DPPS/PEG (35:45:10:10); PS:Vinorelbine (1:1)
Tumor volume (cm3)
0.0
0.1
0.2
0.3
0.4
0.5
10
15
20
25
30
35
40
Days post tumor cell inoculation

FIGURE 26

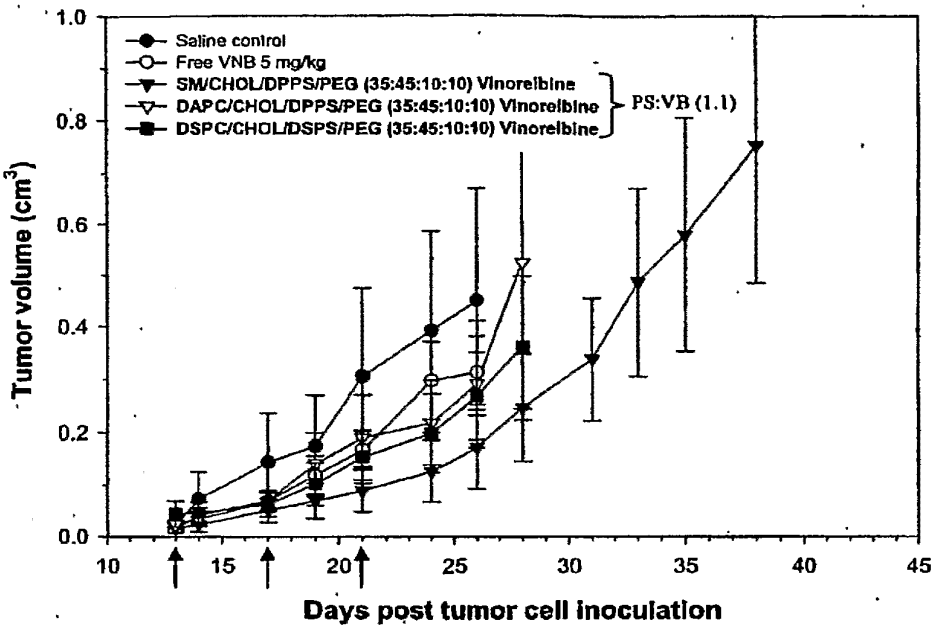

Saline control
Free VNB 5 mg/kg
SM/CHOL/DPPS/PEG (35:45:10:10) Vinorelbine
DAPC/CHOL/DPPS/PEG (35:45:10:10) Vinorelbine
DSPC/CHOL/DSPS/PEG (35:45:10:10) Vinorelbine
PS:VB (1.1)
Tumor volume (cm3)
Days post tumor cell inoculation
1.0
0.8
0.6
0.4
0.2
0.0
10
15
20
25
30
35
40
45

FIGURE 27

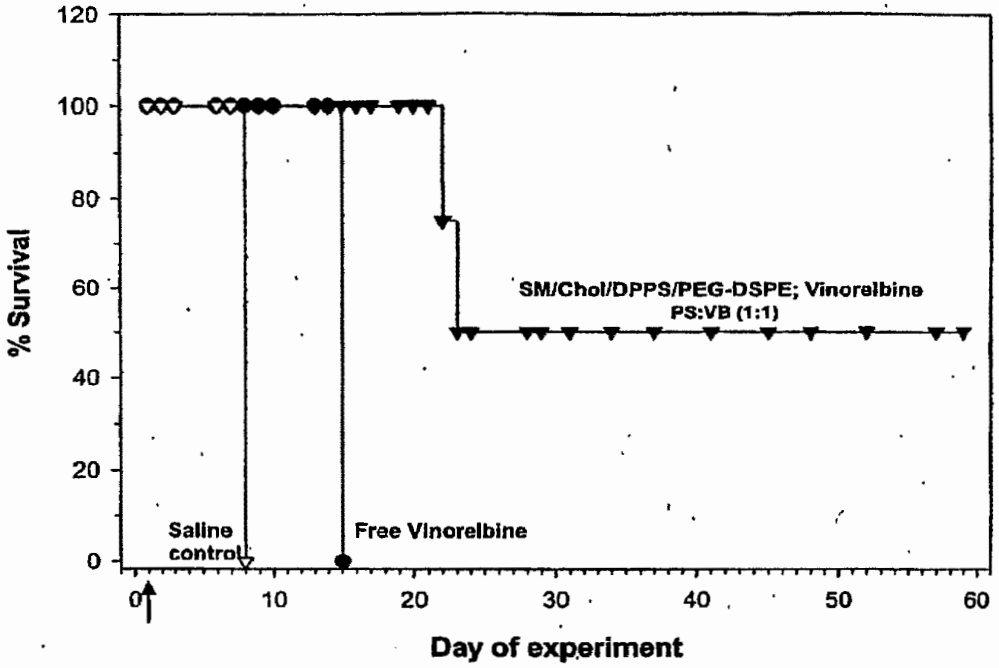

120
100
80
60
40
20
0
% Survival
SM/Chol/DPPS/PEG-DSPE; Vinorelbine
PS:VB (1:1)
Saline
control
Free Vinorelbine
0
10
20
30
40
50
60
Day of experiment

FIGURE 28

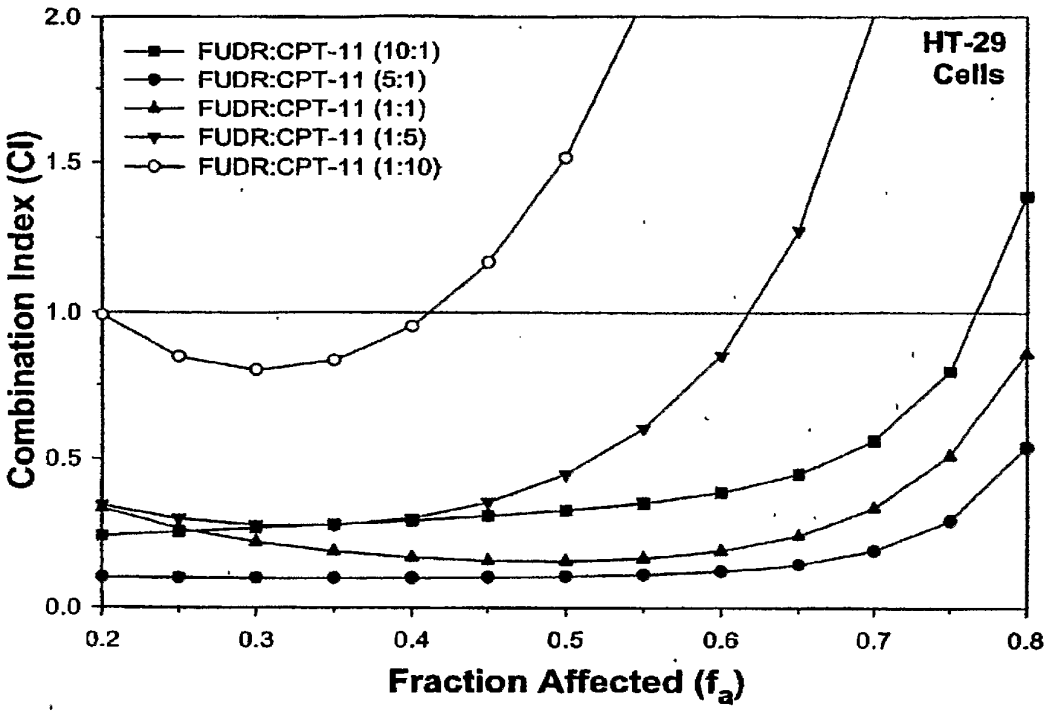

FUDR:CPT-11 (10:1)
FUDR:CPT-11 (5:1)
FUDR:CPT-11 (1:1)
FUDR:CPT-11 (1:5)
FUDR:CPT-11 (1:10)
HT-29
Cells
Combination Index (CI)
Fraction Affected (f_a)
2.0
1.5
1.0
0.5
0.0
0.2
0.3
0.4
0.5
0.6
0.7
0.8

FIGURE 29

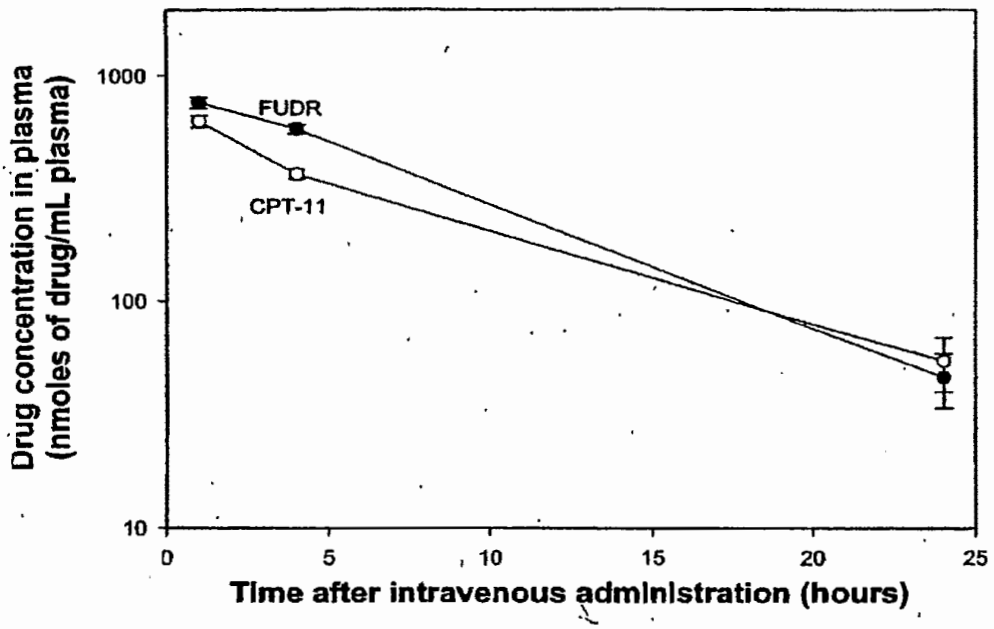

Drug concentration in plasma
(nmoles of drug/mL plasma)
1000
100
10
FUDR
CPT-11
0
5
10
15
20
25
Time after intravenous administration (hours)

FIGURE 30

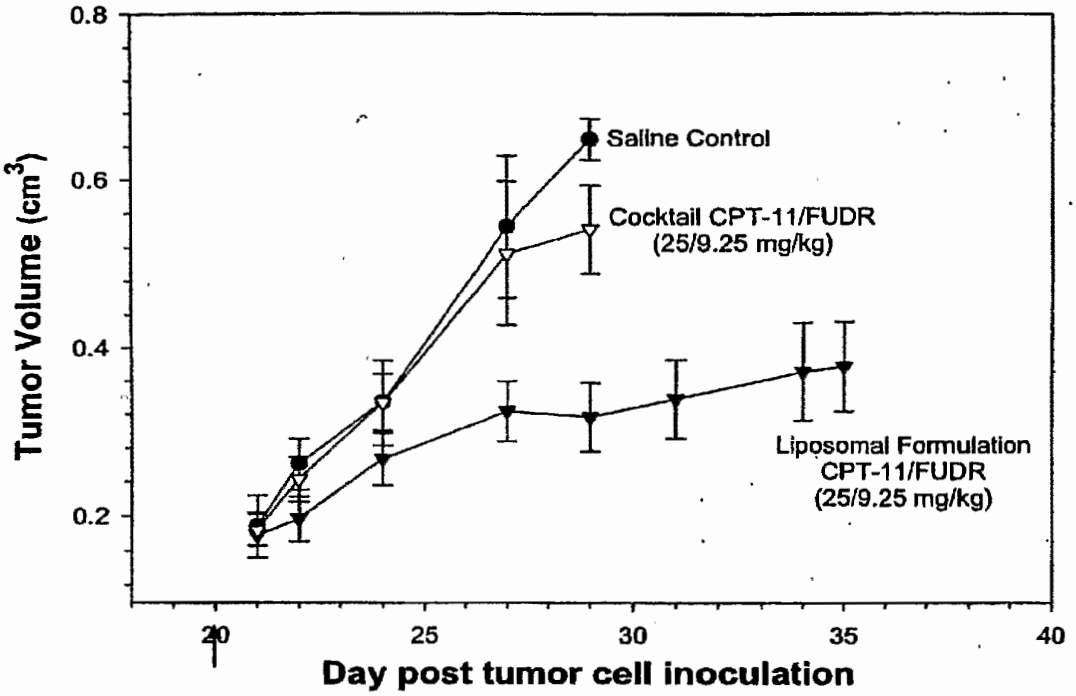

0.8
0.6
0.4
0.2
Tumor Volume (cm3)
Saline Control
Cocktail CPT-11/FUDR
(25/9.25 mg/kg)
Liposomal Formulation
CPT-11/FUDR
(25/9.25 mg/kg)
20
25
30
35
40
Day post tumor cell inoculation

FIGURE 31

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(54) **Title:** IMMUNOLIPOSOMES FOR TREATMENT OF CANCER

(57) **Abstract:** The present invention relates to immunoliposomes for multiple treatment of human patients suffering from cancer, particularly a cancer represented by a locally advanced or metastatic tumor and to compositions used in said method. The invention further relates to the use of immunoliposomes for the treatment of multi-drug resistance in cancer therapy.

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IMMUNOLIPOSOMES FOR TREATMENT OF CANCER

The present invention is in the area of cancer treatment. In particular, the invention relates to first- and higher-line treatment of human patients suffering from cancer, particularly a cancer represented by a locally advanced or metastatic tumor and to compositions used in said method.

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor of the ErbB family that is abnormally activated in many epitheiiai tumors. Receptor activation leads to recruitment and phosphorylation of severaf downstream intracellular substrates, ieading to mitogenic signaling and other tumor-promoting cellular activities. In human tumors, receptor overexpression correlates with a more aggressive clinical course *(1, 2)*. Monoclonal antibodies directed at the iigand-binding extracellular domain and low-molecular weight inhibitors of the receptor's tyrosine kinase are currently in advanced stages of clinicai development.

Among available anti-EGFR MAbs, the one best characterized is the chimeric human:muriπe MAb cetuximab. Cetuximab is a potent inhibitor of the growth of cultured cancer cells that have an active autocrine EGFR loop. A series of phase i, phase II and phase III studies of cetuximab given alone or in combination either with chemotherapy or radiation have now been completed. Cefuxïmab was found to be safe but showed some side effects including an acneiform skin rash in up to 40-70% of afi treated patients and anaphylactoid or anaphylactic reaciions that occurred in 2% of patients. Nonneutraüzing human antibodies against chimeric antibodies were detected in 4% of patients. The optimal biologic dose, as determined by saturation of antibody clearance, was found to be in the range of 200 to 400 mg/m2 per week (3). Cetuximab is now considered part of standard therapy in patients with colorectal cancer and in head&neck tumors in many countries.

Doxorubicin is one of the most widely used anticancer drugs for the treatment of solid tumors and hematologic malignancies. ft is active against a variety of cancer types, and

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is used extensively as a single agent and in combination chemotherapy regimens. in addition to its pivotal role in the treatment of breast cancer, doxorubicin has also demonstrated antitumor activity in ovarian, cervical, endometrial, gastric, bladder, and small-ceil lung cancer, uterine sarcoma, acute lymphoblastic leukemia, Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, and soft tissue and bone sarcomas. While doxorubicin displays an excellent antitumor activity profile, its use in ciinical practice is limited by drug-associated toxicities, particularly myelo suppression and cardiotoxicity (citation: "Principals and Practice of Oncology, DeVita, 6th edition").

Liposomal encapsulation of doxorubicin was used to alter the tissue distribution and pharmacokinetics of the drug and to increase its therapeutic index. Pegyiated liposomal doxorubicin (DOXYL, Ortho Biotech Products LP, Bridgewater, NJ; CAELYX, Schering Plough, Keniiworth, NJ) is a new formulation of doxorubicin. Pegylation protects the liposomes from detection by the mononuclear phagocyte system and increases circulation time, allowing for more targeted delivery of doxorubicin to the tumor ceils.

Pegyiated liposomal doxorubicin has demonstrated efficacy as a single agent in patients with metastatic or recurrent breast cancer, with objective response rates ranging from 9% to 33% *(4, 5).* In comparison with conventional doxorubicin, pegyiated liposomal doxorubicin has a similar efficacy profile and an improved safety profile, with a significantly reduced incidence of cardiotoxicity and significantly fewer cardiac events, as well as a reduced incidence of myelosuppression, mucositis, nausea, vomiting, and alopecia.

On the other hand, pegyiated liposomal doxorubicin is associated with palmar plantar erythema (PPE = hand-foot syndrome), a toxicity rarely or never seen with free doxorubicin.

in addition b its use in breast cancer, liposomai doxorubicin plays a well established role in the treatment of Kaposi's sarcoma *(6,7)* and recurrent ovarian cancer (S), and has also been successfully used in patients with different types of lymphomas, multiple myeloma, soft tissue sarcoma, glioma, melanoma, mesothelioma, transitional cell carcinoma of the urothelial tract, and in endometrial, pancreatic, gastric, small-eel! and non-smaSI-cell lung, hepatocellular, endometrial, renal cell, head and neck, and cholangiocarcinoma (overview in: (9)).

For preclinical studies anti-EGFR immunoliposomes were constructed by using Fab' fragments of the chimeric MAb cetuximab (C225, cetuximab, erbitux, ImClone Systems Corp., NY, USA; Merck KGaA, Darmstadt, Germany), which were covaientiy conjugated

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to the liposome membrane. This approach was designed to provide maximal drug delivery to cancer cells via a receptor-targeted and internalizing drug carrier that is stable, non-immunogenic, long-iived with extended blood and tissue residence times and capable of delivering large payioads of diverse types of drugs. In parallel with MAb fragment optimization, conjugation methodology was also optimized. A new micellar incorporation method was developed involving 2-step conjugation of MAb fragments to preformed drug loaded liposomes *(10).* First, MAb fragments (Fab') were covalently conjugated to derivatized PEG-phosphatidyl-ethanolamine (MAL-PEG-DSPE) linkers in solution, resulting in immunoconjugates prone to spontaneous micelle formation. Next, the conjugates were incorporated into drug-preloaded liposomes by controlled heating, resulting in MAb fragments covalently conjugated to the termini of PEG chains and anchored to the liposome.

When Fab' of C225 was present at only moderate density on immuno üposomes (30 Fab` per liposome), these immunoliposomes displayed highly efficient binding and internalization in a panel of EGFR or EGFRvIII overexpressing cancer celi lines, as indicated by fluorescence microscopy and FACS *(11).* These included epidermoid cancer cells (A431), breast cancer cells (MDA-MB-468), malignant glioma cells (U87), and EGFRvIII stable transfectants NR6-M cells. In contrast, irrelevant tmmunoiiposomes (anti-HER2) and control liposomes (no MAb) did not bind to or accumulate in A431 , MDA-MB468, U87 or NR6-M cells. Also, anti-EGFR immunofiposomes did not detectably bind b or accumulate in non-EGFR-overexpressing cells (breast cancer cell lines SKBR-3 or SvICF-7).

Under *in vitro* conditions, quantitative studies of immunoliposome uptake, internalization, and intracellular drug delivery were performed using anti-EGFR immunoliposomes loaded with the pH-sensitive probe (HPTS). This method allows quantitative analysis of the kinetics of immunoliposome uptake at neutral pH (surface-bound) versus at acidic pH (endocytosis-associated) *(12).* In MDA-MB-468 cells, anti-EGFR immunoliposomes bound within 5 minutes, followed by intracellular accumulation beginning at 15 minutes and increasing up to 240 minutes. Total uptake of EGFR-targeted immunoliposomes in MDA-MB-468 cells when present at saturating concentrations was 1.70 fmo! phospholipid/cell, which corresponds to uptake of 13,000 liposomes/celi, Uptake of non-targeted liposomes in MDA-MB-468 cells was <300 Hposomes/cell, indicating a >43-fold increase due to targeted delivery. Uptake of anti-

EGFR immunoüposomes in non-EGFR overexpressing MCF-7 cells was 450 ILs/celi, indicating a 28-foid greater accumulation in EGFR-overexpressing MDA-MB468 cells.

In vivo, anti-EGFR immunoitposomes (ILs) showed extremely long circuiation as stable constructs in norma! adult rats following a single Lv. dose, with pharmacokinetics that were indistinguishable from those of stericaily stabilized ("stealth") liposomes *(13).* Moreover, repeat administrations revealed no increase in clearance, further confirming that immunoliposomes retain the long circulation and non-immunogenicity characteristic of stealth liposomes. The potential therapeutic efficacy of anti-EGFR immunoliposomes loaded with a variety of anti-cancer agents (C225-ILs-dox) was evaluated In a series of tumor xenograft models (MDA-MB-468, U-87 and U-87vIII) (13).

The feasibility of the anti-EGFR immunαüposomai system (ILs) for use in human therapy in a clinical set-up has not been demonstrated yet. One of the main concerns in this regard relates to the known toxicities of anti-EGFR immunoliposomes such as, for example, iiposomally encapsulated doxorubicin (Doxii, Caelyx). Here the most prominent toxic side-effect is palmar plantar erythema (PPE = hand foot syndrome), which can be observed at a dosage of 40-50 mg/m2 in form of a short infusion every 4 weeks, which is standard in routine oncology practice. Similarly, an important side effect of anti-EGFR antibodies such as Cetuximab is skin toxicity, usually manifesting itself as an acneiform rash of the face and trunk. This side effect is probably a consequence of the fact that the epidermis expresses EGFR at a relatively high level. Therefore, one of the main safety concerns of using anti-EGFR immunoliposomes in a clinical set-set up is that directing said liposomes to EGFR-overexpressing cells via an anti-EGFR antibody such as, for example, Cetuximab might also increase the skin toxicity of the drug.

Further, it has also not yet been demonstrated and is very much unpredictable, whether an anti-EGFR immunoliposome (ILS) encapsulating a chemotherapy drug such as, for example, doxorubicin, vinorelbine or methotrexate, can be used for therapeutic application in a group of patients, which had already received, but not responded to one or multiple standard treatments (first line, second line, third line, etc), i.e., in a group of non-responders.

One of the potential reasons for the observed lack of responsiveness in multi-line treatment is the development of a multi-drug resistance of the cancer cells.

Drug resistance continues to be a major challenge in cancer treatment. Intrinsic or acquired drug resistance occurs frequently in most cancers, and often involves

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resistance to multiple agents simultaneously (multidrug resistance, MDR). A number of mechanisms for drug resistance have been described. These include: overexpressed drug export pumps, such as P-glycoprotein (PGP) and multidrug-resistance protein (MRP); decreased drug uptake, such as altered foiate carriers; inactivation of drugs, such as via glutathione-med iated reduction; overexpression of target enzymes, such as upreguiated thymidylate synthase; altered drug targets, such as topoisomerase Ii; increased DNA repair capacity; reduced ability to undergo apoptosis; and others (reviewed in (30) and (31)). Among these mechanisms, the role of PGP in multidrug resistance has been one of the most intensiveiy studied. PGP, encoded by the *MDR1* gene, is a member of the ABC (ATP-Binding Cassette) transport protein family and is frequently over-expressed in the MDR phenotype. Other membrane-bound transporters capable of mediating drug efflux include multi-drug resistance protein MRP and other related proteins ((32), (33) and (34)). These proteins actively transport a variety of heterocyclic substrates, including cytotoxic drugs such as anthracycli πes, vinca alkaloids, mitoxantrone, paclitaxel, and others out of the cell or into other cellular compartments ((32), (33) and (34)).

Specific inhibitors of these resistance mechanisms have been widely pursued as a means to restore drug sensitivity (for review, see (35)). Although still actively under investigation, specific resistance inhibitors have yet to gain registration for clinical use. Progress towards therapeutic success has been hampered by such issues as inadequate specificity, both predictable and unforeseen toxicities, uncertainty about the true prevalence and contribution of the known resistance mechanisms, paucity of predictive assays to identify tumors dependent upon particular mechanisms, and multiplicity and redundancy of resistance mechanisms ((35)).

There is therefore a need for providing an alternative strategy for a safe therapeutic treatment of patients which have developed cancer, particularly of patients belonging to the group of non-responders, that is patients which are not, or no longer, responsive to a conventional cancer chemotherapy. in particular, there is a need for providing alternative strategies for overcoming intrinsic or acquired drug resistance in cancer therapy.

This need for providing afternative strategies could be satisfied within the scope of the present invention by providing a therapeutic approach which is based on a drug delivery system comprising EGFR-targeted immunoiiposomes, which show extensive internalization in the cytoplasm of EGFR-overexpressing celis (up to 30,000 iLs/cell) but

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not in non-overexpressi πg ceils and aiso marked cytotoxicity when encapsulating any of several chemotherapy drugs (doxorubicin, vinoreibine and methotrexate).

It was now surprisingly found within the scope of the present invention that administration in a ciinical set-up of an anti-EGFR irnmunoïposome, particularly an immunoiiposome comprising any of several chemotherapy drugs such as, for example, doxorubicin, vinoreibine, or methotrexate, to a human patient who is suffering from cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor, and who is chemotherapy naïve, particularly to a human patient who has received, but not responded or stopped to respond to at least one standard treatment (fist line), particularly to at least two standard treatments (second line), particularly to at least three standard treatments (third line), but especially to all available standard treatments (multi-line), not only resulted in a stabilization of the disease, particularly in a partial response, but especially in a complete response, but also showed no or substantially no side effects, particularly no or substantially no palmar plantar erythema (PPE = hand foot syndrome) and/or skin toxicity.

in one embodiment, an immunoiiposome according to the invention and as described herein before is provided comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor, for first- to multi-line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up.

In one embodiment, the invention relates to an immunoiiposome according to the invention and as described herein before comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor, for second-line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up.

In one embodiment, the invention relates to an immunoiiposome according to the invention and as described herein before comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor, for third-line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up.

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In one embodiment, the invention relates to an immunoliposome according to the invention and as described herein before comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor, for fourth-line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up.

In one embodiment, the invention relates to an immunoliposome according to the invention and as described herein before comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor, for fifth-line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up.

In one embodiment, the invention relates to an immunoliposome according to the invention and as described herein before comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor, for sixth-line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up.

in one embodiment, the invention relates to an immunoliposome according to the invention and as described herein before comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor, for seventh- and higher-line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up.

In one embodiment, the invention relates to an immunoliposome according to the invention and as described herein before comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor, for treatment, particularly for multi-line treatment, of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor, and is chemotherapy naïve, particularly a patient, who has received, but not responded to, at least one standard treatment, particularly to at least two standard treatments, particularly to at least three standard treatments, but especially to all available standard treatments.

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In one embodiment, the invention relates to an immunoliposome according to the invention and as described herein before for treatment, particularly for multi-line treatment, of a human patient who has a locally advanced or metastatic tumor as described herein before, wherein said tumor is still progressing.

in one embodiment, an immunoliposome according to the invention and as described herein before is provided for treatment, particularly for multi-line treatment, of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor as described herein before, wherein the liposome encapsulates an anti-cancer compound, particularly a cytostatic compound, particularly a compound selected from the group consisting of daunomycin, idarubicin, mitoxantrone, mitomycin, cisplatin and other Platinum analogs, vincristine, epirubicin, aelactnomycin, methotrexate, etoposide, doxorubicin, epirubicin, vinoreibine cytosine arabinoside, fluorouracil and other fiuorinated pyrimidines, purines, or nucleosides, especially gemcitabine, bleomycin, mitomycin, pficamycin, dactinomycin, cyclophosphamide and derivatives thereof, thiotepa, BCNU, paclitaxel, docetaxel and other taxane derivatives and isolates, camptothecins, polypeptides, a nucleic acid, a nucleic acid having a phosphorothioate internucleotide linkage, and a nucleic acid having a polyamide intemudeotide linkage, but especially a compound selected from the group consisting of doxorubicin, epirubicin and vinoreibine, particularly doxorubicin.

in one embodiment, the invention relates to an immunofiposome according to the invention and as described herein before for treatment, particularly for mufti-line treatment, of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor as described herein before, wherein the non-responsiveness of the patient is caused by muffi-drug resistance mechanisms..

In one embodiment, the invention relates to an immunoliposome according to the invention and as described herein before for treatment, particularly for multi-line treatment, of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor as described herein before and who has developed a multi drug resistance.

In one embodiment, an immunoliposome according b the invention and as described herein before is provided for treatment, particularly for multi-line treatment, of a human patient belonging to the group of non-responders, who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor as described herein before, particularly to a patient who has developed a multi drug resistance, wherein said

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immunoliposome has an IC50, determined in a standard MTT assay, of between 1,0 µg /ml and 5.0 µg /ml, particularly of between 0.8 µg /ml and 3.5 µg /mf, particularly of between 0.7 µg /ml and 2.5 µg /ml, particularly of between 0.6 µg /rnl and 2.0 µg /ml, particularly of between 0.5 µg /ml and 1.5 µg /ml, particularly of between 0.4 µg /mi and 1.0 µg /ml, particularly of between 0.3 µg /ml and 0.5 µg /ml, particularly of between 0.2 µg /mi and 0.4 µg /ml. The immunofiposorne is particularly an immunoliposome comprising doxorubicin.

in one embodiment of the invention, an immunoliposome according to the invention and as described herein before is provided for treatment, particularly for multi-line treatment, of a human patient belonging to the group of non-respo πders who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor as described herein before, particularly to a patient who has developed a multi drug resistance mechanisms, wherein said immunoliposome has a cytotoxicity which is between 3-fold to 5-fold, between 5-fold to 20-fold, between 10-fold to 30-foid, between 15-fold to 40-fold, between 20-foid to 50-fold, between 25-fold to 60-fold, between 30-fold b 70-fofd, between 35~foid to 80-fold, between 40-fold to 90-fold, between 50-fold to 100-fold higher, between 80-fold to 150-fold, between 120-fold to 250-fold higher than that of the free anti-cancer drug.

in one embodiment of the invention, an immunoiiposome is provided for treatment, particularly for multi-Sine treatment, of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor as described herein before, particularly a EGFR-positive tumor, wherein said treatment leads to a stabilization of the disease, particularly to a partial response, but especially to a complete response.

In one embodiment of the invention the anti-EGFR immunoliposome is given at a dose level of 10 mg/m^2 and 40 rng/m^2 body surface, particularly between 30 mg/m^2 and 50 mg/m^2, particularly between 40 mg/m2 and 60 mg/m2, particularly between 50 mg/m2 and 70 mg/m^2, particularly between 60 mg/m^2 and 80 mg/m^2, particularly between 70 mg/m^2 and 90 mg/m^2, particularly between 75 mg/m^2 and 100 mg/ m^2, given as a short infusion every 2 to 6 weeks, particularly every 3 b 5 weeks, but especially every 4 weeks . By a short infusion an infusion time of at least 10 min, particularly of at feast 20 min, particularly of at least 30 min, particularly of at least 40 min, particularly of at least 60 min, particularly of at least 90 min, particularly of at least 120 min, particularly of at least 240 min is meant.

In one embodiment, the invention relates to an immunofiposome as described herein before for treatment, particuiariy for multi-line treatment, of a human patient who is suffering from cancer, particuiariy a cancer represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor, wherein the treatment shows no or substantially no toxic side effects, particularly no or substantially no palmar plantar erythema (PPE = hand foot syndrome) and/or skin toxicity.

In one embodiment, the invention relates to an immunoiiposome as described herein before, particuiariy an immunoiiposome comprising a doxorubicin compound, for treatment, particularly for multi-line treatment, of a human patient who is suffering from cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particuiariy a EGFR-positive tumor, wherein the treatment shows no or substantially no toxic side effects, particularly no or substantially no palmar plantar erythema (PPE = hand foot syndrome) and/or skin toxicity at an immunoiiposome concentration of between 5 mg/m^2 and 20 rng/m^2 of body surface, particularly between 10 mg/m^2 and 40 mg/m^2, particularly between 30 mg/m^2 and 50 mg/m^2, particularly between 40 mg/m^2 and 60 mg/m^2, particularly between 50 mg/m^2 and 70 mg/m^2, particularly between 60 mg/m^2 and 80 mg/m^2, particuiariy between 70 mg/m^2 and 90 mg/m^2, particularly between 75 mg/m^2 and 100 mg/ m^2.

In one embodiment of the invention, an immunoiiposome is provided for treatment, particuiariy for muiti-iine treatment, of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor as described herein before, wherein the antibody or antibody fragment is covalently bound to the liposome membrane, particularly covalentiy conjugated to the terminus of a linker molecule anchored to the liposome. The linker molecule is particuiariy a hydrophiiic polymer, but especially a polyethylene glycol.

in one embodiment of the invention, the immunoiiposome according to the invention and as described herein, which is provided for treatment, particularly for multi-line treatment, of a human patient who has cancer, particuiariy a cancer represented by a locally advanced or metastatic tumor as described herein before, comprises a monoclonal antibody directed to the ligand-binding extracellular domain of the EGF receptor, particularly a chimeric antibody such as, for example, chimeric MAb C225 or a humanized antibody such as, for example, humanized MAb EMD72000.

In one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said

imrnunoliposome, wherein the cancer to be treated is a breast, ovarian, cervical, endometrial, gastric, bladder cancer, a uterine sarcoma, a multiple myeloma, and soft tissue and bone sarcomas.

In one embodiment, an imrnunoliposome according to the invention and as described herein before is provided for treatment, particularly for multi-line treatment, of a human patient in a clinical set-up, wherein said patient is suffering from a cancer selected from the group consisting of Kaposi's sarcoma, recurrent ovarian cancer, soft tissue sarcoma, glioma, melanoma, mesothelioma, transitional cell carcinoma of the urothelial tract, endometrial, pancreatic, small-ceil and non-small-cell lung, hepatocellular, renal cell, esophageal, colorectal, anal, vaginal, vulvar, prostate, basal ceil carcinoma of the skin head and neck, and cholangio carcinoma, which cancer is particularly represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor,

In one embodiment, an immunoliposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoliposome, for treatment, particularly muiti-iine treatment, of a human patient in a clinical set-up, wherein said patient is suffering from a cancer selected from the group consisting of prostate, pancreatic, kidney, oesophageal, colon, and rectal cancer, which cancer is particularly represented by locally advanced or metastatic tumor, particularly a EGFR-positive tumor.

In one embodiment, an immunoliposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoliposome, for multi-line treatment, particularly second-line, particularly third line, particularly fourth-line treatment of a human patient in a clinical set-up, wherein said patient is suffering from a prostate cancer with a tumor that has progressed on hormonal and/or docetaxel and/or mitoxanfhrone treatment.

in one embodiment, an immunoliposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoliposome, for multi-line treatment, particularly for second-üne, particularly third line, particularly fourth-iine treatment of a human patient in a clinical set-up, wherein said patient is suffering from a pancreatic cancer or a gall bladder cancer with a tumor that has progressed on gemcitabine and/or capecitabine and/or oxaliplatin treatment.

in one embodiment, an immunoliposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoliposome, for multi-line treatment, particularly for second-line, particularly third

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line, particularly fourth-line, particularly fifth-line treatment of a human patient in a clinical set-up, wherein said patient is suffering from a kidney cancer with a tumor that has progressed on interferon and/or capecitabine and/or sunitinib and/or sorafinib treatment.

in one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoiiposome, for muiti-line treatment, particularly for second-line, particularly third line, particularly fourth-line, particularly fifth-line treatment of a human patient in a clinical set-up, wherein said patient is suffering from a urothelial cancer with a tumor that has progressed on cis- or carboplatinum and/or gemcitabine and/or doxorubicin and/or methotrexate and/or vincristin.

In one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoiiposome, for multi-line treatment, particularly for second-line, particularly third line, particularly fourth-line, particularly fifth-line treatment of a human patient in a clinical set-up, wherein said patient is suffering from a non-small cell iung cancer with a tumor that has progressed on cis- or carboplatinum and/or gemcitabine and/or vinorelbine and/or, pemetrexed and/or docetaxel and/or gefitinib.

in one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoiiposome, for muitMïne treatment, particularly for second-line, particularly third line, particularly fourth-fine, particularly fifth-line treatment of a human patient in a clinical set-up, wherein said patient is suffering from a small cell fung cancer with a tumor that has progressed on cis- or carboplatinum and/or etoposid and/or irinotecan and/or doxorubicin and/or vincristin and/or cyclophosphamide and/or topotecan.

In one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoiiposome, for multi-line treatment, particularly for second-Sine, particularly third line, particularly fourth-line, particularly fifth-line treatment of a human patient in a ciinica! set-up, wherein said patient is suffering from a mesothelioma with a tumor that has progressed on cis- or carbopiatinum and/or gemcitabine and/or pemetrexed.

In one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoiiposome, for muiti-line treatment, particularly for second-fine, particularly third

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line, particularly fourth-line, particularly fifth-line treatment of a human patient in a ciinical set-up, wherein said patient is suffering from breast cancer with a tumor that has progressed on on cis- or carboplatinum and/or doxorubicfn and/or vincristin and/or cyclophosphamide and/or paclitaxel and/or docetaxel and/or gemcitabine and/or vinorelbine and/or capecitabine and/or mitomycin and/or methotrexate and/or mitoxaπthrone and/or bevacizumab and/or trastuzumab.

In one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunolipαsome, for multi-line treatment, particuiarly for second-line, particularly third iine, particularly fourth-line treatment of a human patient in a clinical set-up, wherein said patient is suffering from a esophageal cancer with a tumor that has progressed on cisplatinum and/or 5-FU and/or docetaxe! and/or cetuximab treatment.

In one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoiiposome, for muiti-iine treatment, particuiarly for second-line, particularly third line, particularly fourth-line, particularly fifth-line treatment of a human patient in a clinical set-up, wherein said patient is suffering from a brain tumor that has progressed on temozolomide and/or bevacizumab and/or irinotecan and/or vincristin and/or procarbacine and/or CCNU and/or BCNU.

In one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoiiposome, for multi-line treatment, particularly for second-line, particularly third line treatment of a human patient in a cfinicai set-up, wherein said patient is suffering from a hepatocellular cancer with a tumor that has progressed on sunitinib and/or sorafenib.

in one embodiment, an immunoiiposome is provided according to the invention and as described herein before, or a pharmaceutical composition comprising said immunoiiposome, for multi-line treatment, particularly for second-line, particularly third line, particularly fourth-line, particuiarly fifth-line, particularly sixth-line, particularly seventh-line treatment of a human patient in a clinical set-up, wherein said patient is suffering from a colon and/or rectal cancer with a tumor that has progressed on cetuximab and/or Bevacizumab and/or oxaliplatin and/or irinotecan and/or capecitabine and/or 5-FU treatment.

In one embodiment of the invention, an immunoiiposome is provided for treatment,

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particularly for multi-line treatment, of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor as described herein before, wherein a response rate is achieved of between 5% and 10%, particularly of between 7% and 15%, particularly of between 9% and 20%, particularly between 12% and 25%, particularly between 18% and 30%, particularly between 22% and 35%, particularly between 28% and 40%, particularly between 32% and 45%, particularly between 38% and 50%, particularly between 42% and 55%, particularly between 48% and 60%, particularly between 52% and 60%, particularly between 52% and 70%, particularly between 52% and 75%, particularly between 58% and 80%, particularly between 62% and 85%, particularly between 68% and 90%, particularly between 72% and 95%, and up to 100%.

In one embodiment, the invention relates to a pharmaceutical composition comprising an immunoliposome according to the invention and as disclosed herein before, together with a pharmaceutically acceptable carrier or excipient or a diluent, for first- to multi-line, particularly for second-line, particularly third-line, particularly fourth-line, particularly fifth-line, particularly sixth-line, particularly seventh- and higher- line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up, particularly a human patient belonging to the group of non-responders, particularly a human patient belonging to the group of non-responders who has developed a multidrug resistance.

In one embodiment, the invention relates to a method of first- to multi-line, particularly of second-line, particularly of third-line, particularly of fourth-line, particularly of fifth-line, particularly of sixth-line, particularly of seventh- and higher- line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient, particularly a human patient belonging to a group of non-responders, particularly in a human patient belonging to the group of non-responders who has developed a multidrug resistance, in a clinical set-up by administering to said human patient an immunoliposome or a pharmaceutical composition according to the invention and as disclosed herein before.

In one embodiment, the invention relates to a method of treating a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor, and is chemotherapy naïve, particularly a patient, who has received, but not responded to, at least one standard treatment, particularly to at least two standard treatments, particularly to at least three standard

treatments, but especially to all available standard treatments with an immunoliposome or a pharmaceutical composition according to the invention and as disclosed herein before.

in particular, the invention relates to a method of treating a human patient who has developed a multi-drug resistance.

In one embodiment, the invention relates to a method of using an irnmunoliposome or a pharmaceutical composition according to the invention and as disclosed herein before for the preparation of a medicament for use in first- to multi-line, particularly second-line, particularly third-fine, particularly fourth-line, particularly fifth-line, particularly sixth-line, particularly seventh- and higher- line treatment of cancer, in a clinical set-up, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient, particularly in a human patient belonging to the group of non-responders, particularly in a human patient belonging to the group of non-responders who has developed a multidrug resistance.

In one embodiment, the invention relates to a method of using an imrnunoiiposome or a pharmaceutical composition according to the invention and as disclosed herein before for the preparation of a medicament for use in the treatment of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor, and is chemotherapy naive, particularly a patient who has received, but not responded to, at least one standard treatment, particularly to at least two standard treatments, particularly to at least three standard treatments, but especially to all available standard treatments.

In particular, the invention relates to a method of using an immunoliposαme or a pharmaceutical composition according to the invention and as disclosed herein before, for the preparation of a medicament for use in the treatment of a human patient who has developed a multi-drug resistance.

In one embodiment, an immunoliposome is provided according to the present invention and as described herein comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor and further encapsulating in the liposome an anti-fumor compound, or a pharmaceutical composition comprising such an immunoliposome, for the treatment of multi-drug resistance in a patient or a group of patients which have developed such a multi-drug resistance.

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In one embodiment, the invention relates to a pharmaceutical composition comprising an immunoliposom θ according to the present invention and as described herein together with a pharmaceutically acceptable carrier or excipient or a düuent for the treatment of cancer, particularly for the treatment of breast cancer or a colonrectai cancer, or both, in a patient or a group of patients who have developed a multi-drug resistance, particulariy a multi-drug resistance against treatment with one or more anti-cancer drugs selected from the group consisting of docetaxel, mitoxanthrone, gemcitabine, capecitabine, oxalipiatin. interferon, sunitinib, sorafinib. cis- or carboplatinum, doxorubicin, methotrexate, vincristin., vinorelbine, pemetrexed, gefitinib, etoposid, irinotecan, cyclophosphamide, topotecan, cyclophosphamide, paclitaxel, mitomycin, bevacizumab, trastuzumab, 5-FU $_1$ cetuximab, iemozoiomide, bevacizumab, procarbacine, CCNU, and BCNU.

in one embodiment, said multi-drug resistance comprises one or more anti-cancer drugs selected from the group consisting of docetaxei, mitoxanthrone, gemcitabine, capecitabine, oxalipSatin, sunitinib, sorafinib, cisplatinum, 5-FU, cetuximab, Bevacizumab, oxalipiatin and ir notecan.

sn one embodiment, a pharmaceutical composition is provided comprising an immuno üposome according to the present invention and as described herein together with a pharmaceutically acceptable carrier or excipient or a diluent for treatment, particularly for multi-line treatment, of cancer, particularly for the treatment of breast cancer or a colonrectai cancer, or both, wherein said immunofiposome encapsulates doxorubicin and further comprises antibody MAb C225 or antibody EMD72000 or a fragment thereof, which still exhibits the specific binding properties of of one or both of said antibodies.

In one embodiment, a pharmaceutical composition is provided comprising an immunoliposome according to the present invention and as described herein together with a pharmaceutically acceptable carrier or excipient or a diluent for the treatment of cancer, particularly for the treatment of breast cancer or a colonrectai cancer, or both, in a patient or a group of patients who have developed a multi-drug resistance, particularly a multi-drug resistance against treatment with one or more anti-cancer drugs selected from the group consisting of docetaxel, mitoxanthrone, gemcitabine, capecitabine, oxalipiatin, interferon, sunitinib, sorafinib, cis- or carboplatinum, doxorubicin, methotrexate, vincristin, vinorelbine, pemetrexed, gefitinib, etoposid, irinotecan, cyclophosphamide, topotecan, cyclophosphamide, paciitaxei, mitomycin, bevacizumab,

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trastuzumab, 5-FU, cetuximab, temozolomide, bevacizumab, procarbacine, CCfMU, and BCNU, wherein said immunoliposome encapsulates doxorubicin and further comprises antibody MAb C225 or antibody EMD72000 or a fragment thereof, which still exhibits the specific binding properties of of one or both of said antibodies.

DEFINITIONS

The term "comprise" is generafly used in the sense of include, that Is to say permitting the presence of one or more features or components.

As used in the specification and claims, the singular form "a", "an" and "the" include plura! references unless the context clearly dictates otherwise. For example, the term "a patient" includes a plurality of patients. The term "an irnmunoiiposome" includes a piuraiity of immunofipos αmes, including mixtures thereof.

The term "EGF Receptor" or "EGFR", "ErbB1 ", "HER1 " is an art recognized term and used herein synonymously and is understood to refer to a receptor protein which is a member of the class I family of Receptor Tyrosine Kinases (RTKs), which includes EGFR (ErbB1 , HER1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). As a target antigen, EGFR is a readily accessible ceil surface receptor, which is overexpressed in many human solid tumors. Also included in this definition are mutants of EGFR, particularly Class IEI mutants such as, for example, EGFRvHI, which contains a deletion in exons 2-7 within the ECD, resulting in an in-frame deletion of 801 bp of the coding sequence and the generation of a novel glycine residue at the fusion junction.

The term "first-line treatment" or "first-line therapy" as used herein is an art recognized term and is understood to refer to the first chemotherapy treatment of cancer, which may be combined with surgery and/or radiation therapy, also called primary treatment or primary therapy.

The term "second-Sine treatment" or "second-line therapy" as used herein is an art recognized term and is understood to refer to a chemotherapy treatment that is given when initial or primary treatment (first-line or primary therapy) doesn't work, or stops working.

The term "third-line, fourth-line, fifth-line, etc, treatment" or "third-line, fourth-line, fifth-line, etc, therapy" as used herein is an art recognized term and is understood to refer to a chemotherapy treatment that is given when initial treatment and any of the following

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treatments (first-line, second-line, third-line, etc, therapy) doesn't work, or stops working.

The term **"multi-line"** treatment is a general term and understood herein to refer to any higher-line treatment that follows an initial or primary treatment (first-iine or primary therapy), which doesn't work, or has stopped working.

The term "substantially no side effect" or "substantially no adverse side effect" as used herein is an art recognized term and understood to refer to mild to moderate drug-related effects or toxicities, which are not dose limiting.

The term E GFR-positive tumor" as used herein is understood to refer to a tumor that contains at least 1%, particularly at least 2%, 3%, 4% or 5%, particularly at least 10%, EGFR positive cells, detected e.g. by an immunohistochernistry test such as, for example, the FDA approved EGFR pharmaDx kit ("DAKO" test; DAKO Notrth America, Inc), the Zymed EGFR kit or the Ventana EGFR 3C6 antibody. In particular, said EGFR positive cells overexpress the EGFR antigen and/or mutants of EGFR, particularly Class HI mutants such as, for example, EGFRvIII.

"A pharmaceutically effective amount" refers to a chemical material or compound which, when administered to a human or animal organism, induces a detectable pharmacologic and/or physiologic effect.

The respective pharmaceutically effect amount can depend on the specific patient to be treated, on the disease to be treated and on the method of administration. Further, the pharmaceutically effective amount depends on the specific protein used, especially if the protein additionally contains a drug as described or not. The treatment usually comprises a multiple administration of the pharmaceutical composition, usually in intervals of several hours, days or weeks. The pharmaceutically effective amount of a dosage unit of the immunoiiposome according to the present invention usually is in the range of between 5 mg/m$^{\Xi}$ and 100 mg/m^2 of body surface of the patient to be treated.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

The terms "antibody" or "antibodies" as used herein is an art-recognized term and is understood to refer to molecules or active fragments of molecules that bind to known antigens, particularly the terms "antibody" or "antibodies" refer to immunoglobulin

molecules and to immunoiogicaily active portions of immunoglobulin molecules, i.e molecules that contain a binding site that immunospecifically binds an antigen. The immunoglobulin according to the invention can be of any type (IgG, IgIvI, igD, IgE, IgA and IgY) or class (IgGI IgG2, IgG3, SgG4, !gA1 and ïgA2) or subclasses of immunoglobulin molecule.

"Antibodies" are intended within the scope of the present invention to include monoclonal antibodies, polyclonal, chimeric, single chain, bispecific, simianized, human and humanized antibodies as well as active fragments thereof. The term "fragment" refers to a part or portion of an antibody or antibody chain comprising fewer amino acid residues than an intact or complete antibody or antibody chain. Examples of active fragments of molecules that bind to known antigens include separated light and heavy chains, Fab, Fab/c, Fv, Fab\ and $F(ab'\}_2$ fragments, including the products of an Fab immunoglobulin expression library and epitope-binding fragments of any of the antibodies and fragments mentioned above. Fragments can be obtained *via* chemical or enzymatic treatment of an intact or complete antibody or antibody chain. Fragments can also be obtained by recombinant means. Exemplary fragments include Fab, Fab', F(ab')2, Fabc and/or Fv fragments. The term "antigen-binding fragment" refers to a polypeptide fragment of an immunoglobulin or antibody that binds antigen or competes with intact antibody *{i.e.,* with the intact antibody from which they were derived) for antigen binding (Ae., specific binding).

Antibody-binding fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and singie-chain antibodies.

These active fragments can be derived from a given antibody by a number of techniques. For example, purified monoclonal antibodies can be cleaved with an enzyme, such as pepsin, and subjected to HPLC gel filtration. The appropriate fraction containing Fab fragments can then be collected and concentrated by membrane filtration and the like. For further description of general techniques for the isolation of active fragments of antibodies, see for example (14); (15).

A "chimeric antibody" is an antibody in which one or more regions of the antibody are from one species of animal and one or more regions of the antibody are from a different species of animal. A preferred chimeric antibody is one which includes regions from a primate immunoglobulin. A chimeric antibody for human clinical use is typically understood to have variable regions from a non-human animal, e.g. a rodent, with the

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constant regions from a human. in contrast, a humanized antibody uses CDRs from the non-hurnan antibody with most or alf of the variable framework regions from and ail the constant regions from a human immunoglobulin. A human chimeric antibody is typicaliy understood b have the variable regions from a rodent. A typical human chimeric antibody has human heavy constant regions and human light chain constant regions with the variable regions of both the heavy and light coming from a rodent antibody, A chimeric antibody may include some changes to a native amino acid sequence of the human constant regions and the native rodent variable region sequence. Chimeric and humanized antibodies may be prepared by methods well known in the art including CDR grafting approaches (see, e.g., U.S. Patent Nos. 5,843,708; 6,180,370; 5,693,762; 5,585,089; 5,530,101), chain shuffling strategies (see e.g., U.S. Patent No. 5,565,332; (16), molecular modelling strategies (U.S. Patent No, 5,639,641}, and the like.

A "humanized antibody" refers to a type of engineered antibody which incorporates at least one humanized immunoglobulin or antibody chain or fragment thereof, particuiariy at least one humanized light or heavy chain. Said humanized immunoglobulin or antibody chain or fragment thereof, but particularly the at least one humanized light or heavy chain is derived from a non-human source, particularly a non-human antibody, typically of rodent origin. Said non-human contribution to the humanized antibody is typically provided in form of at least one CDR region which is interspersed among framework regions derived from one (or more) human immunoglobulin(s). In addition, framework support residues may be altered b preserve binding affinity.

The humanized antibody may further comprise constant regions (e.g., at least one constant region or portion thereof, in the case of a light chain, and preferably three constant regions in the case of a heavy chain).

Methods to obtain "humanized antibodies" are well known to those skilled in the art. (17).

A "humanized antibody" may also be obtained by a novel genetic engineering approach that enables production of affinity-matured human-like polyclonal antibodies in large animals such as, for example, rabbits (http://www.rctech.com/bioventures/-therapeutic.php).

The term "immunoliposome dosage" or "immunoliposome concentration" generally refers to the concentration of the anti-cancer agent entrapped in the liposome.

A "liposome" refers to a small, spherical vesicle composed of lipids, particularly vesicle-forming lipids capable of spontaneously arranging into lipid bilayer structures in water with its hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and its head group moiety oriented toward the exterior, polar surface of the membrane. Vesicle-forming lipids have typically two hydrocarbon chains, particularly acyi chains, and a head group, either polar or nonpolar. Vesicle-forming iipids are either composed of naturally-occurring iipids or of synthetic origin, including the phospholipids, such as phosphatidylcholine, phosphatidyiethanolamine, phosphatidic acid, phosphatidyiinositoi, and sphingomyelin, where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. The above-described lipids and phospholipids whose acyl chains have varying degrees of saturation can be obtained commerciaiiy or prepared according to published methods. Other suitable lipids for use in the composition of the present invention include glycolipids and sterols such as cholesterol and its various analogs which can also be used in the liposomes.

Cationic lipids, which typically have a lipophilic moiety, such as a sterol, an acyl or diacyi chain, and where the lipid has an overall net positive charge can also be suitably used in liposomes. The head group of the lipid typically carries the positive charge. Exemplary cationic lipids include 1,2-dioleyloxy-3-(trirnethyiamino) propane (DOTAP); N-[1~(2,3,-ditetradecyloxy)propyi]-N,N-dimethyi-N-hydroxyethylammonium bromide (DMRIE); N-[1-(2,3,-dioleyloxy)propyi]-N,N-dimethyl-N-hydroxy ethylammonium bromide (DORIE); N~[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethyiammonium chloride (DOTMA); 3 [N-(N\N'-dimethylarninoethane) carbamoiyjcholesterol (DC-Choi); and dimethyldioctadecylammonium (DDAB). The cationic vesicle-forming lipid may also be a neutral fipid, such as dioleoylphosphatidyl ethanolamine (DOPE) or an amphipathic lipid, such as a phospholipid, derivatized with a cationic iipid, such as polylysine or other polyamine lipids.

The liposomes can include a vesicle-forming iipid derivatized with a hydrophilic polymer to form a surface coating of hydrophilic polymer chains on the liposomes surface. A vesicle-forming lipid, in particular a phospholipid, such as distearoyl phosphatidylethanolamine (DSPE), may be covaiently attached to a hydrophilic polymer, which forms a surface coating of hydrophilic polymer chains around the liposome. Hydrophilic polymers suitable for derivatization with a vesicle-forming lipid include polyvinylpyrrolidone, polyvinylmethyl βther, polymethyloxazoiine,

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polyethyioxazoSine, polyhydroxypropyloxazoline, polyhydroxypropylmethacrylamide, pofymethacrylamide, pσfydimethyiacrylamide, polyhydroxypropylmethacrylate. poty-hydroxyethylacryiate, hydroxymethylcelluiose, hydroxyethylceliulose, pαlyetbyle πe-glycol, polyaspartamide and hydrophiSic peptide sequences. The polymers may be employed as homopolymers or as block or random copolymers.

A preferred hydrophific polymer chain is polyethyienegiycol (PEG), preferably as a PEG chain having a molecular weight between 200-20,000 daltons, more preferably between 500-10,000 daltons, still more preferably between 750-5000 daltons. Methoxy or ethoxy-capped analogues of PEG are also preferred hydrophilic polymers, commercially available in a variety of polymer sizes, e.g., 120-20,000 Daltons.

Additional polymer chains added to the lipid mixture at the time of liposome formation and in the form of a lipid-polymer conjugate result in polymer chains extending from both the inner and outer surfaces of the liposomal lipid bilayers. Addition of a lipid-polymer conjugate at the time of liposome formation is typically achieved by including between 0.5-20 mofe percent of the polyrner-derivatized lipid with the remaining liposome forming components, e.g., vesicle-forming lipids.

Preparation of veside-forming lipids derivatized with hydrophilic poiymers has been described, for example in U.S. Pat. No. 5,395,619, in U.S. Pat. No. 5,013,556, in U.S. Pat. No. 5,631 ,018 and in WO 98/07409. It will be appreciated that the hydrophilic polymer may be stabiy coupled to the lipid, or coupled through an unstable linkage, which allows the coated liposomes to shed the coating of polymer chains as they circulate in the bloodstream or in response to a stimulus.

An "internalizing antibody" is an antibody that, upon binding to a receptor or other ligand on a ceil surface, is transported into the cell, for example, into a Sysozyme or other organelle or into the cytoplasm.

The present invention relates to an immunoiiposome comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor and comprises at least one anti-tumor agent, for first- to multi¬line, particularly to second-line, particularly to third line, particularly to fourth-line, particularly to fifth-line, particularly to sixth-line, particularly to seventh- and higher-line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up.

The immunoliposome composition of the invention thus also includes an antibody or antibody fragment including Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and single-chain antibodies that specifically recognizes and bind to EGF receptor on the surface of a tumor derived cell. In another embodiment, the antibody comprises at least one binding domain which specificaliy binds the EGR receptor on the surface of a tumor-derived cell. In an alternate embodiment, the antibody is a single chain antibody comprising at least one binding domain which specifically binds EGF receptor on the surface of a tumor-derived ceil.

Antibodies may be attached to a liposome by covalent methods known in the art. For attaching an antibody covaiently to a liposome, a derivatized lipid containing an end-functionalized polyethylene glycol chain is incorporated into liposomes. After liposome formation, the end-funcfionalized group can react with an antibody for antibody coupling to a liposome.

There are a wide variety of techniques for attaching a selected hydrophilic polymer to a selected iipid and activating the free, unattached end of the polymer for reaction with a selected iigand, and in particular, the hydrophilic polymer polyethyleneglycol (PEG) has been widely studied {18; 19; 20; 21; 22).

Generally, the PEG chains are functionaiized to contain reactive groups suitable for coupling with, for example, suifhydryis, amino groups, and aldehydes or ketones (typicaüy derived from mild oxidation of carbohydrate portions of an antibody) present in a wide variety of ligands. Examples of such PEG-termina! reactive groups include maleimïde (for reaction with sulfhydryl groups), N-hydroxysuccinimide (NHS) or NHS-carbonate ester (for reaction with primary amines), hydrazide or hydrazine (for reaction with aldehydes or ketones), iodoacetyi (preferentially reactive with sulfhydryl groups) and dithiopyridine (thiol-reactive). Liposomes carrying an entrapped agent and bearing surface-bound targeting ligands., i.e., targeted, therapeutic liposomes, are prepared by any of these approaches, A preferred method of preparation is the insertion method, where preformed liposomes and are incubated with the targeting conjugate to achieve insertion of the targeting conjugate into the liposomal biiayers. In this approach, liposomes are prepared by a variety of techniques, such as those detailed in (23), and specific examples of liposomes prepared in support of the present invention will be described below. Typically, the liposomes are multilamellar vesicles (MLVs), which can be formed by simple lipid-film hydration techniques. In this procedure, a mixture of liposome-forming lipids of the type detailed above dissolved in a suitable organic

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solvent is evaporated in a vessel to form a thin film, which is then covered by an aqueous medium. The lipid film hydrates to form MLVs, typically with sizes between about 0.1 to 10 microns. The liposomes can include a vesicle-forming lipid derivatized with a hydrophilic polymer to form a surface coating of hydrophilic poiymer chains on the liposomes surface. Addition of a lipid-polymer conjugate is optional, since after the insertion step, described below, the liposomes will inciude lipid-polymer-targeting ligand. Additional polymer chains added to the lipid mixture at the time of liposome formation and in the form of a lipid-poiymer conjugate result in poiymer chains extending from both the inner and outer surfaces of the liposomal lipid bifayers. Addition of a **lipid-**polymer conjugate at the time of liposome formation is typically achieved by including between 0.5-20 mole percent of the polymer-derivatized lipid with the remaining liposome forming components, e.g., vesicle-forming lipids, Exemplary methods of preparing polymer-derivatized lipids and of forming polymer-coated liposomes have been described in U.S. Pat. Nos. 5,013,556, 5,631 ,018 and 5,395,61 9, which are incorporated herein by reference. It will be appreciated that the hydrophilic polymer may be stably coupled to the iipid, or coupied through an unstable linkage, which allows the coated liposomes to shed the coating of poiymer chains as they circulate in the bloodstream or in response to a stimulus.

Alternatively, an antibody-lipid derivative may be first formed and then incorporated into a liposome. As an example, an antibody is coupled to the maieimide group of a free DSPE-PEG molecule. The antibody-coupled DSPE-PEG molecule is then employed to form vesicles.

After formation of the liposomes, a targeting ligand is incorporated to achieve a ceil-targeted, therapeutic liposome. The targeting ligand is incorporated by incubating the pre-formed liposomes with the iipid-polymer-ligand conjugate, prepared as described above. The pre-formed liposomes and the conjugate are incubated under conditions effective to association with the conjugate and the liposomes, which may include interaction of the conjugate with the outer liposome biiayer or insertion of the conjugate into the liposome biiayer. More specifically, the two components are incubated together under conditions which achieve associate of the conjugate with the liposomes in such a way that the targeting ligand is oriented outwardly from the liposome surface, and therefore available for interaction with its cognate receptor. It wil! be appreciated that the conditions effective to achieve such association or insertion are determined based on several variables, including, the desired rate of insertion, where a higher incubation

temperature may achieve a faster rate of insertion, the temperature to which the ligand can be safely heated without affecting its activity, and to a lesser degree the phase transition temperature of the lipids and the lipid composition. It will also be appreciated that insertion can be varied by the presence of solvents, such as amphipathic solvents including polyethyleneglycol and ethanol, or detergents.

The targeting conjugate, in the form of a lipid-poiyrner-ligand conjugate, will typicaily form a solution of micelles when the conjugate is mixed with an aqueous solvent. The micellar solution of the conjugates is mixed with a suspension of pre-formed liposomes for incubation and association of the conjugate with the liposomes or insertion of the conjugate into the liposomal lipid bifayers. The incubation is effective to achieve associate or insertion of the iipid-poiymer-antibody conjugate with the outer bilayer leaflet of the liposomes, to form an immunoliposome.

After preparation, the immunoliposomes preferably have a size of less than about 200 nm, preferably of between about 85-120 nm, and more preferably of between 90-1 10 nm, as measured, for exampie, by dynamic light scattering at 30[deg.] or 90[deg.].

Liposome compositions are typicaily prepared with lipid components present in a moiar ratio of about 30-75 percent vesicie-forming lipids, 25-40 percent cholesterol, 0.5-20 percent polymer derivatized lipid, and 0.0001-1 0 mole percent of the lipid derivative employed for antibody coupiing.

Generally, a therapeutic drug is incorporated into liposomes by adding the drug to the vesicle forming lipids prior to liposome formation, as described below, to entrap the drug in the formed liposome. If the drug is hydrophobic the drug is added directly to the hydrophobic mixture. If the drug is hydrophilic the drug can be added to the aqueous medium which covers the thin film of evaporated lipids.

The liposomes to be used in the present invention include an anti-tumor agent. Antitumor compounds contemplated for use in the invention include, but are not limited to, plant alkaloids, such as vincristine, vinblastine and etoposide; anthracycline antibiotics including doxorubicin, epirubicin, daunorubicin; fluorouracil; antibiotics including bleomycin, mitomycin, plicamycin, dactinomycin; topoisomerase inhibitors, such as camptothecin and its analogues; and platinum compounds, including cisplatin and its analogues, such as carboplatin. Other traditional chemotherapeutic agents suitable for use are known to those of skill in the art and include , asparaginase, busuffan, chlorambucil, cyclophosphamide, cytarabine, dacarbazine, estramustine phosphate sodium, floxuridine, fluorouracil (5~FU), hydroxyurea (hydroxycarbamide),

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ifosfamide, lornustine (CCNU), mechlorethamine HCl (nitrogen mustard), rne!pha!an, mercaptopurine, methotrexate (MTX), mitomycin, mitotane, mitσxantrone, , procarbazine, streptozocin, , thioguanine, thiotepa, amsacrine (m-AMSA), azacitidine, , hexamethylmeiamine (HMM), , mitoguazone (methyl-GAG; methyl giyoxa! bis-guanyihydrazone; MGBG), , semustine (methyi-CCNU), teniposide (VM-26) and vindesine sulfate.

in one embodiment of the invention, the liposomes have a size suitable for extravasation into a solid tumor. This is particularly useful where the liposomes also include a surface coating of a hydrophilic polymer chain to extend the blood circulation lifetime of the liposomes. Liposomes remaining in circulation for longer periods of time, e.g., more than about 2-5 hours, are capable of extravasating into tumors and sites of infection, which exhibit compromised leaky vasculature or endothelial barriers. Such liposomes are typically between about 40-200 nm, more preferably between 50-150 nm, most preferably between 70-120 nm.

The selected agent is incorporated into liposomes by standard methods, including (i) passive entrapment of a water-soiubSe compound by hydrating a lipid film with an aqueous solution of the agent, (ii) passive entrapment of a lipophilic compound by hydrating a lipid film containing the agent, and (Hi) loading an ionizable drug against an inside/outside liposome pH gradient. Other methods, such as reverse-phase evaporation, are also suitable.

Alternatively, the drug may be incorporated into preformed liposomes by active transport mechanisms. Typically, in this case drug is taken up in liposomes in response to a potassium or hydrogen ion concentration differential (Mayer, 1986; Mayer 1989).

After liposome formation, the liposomes can be sized to obtain a population of liposomes having a substantially homogeneous size range, typically between about 0,01 to 0.5 microns, more preferably between 0.03-0.40 microns. One effective sizing method for REVs and MLVs involves extruding an aqueous suspension of the liposomes through a series of polycarbonate membranes having a selected uniform pore size in the range of 0.03 to 0.2 micron, typically 0.05, 0.08, 0.1 , or 0.2 microns. The pore size of the membrane corresponds roughly to the largest sizes of liposomes produced by extrusion through that membrane, particularly where the preparation is extruded two or more times through the same membrane. Homogenizati σn methods are also useful for down-sizing liposomes to sizes of 100 nm or less (24).

Liposomes carrying an entrapped agent and bearing surface-bound targeting figands, i.e., targeted, therapeutic liposomes, may be prepared by any of these approaches. A preferred method of preparation is the insertion method, where pre-formed liposomes and are incubated with the targeting conjugate to achieve insertion of the targeting conjugate into the liposomaf biiayers. In this approach, liposomes are prepared by a variety of techniques, such as those detailed in (23), and specific examples of liposomes prepared in support of the present invention will be described beiow. Typïcaliy, the liposomes are multilamellar vesicles (MLVs) or unilamellar vesicles (ULVs).

MLVs can be formed by simple iipid-filrn hydration techniques. In this procedure, a mixture of ltposome-forming lipids of the type detailed above dissolved in a suitable organic solvent is evaporated in a vessel to form a thin film, which is then covered by an aqueous medium. The lipid film hydrates to form MLVs, typically with sizes between about 0.1 to 10 microns.

ULVs can be formed by the repeated freeze-thawing method. In this method 1-2-oleoy!~ 3-sn-glycerophosphoch σiine and Choi, or DSPC and Choi (molar ratio 3:2) is mixed with mPEGDSPE (0.5-5 mof% of phospholipid). Liposomes are subsequently extruded several times through polycarbonate filters with defined pore sizes of 0.1 , ɑ.08 and 0,05 µm. This yields liposomes typically with sizes of 70-120 nm diameters. The size of the liposomes may be determined by dynamic light scattering. Liposome concentration can be measured using a standard phosphate assay.

The anti-EGFR immunoliposomes obtainable by any of the above described methods has clinical relevance and can be used in second and higher-line treatment of human patients suffering from cancer, particularly a cancer represented by a locally advanced or metastatic tumor. The immunoliposome contemplated for use in the present invention comprises an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor. In particular, the immunoitposome comprises a Fab, Fab', $F(ab')_2$, Fabc, Fv fragment, or is a single-chain antibody.

The immunoiiposome contempiated for use in the present invention further comprises an anti-tumor agent, particularly anti-tumor agent selected from the group consisting of doxorubicin, epirubicin and vinorelbine, particularly doxorubicin.

The immunoliposome according to the invention may be administered to a human patient in form of a pharmaceutical composition comprising said immunoliposome together with a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

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Formulation of the pharmaceutical composition according to the invention can be accompiished according b standard methodology known to those skilled in the art.

The immunoliposome according to the invention or a pharmaceutical compositions comprising said immunoiiposome may be administered to a subject in the form of a solid, liquid or aerosol at a suitable, pharmaceutically effective dose. Examples of solid compositions include püls, creams, and implantable dosage units. Pills may be administered orally. Therapeutic creams may be administered topically. Implantable dosage units may be administered locally, for example, at a tumor site, or may be implanted for systematic release of the therapeutic composition, for example, subcutaneously. Examples of liquid compositions include formulations adapted for infusions, formulations adapted for injection intramuscularly, subcutaneousiy, intravenously, intra-arterially, and formulations for topical and intraocular administration. Examples of aerosol formulations include inhaler formulations for administration to the lungs.

The immunoliposome according to the invention or a pharmaceutical compositions comprising said immunoliposome may be administered by standard routes of administration. In general, the composition may be administered by topical, oral, rectal, nasal, interdermal, intraperitoneal, or parenteral (for example, intravenous, subcutaneous, or intramuscular) routes. In addition, the composition may be incorporated into sustained release matrices such as biodegradable polymers, the polymers being implanted in the vicinity of where delivery is desired, for example, at the site of a tumor. The method includes administration of a single dose, administration of repeated doses at predetermined time intervals, and sustained administration for a predetermined period of time.

It is well known to those skilled in the pertinent art that the dosage of a pharmaceutical composition will depend on various factors such as, for example, the condition of being treated, the particular composition used, and other clinical factors such as weight, size, sex and general health condition of the patient, body surface area, the particular compound or composition to be administered, other drugs being administered concurrently, and the route of administration.

The immunoliposome according to the invention or the composition comprising said immunoliposome may be administered in combination with an biologically active substance or compound or other compositions comprising said biologically active substance or compound, particularly an anti-tumor compound, particularly at least one

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cytostatic compound, particularly a compound selected from the group consisting of particularly a compound selected from the group consisting of daunomycin, idarubicin, mitoxantrone, mitomycin, cisplatin and other Platinum analogs, vincristine, epirubicin, aclacinomycin, methotrexate, etoposide, doxorubicin, cytosine arabinoside, fluorouracil and other ffuorinated pyrimidines, purines, or nucleosides, especially gemcitabine, bleomycin, mitomycin, plicamycin, dactinomycin, cyclophosphamide and derivatives thereof, thiotepa, BCNU, paclitaxel , docetaxel and other taxane derivatives and isolates, camptothecins, polypeptides, a nucleic acid, a nucleic acid having a phosphorothioate internucleotide linkage, and a nucleic acid having a polyamide intemucleotide linkage, but especially doxorubicin, epirubicin and vinorelbine, together with an antibody according to the present invention and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

Pharmaceutically active matter, particularly the anti-tumor compounds which are entrapped in the immunoliposome, may be present in amounts between 0.1 mg/m^2 ng and 2.5 g/m^2 of body surface and per dose. Generally, the regime of administration should be in the range of between 0.5 mg/m^2 and 1000 mg/m^2 of the anti-tumor compound according to the invention, particularly in a range of between 1.0 mg/m^2 to 500 mg/m^2, and particularly in a range of between 5.0 mg/m^2 and 250 mg/m^2, particularly in a range of between 10.0 mg/m^2 and 150 mg/m^2, with all individual numbers failing within these ranges also being part of the invention. If the administration occurs through continuous infusion a more proper dosage may be in the range of between 0.01 µg and 10 mg units per kilogram of body weight per hour with all individual numbers falling within these ranges also being part of the invention.

The antibody concentration of the immunoliposome is in a range of between 1 µg to 150 µg of antibody or antibody fragment per µmol phospholipid, particularly in a range of 5 µg to 100 µg of antibody or antibody fragment per µmol phospholipid, particularly in a range of 10 µg to 100 µg of antibody or antibody fragment per µmol phospholipid, particularly in a range of 20 µg to 50 µg of antibody or antibody fragment per µmol phospholipid, particularly in a range of 30 µg b 40 µg of antibody or antibody fragment per µmol phospholipid.

The immunoliposomal preparation of the present invention may be prepared in the form of a pharmaceutical composition containing the isolated and purified immunoiiposome dissolved or dispersed in a pharmaceutically acceptable carrier well known to those

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skilled in the art, for parenteral administration by, e. g., intravenous, subcutaneous or intramuscular injection or by intravenous drip infusion.

As to a pharmaceutical composition for parenteral administration, any conventiona! additives may be used such as excipients, adjuvants, binders, disintegrants, dispersing agents, lubricants, diluents, absorption enhancers, buffering agents, surfactants, solubilizing agents, preservatives, emulsifiers, isotonizers, stabilizers, soiubilizers for injection, pH adjusting agents, etc.

Acceptabie carriers, diluents and adjuvants which faciiitates processing of the active compounds into preparation which can be used pharmaceutically are non-toxic 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; benzaïkonium chloride, benzethonium chloride; phenol, butyl orbenzyl alcohol; alky! parabens such as methyl or propyl paraben; catechol; resorcino!; cyciohexanol; 3-pentanol; and m-cresol); !ow 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, giutamine, asparagine, hJstidine, argintne, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, rnannitol, 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 giycoi (PEG).

The form of administration of the pharmaceutical composition may be systemic or topical. For example, administration of such a composition may be various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, buccal routes or via an implanted device, and may also be delivered by peristaltic means.

Administration will generally be parenteral^, eg intravenously, particularly in form of an infusion. Preparations for parenteral administration include sterile aqueous or non¬ aqueous solutions, suspensions and emulsions. Non-aqueous solvents include without being limited to it, propylene giycoi, polyethylene glycol, vegetable oil such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous solvents may be chosen from the group consisting of water, alcohol/aqueous solutions, emulsions or suspensions including saline and buffered media. Parenteral vehicles include sodium

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chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose) *and* others. Preservatives may also be present such as, for example, antimicrobials, antl-oxidants, chelating agents, inert gases, etc.

The pharmaceutical composition may further comprise proteinaceous carriers such as, for example, serum albumine or immunoglobuitne, particularly of human origin. Further biologically active agents may be present in the pharmaceutical composition of the invention dependent on its intended use.

In one aspect of the invention, immunoliposomes (ILs) as described herein were generated that bind EGFR b provide efficient antibody-directed intracellular delivery of anticancer drugs into target ceils to study whether it is possible by this approach to overcome drug resistance mechanisms, which remain an important obstacle towards better outcomes in cancer therapy.

ILs may be constructed modulariy with various MAb or MAb fragments, including chimeric antibodies such as, for example, Fab' from C225 (cetuximab, Erbttux®) or humanized antibodies, such as, for example, EMD72000, covalentiy linked to stabilized liposomes containing various drugs or probes.

EGFR-overexpressing cells that also feature mdr-mediated multidrug-resistance such as, for example, human breast cancer ceil line MDA-MB-231/mdr or colorectal cancer cell line HT-29/mdr, can then be treated with the so-produced ILs.

In the multidrug resistant eel! lines, ILs loaded with doxorubicin (dox) could be shown to produce 15-86-fo!d greater cytotoxicity than free doxorubicin (e.g. IC50 of ILs-dox in HT-29/mdr cells = 0.37 vs. IC50 of free dox = 6.0 (µg dox/ml). In non-resistant HT-29 cells tmmunoliposomal cytotoxicity of doxorubicin was comparable to that of the free drug (IC50 = 0.23 vs. 0.36 µg dox/ml), while markedly more cytotoxic than the non-target θd liposomal doxorubicin (!C50 > 27 µg dox/ml). Interestingly, intracellular distribution studies in MDA-MB-231/mdr cells revealed distinctive differences between free dox and immunoliposoma! dox delivery. While free dox was efficiently pumped out of this multidrug resistant tumor cells, immunoiiposomai dox at the identical concentration reached 3.5-8 times higher accumulation of dox in the cytoplasrna and 3.5-4.9 times in the nuclei.

Finally, *in vivo* therapy studies in the MDA-MB-231/mdr xenograft model confirmed the ability of anti-EGFR ILs-dox to efficiently target multidrug resistant cells. While free dox

failed to show any activity at its MTD in this highly rnuitidrug-resistant tumor model, anti-EGFR !Ls-dox showed impressive antitumor effects, clearly superior to ail other treatments.

The immunoüposomes according to the present invention and as disclosed herein thus provide efficient and targeted drug delivery to EGFR-overexpressing tumor cells and show potent activity even against multidrug-resistant cells.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. it is to be understood that the invention includes all such variations and modifications without departing from the spirit or essential characteristics thereof. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features. The present disclosure is therefore to be considered as in all aspects illustrated and not restrictive, the scope of the invention being indicated by the appended claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.

The foregoing description wil! be more fully understood with reference to the following Examples. Such Examples, are, however, exemplary of methods of practising the present invention and are not intended to limit the scope of the invention.

EXAMPLES

The target population are patients with EGFR-overexpressing solid tumors who have received all available standard treatments.

In particular, the patients are suffering from the following cancers and the tumor has progressed on the following treatments:

Prostate cancer with tumors progressed on hormonal treatment, docetaxel, mitoxanthrone.

Pancreatic and gall bladder cancer with tumors progressed on Gemcitabine, Capecitabine, oxaliplatin

Kidney cancer with tumors progressed on interferon, capecitabine, sunitinib, sorafinib.

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Urotheiiaf cancer with tumors progressed on cis- or carboplatinum, gemcitabine, doxorubicin, methotrexate, vincristin,

Non-smali ceil lung cancer with tumors progressed on cis- or carboplatinum, gemcitabine, vinorefbine, pemetrexed, docetaxei, gefitintb.

Small ceil lung cancer with tumors progressed on cis- or carboplatinum, etoposid, irinotecan, doxorubicin, vincristin, cyclophosphamide, topotecan.

Mesothelioma with tumors progressed on cis- or carboplatinum, gemcitabine, pemetrexed.

Breast cancer with tumors progressed on cis- or carboplatinum, doxorubicin, vincristin, cyclophosphamide, paciitaxel, docetaxei, gemcitabine, vtnorelbine, capecitabine, mitomycin, methotrexate, mitoxanthrone, bevacizumab, trastuzumab.

Esophageal cancer with tumors progressed on cis- or carboplatinum, 5-FU, docetaxei

Head&Neck cancer with tumors progressed on cis- or carbopiatinum, 5-FU, docetaxei, cetuximab.

Brain tumors with tumors progressed on temozolomide, bevacizumab, irinotecan, vincristin, procarbacine, CCNU, BCNU.

Hepatocellular cancer with tumors progressed on sunitinib, sorafenib.

Colon and rectal cancer with tumors progressed on Cetuximab, Bevacizumab, oxaliplatin, irinotecan, capecitabine, 5-FU

The therapeutic compound tested in the trial is C225-ILs-dox, a construct in which the EGFR-specific antibody C225 is covalently bound to the lipid membrane of doxorubicin-containing liposomes. The rationale to use this compound is the fact that doxorubicin is one of the most active agents in many human tumors, and that a high percentage of these malignancies do express EGFR.

A; PROTOCOL OF PHASE I STUDY CC1

1. SELECTiON CRITERIA

1-1. Total Number of Patients

Approximately 30 patients.

1-2. Inclusion Criteria

Prior to enrollment in the study candidates must meet ALL the foilowing criteria:

1. Histologically proven locally advanced or metastatic solid tumor.
2. ECOG Performance ≤ 2.
3. No additional standard therapy available for the patient.

4. EGFR overexpression {according to DAKO EGFR pharmDx - Test) determined in the most recently evaiuable tumor tissue.
5. No concomitant anti-tumor therapy (steroids are permitted - in breast cancer and prostate cancer, steroid dose needs to remain stable during the study period).
6. At least four weeks since termination of any previous anti-tumor treatment (6 weeks in the case of nitrosoureas or mitomycin C).
7. In patients with previous anthracycline exposure, a norma! echocardiogram (LVEF > 50%) is required.
8. Age ≥ 18.
9. Mate or fema ïe.
10. Female and male patients of reproductive age must be using effective contraception.
11. Wiiling and able to sign an informed consent prior to participation in the study and to comply with the protocol for the duration of the study.

1-3. Exclusion Criteria

Candidates must be excluded from the study if ANY of the foiiowing criteria are met:

1. Pregnancy and/or breastfeeding.
2. Patients with the following laboratory values
   - neutrophils < 1.5 x 10^9/L
   - platelets < 100 x 10^9/L
   - serum creatine > 3.0 x upper normal limit
   - ALAT, ASAT > 3.0 x upper normal limit (5.0 x in patients with liver metastases as the only likely cause of enzyme alteration)
   - alkaline phosphatase > 3.0 x upper norma! limit (5.0 x in patients with liver or bone metastases as the only iikeSy cause of enzyme alteration)
   - bilirubin > 3.0 x upper normal limit
3. Participation in any investigational drug study within 4 weeks preceding treatment start.
4. Patients with clinically significant and uncontrolled renal- or hepatic disease.
5. Clinically significant cardiac disease: congestive heart failure (New York Heart Association class ili or IV); symptomatic coronary artery disease; cardiac arrhythmia not well controiled with medication; myocardial infarction within the last 12 months.
6. Any serious underlying medical condition (at the judgement of the investigator) which could impair the ability of the patient to participate in the trial (e.g. active autoimmune disease, uncontrolled diabetes, etc.).
7. Any concomitant drugs contraindicated when administering Erbitux™ or Caelyx™ according to the Swissmedic-approved product information.
8. A cumulative doxorubicin dose of > 300 mg/m^2 BSA (or cardiotoxic anthracycline-equivalent).
9. Patients with a history of uncontrolled seizures, central nervous system disorders or psychiatric disability judged by the investigator to be clinically significant and precluding informed consent or interfering with compliance.

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10. Brain metastases.

2. SAFETY PARAMETERS

2.1 . Adverse Events (Primary Objective)

Ali adverse events encountered during the cfinica! study will be recorded in the patients' history/fiie.

The intensity of ciinica! adverse events will be graded according to the NCi CTC grading system Version 3.0 (http://ctep.info.nih.gov/ reporting/ctchtml).

2.2. Laboratory Parameters

Prior to study onset, the normal values of the participating laboratories have to be recorded. The following laboratory procedures have to be carried out during the study;

every week (before new administration of study medications if appropriate):
- hemoglobin - leukocytes count including differentia! blood count
- platelet count

every 4 weeks (before new administration of study medications):
- ASAT - ALAT
- bilirubin
- alkaline phosphatase
- serum creatine
- LDH
- caicium
- urine analysis ("U-Status": detection of erythro-, leuco- and proteinuria)

Pharmacokinetic study onjy during cyjcie 1:

- Blood sample (2 x 7.5 ml serum tubes) at 0, 24, 48, 96 h and on day 8

2.3. **Vital** signs and physical examination

The following vital signs and results of physical examination have to be documented prior to study start:

- body temperature
- blood pressure
- heart rate
- height (once at screening)
- weight
- performance status (ECOG)

2-4. Special Investigation

For pharmacokinetic studies, a blood sample (2 x 7.5 ml serum tubes) will be drawn at 0, 24, 48 and 96 hours as well as on day 8. Plasma will be separated from whole blood by centrifugation and frozen at -80 °C for further analysis. Doxorubicin concentration will be determined by fluorescence. Due to rapid clearance of free doxorubicin, this simple analysis provided an excellent measurement of circulating intact C225-iLs-dox. Pharmacokinetic parameters will be determined by noncompartmental pharmacokinetics data analysis using PK Solution 2.0 software (Summit Research Serviced, Montrose, CO, USA).

2.5. Dose Modification for Toxicity

This is a dose escalation study (Phase I). For details see also section 10.3.2.

In an individual patient who experiences toxicity (DLT) but benefits from therapy, continuation of treatment at a reduced dose, determined according to the clinical judgment of the primary investigators, is an option (off study).

If possible, toxicities should be managed symptomatically. If toxicity occurs, the appropriate treatment will be used to ameliorate signs and symptoms including antiemetics for nausea and vomiting, antidiarrhoeais for diarrhoea, antipyretics and antihistamines for drug fever and 50% DMSO ointment for skin toxicity.

2.6. Supportive Measures

2.6. 1. Nausea/Vomiting

A prophylactic antiemetic treatment should be given to the patients from the first cycle on. The use of a 5-HT3-receptor~antagonist is recommended. More aggressive antiemetic prophylaxis should be given to any patient who experiences grade ≥ 3 nausea/vomiting in a preceding cycle.

If, despite appropriate medication, grade ≥ 3 nausea/vomiting persists, the patient must be withdrawn from the study.

2.6.2. Diarrhea

No prophylactic anti-diarrhea treatment is recommended for the first cycle. However, following the first episode of diarrhea, the patient should receive symptomatic treatment with loperamide: 4 mg following the first episode, then 2

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mg following each new episode until recovery of diarrhea (no more than 16 mg daily).

if, despite the appropriate medication, grade ≥ 3 diarrhea persists, the patient must be withdrawn from the study.

2.6.3. Palmar plantar erythema (PPE = hand foot syndrome)

A prophylactic treatment should be given to the patients from the first cycle. The patient should receive 8 mg of dexamethason BID orally on days - 1 ~4, 4 mg BID on day 5 and 4 mg on day 6. Additionally, patients should receive 150 mg pyridoxin (Vitamin B6) daily during the treatment period (orally) *{20).* If, despite the appropriate medication, grade 2 or 3 PPE occurs, administration of C225-ILs-dox should be interrupted for a maximum of 14 days. Once the PPE decreases in severity to CTC grade 1, the patient may continue treatment (if not defined as DLT).

If, despite prophylactic and symptomatic treatment grade 2 or 3 toxicity remains, the patient must be withdrawn from the study.

3. DISEASE EVALUATION (EFFICACY CRITERIA)

3.1. Overall Response Rate (Secondary Objective)

Although response rate is not the primary endpoint of this trial, patients with measurable disease will be assessed by standard criteria. Tumor assessments will be done during screening and after 2, 4 and 6 cycles of treatment. After treatment compietion, an assessment is performed every 3 months for the first year and then according to clinical needs. If progression is documented, no further assessments will have to be performed within the study. In responding patients, the response must be confirmed a minimum of 4 weeks after the response has first been recorded.

The primary efficacy criteria is the overall response rate which will be assessed according the RECIST criteria for reporting results of cancer treatment given in appendix 1.

Consistency of consecutive CT-scans and X-rays (e.g. the use of contrast etc.) must be ensured during all assessments for each patient with the same technique being used throughout the treatment period for evaluating the iesions.

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3.2. Time to Progression

Time to progression will be measured from the time the patient has started treatment, to the time the patient is first recorded as having disease progression.

Disease progression must be adequately documented and assessed according to RECIST criteria.

4. STUDY PROCEDURES

4.1. Screening

informed consent must be given before any study specific screening procedures are performed.

The screening procedure may be done in two stages. The first group of assessments may be done at any time within 4 weeks prior to treatment start on day 1. The second group must be done within 7 days prior to treatment stat. if the assessments are undertaken on day 1 they must be completed prior to study drug administration.

Assessments Day -28 to Day 1 (first day of C-225-ILs-dox, prior to drug administration)

Assessment	Includes
Patient's informed consent	Written consent
Demographic data	Date of birth
	Race
	Sex
History of malignant disease	Primary diagnosis
	Histology
	Location of metastases
Medical history	Concomitant non-malignant disease
	Treatment for non-rnalignant con¬ comitant disease (^concomitant medication)
General physicai examination	Total body examination
Special examinations	ECG, Echocardiography
	Pregnancy test if requested
Tumor measurement/ assess¬ ment	CT scan, MRI scan, ultrasound, or X-ray; clinical measurement in case of skin or palpable [ymphnode metastases
Special examination	EGFR overexpression tmmunohisto-chemistry (DAKO)

Assessments Day -7 to Day 1 (first day of C225-ILs-dox, prior to drug administration)

Assessment	Includes
Vital signs and physical measurements	Body temperature Blood pressure Heart rate Height Weight Performance status (ECOG) Physical examination
General laboratory tests	Hematology Blood chemistry

4.2. During Treatment

Tumor assessments will be done during screening and after 2, 4 and 6 cycles of treatment. After treatment completion, an assessment is performed every 3 months for the first year and thereafter according to clinical needs. If progression is documented, no further assessments will have to be performed within the study. In responding patients, the response must be confirmed a minimum of 4 weeks after the response has first been recorded.

Laboratory safety assessments:

- Hemoglobin, leukocytes and thrombocytes will be analyzed weekly during the first cycle and every two weeks during subsequent cycles if not clinically indicated otherwise.
- Transaminases, bilirubin, alkaline phosphate, creatinine, calcium, LDH and urine status will be analyzed every 4 weeks.

Adverse events will be recorded at each visit.

An echocardiography will be performed before, after 2 and 6 cycles of treatment (or at the end of study), and if clinically indicated in all patients.

5. STUDY DESIGN

This is a single center, open study.

The recruitment of patients will be performed in two stages. First, patients will be enrolled according to section 10.3.2. {dose regimen and dose adjustment). The second stage allows an additional recruitment of up to 6 additional patients on the dose level defined as the MTD.

Patients having completed the treatment phase (24 weeks) and showing complete or partial response as well as stable disease will enter the observation phase of the study. This phase will end 12 months after the last patient has been included.

At any time during treatment phase or observation phase, patients with signs of disease progression according to RECiST criteria for reporting resuits of cancer treatment given in appendix 1 or having discontinued treatment due to unacceptable toxicity wiil go off study and be treated at the investigator's discretion.

6. STUDY MEDICATION

6.1 . Drug Names, Formulation, Storage

C225-IL-dox will be supplied for use as a solution of 10 mg doxorubicin per 20 m! vial for parenteral administration (0.5 mg doxorubicin/ml). C225-!Ls-dox should be stored at 2-8 ^{0}C.

6.1,1. Liposome Preparation

Liposomes were prepared by a lipid film hydration-extrusion method using repeated freeze-thawing to hydrate the lipid films (23). Liposomes were composed of 1^-distearoyi-sn-glycero-S-phosphocholine (DSPC) and cholesterol (motar ratio 3:2) with methoxy polyethylene glycol (mPEG)-1 ,2-distearoy!-3-sn-giycerophosphoethanoi-amine (DSPE; 0.5-5 mol% of phospholipid; Avanti Polar Lipids; Alabaster, AL). Following hydration, liposomes were extruded 10 times through polycarbonate filters (0.1 µm pore size). Liposome size was determined by dynamic Sight scattering (typically 80-100 nm). Phospholipid concentration was measured by phosphate assay (25).

For liposomes loaded with ADS645WS (American Dye Source, Quebec, Canada), the fluorescent dye (5mmol/L) was dissolved in buffer for rehydration of the dried lipids. After passive loading, unencapsulated dye was removed using Sephadex G-75 chromatography.

For encapsulation of chemotherapeut ïc drugs doxorubicin (Bedford Laboratories, Bedford, OH) and epirubicin (Pharmacia, Kalamazoo, MI), a standard remote-loading method using ammonium sulphate was done (26, 27). For encapsulation of vinorelbine, liposomes were prepared as described following hydration in a solution of tπethylammonium sucrose octasuifate (TEA_8SOS; 0.65 mol/LTEA, pH 5.2-5.5). Unentrapped TEA_8SOS was removed on a Sepharose CL-4B size exclusion column. Vinoreibine was added at a drug-to-phosphoiipid ratio of 350 g drug/mot phospholipid and the pH adjusted to 6.5 with 1 N HCi before initiation of ioading at 6O^{0}C for 30 minutes. The resulting liposomal vinorelbine was purified on a Sephadex G-75 column to remove unencapsulated drug.

6.1.2. Preparation of Monoclonal Antibody Fragments and Imrnunoliposomes

Intact C225 rnAb (cetuximab. Erbitux; ImClone Systems, in., New York, NY) was cleaved and reduced as previously described (11). Fab' fragments were covalentiy conjugated to maleimide groups at the termini of PEG-DSPE chains (Mal-PEG-DSPE; Nektar, Huntsville, AL; ref. 8). Conjugation efficiencies were typically 30% to 50% for C225-Fab\ For incorporation into preformed liposomes or commercial PLD (Doxil, Alza Pharmaceuticals, Paio Alto, CA), mAb conjugates were incorporated into liposomes by coincubation at 55°C for 30 minutes at protein/iiposome ratio of 30 μg Fab'/μmol phospholipid, resulting in incorporation efficiencies of 70% to 80% (11)

6.1.3. Formulation

C225-!Ls-dox wii! be prepared in the pharmacy of the University Hospital of Basel (Prof. C. Surber). C225-ILs-dox will be stored in HEPES-Buffered-Saiine (0.9 % NaCi; HEPES 2 mM) at a pH of 6-7 in a concentration of 0.5 mg doxorubicin/mi. C225-iLs-dox will be added to 250 ml of 5% glucose for injection (500 ml for dose ïevels 50 mg/m2 and above). This formulation must be used within 24 hours after dilution in glucose. Diluted C225-ILs-dox should be a clear and reddish solution without any signs of aggregation.

6.1.4. Storage Requirement

Vials of C225-ILs-dox have to be stored in the refrigerator at a temperature ranging from 2° - 8^{0}C to ensure optima! retention of physical and biochemicai integrity. It is important not to freeze the study drug, since liposomes would be

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disrupted. C225-ILs~dox may be sensitive to shear-induced stress (e.g. agitation or rapid expulsion from a syringe). Vigorous handling (such as shaking) of C225-iLs-dox solution may results in aggregation of the protein and may create cloudy solutions. Vials are designed for single use only.

6.2. Packaging and Labeling

The vials of the study medication C225-ILs-dox are labeled as follows:

FOR CLINICAL TRIAL USE ONLY
Study CC1
C225-ILs-d ox
Total content: 20 ml at 0.5 mg doxorubicin/ml = 10 mg/vial
Store between 2-8 ^{0}C (DO NOT FREEZE)
Expiry date:
Batch ID;
investigator name:
Patient identification:

6.3. Study Treatment

6.3.1. Rationale for Dose Selection

The standard dose of Caeiyx used in numerous phase II and III trials and also in routine oncology practice is 40-50 mg/m2 given as a short infusion every 4 weeks. One of the main toxicities of the drug given at that dosage is palmar plantar erythema (PPE = hand foot syndrome). Similarly, an important possible side effect of Cetuximab is skin toxicity, usually manifesting itself as an acneiform rash of the face and trunk. This side effect is probably a consequence of the fact that the epidermis expresses EGFR at a relatively high level. Therefore, the main safety concern of this study is that directing Caeiyx to EGFR-overexpessing cells via the anti-EGFR antibody Cetuximab might also increase the skin toxicity of the drug.

Treatment within this phase I study was at a very low dose of Caeiyx, i.e. a 10th of the standard dose of the drug (corresponding to an antibody (Cetuximab) dosage of approx. 0.9 mg/m2 compared to 250 mg/m2 (loading dose 400 mg/m2) in established clinical regimens), and to escalate dosage in small increments.

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6.3.2. Dosage Regimen and Dose Adjustment

Patients will be treated in cohorts of three patients each at the foitowing dose levels (quantification and dose levels of C225~!Ls-dox are defined in mg doxorubicin):

Level 1 = 5 mg/m2
Level 2 = 1Q mg/m2
Level 3 = 20 mg/m2
Level 4 = 30 mg/m2
Level 5 = 40 mg/rn2
Level 6 = 50 mg/m2
Level 7 = 60 mg/rn2
Level 8 = 70 mg/m2
Level 9 = 80 mg/m2

At each dose level, 3 patients may be enrolled simultaneously. Escalation to the next higher dose will be allowed after patient 3 of a given dose level has received at least one full cycle of therapy if no dose limiting toxicity (DLT) occured at a given dose level The decision to enter a next dose level will be made by a team after reviewing all available toxicity data of the previous groups. A DLT is defined as any grade 4 toxicity, any grade 3 toxicity lasting more than one week or/and febrile neutropenia grade 3 (defined as neutrophils < 1.0 x 10e9/l and fever > 38.5 °C). Nausea, vomiting, anorexia, and alopecia (grade 2) will be excluded as dose limiting toxicities. Similarly, adverse events that are clearly related to the primary tumor, such as progression of disease will not be considered as DLTs. In addition, preexisting toxicities must be taken into account when defining and analyzing DLTs.

Examples of grade 3 toxicities considered as DLT:

- In the case of PPD (Hand Foot Syndrome), grade 3 toxicity is defined as ulcerative dermatitis or skin changes with pain interfering with function, and therefore considered as DLT.
- in the case of diarrhea, grade 3 toxicity is defined as increase of > 7 stools per day over baseline; incontinence; i.v. fluids > 24 hrs and/or hospitalization, and therefore considered as DLT.
- In the case of left ventricular function, grade 3 toxicity is defined as symptomatic cardiac dysfunction responsive to intervention and/or a decrease of the ejection fraction < 40 %, and therefore considered as DLT.

if a DLT occurs at any dose level, the following rules will apply:

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Number of Patients with DLT at a given Dose Level	Escalation Decision Rule
0 out of 3	Enter 3 patients at the next dose level
≥ 2	Dose escalation will be stopped. This dose level will be declared the maximally administered dose (highest dose administered). Three (3) additional patients will be entered at the next lowesr dose level if only 3 patients were treated previously at that dose.
1 out of 3	Enter at least 3 more patients at this dose level. If 0/3 or 1/3 of these 3 patients experience DLT, proceed to the next dose level. If 2/3 or more of this group suffer DLT, then dose escalation is stopped, and this dose is declared the maximally administered dose. Three (3) additional patients will be entered at the next lower dose level if only 3 patients were treated previously at that dose.
≤ 2 out of 6 at highest dose level below the maximally administered dose	This is generally the recommended phase 2 dose. At least 6 patients must be entered at the recommended phase 2 dose.

Sequential dose escalation will be allowed until a DLT is observed in 3/3-6 patients treated at the same dose level. At this point no further dose escalation will be allowed. The maximum tolerated dose (MTD) for potential future studies will than be defined as the dose level below the one at which the dose escalation had to be stopped.

In an individual patient who experiences toxicity, continuation of treatment at a reduced dose, determined according to the clinical judgment of the primary investigators and following the rules detailed in chapter 6.5, will be an option.

6.3.3. Treatment Duration

Patients will be treated until disease progression but for a maximum of 6 cycles.

6.4. Concomitant Treatment

All concomitant medication(s) must be reported in the case report form.

7. PREMATURE WITHDRAWAL

Patients may withdraw from the study at any time and for whatever reason, without affecting their right to appropriate treatment. The investigator has the right to withdraw a patient for any reason which is in the best interest of the patient, including intercurrent illness, adverse events, treatment failure or protocol violations.

A patient who becomes pregnant during the study will be withdrawn. The reason for drop out should be coded as protocol violation and pregnancy shouid be reported as Serious Adverse Event.

Although withdrawals shouid be avoided if at all possible, it is understood that withdrawals may occur during a study. Whenever a patient is withdrawn from a study, for whatever reason, a final study evaluation must be completed for that patient, staging the reason for withdrawal. All documentation concerning the patient must be as complete as possible.

8. WARNINGS AND PRECAUTIONS

Any adverse event that is considered SERIOUS must be reported IMMEDIATELY (within one working day) to Dr. Christoph Mamot or Prof. Christoph Rochütz (both Division of Oncology, University Hospital of Basel; affiliation see title page of this protocol).

C225-ILs-dox therapy should only be initiated under supervision of a physician experienced in the treatment of cancer patients. Since this is a single center study performed at the Division of Oncology at the University Hospital in Basel only physicians of this division will perform the treatment in close coilaboration with the investigators.

Regarding skin toxicity piease also refer to 10.3.1 (rationale for dose selection).

9. STATISTICAL METHODS AMD CONSIDERATIONS

9.1 . General Considerations

This is a phase I study b evaluate the safety of C225-ILs-dox in patients with advanced solid tumors. Efficacy is a secondary endpoint of this study, therefore tumor measurements before, during and after therapy will provide some preliminary data also on tumor response to C225-Il_s-dox. However, analysis of efficacy wiü be purely descriptive and no formal statistica! tests will be performed.

9.2. Sample Size

The sample size for this trial is based on a study design used to provide a safety stopping ruie in the event that dose-ümiting toxicity (DLT) is encountered during the trial. The study plan is to enroll 3 patients at each dose level, with a maximum of another three additional patients to be entered sequentially at each of these dose levels depending on toxicity. The trial will be terminated when three out of three to six patients experience DLT at a particular dose ieve! (DLT dose).

If the true toxicity rate at a dose level is ,,P", then the probability of declaring the dose as toxic (DLT dose) is as follows:

Toxicity Rate (P)	Probability of Detecting DLT Dose
0.2	0.099
0.3	0.256
0.4	0.456
0.5	0.656
0.6	0.821
0.7	0.930
0.8	0.983

9.3. Primary and Secondary Analyses

9.3.1. Primary Variables

The adverse event profile of the patients for each dose level will be summarized in terms of frequency and number of events. Similarly, the number and proportion of patients who experience DLT will also be summarized. Listings of all adverse events and laboratory data will be provided.

9.3.2. Secondary Variables

The proportion of patients belonging to each of the response categories (see 7.2.1 .) will be tabulated.

10. ETHSCAL CONSfDERATSONS

This protocol has been written, and the trial is to be performed in accordance with the Declaration of Helsinki, the Guidelines of Good Clinical Practice issued by !CH and Swiss regulatory authorities' requirements.

Before entering any patients into this **trial,** the investigator has to make sure that the trial has been approved by the focal ethics committee and that Swissmedic has opened the center.

10.1 .Informed Consent and Patient information

informed consent shall be obtained on a written form approved by the local ethics committee and signed by the patient. Two informed consents have to be signed, one of which will be handed to the patient.

In seeking informed consent, the patient information provided in the appendix should be used (amended according to the requirements of the local ethics committee) and one copy shouid be handed to the patient.

The informed consent procedure must conform to the guidelines on Good Clinical Practice issued by iCH and Swissmedic.

AH patients will be informed of the aims of the trial, the possible adverse experiences, how to react in case an adverse event occurs, and the procedures and possible hazards to which he/she will be exposed. They will be informed as to the strict confidentiality of their patient data, but they need to know that their rnedicai records may be reviewed for trial purposes by authorized individuals other than their treating physician.

An investigator must provide the patient with sufficient opportunity to consider whether or not to participate and minimize the possibility of coercion or undue influence. The information provided shall be in a language intelligible to the patient and may not include any content that appears to waive any of the patient's legal rights, or appears to release the investigator, the sponsor, or the institution from liability for negligence.

It will be emphasized that participation is voluntary and that the patient is allowed to refuse further participation in the protocoS whenever he/she wants. This will not prejudice the patient's subsequent care.

In case new data becomes available that shifts the risk/benefit ratio, the patient shoufd reconsent.

11.

11. APPENDICES

11.1. Recist Criteria

Response Evaluation Criteria in Solid Tumors (RECIST) (29)

Ali patients with measurable disease wii! be evaluated for response.

Measurable disease - the presence of at least one measurable lesion. If the measurable disease is restricted to a solitary lesion, its neoplastic nature should be confirmed by cytology/histology.

- Measurable lesions: lesions that can be accurately measured in at least one dimension with longest diameter ≥ 20 mm using conventional techniques or ≥ 10 mm with spiral CT scan.
- Non-measurable lesions: all other lesions, including small lesions (longest diameter < 20 mm with conventional techniques or < 10 mm with spiral CT scan), i.e. bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusion, inflammatory breast disease, lymphangitis cutis/pulmonis, cystic lesions, and also abdominal masses that are not confirmed and followed by imaging techniques.

Evaluation of Lesions

Evaluation of Target Lesions [A]

- Complete Response (CR): Disappearance of all target lesions
- Partial Response (PR): At least a 30% decrease in the sum of the longest diameter (LD) of target lesions taking as reference the baseline sum LD.

[A] All measurable lesions up to a maximum of 10 lesions representative of all involved organs should be identified as target lesions and recorded and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter) and their suitability for accurate repetitive measurements (either by imaging techniques or clinically). A sum of the long distance (LD) for ail target lesions will be calculated and reported as the baseline sum LD. The baseline sum LD will be used as reference to further characterize the objective tumor response of the measurable dimension of the disease.

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- Progression (PD): At least a 20% increase in the sum of LD of target lesions taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions.
- Stable Disease (SD); Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD taking as references the smallest sum LD since the treatment started.

Evaluation of Non-Target Lesions [B]

- Complete Response (CR): Disappearance of ail non-target lesions and normalization of tumor marker ievel.
- Non-Complete Response; Persistence of one or more non-target lesions (non-CR) or/and maintenance of tumor marker leve! above the normal limits.
- Progression (PD): Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions.[C]

Note:

« Tumor markers alone cannot be used to assess response. If tumor markers are initially above the upper normal limit, they must normalize for a patient to be considered in complete clinical response when ail lesions have disappeared.

- Cytology and histology: If the measurable disease is restricted to a solitary lesion, its neoplastic nature should be confirmed by cytology/histology.

These techniques can be used to differentiate between PR and CR in rare cases (for example, residual lesions in tumor types such as germ celi tumors, where known residual benign tumors can remain).

The cytologicai 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 is mandatory to differentiate between response or stable disease (an effusion may be a side effect of the treatment) and progressive disease.

Evaluation of best overall response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for

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[B] All other lesions (or sites of disease) should be identified as non-target lesions and should also be recorded at baseline. Measurements are not required, but the presence or absence of each should be noted throughout follow-up.

[c] Although a clear progression of "non-target" lesions oniy is exceptional, in such circumstances, the opinion of the treating physician should prevail, and the progression status should be confirmed at a later time by the review panel (or trial chair).

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progressive disease the smallest measurements recorded since the treatment started). The patients' best response assignment will depend on the achievement of both measurement and confirmation criteria.

Target Lesions	Non-Target Lesions	New Lesions Response	Overall
CR	CR	No	CR
CR	Non-CR/Non-PD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Note:

- Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be reported as "symptomatic deterioration". Every effort should be made to document the objective progression even after discontinuation of treatment.
- In some circumstances, it may be difficult to distinguish residual disease from normal tissue. When the evaluation of complete response depends upon this determination, it is recommended that the residual lesion be investigated (fine needle aspirate/biopsy) before confirming the complete response status.

Guidelines for evaluation of measurable disease

All measurements should be recorded in metric notation using a ruler or calipers. All baseline evaluations should be performed within 14 days before registration according to the schedule of assessments.

Note: Tumor lesions in a previously irradiated area are not optimally considered measurable disease.

The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. CT and MRI are the best currently available and reproducible methods to measure target lesions selected for response assessment. Imaging-based evaluation is preferred to evaluation by clinical examination when both methods have been used to assess the antitumor effect of a treatment.

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- Clinical lesions will only be considered measurabie when they are superficial (e.g. skin nodules, palpable lymph nodes). In the case of skin lesions, documentation by color photography including a ruler to estimate the size of the lesion is recommended.

- Lesions on chest X-ray are acceptabie as measurabie lesions when they are clearly defined and surrounded by aerated iung. However, CT is preferable.

- Conventional CT and MRI should be performed with cuts of 10 mm or Jess in slice thickness contiguously. Spiral CT should be performed using a 5 mm contiguous reconstruction algorithm. This applies to the chest, abdomen, and pelvis. Head & neck extremities usually require specific protocols.

- When the primary endpoint of the trial is objective response evaluation, ultrasound (US) should not be used to measure tumor lesions that are clinically not easily accessible. It is a possible alternative to clinical measurements of superficial palpable nodes, subcutaneous lesions, and thyroid nodules. US might also be useful to confirm the complete disappearance of superficial lesions usually assessed by clinical examination.

Confirmatory measurement/duration of response

Confirmation

In order to be assigned a status of PR or CR, changes in tumor measurements must be confirmed by repeat studies that should be performed 4 weeks after the criteria for response are first met. in the case of SD, follow-up measurements must have met the SD criteria at least once after tria! at 7 weeks (see Schedule of assessments, appendix 23.18).

Duration of overall response

The duration of overall response is measured from the time measurement criteria are met for CR/PR (whichever is first recorded) until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurements recorded since the treatment started).

The duration of overall complete response is measured from the time measurement criteria are first met for CR until the first date that recurrent disease is objectively documented.

Duration of stable disease

Stable disease is measured from the start of treatment until the criteria for progression are met, taking as reference the smallest measurements recorded since the treatment started.

11.2. Eligibility Forms

Inclusion Criteria

Prior to enrollment in the study candidates must meet ALL the following criteria (check each box if OK):

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1. Histoiogicaily proven locally advanced or metastatic solid tumor.
2. ECOG Performance ≤ 2.
3. No additional standard therapy available for the patient.
4. EGFR overexpression (according to DAKO EGFR pharmDx - Test) determined in the most recently evaluable tumor tissue.
5. No concomitant anti-tumor therapy (steroids are permitted - in breast cancer and prostate cancer, steroid dose needs to remain stable during the study period).
6. At least four weeks since termination of any previous anti-tumor treatment (6 weeks in the case of nitrosoureas or mitomycin C).
7. in patients with previous anthracycline exposure, a normal echocardiogram (LVEF > 50%) is required.
8. Age ≥ 18.
9. Male or female.
10. Female and male patients of reproductive age must be using effective contraception.
11. Willing and able to sign an informed consent prior to participation in the study and to comply with the protoco! for the duration of the study.

Exclusion criteria

Candidates must be excluded from the study if ANY of the folfowing criteria are met (check each box if OK):

1. Pregnancy and/or breastfeeding.
2. Patients with the following laboratory values

   -neutrophils < 1.5 x 10^9/L

   -platelets < 100 x 10^9/L

   -serum creatine > 3.0 x upper norma! limit

   -ALAT, ASAT > 3.0 x upper normal limit (5.0 x in patients with liver metastases as the only likely cause of enzyme alteration)

   -alkaline phosphatase > 3.0 x upper normal limit (5.0 x in patients with liver or bone metastases as the only likely cause of enzyme alteration)

   -bilirubin > 3.0 x upper normal limit
3. Participation in any investigational drug study within 4 weeks preceding treatment start.
4. Patients with clinically significant and uncontrolled renal- or hepatic disease.
5. Clinically significant cardiac disease: congestive heart failure (New York Heart Association class III or IV); symptomatic coronary artery disease; cardiac arrhythmia not well controlled with medication; myocardial infarction within the last 12 months.
6. Any serious underlying medical condition (at the judgement of the investigator) which could impair the ability of the patient to participate in the trial (e.g. active autoimmune disease, uncontrolled diabetes, etc.).

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7. Any concomitant drugs contraindicated when administering Erbitux™ or Caelyx™ according to the Swissmedic-approved product information.

8. A cumulative doxorubicin dose of > 300 mg/m2 BSA (or cardiotoxic anthracycline-equivaient).

9. Patients with a history of uncontrolled seizures, central nervous system disorders or psychiatric disability judged by the investigator to be ciinicaliy significant and precluding informed consent or interfering with compliance.

10. Brain metastases.

12. RESULTS

Preliminary results of the phase I trial are included in the following table. These results show that no or very little drug-related toxic effects can be observed up to a concentration of 50 mg/m^2. Particularly, no skin toxicity, particularly no palmar plantar erythema, was found at even the higher doses, while at the same time, clear signals of efficacy were observed, even at the lowest dose used.

Table 1: Preliminary results of the phase I trial

Pat.No.	Dose (per m2)	Tumor	Cycles	Grade 3/4 Toxicity	Efficacy (best response)
1	5 mg	Prostate	2	none	PD*
2	5 mg	Pancreatic	2	none	PD
3	5 mg	Renal cell	2	none	PD
4	10 mg	Pancreatic	1	none	n.e.
5	10 mg	Esophageal	3	none	SD
6	10 mg	Colorectal	6	none	SD
7	20 mg	Colorectal	2	none	PD
8	20 mg	Pancreatic	1	none	PD
9	20 mg	Head and Neck	4	none	SD
10	30 mg	Mesothelioma	6	none	PR
11	30 mg	Prostate	2	none	PD

12	30 mg	Pancreatic	2	none	PD
13	40 mg	Bladder	2	none	PD
14	40 mg	Bladder	6	none	SD (MR)
15	40 mg	Renal cell	1	none	n.e.
16	50 mg	Hepatocellular	2	Neutropenia Grade 3	n.e.

{* Minimal tumor progression after 2 cycles. Retrospectively, PSA decrease and remission of lung metastases for 18 months).

(no skin toxicity at all in all 16 patients treated so far)

PD Progression
SD Stable Disease
SD (MR) Stable Disease (Minimal Response)
PR Partial Response
n.e. not evaluated

B MULTS DRUG RESISTANCE STUDY

1. MATERIALS

1-1 Reagents

Reagents for liposome preparation included: DilCi $_8$(3)-DS (MoJecuiar Probes; Leiden, Netherlands); DSPC, cholesterol, and mPEG-DSPE (Avanti Polar Lipids; Alabaster, AL, USA); Mal-PEG(2000/3400)-DSPE (Nektar; Huntsville, AL, USA); organic solvents, and other chemicals of reagent purity (Sigrna-Aldrich AG; Buchs, Switzerland).

Doxorubicin (Adriblastin RD®; Pfizer AG, Zurich, Switzerland) and pegylated liposomal doxorubicin (Caelyx®, Essex Chemie AG, Luzern, Switzerland) were obtained commercially from the pharmacy.

Immunoiiposomes contained either Fab' derived from C225 (cetuximab, Erbitux) or EMD72000 (matuzumab; both Merck KGaA, Darmstadt, Germany). Both monoclonal antibodies are recombinant lgGi that bind b the extracellular domain (ECD) of EGFR and thereby block activation by EGFR Sigands such as EGF and TGF-α (36). While MAb

C225 is a chimeric MAb, EMD7200G is a humanized MAb derived from transgenic mice (37).

MAb EMD72000 was kindly provided by Merck KGaA, Darmstadt, Germany,

1.2. Ceil lines

MDA-MB-231 human breast cancer and colorectal cancer ceil lines HT-29 cancer cell lines were obtained from the department of research at the University of Base! or the American Type Culture Collection (ATCC). The resistant versions of theses ceii lines were provided by Susan Bates (MDA-MB-231 Vb100; NIH, Bethesda, USA) and by Dr. Schafer (HT-29 RDB; Charite, Berlin, Germany). MDA-MB-231 cells were maintained in "Improved MEM Zinc Option" medium (invitrogen AG, Basei, Switzerland) and HT-29 in RPM1-1640 (Sigma-Aldrich AG, Buchs, Switzerland) supplemented with 10 % fetal calf serum, 100 iU/ml peniciilin and 100 µg/ml streptomycin In a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C.

2. LIPOSOME PREPARATION, LOADING AND ANTIBODY INCORPORATION

2.1 Liposome preparation

Unilamellar liposomes were prepared according to the repeated freeze-thawing method (23) using DSPC and Cholesterol (molar ratio 3:2) with mPEG-DSPE (0.5-5 moi% of phospholipid). Briefly, liposomes were subsequently extruded 10 times through polycarbonate filters with defined pore sizes of 0.1 µm, yielded liposomes of 90-120 nm diameter as determined by dynamic light scattering. Liposome concentration was measured utilizing a standard phosphate assay.

For uptake and internalization studies, liposomes were labeled with 0.1-0.3 mol% DilCi $_S$(3)-DS, a fluorescent lipid that can be stably incorporated into liposomal membranes ((38) (39))..

For encapsulation of doxorubicin, the remote-loading method using ammonium sulfate was performed ((27)(26)}. First, dry lipids were rehydrated in 250 mM ammonium sulfate at pH 5.5, followed by extrusion as described above. Free ammonium sulfate was removed by size-exclusion chromatography using a Sephadex G-75 column/HEPES buffered saline (pH 7.0). Liposomes were then incubated with doxorubicin for 30 min at 60 °C. Under these conditions, loading efficiencies were

typically in the range of 95-100 % when 150 µg drug per µmol phospholipid was used. All unencapsulated doxorubicin was removed by size-exclusion chromatography using a Sephadex G-75 column. In addition, pegylated liposomal doxorubicin (PLD/Caelyx®/Doxil®) was obtained commercially.

2.2 MAb fragment preparation, conjugation, and liposome incorporation

For C225- and **EMD720Q0-Fab**\ intact MAbs were incubated with pepsin (weight ratio 1:20) in 0.1 M sodium acetate (pH 3.7) at 37 ^{0}C for 3 h, followed by dialysis against HEPES-buffered saline (pH 6.0). The resulting F{ab)a were reduced with 2-mercapt σ-ethyïamïne or 2-mercaptoethanoi under argon for 15 min at 37 ^{0}C, and then recovered by gel filtration using Sephadex G-25. Reduction efficiency was typically 70-90 %.

Fab' were conjugated to Mal-PEG-DSPE as described previously ((1 1) (12)). Conjugation efficiencies were evaluated by SDS-PAGE, allowing comparison of free MAb fragment vs. conjugate; conjugation efficiences were typically 30-50 % for C225 and 40-60 % for EMD72000. For incorporation into preformed liposomes, including prepared liposomal drugs and probes or commercial pegylated liposomal doxorubicin, MAb fragment conjugates (Fab'-Mal-PEG-DSPE) which form micellar solutions, were incorporated into liposomes by coincubation at 55 ^{0}C for 30 min. As a result, the conjugates become attached to the outer lipid layer of the liposomes via hydrophobic DSPE domains. Unincorporated conjugates and free drug were separated from immunoiiposomes by Sepharose CL-4B gel filtration. When $DiIC_{18}$(3)-DS-labeled liposomes were used, <5% of the fluorescence was co-associated with the micelle fraction, indicating minima! transfer of this marker. Incorporation efficiency of conjugated MAb fragments was estimated by SDS-PAGE using a series of protein standards and gel scanning and quantitation as described. For both, C225 and EMD72000, typically 75-85 % of added MAb conjugate was incorporated into immunoiiposomes, corresponding to 30-40 Fab' fragments per liposome.

3. STUDY DESIGNS

3-1 Binding and internalisation study

For flow cytometry studies, 250,000 cells were co~incubated in 12-weti plates with saline (control), liposomes or EGFR-targeted immunoiiposomes labeled with DiiCie(3)-DS for 2 h at 37 ^{0}C, washed extensively with PBS, foilowed by detaching and storing on ice

until subjected to flow cytometry. Fluorescence microscopy studies were performed accordingly except detaching the ceils from the 12-we!l plates.

Immunoliposomes containing C225-Fab' showed an approximately 2 orders-of-magnitude greater accumuiation in the human breast cancer cell line MDA-MB-231 than did control liposomes, which produced only background levels of fluorescence in these cells. A similar pattern was found in the multi-drug resistant subcell line MDA-MB-231 VbIOO.

Binding and uptake of C225-Fab'-containing immuno üposomes was also evaiuated in EGFR-overexpressing colon cancer HT-29 ceüs and its multi-drug resistant subceil line HT-29 RDB. Here, immunoliposomes showed a more than 1 order-of-magnitude greater uptake in EGFR-overexpressing HT-29 cells, and comparable findings in the mdr celi line HT-29 RDB. In the non-EGFR overexpressing control celi line MCF-7 there was no difference in uptake/binding between non-targeted liposomes and anti-EGFR immunoliposomes (data not shown). These results indicate a high selectivity for immunoiiposorne uptake in both isogenic cell lines regardless of their mdr features.

The observation of minimal fluorescence uptake in target ceils after incubation with control liposomes is consistent with the non-reactive properties of pegylated liposomes ((12) (40)). and also confirms that DilCi $_S$(3)~DS can be used as a stable liposome-based marker without significant exchange into cell membranes.

3.2 Cytotoxicity studies

Specific cytotoxicity of EGFR-targeted immunoliposomes containing doxorubicin was evaluated in target cells plated at a density of 8,000 cells per well in 96-well plates and allowed to grow overnight. Immunoliposomes or control treatments were applied for 2 h at 37 °C, followed by washing with PBS and re-adding growth media. Ceils were further incubated at 37 °C for 3 days and analyzed for ceil viability using 3-{4,5dimethylthiazol-2~y!)-2,5-diphenyl tetrazolium bromide (MTT) staining (41), For the cytotoxicity studies using the efflux pump inhibitor verapamil, this compound was added to the media at a concentration of 100 µM during the complete experiment,

In EGFR-overexpressing HT-29 wilde type colon cancer cells, EGFR-targeted immunoliposomal doxorubicin showed substantial in vitro cytotoxicity following treatment for 2 h (IC_{50} = 0.25 µg/ml), which approached that of free doxorubicin (IC_{50} = 0.3 µg/ml) {Table 2). Thus, EGFR-targeted immunoliposome delivery of doxorubicin was as efficient as the rapid diffusion of free doxorubicin, a smali, amphipathic molecule

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that readily transverses ceil membranes In vitro. On the other hand, EGFR-targeted immunoliposoma! doxorubicin, derived by conjugation of C225-Fab" to PLD, showed a much greater cytotoxicity than non-targeted PLD itself (IC_{50} not reached) in HT-29 ceils, indicating that delivery was antibody-dependent (Table 2). Notably, similar treatment with the antibody C225 alone for 2 h showed no cytoxicity in this assay, confirming that immunoliposome activity was due to targeted drug delivery and not related b potential antiproliferative effects of C225 during this brief incubation time. Furthermore, immunolfposomes containing C225-Fab' but lacking encapsulated drug ("empty immunoliposomes") similarly showed no cytotoxicity under these assay conditions. Also no effects of G225-immunoiiposomes~dox have been seen in MCF-7 cells, which lack the EGF receptor (negative control; data not shown).

The identical experiment was performed in the multi-drug resistant sub eel! line HT-29 RDB. Notably, in this cei! line immunoliposoma] delivery of doxorubicin (IC_{50} = 0.5 µg/ml) was superior to that of the free drug (IC_{50} = 9.5 µg/ml; = 19-fold) and also liposomal drug (IC_{50} not reached). To sum up this part of our studies, while free doxorubicin was much less cytotoxic in the multi-drug resistant variant of the HT-29 cell line compared to the wild type, there was almost no difference for the immunoiiposoma! compound regardless of different mdr features in this cell lines, indicating that immuno üposomes are able to bypass multi-drug resistance mechanisms in this setting.

Immunoliposome-mediated cytotoxicity with doxorubicin was also evaluated in EGFR-overexpressing human breast cancer cell line MDA-MB-231 Vb100 featuring multi-drug resistance and compared to results with its parental cell line MDA-MB-231 lacking mdr. In the parental wild-type MDA-MB-231 , ILs containing C225~Fab' were as efficient in delivering doxorubicin as free doxorubicin itself, which again can easily penetrate the ceii membrane, and clearly more cytotoxic than non-targeted liposomal doxorubicin/PLD (IC_{50} = 0.3 vs. 0.6 vs. 120 µg/ml.

Interestingly, in the highly drug resistant MDA-MB-231 Vb 100 cell line, ILs loaded with doxorubicin (dox) produced a 216-fold greater cytotoxicity than free dox, and were also markedly more cytotoxic than the non-targeted liposomal doxorubicin (IC_{50} = 0.6 vs. 130 vs. >900 µg/ml.

The same experiment in resistant MDA-MB-231 VbIOO cells was repeated in the presence of verapamil. This substance is abie to inhibit efflux pumps and therefore reverse specific mechanisms of multi-drug resistance. In fact, by adding verapamil to this experiment the multi-drug mechanism could be converted effectively and as a result

free doxorubicin was as efficient as in the wild-type MDA-MB-231 cell line (IC_{50} = 0.9 µg/ml). In contrast, the addition of verapamil did not further increase the efficacy of doxorubicin delivered by anti-EGFR immunoliposomes (IC_{50} = 0.5 µg/ml), thus confirming our finding that Hs are able to overcome multi-drug mechanisms and that this delivery system is unaffected by the presence of efflux pumps.

Table 2; Summary Results of Cytotoxicity Study

	HT-29 WT (IC_{50})	*C225-ILs-dox vs. free dox*	*HT-29 RDB (IC_{50})*	*C225-ILs-dox vs. free dox*
free dox	0.3	**1.2 - fold**	9.5	**19 - fold**
C225-ILs-dox	0.25		0.5	
PLD	> 31		> 31	

PLD non-targeted liposomal doxorubicin
C225-ILs-dox C225 antibody targeted liposomal doxorubicin

cL3 Accumulation of doxorubicin in the cytoplasma and nuciei

For comparative accumuiation studies, tumor ceils (HT-29, HT-29 RDB, MDA-MB-231 or MDA-MB-231 Vb100) have been plated at a density of 200,000 cells per well in 12-weil plates. Free doxorubicin, non-targeted liposomal doxorubicin (PLD) and imrnunoliposomal doxorubicin have been applied at a doxorubicin concentration of 3 µg/ml for 2 h at 37 ^{0}C, followed by 2 washing rounds with media. Verapamil was added to the experiment in a concentration of 0, 10 or 100 µM. After another 2 h incubation without any treatment cells were analyzed as follows:

After removing the media, cells were washed once with 1 ml of culture medium containing FCS, followed by 1 ml of PBS with calcium and magnesium at room temperature (RT) for 3 min. The PBS was replaced by 400 µl C100T solution (100 ml containing 2.1 g citric acid and 0.5 ml Tween 20. Shaking for 10-15 min at 300 cycles/min resulted in solubilization of the cell membrane and released the nuclei, as confirmed by microscopy. The complete content of each well was transferred to transparent 0.5 ml PCR tubes and centrifuged at RT and 1200 rcf for 5 min. This way, the nuclei will sediment at the tips of the tubes, which is crucial for further processing. For the determination of doxorubicin in the cytopiasma, 350 µl from the supernatant were removed and mixed with 350 µl acid methanol (methanol containing 1 M orthophosporic acid). For nuciear accumulation of doxorubicin the pellet with the nuclei

was washed twice with 500 µi PBS containing 1% C100T and using subsequent centrifugation as described before. After careful removing of the final supernatant, doxorubicin from the pellets was extracted overnight by 400 µl 50% acid methanol.

From both cytoplasmic and nuclear extracts, 300 µl were transferred into a 96-well piate and measured by a "SpertraMax Gemini Fluorimeter" (Molecular Devices).

3,4 Tumor xenograft models

Efficacy for non-targeted liposomes versus anti-EGFR immunoliposomes were studied in the MDA-iV1B~231 wild type and resistant breast cancer xenograft tumor model. Swiss nu/nu mice (5-6 weeks; Charles River, France) were injected subcutaneousiy (s.c.) with EGFR-overexpressing MDA-MB-231 tumor cells (1x1 0^7 ceils, wild type or resistant) into the back of the animal. Once tumor xenografts had become established and tumors measured 150-250 mm^3, mice were randomly assigned to different treatment groups (8-10 animais/group, depending on study). All Lv. treatments were performed via tail vein injection, typically in 100-200 µl volume. Liposomes and anti-EGFR immunoiiposomes (C225- and EMD7200G-) were administered intravenously at a dose of 10 mg doxorubicin/kg/dose once weekly for 3 weeks, for a total dose of 30 mg dox/kg. Free drug was injected on the same schedule as liposomes or immunoliposomes intravenously at their MTD of 30 mg dox/kg for doxorubicin. In control groups, saline was administered intravenously at the same injection volume and schedule.

Tumor growth was monitored for a period of 55-100 days post tumor impiantation. Mice were weighted and examined for toxicity three times a week. Tumor measurements were performed 2-3 times weekly using a caliper, and tumor volumes were calculated using the equation: (length X width ^{2}) / 2.

In the wild-type MDA-MB-231 xenograft mode! lacking mdr features, anti-EGFR immunoliposome-dox was administered Lv. at a total dose of 30 mg dox/kg divided into three weekly doses of 10 mg/kg. Anti-EGFR immunoliposomes were either prepared from the anti-EGFR MAb C225 or from EMD72000. Control treatments included: saline; free doxorubicin and non-targeted liposomal doxorubicin (commercial pegylated liposomal doxorubicin; PLD) at the same dose and schedule as immunoliposomes.

Free doxorubicin produced some tumor growth inhibition when compared to saline treatment. Non-targeted iiposome delivery of doxorubicin via PLD at this high dose induced tumor regression and clearly increased efficacy over free drug. Treatment with anti-EGFR immunøiiposome-dox, regardless if C225 or EMD72000 was used, produced

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substantial tumor regressions and was overall the most efficacious treatment. Until day 77, tumor regressions were similar for the PLD, G225-iLs-dox and EMD720GG-!Ls-dox groups. However, during follow-up, tumors treated with untargeted PLD ait started to regrow while tumors treated with immunoliposomal doxorubicin, both C225-!Ls-dox and EMD72000-ILs-dox, did not show growth activity until the end of observation (day 10O), suggesting even a curative potential of anti-EGFR immunoiiposomes as previously reported in other xenograft tumor models.

The same experiment was repeated in the MDA-MB-231 Vb100 xenograft model featuring a very similar EGFR overexpression (data not shown) but additionaliy multi¬ drug resistance. Again anti-EGFR immunoliposome-dox derived either from C225 or EMD72000 were administered i.v. at a total dose of 30 mg dox/kg divided into three weekly doses of 10 mg/kg. Comparators included saline, free doxorubicin and non-targeted iiposomal doxorubicin (commercial pegyiated liposomal doxorubicin; PLD) at the same dose and schedule as immunoiiposomes.

In this highly multi-drug resistant model, free doxorubicin did not show any tumor growth inhibition when compared to saiine treatment. Non-targeted liposome delivery of doxorubicin via PLD at this high dose demonstrated some tumor growth inhibition. interestingly and importantly, treatment with anti-EGFR immunoliposome-dox, regardless if C225 or EMD72000 was used, produced substantial tumor regressions and was overall the most efficacious treatment. C225-ILs-dox seemed to be moderately more officious compared to EMD72000-ILs-dox. However, this was only a trend and statistically not significant. Overall, the results of this experiment demonstrate that anti-EGFR immunoiiposomes are effective even against muiti-drug resistant tumors and can overcome mdr mechanisms, (see Table 3)

in both models, anti-EGFR immunoliposome-dox were well-toierated by the mice. Treatment with anti-EGFR immunoliposome-dox was associated with no major weight loss:

Table 3: Results of Tumor Xenograft Study

IC 50 (ug/ml)	MDA-231 WT	MDA-231 Vb100	MDA-231 Vb100 verapamil
PLD	120	>900	740
free dox	0.6	130	0.9
C225-ILs-dox	0.3	0.6	0.5

3.5 Statisticai Analysis

To evaluate the statistical significance of the results, tumor volumes were analyzed and different treatment groups were compared using Student's t-test (2-samp!e individual t-test) for each time point, In addition, a multivariate (rank) test was performed based on the sums of ranks for each mouse. Tumor size at each time point after last treatment was ranked across al! mice for that day and the ranks were summed. The sum of the ranks was compared in each case for two treatments by a 2-sampie t-test (42).

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and WO 98/07409

CLAIMS

1. An imnnunoliposome comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor and further encapsulating in the liposome an anti-tumor compound, for multi-üne treatment of cancer, particuiarly a cancer represented by a locally advanced or metastatic tumor, in a human patient in a clinical set-up.

2. An immunoliposome according to claim 1, wherein said tumor is an EGFR-positive tumor.

3. An immunoliposome according to claim 1 or 2, wherein the liposome encapsulates a cytostatic compound.

4. An immuno üposome according to any of the preceding claims, wherein the anti¬tumor compound is a compound selected from the group consisting of daunomycin, idarubicin, mitoxantrone, mitomycin, cisplatin and other Platinum analogs, vincristine, epirubicin, aclacinomycin, methotrexate, etoposide, doxorubicin, cytosine arabinoside, fluorouracil and other fluorinated pyrimidines, purines, or nucleosides, especially gemcitabine, bleomycin, mitomycin, pücamycin, dactinor πycin, cyclophosphamide and derivatives thereof, thiotepa, BCNU, paclitaxei, docetaxel and other taxane derivatives and isolates, camptothecins, polypeptides, a nucleic acid, a nucleic acid having a phosphorothioate internucleotide linkage, and a nucleic acid having a polyamide internucleotide linkage.

5. An immunoiipos αme according to claim 4, wherein the cytotoxic compound is a compound selected from the group consisting of doxorubicin, epirubicin and vinorelbine.

6. An immunoiiposome according to any of the preceding claims, for second-line treatment of a human patient.

7. An immunoliposome according to any of the preceding claims, for third-line treatment of a human patient.

8. An immunoliposome according to any of the preceding claims, for fourth-line treatment of a human patient.

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9. An irnmuπoliposome according to any of the preceding claims, for fifth-line treatment of a human patient.

10. An immunoliposome according to any of the preceding claims, for sixth-iine treatment of a human patient.

11. An immunoliposome according to any of the preceding claims, for seventh-line treatment of a human patient.

12. An immunoiiposome according to any of the preceding claims for muiti-line treatment of a group of patients which have received, but not responded to, at! available standard treatments.

13. An immunoliposome according to any of the preceding claims, wherein the tumor is still progressing.

14. An immunoiiposome according to any of the preceding claims, wherein the patient has developed a multi-drug resistance.

15. An immunoliposome according to any of the preceding claims, wherein the treatment leads to a stabilization of the disease.

16. An immunoiiposome according to any of the preceding claims, wherein the treatment leads to a partial response.

17. An immunoliposome according to any of the preceding claims, wherein the treatment leads to a complete response,

18. An immunoiiposome according to any of the preceding claims, wherein the treatment shows no or substantially no toxic side effects.

19. An immunoliposome according to any of the preceding claims, wherein the treatment does not show skin toxicity.

20. An immunoliposome according to any of the preceding claims, wherein the treatment does not show palmar plantar erythema (PPE = hand foot syndrome).

21. An immunoliposome according to any of the preceding claims, wherein the treatment shows no or substantially no toxic side effects at a concentration of between 5 mg/m^2 and 80 mg/m^2,

22. An immunoiiposome according to any of the preceding claims, wherein the treatment shows no or substantiatiy no toxic side effects at a concentration of up to 40 mg/m^2.

23. An immunoiiposome according to any of the preceding claims, wherein the antibody or antibody fragment is covaiently bound to the liposome membrane.

24. An immunoiiposome according to any of the preceding claims, wherein the antibody is covalentiy conjugated to the terminus of a linker rnoiecuie anchored to the liposome.

25. An immunoiiposome according to the previous claim, wherein the linker molecule is a polyethylene glycol.

26. An immunoiiposome according to any of the preceding claims, wherein the antibody is a monoclonal antibody directed to the ligand-binding extracellular domain of the EGF receptor.

27. An immunoiiposome according to any of the preceding claims for the treatment of a cancer in a human patient clinical set-up selected from the group consisting of Kaposi's sarcoma, recurrent ovarian cancer, soft tissue sarcoma, glioma, melanoma, mesotheiioma, transitional cell carcinoma of the urothelial tract, endometrial, pancreatic, small-cell and non-smalt-cell lung, hepatocellular, renal cell, esophageal, colorectal, anal, vaginal, vulvar, prostate, basal cell carcinoma of the skin head and neck, and choiangio carcinoma, which cancer is particularly represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor.

28. An immunoiiposome according to any of the preceding claims for the treatment of a cancer in a human patient clinical set-up selected from the group consisting of prostate, pancreatic, kidney, urothelial, oesophageal, head and neck, colonrectal, a hepatocellular cancer and a mesotheiioma, which cancer is particularly represented by locally advanced or metastatic tumor, particularly a EGFR-positive tumor.

29. An immunoiiposome according to any of the preceding claims for multi-line treatment of a cancer in a human patient in a clinical set-up, wherein said patient is suffering from a prostate cancer with a tumor that has progressed on hormonal and/or docetaxel and/or mitoxanthro πe treatment.

30. An immunoiiposome according to any of the preceding claims for multi-line treatment of a cancer in a human patient in a clinical set-up, wherein said patient is suffering from a pancreatic cancer with a tumor that has progressed on gemcitabine and/or capecitabine and/or oxalipiatin treatment.

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31. An immunoiiposome according to any of the preceding claims for muiti-line treatment of a cancer in a human patient in a clinical set-up, wherein said patient is suffering from a kidney cancer with a tumor that has progressed on interferon and/or capecitabine and/or sunitinib and/or sorafinib treatment.

32. An immunoiiposome according to any of the preceding claims for multi-line treatment of a cancer in a human patient in a clinical set-up, wherein said patient is suffering from an esophageal cancer with a tumor that has progressed on cispiatinum and/or 5-FU and/or docetaxel and/or cetuximab treatment.

33. An immunoiiposome according to any of the preceding claims for multi-line treatment of a cancer in a human patient in a clinical set-up, wherein said patient is suffering from a colon and/or rectal cancer with a tumor that has progressed on cetuximab and/or Bevacizumab and/or oxaliplatin and/or irinotecan and/or capecitabine and/or 5-FU treatment.

34. An immunoiiposome according to any of the preceding claims for multi-line treatment of a cancer in a human patient in a clinical set-up, wherein said patient is suffering from a urothelial cancer with a tumor that has progressed on cis- or carboplatinum and/or gemcitabine and/or doxorubicin and/or methotrexate and/or vincristin.

35. An immunoiiposome according to any of the preceding claims for muiti-line treatment of a cancer in a human patient in a clinical set-up, wherein said patient is suffering from a mesothelioma with a tumor that has progressed on cis- or carboplatinum and/or gemcitabine and/or pemetrexed.

36. An immunoiiposome according to any of the preceding claims for multi-line treatment of a cancer in a human patient in a ciinicai set-up, wherein said patient is suffering from a hepatocellular cancer with a tumor that has progressed on sunitinib and/or sorafenib.

37. An immunoiiposome according to any of the preceding claims for the treatment of a human patient in a clinica! set up who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor, wherein a response rate is achieved of between 5% and 95%.

38. A pharmaceutical composition comprising an immunoiiposome according to any of the preceding claims together with a pharmaceutically acceptable carrier or excipient or a diluent for first- to mufti-line, particularly for second-line, particularly

third-line, particularly fourth-line, particularly fifth-line, particularly sixth-line, particularly seventh- and higher- iine treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a ciinical set-up.

39. A method of first- to multi-line, particularly of second-line, particularly third-line, particularly fourth-line, particularly fifth-line, particularly sixth-line, particularly seventh- and higher -line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a clinical set-up by administering to said human patient an immunoliposome or a pharmaceutical composition according to any of the preceding claims.

40. A method of treating a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor, and is chemically naïve, particularly a patient, who has received, but not responded to, at least one standard treatment, particularly to at least two standard treatments, particularly to at least three standard treatments, but especially to all available standard treatments, by administering to said human patient an immunoliposome or a pharmaceutical composition according to any of the preceding claims.

41. A method of using an immunoliposome or a pharmaceutical composition according to any of the preceding claims for the preparation of a medicament for use in first- to multi-line, particularly second-line, particularly third-line, particularly fourth-line, particularly fifth-line, particularly sixth-iine, particularly seventh- and higher- line treatment of cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly an EGFR-positive tumor, in a human patient in a cünicai set-up.

42. A method of using an immunoliposome or a pharmaceutical composition according b any of the preceding claims for the preparation of a medicament for use in the treatment of a human patient who has cancer, particularly a cancer represented by a locally advanced or metastatic tumor, particularly a EGFR-positive tumor, and is chemotherapy naive, particularly a patient who has received, but not responded to, at least one standard treatment, particularly to at least two standard treatments, particularly to at least three standard treatments, but especially to all available standard treatments.

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43. An immuno üposome comprising an antibody or an antibody fragment, which recognizes and binds to an EGF receptor antigen on the surface of a solid tumor and further encapsuiating in the liposome an anti-tumor compound, for treatment of muiti-drug resistance in a patient or a group of patients which have developed such a multi-drug resistance.

44. A pharmaceutical composition comprising an immunoliposome according to any of the preceding claims together with a pharmaceutically acceptable carrier or excipient or a diluent for the treatment of cancer in a patient or a group of patients who have developed a muiti-drug resistance.

45. A pharmaceutical composition according to claim 44, for the treatment of breast cancer.

48. A pharmaceutical composition according to claim 44, for the treatment of a coionrecfal cancer.

47. A pharmaceutical composition according to claim 44, wherein said multi-drug resistance comprises one or more anti-cancer drugs selected from the group consisting of docetaxei, mitoxanthrone, gemcitabine, capecitabi πe, oxaliplatin, interferon, sunitinib, sorafinib, cis- or carboplatinum, doxorubicin, methotrexate, vincristin, vinoreibine, pemetrexed, gefitinib, etoposid, irinotecan, cyclophosphamide, topofecan, cyclophosphamide, paclitaxel, mitomycin, bβvacizumab, trastuzumab, 5-FU, cetuximab, temozoiom ïde, bevacizumab, procarbacine, CCNU, and BCNU

48. A pharmaceutical composition according to claim 47,, wherein said mufti-drug resistance comprises one or more anti-cancer drugs selected from the group consisting of docetaxei, mitoxanthrone, gemcitabine, capecitabine, oxalipSatin, sunitinib, sorafinib, cisplatinum, 5-FU, cetuximab, Bevacizumab, oxaüpiatin and irinotecan.

49. A pharmaceutical composition comprising an immunoiiposome according to any of the preceding claims, wherein said immunoliposome encapsulates doxorubicin and further comprises antibody MAb C225 or antibody EMD72000 or a fragment thereof, which stiil exhibits the specific binding properties of one or both of said antibodies. .

INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
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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)
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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 bass and where practical search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
X	NAMOT CHRISTOPH ET AL: "Epidermal growth factor receptor-targeted Immunol iposomes significantly enhance the efficacy of multiple anticancer drugs in vivo" CANCER RESEARCH, vol. 65, no. 24, December 2005 (2005-12), pages 11631-11638, XP002509421 ISSN: 0008-5472 cited 1n the application	1-5,13, 15-18, 23-27, 38-41,49
Y	abstract page 11631, column 2, paragraph 3 page 11632, column 1, paragraph 2 page 11632, column 2, paragraph 3 page 11634, column 1, last paragraph - page 11636, column 2, paragraph 1 page 11637, column 2, last paragraph -/--	6-12,14, 29-36, 43-48

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

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	MAMOT CHRISTOPH ET AL: "Anti-EGFR Immunoliposomes mediate specific and efficient drug delivery to target cells and can overcome multidrug-resistance." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol . 46, April 2005 (2005-04) , page 334, XP001537040 & 96TH ANNUAL MEETING OF THE AMERICAN-ASSOCIATION-FOR-CANCER-RESEARCH; ANAHEIM, CA, USA; APRIL 16 -20, 2005 ISSN: 0197-016X abstract	6-12, 14, 29-36, 43-48
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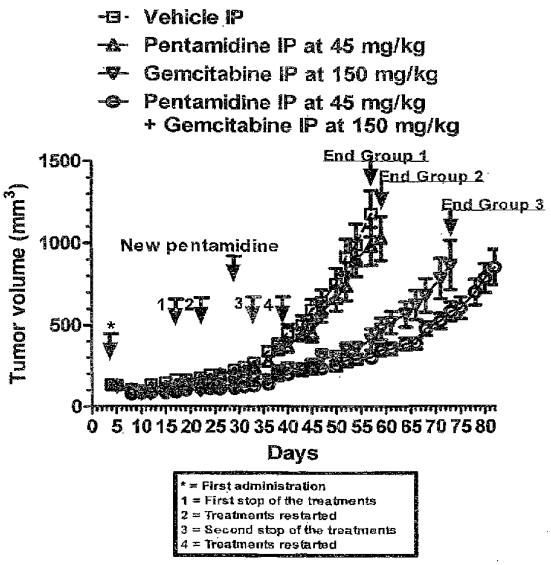
[Continued on next page]

(54) Title: PENTAMIDINE COMBINATIONS FOR TREATING CANCER

(57) Abstract: The present invention relates to the treatment of cancer, e.g., ovarian cancer, breast cancer, pancreatic cancer or colon cancer, with pentamidine and (a) oxaliplatin, (b) gemcitabine, (c) taxol, (d) 5-fluorouracil or (e) CPT 11.

Figure 3

Effect of Pentamidine in combination with Gemcitabine on tumor size (mm^3) over time



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LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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