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(54) **METHOD OF PRODUCING A CATIONIC LIPOSOMAL PREPARATION COMPRISING A LIPOPHILIC COMPOUND**

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ABSTRACT

A method for producing a cationic liposomal preparation comprising a lipophilic active compound with physical and chemical stability during manufacturing, storing and reconstituting, and further a cationic liposomal preparation obtainable by this method as well as specific cationic liposomal preparations as well as pharmaceutical compositions are disclosed.

Fig. 1: Liposomal diameter and PI values for LipoPac™ (Batch GB 100)

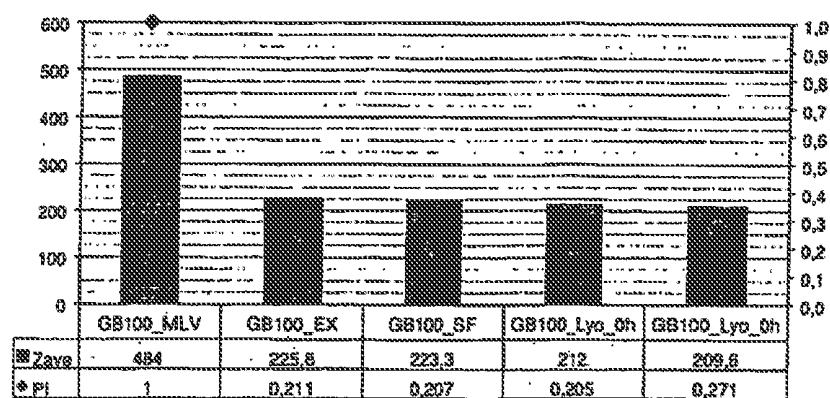


Fig. 2: Liposomal diameter and PI values for LipoPac™ (Batch GB 261)

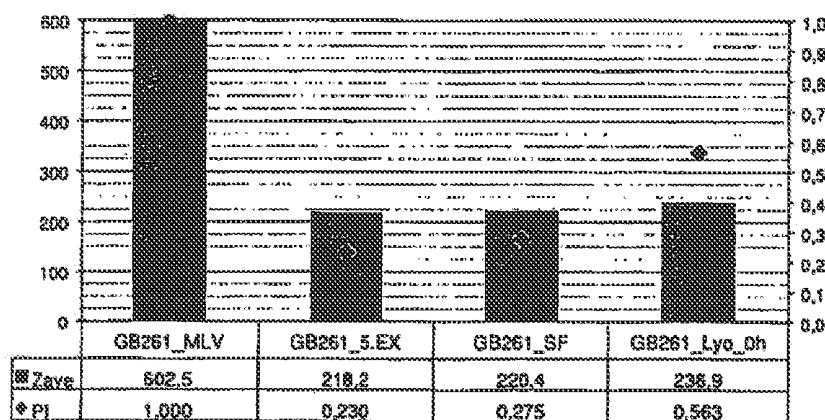


Fig 3: Storage stability as determined by PCS measurements

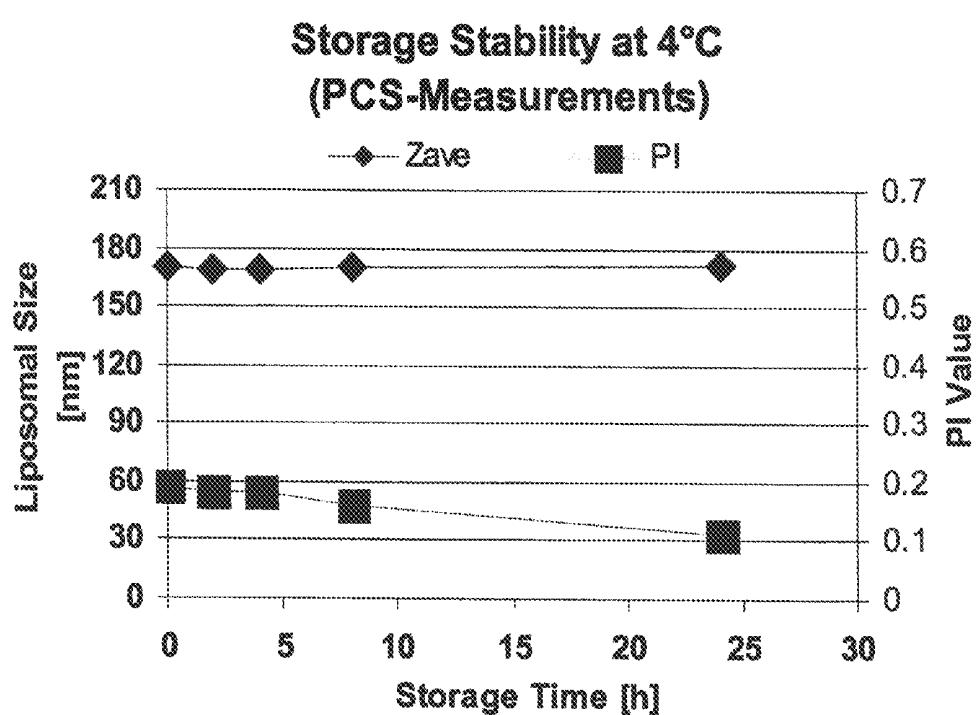


Figure 3A

Fig 3: Storage stability as determined by PCS measurements

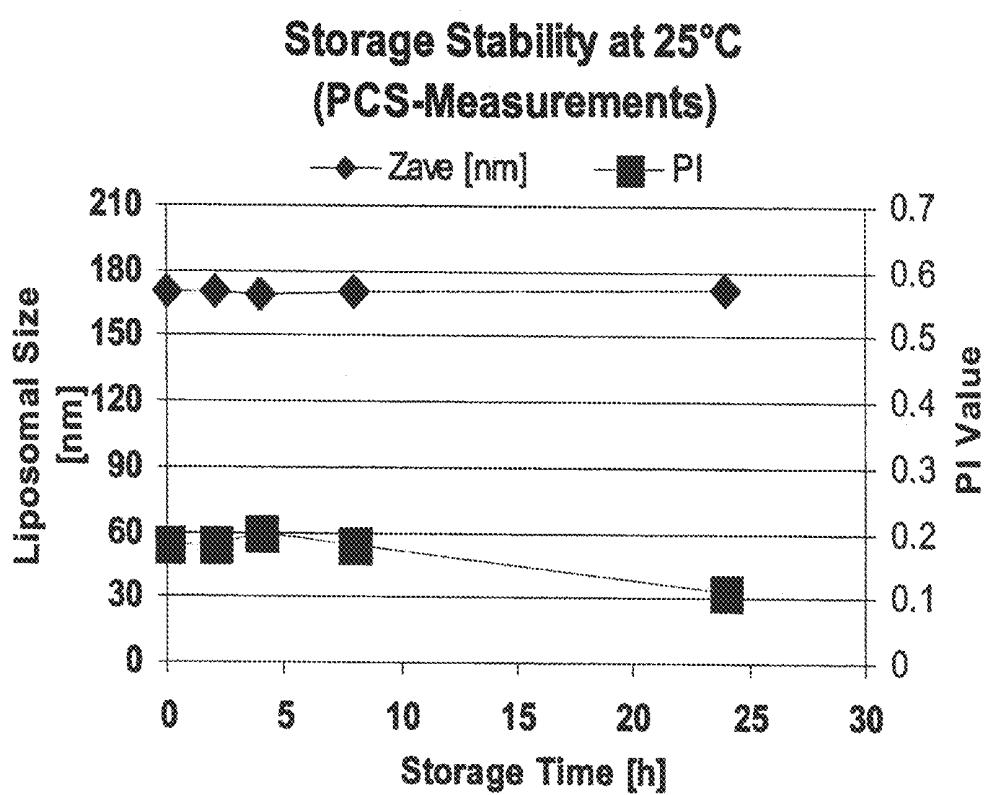


Figure 3B

Fig 3: Storage stability as determined by PCS measurements

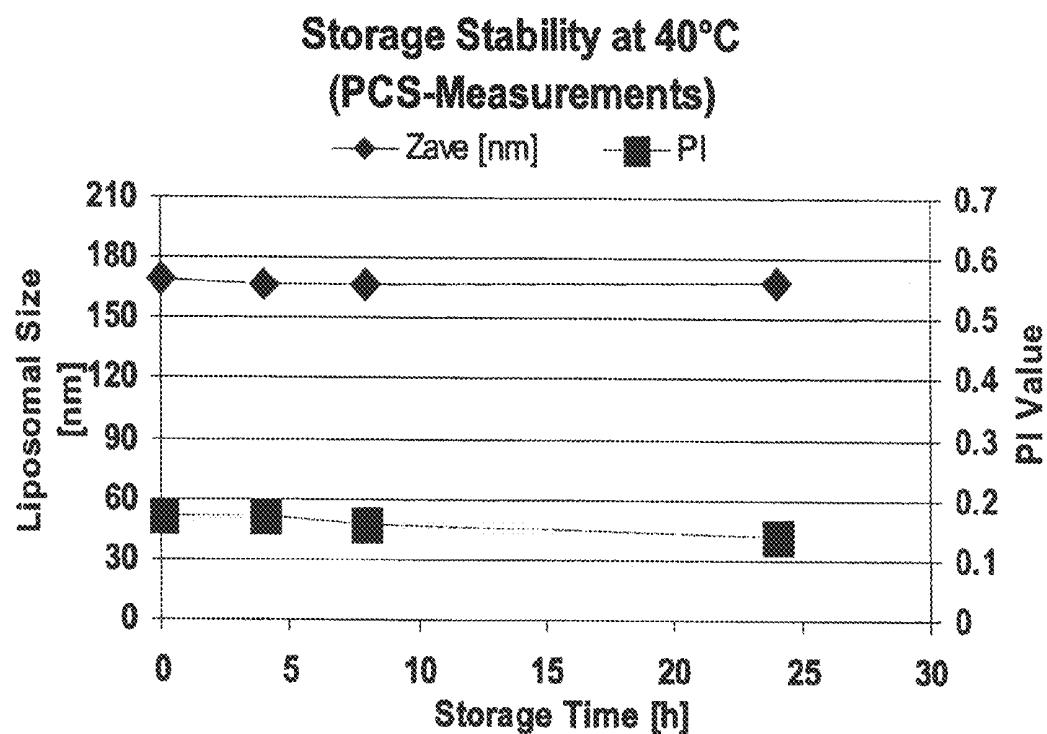


Figure 3C

Fig 4: Particle counts (0-8h)

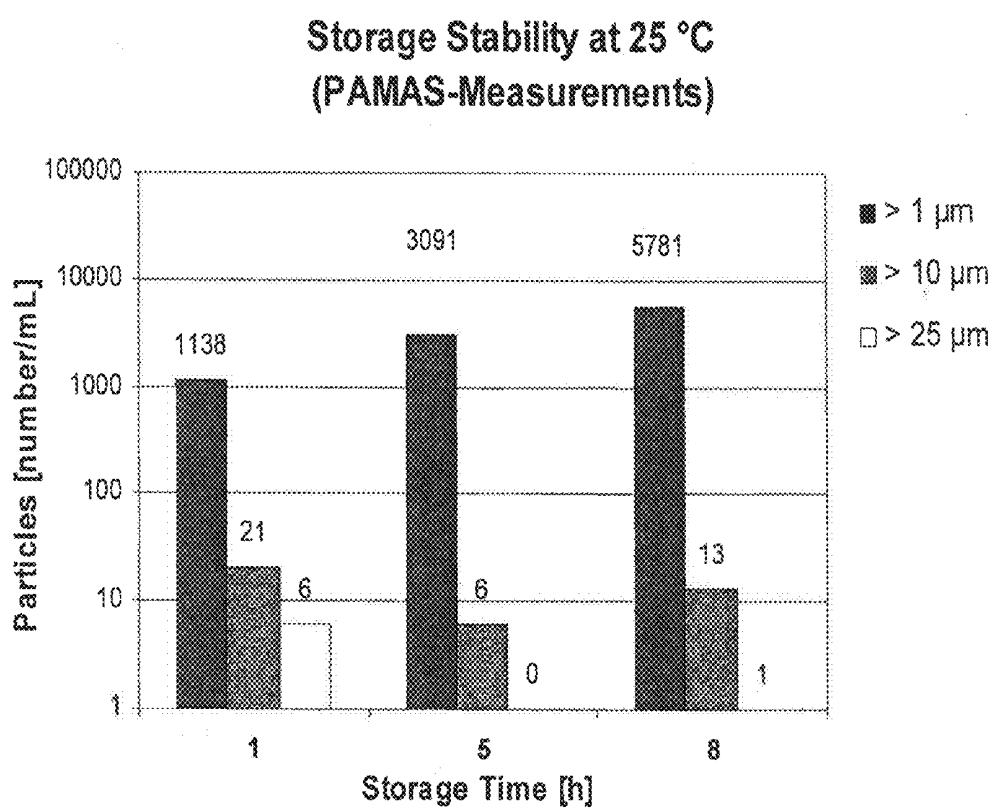


Fig 5: Therapeutic efficacy of LipoDoc™ vs. Taxotere® in A-375 melanoma in nude mice

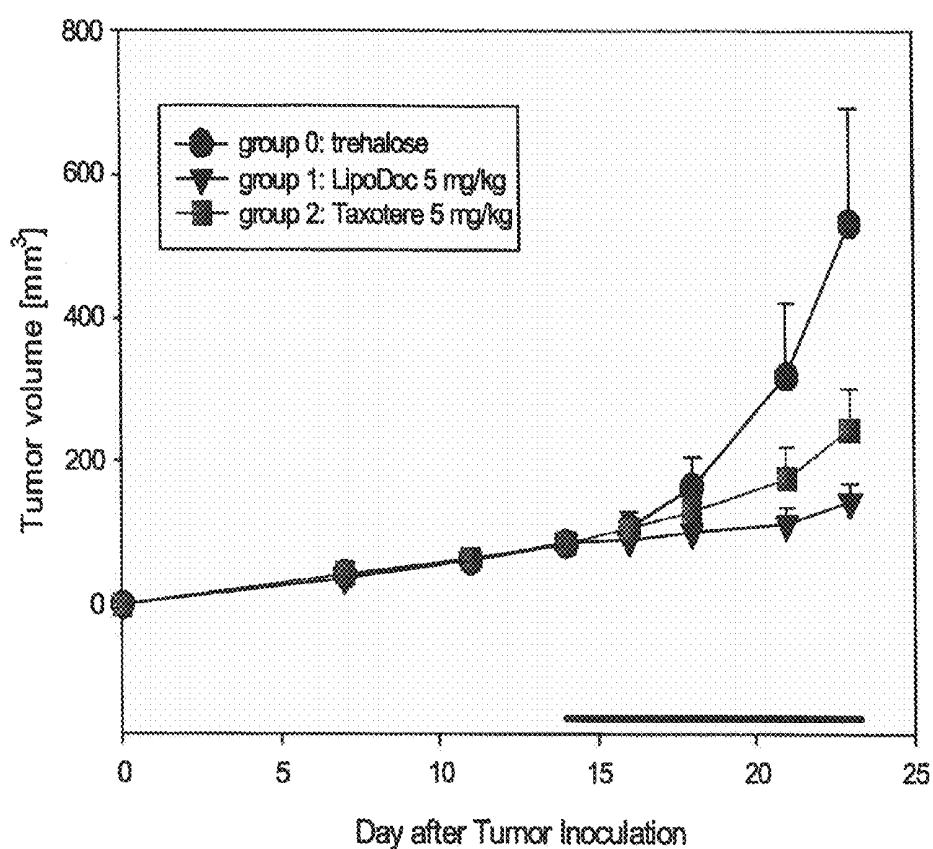


Fig 6: Therapeutic efficacy of LipoPac™ vs. Taxol® in A-375 melanoma in nude mice

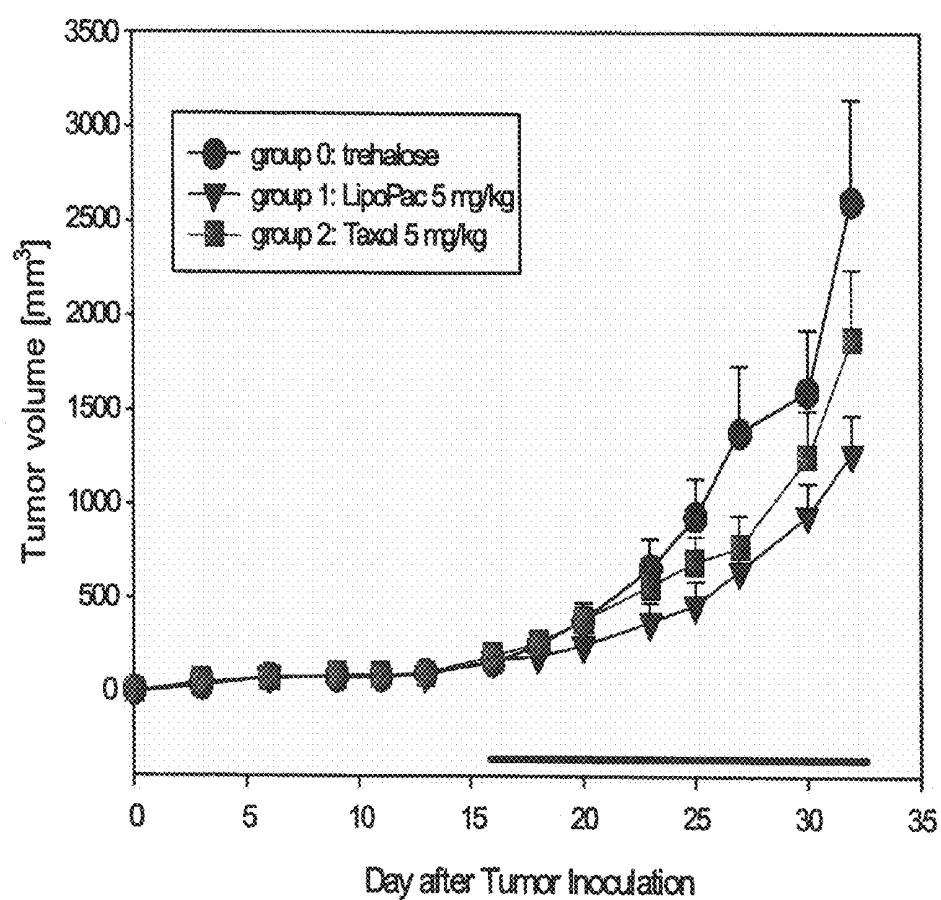


Fig 7: Therapeutic efficacy of LipoPac™ vs. Taxol® in B-16 melanoma in C57/BL6 mice

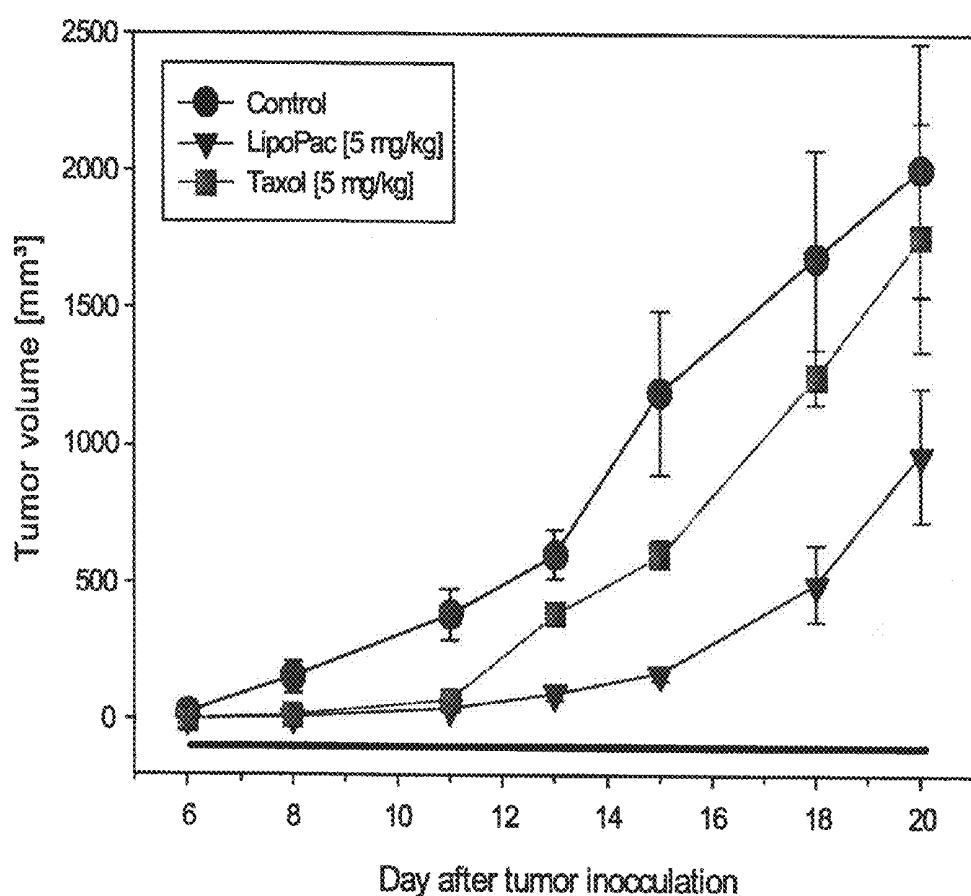
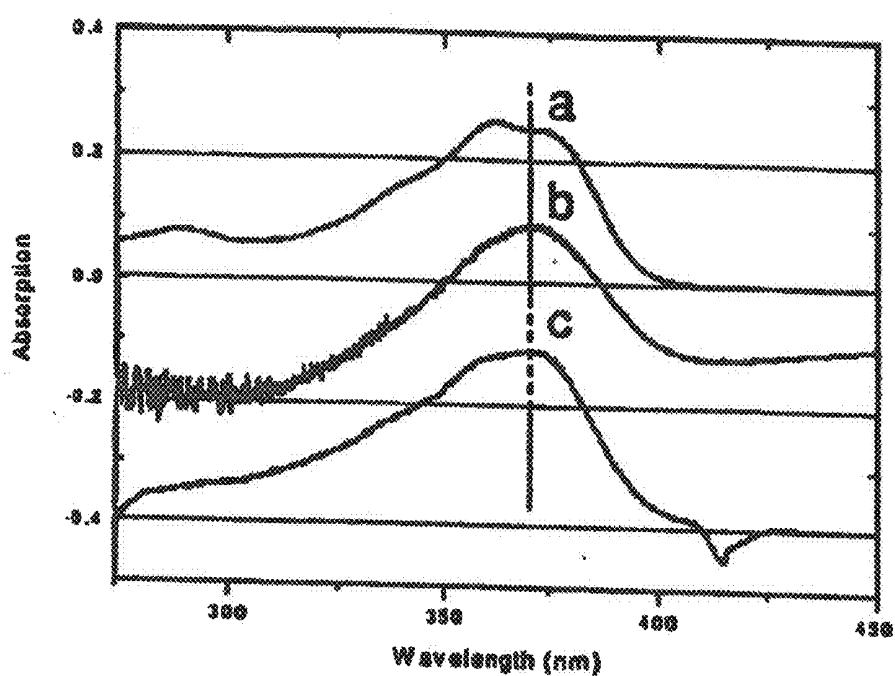


Fig 8: UV-VIS Spectra of Camptothecin



METHOD OF PRODUCING A CATIONIC LIPOSOMAL PREPARATION COMPRISING A LIPOPHILIC COMPOUND

[0001] The present application is a continuation of U.S. Ser. No. 13/278,801, filed Oct. 21, 2011, now U.S. Pat. No. 8,663,606, which is a continuation of U.S. Ser. No. 12/859,000, filed Aug. 18, 2010, now U.S. Pat. No. 8,075,913, which is a divisional of U.S. Ser. No. 11/018,574, filed Dec. 22, 2004, now U.S. Pat. No. 7,794,747, which is a Continuation-in-Part of PCT/EP2003/006759, filed Jun. 26, 2003, which claims the benefit of priority of U.S. Provisional Application No. 60/391/245, filed Jun. 26, 2002; U.S. Provisional Application No. 60/391,246, filed Jun. 26, 2002; European Application No. EP 02018724.1, filed Aug. 21, 2002; and European Application No. EP 03004744.3, filed Mar. 4, 2003, all of which are herein incorporated by reference in their entirety.

DESCRIPTION

[0002] The present invention relates to a method for producing a cationic liposomal preparation containing a lipophilic active compound, e.g. a taxane, having high stability which is suitable for therapeutic applications.

[0003] Liposomes are small, spherical vesicles composed primarily of various types of lipids, phospholipids and other lipophilic components. The lipid components normally form a bilayer, where the polar end of the amphiphile is in contact with the surrounding solution, which is typically an aqueous solution. The non-polar, hydrophobic end of the amphiphile is in contact with another non-polar, hydrophobic end of another amphiphile thereby forming the lipid bilayer. Depending on the type of amphiphiles used, the liposome membrane can be classified according to their outer charge into net neutral, negatively and positively charged membranes.

[0004] Liposomes have been developed for many therapeutic and diagnostic applications. Among others they are used to deliver molecules which are not sufficiently soluble in water. These lipophilic molecules are incorporated into the liposome bilayer or have been chemically linked to the lipid bilayer.

[0005] Paclitaxel, the most prominent representative of the taxane family, is such a highly lipophilic compound. Paclitaxel is known as Taxol® which is the drug formulated in polyethoxylated castor oil (Cremophor® EL) and absolute ethanol. Additionally, paclitaxel has been formulated in liposomes.

[0006] Before Taxol® is applied to humans, the pharmaceutical carrier with the therapeutic compound is diluted in a suitable aqueous solution. The carrier, however, has been observed to cause serious, life-threatening anaphylactic reactions in animals and humans, and is physically incompatible with some intravenous infusion systems. Therefore, several attempts have been made to eliminate the Cremophor® EL by reformulating the drug in a better tolerated vehicle. Liposomes have been clinically characterized during the last decades and are known as a safe and well tolerated drug delivery system. Liposomes may consist of naturally occurring lipids bearing a polar head group which is neutrally or negatively charged. Positively charged diacylglyceride lipids do not occur in nature.

[0007] Sharma et al. [1] manufactured neutral paclitaxel-liposomes according to the so-called film method. The lipids, phosphatidylcholine (PC) and phosphatidylglycerol (PG)

were dissolved together with paclitaxel in chloroform. The chloroform was evaporated at 40° C. and the paclitaxel-lipid film dissolved in tert-butanol. The solution was aliquoted and lyophilized. The powder was hydrated with buffer (NaCl/Tes/EDTA: 140 mM/10 mM/0.1 mM) giving a crude liposome suspension, which was further processed in a bath sonicator at 20° C. Chemical stability of the drug has been shown in these formulations for more than 2 months at 4° C. and room temperature. The pH is specified in the physiological range of pH 7-7.5.

[0008] In U.S. Pat. No. 6,090,955 Rerska et al. described manufacturing of neutral paclitaxel liposomes from egg phosphatidylcholine according to the film method. The crude liposome suspension, pH 7.2-7.4, consisting of multi-layered vesicles (MLV) was homogenized with a high-pressure homogenizer. For longer storage gel formation or lyophilisation is suggested. However, no data were presented on stability, e.g. chemical stability of paclitaxel was not addressed.

[0009] Recently, it has been reported that cationic liposomes represent not only another variety of a liposomal carrier system but also show a specific targeting effect to neoangiogenic areas in blood vessels [2]. Cationic liposomes have been used frequently for gene delivery, but little is known about their formulation characteristics for other compounds as compared to neutral or anionic liposomes.

[0010] Campbell et al. [3] formulated paclitaxel liposomes with varying content of cationic lipid finding increased physical stability of the paclitaxel-containing liposomes. The liposomes were manufactured according to the film-method. The film was hydrated with water, which was heated to a temperature of 5-10° C. above the phase transition temperature of the respective phospholipid which was used. The liposomal suspension was then sonicated in a bath-type sonicator. The resulting liposomal diameter was in the range of 500-800 nm. It was noted that the physical stability of these liposomes was for a maximum of 3 days. The conditions like temperature and pH in which these liposomes were kept were not disclosed. For a pharmaceutical formulation a stability of a few days is, however, not sufficient if applied for clinical purposes.

[0011] Another class of highly lipophilic molecules are the epothilones, specifically epothilone A and B. For both compounds, lack of stability at low pH has been described and is attributed to acid catalyzed ring opening reactions of the epoxide moiety. This lead to reaction products which had lost their exceptional cytotoxic properties. [9] The apparent instability of epothilone A and B does not allow the development of oral formulations of epothilone A or B, since stomach pH is around 1-3 and would rapidly degrade the cytostatic epothilone A or B [10].

[0012] The plasma half life especially of epothilone B has been reported to be extremely low due to its metabolic degradation via esterases. [11, 12] This holds true also for other epothilones; in murine plasma, the approximate in vitro half life of desoxy-epothilone B (epothilone D) was found to be 20 min, in human plasma the half life was around 3 h [13]. This does not allow a continuous high level drug exposure of the tumor and unsatisfactory in vivo antitumor activity of epothilones A and B have been attributed to their poor metabolic stability [12].

[0013] Liposomal compositions of epothilones A or B have been described. [WO 01/10412 A1]. Here, the general instability of these epothilones is referred to and this is given as a rationale for liposomal loading. However, no data is pre-

sented to support that the stability of liposomal epothilones is enhanced over that of nonliposomal epothilones.

[0014] Most of the preparation steps for the manufacturing of liposomes are performed in an aqueous environment (formation of the vesicles, homogenisation and/or removal of undesired components, reconstitution of lyophilized formulations). During these steps the liposomal components as well as active ingredients which are loaded into the liposomal membrane are prone to degradation.

[0015] The physicochemical stability of liposomes containing drugs is a limiting factor for the development of a pharmaceutical product with a shelf life sufficient for storage, distribution and application to humans after manufacturing.

[0016] One approach to increase the physicochemical stability of drug-loaded liposomes is to remove water quantitatively from the liposomal suspension. Methods that have been successfully applied to remove water from liposomes are freeze-drying, spray-drying or evaporation. Typically, a liposomal suspension is manufactured by dispersing the amphiphile compounds in an aqueous environment. Immediately after manufacturing of the aqueous bulk material, the suspension is dehydrated by any suitable method and stored until application in dried state. During the drying process a stabilizing agent may be used to maintain the liposome structure. Water that is usually associated with the polar liposomal surface is replaced by the stabilizing agent during drying to maintain the liposomal physicochemical characteristics. The drug stays loaded or strongly associated in/with the liposomal membrane. Release of compounds is well controlled. In an optimal case, liposomal size and size distribution is not affected by the process and loaded compounds and lipids stay chemically intact. However, dehydrating of a cationic liposomal preparation comprising a lipophilic active compound was not disclosed yet.

[0017] Thus, the underlying problem of the present invention was to provide an improved method for producing a cationic liposomal preparation comprising a lipophilic active compound with improved physicochemical stability and pharmaceutical applicability.

[0018] The solution was to provide a method for producing a cationic liposomal preparation comprising at least one amphiphile selected from cationic lipids in an amount of at least about 30 mol %, optionally at least one further amphiphile in an amount of up to about 69.9 mol %, a lipophilic active compound in an amount of at least about 0.1 mol % and a stabilizing agent in an amount of about 0.1% (m/v) to about 20% (m/v), comprising the steps of

[0019] a) providing

[0020] i. an organic solution comprising an organic solvent, said active compound and said cationic lipid, and optionally said further amphiphile,

[0021] ii. an aqueous solution comprising said stabilizing agent,

[0022] b) preparing a cationic liposomal preparation from said solution a) i. and a) ii., wherein said preparation comprises cationic liposomes in an aqueous medium,

[0023] c) optionally homogenising said preparation at least once and/or

[0024] d) optionally sterile filtrating said preparation,

[0025] e) dehydrating said preparation and

[0026] f) optionally reconstituting said cationic liposomes of step e) in an aqueous solution and

[0027] wherein optionally before step c) and/or d) an ultrafiltration step is included.

[0028] Preferred organic solvents to be used in step a) i. are, although not limited to these examples, selected from the following group: methanol, ethanol, propanol, isopropanol, ethylene glycol, tetrahydrofuran, chloroform, tert.-butanol or diethylether or a mixture of these solvents.

[0029] Any pharmacologically active lipophilic compound may be loaded into cationic liposomes of the present invention. Preferably, the active compound is selected from a therapeutically or diagnostically suitable lipophilic compound such as a cytostatic or cytotoxic agent or an imaging agent such as a dye, fluorescent dye and the like. Preferred therapeutically active compounds are selected from a taxane, from a camptothecin in its lactone form, from other agents interacting with microtubuli such as epothilones, discodermolide, laulimalide, isolaulimalide, eleutherobin, Sarcodictyin A and B, from a statin (e.g., lovastatin), from a depsipeptide, from other drugs such as thalidomide. Preferred diagnostically active compounds are selected from (i) poly-iodinated triglycerides (e.g., 2-oleoylglycerol-1,3-bis[7-(3-amino-2,4,6-triiodophenyl)heptanoate] or poly-iodinated oils such as Lipiodol, (ii) ^{99m}Tc-HMPAO (hexamethyl propyleneamine dioxim) and derivatives thereof, (iii) fluorescent compounds such as rhodamine, (iv) lipid coated ferrite particles, (v) lipid coupled contrast agents for MRI (e.g., Gd chelators such as DOTA or DTPA coupled to a lipid or to a fatty acid), (vi) lipid coupled contrast agents for Xray (e.g. lipid coupled Iopamadol), (vii) lipid coupled chelators such as HYNIC or DTPA for scintigraphically useful nuclides such as ¹¹¹In or ^{99m}Tc, or (viii) lipid coupled fluorescent dyes such as rhodamine or Texas Red.

[0030] In a preferred embodiment the liposomal preparation comprises a taxane, preferably paclitaxel or docetaxel or a lipophilic derivative thereof in an amount of about 1 to about 20 mol %, preferably in an amount of about 2 to about 5 mol % paclitaxel, and preferably in an amount of at least 11 mol % for docetaxel or succinyl-paclitaxel. In a further preferred embodiment said liposomal preparation comprises camptothecin lactone in an amount of about 0.1 mol % to about 1 mol %.

[0031] Useful cationic lipids with respect to the present invention include but are not limited to:

[0032] DDAB, dimethyldioctadecyl ammonium bromide; N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium methylsulfate; 1,2-diacyloxy-3-trimethylammonium propanes, (including but not limited to, dioleoyl (DOTAP), dilauroyloxy, dimyristoyloxy, dipalmitoyloxy, and distearoyloxy); N-[1-(2,3-dioleyloxy)propyl]-N,N-dimethyl amine; 1,2-diacyl-3-dimethylammonium propanes, (including but not limited to, dioleoyl (DODAP), dilauroyl, dimyristoyl, dipalmitoyl, and distearoyl); DOTMA, N-[1-[2,3-bis(oleyl)]propyl]-N,N,N-trimethylammonium chloride, (including but not limited to, dioleoyl (DOTMA), dilauryl, dimyristyl, dipalmityl, and distearyl); DOGS, dioctadecylamidoglycylspermine; DC-cholesterol, 3□-[N—(N',N'-dimethylaminoethane)carbamoyl]cholesterol; DOSPA, 2,3-dioleyloxy-N-(2-sperminecarboxamido)-ethyl)-N,N-dimethyl-1-propanaminium trifluoroacetate; 1,2-diacyl-sn-glycero-3-ethylphosphocholines (including but not limited to dioleoyl (DOEPC), dilauroyl, dimyristoyl, dipalmitoyl, distearoyl, and palmitoyl-oleoyl); □-alanyl cholesterol; CTAB, cetyl trimethyl ammonium bromide; diC14-amidine, N-t-butyl-N'-tetradecyl-3-tetradecylaminopropionamide; 14Dea2;

TMAG, N-(alpha-trimethylammonioacetyl)didodecyl-D-glutamate chloride; O,O'-ditetradecanoyl-N-(trimethylammonioacetyl)diethanolamine chloride; DOSPER, 1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamide; N,N,N',N"-tetramethyl-N,N'-bis(2-hydroxylethyl)-2,3-dioleoyloxy-1,4-butanedi ammonium iodide; 1-[2-(acyloxy)ethyl]2-alkyl (alkenyl)-3-(2-hydroxyethyl)imidazolinium chloride, derivatives as described by Solodin et al. (1995) Biochem. 43:13537-13544, such as DOTIM, 1-[2-(9(Z)-octadecenoyloxy)ethyl]-2-(8(Z)-heptadecenyl-3-(2-hydroxyethyl)imidazolinium chloride; DPTIM, 1-[2-(hexadecanoyloxy)ethyl]-2-pentadecyl-3-(2-hydroxyethyl)imidazolinium chloride; 2,3-dialkyloxypropyl quaternary ammonium compound derivatives, contain a hydroxyalkyl moiety on the quaternary amine, as described e.g., Feigner et al. (1994) J. Biol. Chem. 269:2550-2561, such as: DORI, 1,2-dioleyl-3-dimethyl-hydroxyethyl ammonium bromide; DORIE, 1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DORIE-HP, 1,2-dioleyloxypropyl-3-dimethyl-hydroxypropyl ammonium bromide; DORIE-HB, 1,2-dioleyloxypropyl-3-dimethyl-hydroxybutyl ammonium bromide; DORIE-HPe, 1,2-dioleyloxypropyl-3-dimethyl-hydroxypentyl ammonium bromide; DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxylethyl ammonium bromide; DPRIE, 1,2-dipalmityloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DSRIE, 1,2-disteryloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide.

[0033] In a preferred embodiment the cationic lipid is selected from a quaternary ammonium compound such as N-[1-(2,3-diacyloxy)propyl]-N,N, N-trimethyl ammonium, which may be present as a salt with a pharmaceutically acceptable counter anion e.g. a chloride, bromide, fluoride, iodide, nitrate, sulfate, methyl sulfate, phosphate, acetate, benzoate, citrate, glutamate or lactate. In an even further preferred embodiment the cationic lipid is DOTAP.

[0034] The further amphiphile may be selected from an amphiphile having a neutral or anionic net charge of its hydrophilic moiety (head group). A suitable amphiphile may be selected from sterols or lipids such as phospholipids, lysolipids, lysophospholipids, sphingolipids or pegylated lipids, or any combination thereof. A preferred amphiphile is a neutral lipid, sterol or pegylated lipid such as cholesterol, lanosterol, phytosterol, 1,2-diacyl-sn-glycero-3-phosphoethanolamine, including but not limited to dioleoyl (DOPE), 1,2-diacyl-glycero-3-phosphocholines, sphingomyelin. Most preferred the further amphiphile is diacylphosphatidylcholine. PEGylated lipids refer to lipids bearing one or more polyethylene glycol residues.

[0035] A suitable aqueous solution according to step a) ii) of the present invention comprises water, optionally a buffer substance and a stabilizing agent and has a pH value between about 3 and 7, preferably between about 4 and about 6.5. Suitable buffer substances are selected from e.g. acetic acid, citric acid, Tris, Bis, phosphatic acid, lactic acid and the like.

[0036] The stabilizing agent is preferably selected from a sugar or an alcohol or a combination thereof such as trehalose, maltose, sucrose, glucose, lactose, dextran, mannitol or sorbitol and used in the range of up to about 20% (m/v). Preferably the stabilizing agent is used in the range of about 0.1 (m/v) to about 20% (m/v) and most preferably in the range of about 5 (m/v) to about 15% (m/v) with respect of the total volume of the liposomal dispersion further in step b).

[0037] The preparation of a liposomal dispersion according to step b) can be carried out according to several methods well

known in the art. In a preferred embodiment of the present invention the film method and in a more preferred embodiment the organic solvent injection method is performed.

[0038] According to the film method cationic lipids and optionally amphiphiles and the lipophilic compound are dissolved in an organic solvents or a mixture of different organic solvents that are selected from alcohols (such as ethanol or tert-butanol), halogenated solvents (such as dichloromethane or chloroform) or other suitable organic solvents. After dissolving said compounds in an organic solvent, the organic solvent of the mixture or different organic solvents are evaporated under vacuum to produce a thin film. Instead of producing a thin film from the organic solution containing the cationic lipids, optionally amphiphiles and the lipophilic compound may be dried by lyophilisation or other suitable means so that a homogenous drug-lipid mixture is obtained. An aqueous solution comprising a stabilizing agent is added to rehydrate the lipid film or the dried lipid mixture resulting in a homogeneous dispersion of multilamellar vesicles (MLV).

[0039] The organic solvent injection is performed by dissolving cationic lipids and optionally amphiphiles and the lipophilic compound in a water miscible volatile solvent, such as an alcohol or ether, preferably ethanol, and injecting this solution into an aqueous solution comprising a stabilizing agent. The so-called organic phase comprises cationic lipids and optionally amphiphiles and the lipophilic compound and an organic solvent whereby the organic phase should not exceed about 5% (m/v), preferably at least 2.5% (m/v) in the final liquid mixture.

[0040] The cationic liposomes of the present invention comprise at least an amount of about 30 mol % cationic lipids, preferably about 40 mol %, more preferably about 50 mol %, even more preferred about 60 mol %, about 70 mol %, about 80 mol %, or about up to 99.9 mol % and are characterized by having a positive zeta potential in about 0.05 M KCl solution at about pH 7.5 at room temperature.

[0041] Adjusting the size of liposomes is often performed by sonication in the art. However, in the inventive method homogenising in step c) is preferably performed by extrusion, filtration through membrane filters, high pressure homogenisation and/or high speed homogenization and mostly preferred by extrusion through a membrane with a pore size of about 200 nm under pressure. Membranes with other pore sizes such as 50 nm, 100 nm, 150 nm, 400 nm well known in the art may be used as well. Filtration through membrane filters maybe performed by filtration through membranes composed of PVDF, PES, nylon-filters but also other materials may be used if defined to be suitable. Pore size of membranes shall be in the range of about 200 nm to 450 nm, but pore size is not limited to the sizes mentioned. Different materials and different pore sizes maybe combined in a way to obtain a solution which maybe processed by a sterilizing grade filtration.

[0042] For pharmaceutical use, it is a prerequisite that the liposomal formulation can be sterilised through a sterilizing grade filter after the preparation procedure as they are often intended to be used parenterally in a subject in need thereof. Methods for sterilizing liposomes should be destructive for microorganisms, but should not affect physicochemical characteristics of the liposomal formulation in an unfavorable manner. The preferred way for sterilizing pharmaceutical products is autoclaving, e.g. at 134° C. for a minimum of 5 min or at 121° C. for a minimum of 15 min. Under these harsh

conditions liposomes often show degradation at considerable content, e.g. as agglomeration of liposomes, change of liposomal size or size distribution, hydrolysis/oxidation of lipids, chemical degradation or undesired release of the lipophilic compound from the liposomes. Therefore, sterile filtration and aseptic filling are preferred methods to obtain a pharmaceutical liposomal product for parenteral application. Typically, sterilizing grade filtration is performed once or repeatedly through a membrane with a pore sizes in the range of 0.1 to 0.45 µm. Two to several filters with a defined pore diameter may also be connected in series to achieve a sterilizing grade filtration. Materials commonly used are cellulose derivatives such as cellulose acetate or polyvinyl membranes like PVDF, PES or Nylon but also other materials may be used if defined to be suitable.

[0043] Filtration processes may also be used to remove undesired compounds from the liposomal preparation, such as reagents or solvents used in the manufacturing process, or not liposomally loaded lipophilic compound. The pore size of the filter is preferably between the liposomal diameter (typically >60 nm) and the compound to be removed (typically <5 nm). Depending on the size difference ultra filtration (1-1000 kDa molecular weight cut-off) or micro filtration (0.02-1 µm) may be used. Instead of a depth end filtration more convenient techniques have been developed like dialysis or cross flow filtration.

[0044] A sterilised liposomal preparation can be filled aseptically into appropriate vials, e.g. glass bottles. The filling height of glass bottles is preferred to be in the range of 0.5-10 cm, more preferred in the range of 1.0-5 cm, most preferred in range of 2.0-3.0 cm. Pharmaceutical grade glass bottles maybe in the size of 1 ml to 1000 ml. A liposomal dispersion in an aqueous solution may also be filled into sterile plastic containers or bags.

[0045] After step d), dehydration (step e)) is performed. The formulation is dehydrated and reconstituted prior to use with an aqueous solution such as pure water or a solution of a pH-stabilizing agent. The dehydrating process is an important step in the manufacturing process of cationic liposomes since it may directly influence the quality of the dried liposomal preparation and further of the reconstituted liposomal dispersion. Dehydrating can be performed by freeze drying which can be divided into three different steps, (i) freezing, (ii) primary drying, and (iii) secondary drying which are connected by exactly defined temperature/time/pressure ramps.

[0046] Freezing of a liposomal dispersion is an important step. It is well known that formation of ice crystals is strongly dependent on freezing speed resulting in different pore sizes of the frozen liposomal dispersion. The drying speed in the following drying steps is mainly influenced by the pore size during freezing.

[0047] During primary drying water is removed under vacuum from the frozen dispersion. The temperature of the shelf during freeze-drying as well as the applied vacuum strongly controls the drying process. Choosing an inadequate temperature and pressure may result in several problems during freeze-drying like thawing of the frozen dispersion or phase transition of lipids.

[0048] Also during secondary drying a melting of the product may occur. The time of primary drying as well as temperature and pressure of the secondary drying may strongly affect the quality of the liposomal preparation if the parameters are not in a proper range. The quality of the liposomal

preparation might be affected as the loaded compound may lack sufficient physical or chemical stability or due to aggregation or crystal formation.

[0049] In a preferred embodiment of the present invention dehydrating is performed by freeze-drying. Freezing is preferably performed at atmospheric pressure and the liposomal suspension is frozen to a temperature of about -20 to about -60° C., more preferred to a temperature of about -30° C. to about -50° C. and most preferred to a temperature of about -35° C. to about -45° C. Time is adjusted to ensure complete freezing of the liposomal dispersion and is preferably about 3 to about 10 hours, depending on size, filling height, and type of the glass vessel, wherein the liposomal dispersion is placed.

[0050] Freezing and a primary-drying step are connected by a first temperature ramp. The increment of the temperature is determined by the temperature difference during freezing and primary drying. The time of the temperature ramp is preferably in the range of about 0.1 to about 24 hours, more preferably between about 3 to about 5 hours.

[0051] Primary drying can be performed at a constant temperature or a temperature ramp may be applied. Drying with a constant temperature is preferably performed at a temperature between about 0° C. and about -50° C., more preferably between about -10° C. and about -30° C. An appropriate vacuum is applied to ensure drying of the product. Vacuum shall be at about 1 mbar to about 0.001 mbar, most preferred at about 0.05 mbar to about 0.15 mbar, dependent on the temperature of the shelf. Also the phase diagram of the formulation has to be taken into consideration for choosing an appropriate vacuum for the primary drying step. The time for the primary drying shall be sufficient to ensure sufficient drying of the liposomal preparation and shall be in the range of about 10 hours to about 200 hours, depending on the lyophilisator.

[0052] Primary drying can also be performed using a temperature ramp. The temperature is slowly increased during primary drying. The increase of temperature is preferably in the range of about 0.1 to about 10 K/hour. Temperature can be increased at the beginning or at the end of the primary drying. A pressure rising test can be applied to determine the end of the primary drying.

[0053] Primary drying and secondary drying are connected by a second temperature ramp. The increment of the temperature is determined by the temperature at the end of the primary drying and the temperature at the beginning of the secondary drying. The time of the temperature ramp lies preferably in the range of about 0.5 to about 24 hours, more preferably between about 3 to about 5 hours.

[0054] Secondary drying can be performed at a constant temperature or a temperature ramp. Drying with a constant temperature is performed at a temperature between about 0° C. and about 50° C., preferably between about 10 and about 20° C., more preferred at about 20° C. An appropriate vacuum is applied to ensure drying of the product. Vacuum shall be at about 1 mbar to about 0.001 mbar, preferably at about 0.1 to about 0.001 mbar. The time for secondary drying shall be sufficient to ensure sufficient drying of the liposomal preparation and should be in the range of about 1 hour to about 50 hours. A pressure rising test can be applied to determine the end of the secondary drying.

[0055] Reconstitution behaviour of the dehydrated liposomal preparation such as its reconstitutability, release of the active compound from the liposomal membrane or physico-

chemical properties of the compound e.g. degradation and the like may be dependent on the dehydrating but also on the reconstitution process. An optimal reconstitution behavior is shown when after adding of an aqueous solution a homogeneous liposomal dispersion is formed. A simple reconstitution protocol is favorable, such as adding the aqueous solution followed by gentle shaking. During reconstitution, dried liposomes are resuspended with water while the physicochemical stability of the lipophilic compound in the liposomal membrane is not jeopardized. Reconstitution behaviour may be examined e.g. by visual assessment, microscopy or light blockage measurements.

[0056] The inventive method allows the production of cationic liposomes having a positive zeta potential in about 0.05 M KCl solution at about pH 7.5 at room temperature, preferably having a zeta potential in the range of about 25 mV to 100 mV in about 0.05 M KCl solution at about pH 7.5 at room temperature and more preferably having a zeta potential in the range of about 35 mV to 70 mV in about 0.05 M KCl solution at about pH 7.5 at room temperature.

[0057] Further, PI-values of the inventive cationic liposomal preparation are below about 0.6, preferably below about 0.5, more preferred below about 0.4 and most preferred below about 0.3.

[0058] Cationic liposomes prepared by the inventive method and the cationic liposomes disclosed in the present invention have a diameter in the range of about 20 to about 400 nm, preferably about 100 to about 400 nm and more preferably about 200 to about 300 nm.

[0059] It is a feature of the present invention that the lipophilic active compound does not substantially partition from the liposomal bilayer and does not substantially form aggregates in an inventive liposomal dispersion in a period of at least 0.5 hours, generally at least 1 hour, preferably at least about 2 hours, more preferably at least about 3 hours and most preferably at least about 4 hours in ambient temperature. A cationic liposome in which the lipophilic active compound does not substantially partition from the liposomal bilayer is one in which generally less than about 20%, usually less than about 10%, usually less than about 5%, typically less than about 1% and preferably less than about 0.5% of the total lipophilic active compound amount loaded in the cationic liposome has partitioned from the liposome bilayer.

[0060] Furthermore, the present invention is characterized by a sufficient chemical stability of the lipophilic compound.

BRIEF DESCRIPTION OF DRAWINGS

[0061] FIG. 1 discloses liposomal diameter and PI values for LipoPacTM (Batch GB 100).

[0062] FIG. 2 discloses liposomal diameter and PI values for LipoPacTM (Batch GB 261).

[0063] FIGS. 3A-C disclose storage stability as determined by PCS measurements.

[0064] FIG. 4 discloses particle counts (0-8 h).

[0065] FIG. 5 discloses therapeutic efficacy of LipoPacTM vs. Taxotere[®] in A-375 melanoma in nude mice.

[0066] FIG. 6 discloses therapeutic efficacy of LipoPacTM vs. Taxol[®] in A-375 melanoma in nude mice.

[0067] FIG. 7 discloses therapeutic efficacy of LipoPacTM vs. Taxol[®] in B-16 melanoma in C57/BL6 mice.

[0068] FIG. 8 discloses UV-VIS spectra of camptothecin in CHCl₃/MeOH stock solution (a), in a 10 mM DOTAP/DOPC

liposomal preparation, active compound/lipid ratio 1:1000 (b), and after dissolving a liposomal preparation 1:5 in THF/MeOH/HCl (c).

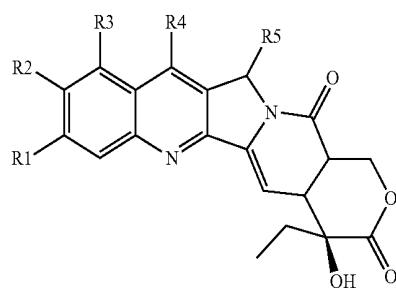
[0069] Unless defined otherwise, all technical and scientific terms used in this specification shall have the same meaning a commonly understood by persons of ordinary skill in the art to which the present invention pertains.

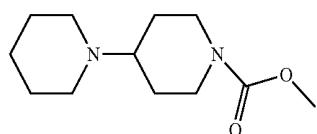
[0070] “About” in the context of amount values refers to an average deviation of maximum+/-20%, preferably +/-10% based on the indicated value. For example, an amount of about 30 mol % cationic lipid refers to 30 mol %+/-6 mol % and preferably 30 mol %+/-3 mol % cationic lipid with respect to the total lipid/amphiphile molarity.

[0071] “Amphiphile” refers to a molecule consisting of a water-soluble (hydrophilic) and an organic solvent-soluble (lipophilic) moiety. A suitable amphiphile of the present invention can be cationic, neutral or anionic with regard to the net charge of the hydrophilic moiety (head group). A cationic amphiphile has a positive net charge, a neutral amphiphile a neutral and an anionic amphiphile an anionic net charge. An amphiphile, such as used in the present invention, is selected from sterols such as cholesterol, phytosterol or lanosterol or lipids such as lysophospholipids, sphingolipids or pegylated lipids such as 1,2-diacyl-sn-glycero-3-phosphoethanolamine, including but not limited to dioleoyl (DOPE), 1,2-diacyl-glycero-3-phosphocholines, sphingomyelin. Pegylated lipids refer to lipids bearing one ore more polyethylene glycol residues.

[0072] “Aqueous solution” refers to any solution comprising water and optionally at least one suitable additive which is completely dissolved in water. Such additives may be buffers or their individual components, sugars, alcohols, stabilizing agents.

[0073] “Camptothecin” refers to any camptothecin or derivatives thereof. A camptothecin derivative is obtained from any chemical derivatization of camptothecin. In the sketch of the molecule, the most frequent derivatization sites are outlined as R₁-R₅. In the table, typical examples for derivatization at the different sites are listed. Any combination of these examples and any other derivatization may be performed. The compound may be present as a hydrochloride. The lactone ring may be seven-membered instead of six-membered.



Name	R1	R2	R3	R4	R5
camptothecin	H	H	H	H	H
9-Nitro-camptothecin	H	H	NO ₂	H	H
9-Amino-camptothecin	H	H	NH ₂	H	H
10-Hydroxy-camptothecin	H	OH	H	H	H
Topotecan	H	OH	N—(CH ₃) ₂	H	H
SN38	H	OH	H	CH ₂ —CH ₃	H
Camptosar ®	H				H CH ₂ —CH ₃ H
Lurtotecan ®		R1 and R2 is:	H	H	
		<div style="border: 1px solid black; padding: 2px;">O—CH₂—CH₂—O</div>			
DX-8951f	H	H	H	H	F

[0074] “Cationic lipid” refers to an amphiphile that has a positive charge (at physiological pH) as measurable by instrumentation utilized at the time of the measurement. Where there are fatty acids or alkyl chains present on the cationic lipid, they could be 12-24 carbons in length, containing up to 6 unsaturations (double bonds), and linked to the backbone by either acyl or ether linkages; there could also only be one fatty acid or alkyl chain linked to the backbone. Where there is more than one fatty acid or alkyl chain linked to the backbone, the fatty acids could be different (asymmetric). Mixed formulations are also possible.

[0075] “Cationic liposomes” can be prepared from the cationic lipids themselves, or in admixture with a further amphiphile such as sterols or lipids like cholesterol, phospholipids, lysolipids, lysophospholipids, sphingolipids or pegylated lipids with a negative or neutral net charge, particularly neutral lipids such as cholesterol; 1,2-diacyl-sn-glycero-3-phosphoethanolamines (including but not limited to dioleoyl (DOPE)); 1,2-diacyl-sn-glycero-3-phosphocholines; natural egg yolk or soy bean phosphatidylcholine (PC), and the like; synthetic mono- and diacyl-phosphoethanolamines. Asymmetric fatty acids, both synthetic and natural, and mixed formulations, for the above diacyl derivatives may also be included.

[0076] “Cationic liposomal preparation or formulation” refers to either a dehydrated liposomal preparation or formulation or a liposomal dispersion.

[0077] “Chemical stability” of the lipophilic compound refers to a significant change of its original chemical structure, and is defined as about 5% potency change from the initial assay value (original compound), preferably about 2% or appearance of specific degradation products exceeding its acceptance criteria with respect to toxicological limits and safety aspects. For lipophilic compounds such as paclitaxel chemical stability can be defined by HPLC/LC-MS/MS and typically means less than 5% degradation products of said compound. Typical degradation products of paclitaxel are e.g. BaccatinIII, 7-Epi-Taxol etc. (Monography of Paclitaxel, USP26, [January-March 2003], USPC, Inc.).

[0078] “Compound loaded into the liposome” or “liposomally loaded compound” or liposomal compound” is used

synonymously and refers to a compound that is either integrated in the lipid bilayer of the liposome or associated with the lipid bilayer of the liposome of the liposomal preparation.

[0079] “Concentration” of x mol % of an amphiphilic or lipophilic compound refers to the mol fraction of this compound of the total lipid concentration. Concentrations of water soluble compounds are given in % (m/m) or % (m/v) of the total preparation.

[0080] “Lipophilic compound” refers to a compound that is characterized by its favorable interaction with the lipophilic part of the liposomal membrane. In liposomal formulations the lipophilic compound is mainly incorporated (embedded) in the membrane or strongly associated with the same. No significant amount is present in the non-liposomal environment, as it would be the case for polar water-soluble compounds.

[0081] “Liposomal dispersion” refers to liposomes within an aqueous solution. The term liposomal suspension may also be used in the same sense as “liposomal dispersion” if not otherwise stated.

[0082] “Liposomes” refer to microscopic spherical membrane-enclosed vesicles (50-2000 nm diameter) made artificially in the laboratory or production plant. The term “liposome” encompasses any compartment enclosed by a lipid bilayer. Liposomes are also referred to as lipid vesicles. In order to form a liposome the lipid molecules comprise elongated non-polar (hydrophobic) portions and polar (hydrophilic) portions. The hydrophobic and hydrophilic portions of the molecule are preferably positioned at two ends of an elongated molecular structure. When such lipids are dispersed in water they spontaneously form bilayer membranes referred to as lamellae. The lamellae are composed of two mono layer sheets of lipid molecules with their non-polar (hydrophobic) surfaces facing each other and their polar (hydrophilic) surfaces facing the aqueous medium. The membranes formed by the lipids enclose a portion of the aqueous phase in a manner similar to that of a cell membrane enclosing the contents of a cell. Thus, the bilayer of a liposome has similarities to a cell membrane without the protein components present in a cell membrane. As used in connection with the present invention, the term liposome includes multilamel-

lar liposomes, which generally have a diameter in the range of 1 to 10 µm and are comprised of anywhere from two to hundreds of concentric lipid bilayers alternating with layers of an aqueous phase, and also includes unilamellar vesicles which are comprised of a single lipid layer and generally have a diameter in the range of about 20 to about 400 nm, preferably about 100 to about 400 nm, more preferably about 200 to about 300 nm. The vesicles can be produced by subjecting multilamellar liposomes to extrusion under pressure through membranes having pores of defined size, or by high pressure homogenization. Further homogenization methods which are suitable are well known in the art.

[0083] “Physical stability” of the lipophilic compound loaded into the liposome refers to the physical state of the compound. The formation of extra-liposomal aggregates (e.g. crystals of the compound) is the most common form of physical instability of a compound. In the case of taxanes, aggregation is visible by the formation of needles of the taxane. Crystallization of a taxane can be measured by visual inspection of liquid liposomal formulation, light microscopy or light blockage measurement or dynamic light scattering. Physical stability of the liposomal dispersion refers also to characteristics such as liposomal size and size distribution or the existence of particles larger than 1 µm. Especially during manufacturing liposomal formulation of a lipophilic compound liposomal characteristics should be maintained.

[0084] “Physicochemical stability” refers to a combination of chemical and physical stability.

[0085] “PI value” refers to the Polydispersity Index which refers to the particle size distribution in a liposomal dispersion as measured by dynamic light scattering techniques, e.g. with a Malvern Zetasizer 1000 or 3000.

[0086] “Stabilizing agent” refers to an agent that stabilizes compound-loaded liposomes during manufacturing to maintain the physicochemical stability of the lipophilic compound and the liposomal formulation. For example for freeze-dried products, cryoprotectants are used as stabilizing agents during manufacturing.

[0087] “Taxane” refers to the class of antineoplastic agents having a mechanism of microtubule action and having a structure that includes the unusual taxane ring structure and a stereospecific side chain that is required for cytostatic activity. Taxane further refers to a variety of known taxane derivatives, including both hydrophilic derivatives, and hydrophobic derivatives. Taxane derivatives include, but not limited to, galactose and mannose derivatives described in International Patent Application No. WO 99/18113; piperazino and other derivatives described in WO 99/14209; taxane derivatives described in WO99/09021, WO 98/22451, and U.S. Pat. No. 5,869,680; 6-thio derivatives described in WO 98/28288; sulfenamide derivatives described in U.S. Pat. No. 5,821,263; and paclitaxel derivatives described in U.S. Pat. No. 5,415,869.

[0088] “Total lipid concentration” refers to the concentration of the sum of amphiphilic compounds and lipophilic compounds.

[0089] “Zeta potential” refers to a surface potential of a particle such as a colloidal particle measured with an instrument such as a Zetasizer 3000 using Laser Doppler microelectrophoresis under the conditions specified. The zeta potential describes the potential at the boundary between bulk solution and the region of hydrodynamic shear or diffuse layer.

[0090] In contrast to the methods disclosed in the art, stability of the loaded active compound during manufacturing steps a) to d) and reconstitution step f) of the present invention is preferably further controlled by any of the following means:

- [0091] controlled (low) pH in the aqueous phase
- [0092] controlled (low) temperature
- [0093] controlled (high) speed of manufacturing and/or application.

[0094] The inventive method allows physical and chemical stabilization of the loaded active compound while the liposome is in an aqueous environment.

[0095] Limitations of processing liposomes on production scale as methods disclosed in the art as mentioned above partially or generally lack the ability of up-scaling in order to fulfill the requirements which are necessary for market production. With the inventive method the large scale production of physicochemically stable cationic liposomes comprising a lipophilic active compound is disclosed for the first time.

[0096] Manufacturing of the cationic liposomes of the present invention, sterile filling and transfer to the freeze-dryer requires 4-18 hours. Afterwards the liposomes are stored e.g. as a freeze-dried powder with a water content of about 0.1 to about 2.5%, preferably about 0.5 to about 1%. Prior to application the freeze-dried powder has to be reconstituted, which means that the liposomes are redispersed in an aqueous solution or water what may lead to physicochemical instability of the liposomal formulation and the loaded lipophilic compound. Therefore, the in-use stability has to cover the time period for reconstitution, transfer to the ward and application to the patient (which is typically several hours) and should cover a minimum of 8 h, ideally 24 h. Thus, the minimum time period for handling the aqueous liposomal preparation is 12 h at refrigerated temperatures (2-8° C.) and additionally 4 h at ambient temperature.

[0097] With the present invention a method is provided in which chemical stability of the cationic liposome comprising an active compound is warranted for the depicted time frame.

[0098] Thus, in a preferred embodiment of the inventive method said liposomal preparation comprising said active compound is physically and chemically stable in any one of the steps b) to d) or f) for at least 12 hours at about 2° C. to about 8° C. and at least about 4 hours at ambient temperature.

[0099] Physical and chemical stability in the context of the present invention relate to the cationic liposome as well as the active compound. Physicochemical stability of the active compound refers to the lipophilic compound which is loaded into the cationic liposome of the liposomal preparation. Loaded means that the compound can be integrated/embedded in the lipid bilayer of the liposome and/or associated inside and/or outside with the liposome.

[0100] Physically stable regarding the loaded compound means that e.g. substantially no aggregation products of the compound are detectable. Physical instability is detectable by means of measurement of non visible particles (e.g. by light blockage measurement), light microscopy and dynamic light scattering (DLS). Chemical stability means that degradation products are below about 5% of the total amount of the compound. Detection of degradation products can be performed e.g. by HPLC.

[0101] Apart from stability considerations the pH of a pharmaceutical dosage form is determined by its mode of application. In general, for an i.v. application (injection, infusion) solutions at physiological pH are preferred. Therefore, non-

buffered aqueous solutions or a physiological buffer in the range of pH 7.0-7.5 are usually used for the manufacturing of paclitaxel liposomes. None of the disclosures dealing with the loading of paclitaxel in cationic liposomes consider the chemical stability of liposomal paclitaxel. Accordingly, the pH is chosen considering a maximum tolerability of the pharmaceutical formulation in the patient, which is at physiological pH.

[0102] The ignorance of the stability issue by the most recent paper published on paclitaxel in cationic liposomes (such as temperature and pH, see [3]) is supported by the fact that the manufacturing process is performed at elevated temperature as has been described above.

[0103] It is known from the scientific literature that paclitaxel in an aqueous buffer is most stable at an acidic pH in the range 3-5 [4]. Nevertheless, data published for paclitaxel formulated with negatively charged or neutral liposomes differ significantly from findings with positively charged liposomes. Sharma and Straubinger [1] reported for neutral and anionic liposomes a chemical stability of more than 3 months at 4° C. and RT. On the other hand it was found by the applicants that decomposition in cationic formulations may occur on a time scale of hours or days. This shows that the compound-loaded liposomal membranes represent a highly complex system, where interactions between the individual components are critical for the physicochemical stability of paclitaxel-loaded liposomes.

[0104] Experiments show that the chemical stability of (phosphoester-)lipids in liposomes is dependent on the pH of the environment (e.g. [5], [7], [8]). Most neutral and anionic liposomes show optimal physicochemical stability at about pH 6.5 and a more or less significant loss of stability with rising or decreasing pH value due to e.g. ester hydrolysis of the lipid structures. Verlooij et al. show, that these results can not be transferred to cationic liposomes composed of DOTAP and DOPE. In these liposomes the DOTAP and the DOPE are most stable at a pH below 6.4 and 6.1, respectively, and hydrolysis rate in this region is almost independent of pH. The authors failed to explain the observed hydrolysis kinetics on basis of their existing model, but suggested that amine-influenced hydrolysis may play an important role in shaping the k-pH profiles.

[0105] However, it is questionable whether one of the above mentioned models applies to a formulation containing another neutral lipid instead of DOPE. The optimum pH for the chemical stability of lipids used for cationic liposome formulations can, therefore, not be concluded from the prior art disclosures.

[0106] In the present invention it was surprisingly found that cationic liposomes comprising paclitaxel are characterized by best chemical stability at acidic pH values. This is in sharp contrast to the data published for neutral and/or anionic liposomes ([1], [6]).

[0107] Thus, in a preferred embodiment the pH value of the aqueous medium in any one of the steps b) to d) and f) of the inventive method is such that said liposomal preparation maintains physical and chemical stability for at least 12 hours at about 2° C. to about 8° C. and at least about 4 hours at ambient temperature, preferably the pH value is between about 3 and about 7 and more preferably between about 4 and about 6.5.

[0108] In another preferred embodiment, the inventive method further comprises cooling to a temperature between about -1° C. and about 15° C., preferably to a temperature

between about 1° C. and about 10° C., most preferably between about 2° C. and about 8° C.

[0109] The invention further provides a method for producing a cationic liposomal preparation comprising a taxane as anti-angiogenic and cytotoxic agent. Such preparation may inhibit angiogenesis and is thus useful in the treatment of a variety of diseases such as cancer, chronic inflammation and the like.

[0110] Thus, another object of the present invention is to provide a method for producing a cationic liposomal preparation comprising at least one amphiphile selected from cationic lipids in an amount of at least about 30 mol %, optionally at least one further amphiphile in an amount of up to about 68 mol %, a taxane in an amount of at least about 2 mol % and a stabilizing agent in an amount of about 0.1% (m/v) to about 20% (m/v),

[0111] comprising the steps of

[0112] a) providing

[0113] i. an organic solution comprising an organic solvent, said taxane and said cationic lipid, and optionally said further amphiphile,

[0114] ii. an aqueous solution comprising said stabilizing agent,

[0115] b) preparing a cationic liposomal preparation from said solution a) i) and a) ii), wherein said preparation comprises cationic liposomes in an aqueous medium,

[0116] c) optionally homogenising said preparation at least once and/or

[0117] d) optionally sterile filtrating said preparation,

[0118] e) dehydrating said preparation and

[0119] f) optionally reconstituting said cationic liposomes of step e) in an aqueous solution and wherein optionally before step c) and/or d) an ultrafiltration step is included.

[0120] In a preferred embodiment of the inventive method said liposomal preparation comprising said taxane is physically and chemically stable in any one of the steps b) to d) or f) for at least 12 hours at about 2 to about 8° C. and at least about 4 hours at ambient temperature.

[0121] Generally, the proportion of a taxane in the cationic liposomal preparation of the present invention is less than about 20 mol %. In some embodiments, the cationic liposomal preparation comprises a taxane in a proportion from about 0.5 mol % to about 20 mol %, preferably from about 2 mol % to about 15 mol %. In other embodiments, a taxane is present in about 1 mol % to about 5 mol %, and in still other embodiments from about 5 mol % to about 15 mol % and more preferably from about 10 mol % to about 13 mol %.

[0122] In a preferred embodiment of the inventive method said liposomal preparation comprises a taxane, preferably paclitaxel or docetaxel or a lipophilic derivative thereof in an amount of about 1 mol % to about 20 mol %, preferably in an amount of about 2 mol % to about 5 mol % for paclitaxel or preferably in an amount of at least 3 mol % for docetaxel or succinyl-paclitaxel and most preferably in an amount of at least 5 mol % for docetaxel or succinyl-paclitaxel.

[0123] It is a feature of the present invention that the taxane does not substantially partition from the liposomal bilayer into the aqueous phase and does not substantially form taxane crystals in a liposomal dispersion in a period of at least 0.5 hours, generally at least 1 hour, preferably at least about 2 hours, more preferably at least about 3 hours and most preferably at least about 4 hours in ambient temperature. A cat-

ionic liposome in which the taxane does not substantially partition from the liposomal bilayer is one in which generally less than about 20%, usually less than about 10%, usually less than about 5%, typically less than about 1% and preferably less than about 0.5% of the total taxane amount loaded in the cationic liposome has partitioned from the liposome bilayer.

[0124] Yet another object of the present invention is to provide a cationic liposomal preparation obtainable by a process of disclosed method.

[0125] Another object of the present invention is to provide a cationic liposomal preparation comprising at least one amphiphile selected from cationic lipids of at least about 30 mol %, optionally at least one further amphiphile of up to about 69.9 mol %, a lipophilic active compound of at least about 0.1 mol % and a stabilizing agent of about 0.1% to about 20% (m/v), characterized in that said liposomal preparation is physically and chemically stable in an aqueous solution for at least 12 hours at 2 to 8° C. and at least 4 hours at ambient temperature.

[0126] In a further preferred embodiment of the preparation of the present invention the lipophilic active compound is selected from a taxane, a camptothecin, a statin, a depsipeptide, thalidomide, other agents interacting with microtubuli such as discodermolide, laulimalide, isolaulimalide, eleutheroxin, Sarcodictyin A and B and in a more preferred embodiment the lipophilic active compound is selected from paclitaxel, docetaxel, camptothecin or any lipophilic derivative thereof.

[0127] In a preferred embodiment of the present invention said liposomal preparation comprises a taxane, preferably paclitaxel or docetaxel or a lipophilic derivative thereof in an amount of about 1 to about 20 mol %, preferably in an amount of about 5 mol % for paclitaxel or preferably in an amount of at least 5 mol % for docetaxel or succinyl-paclitaxel. In a further preferred embodiment said liposomal preparation comprises camptothecin lactone in an amount of about 0.1 mol % to about 1 mol %.

[0128] In a preferred embodiment the inventive preparation comprises a stabilizing agent such as trehalose in the range of about 5% (m/v) to about 15% (m/v) with respect to the total volume of the preparation.

[0129] Yet another object of the present invention is to provide a cationic liposomal preparation comprising at least one amphiphile selected from cationic lipids of at least about 30 mol %, optionally at least one further amphiphile of up to about 65 mol %, paclitaxel of about 5 mol % and a stabilizing agent of about 0.1% (m/v) to about 20% (m/v), characterized in that said liposomal preparation is physically and chemically stable in an aqueous solution for at least 12 hours at 2° C. to 8° C. and at least 4 hours at ambient temperature.

[0130] It is a further object of the present invention to provide a cationic liposomal preparation comprising at least one amphiphile selected from cationic lipids of at least about 30 mol %, optionally at least one further amphiphile of up to about 65 mol %, docetaxel of at least about 5 mol % and a stabilizing agent of about 0.1% (m/v) to about 20% (m/v).

[0131] Another object of the present invention is to provide a cationic liposomal preparation comprising at least one cationic lipid of at least about 30 mol %, optionally at least one further amphiphile of up to about 65 mol %, succinyl-paclitaxel of at least about 5 mol % and a stabilizing agent of about 01% (m/v) to about 20% (m/v).

[0132] It is a feature of the present invention that the cationic liposomes have a positive zeta potential in about 0.05 M

KCl solution at about pH 7.5 at room temperature, preferably a zeta potential in the range of about 25 mV to 100 mV in about 0.05 M KCl solution at about pH 7.5 at room temperature and more preferably a zeta potential in the range of about 35 mV to 70 mV in about 0.05 M KCl solution at about pH 7.5 at room temperature.

[0133] A further feature of the present invention is that any inventive liposomal preparation comprises liposomes with an average particle size of about 50 nm to about 400 nm, preferably about 100 nm to about 300 nm.

[0134] The pharmaceutical composition of the present invention can be in a dry, lyophilized form or in the form of a liquid suspension. The lyophilized form is preferred, because it can be stably stored for periods up to several months or years. Suspensions of the pharmaceutical composition of the present invention in low acidic pH (buffered or acidified) are stable for periods of hours up to months, depending upon the temperature, compound content, and phospholipid constituents.

[0135] Another object of the present invention is to provide a pharmaceutical composition comprising any one of the inventive liposomal preparations together with a pharmaceutically acceptable carrier, diluent and/or adjuvant.

[0136] The pharmaceutical composition of the present invention is active in the field of cancer treatment, wound healing as well as several chronic diseases, and in general in the treatment of diseases associated with enhanced angiogenic activity by administering the composition to patients in an effective amount. The liposomes of the present invention may be administered alone or in combination with suitable pharmaceutical carriers or diluents. Suitable application forms are parenteral routes of administration such as intramuscular, intravenous, intraperitoneal as well as subcutaneous administration. Dosage forms suitable for parenteral administration include solutions, suspensions, dispersions, emulsions and the like well known in the art.

[0137] In light of the foregoing general discussion, the specific examples presented below are illustrative only and are not intended to limit the scope of the invention. Other generic and specific configurations will be apparent to those persons skilled in the art.

EXAMPLES

1. Example 1

Preparation of Paclitaxel Loaded Liposomes (LipoPac™)

[0138] The following example describes manufacturing of paclitaxel loaded liposomes (LipoPac™) which is applicable to a scale of 4 l, 12 l and at least 66 l. All liquid formulations are stoichiometrically composed of

DOTAP-Cl	50 mol %
DOPC	47 mol %
paclitaxel	3 mol %
trehalose-dihydrate	108.2 g/l
ethanol	1.33% (m/m)

[0139] Ethanol is an intermediate product. It is proposed that ethanol is at least partially removed by lyophilisation. Residual amounts of ethanol were determined for Protocol 2 and 3 and found to be below 1%.

[0140] 1.1 Ethanolic Lipid Solution

[0141] An appropriate amount of DOTAP-Cl, DOPC and paclitaxel is dissolved in ethanol to give a final concentration of 400 mM of total lipophilic compounds in ethanol. A clear solution was obtained (ethanolic lipid solution). The ethanolic lipid solution maybe stored overnight at 2-8° C.

[0142] 1.2 Preparation of Trehalose Solution

[0143] An appropriate amount of trehalose-dihydrate is dissolved in water for injection (WFI) and stirred for at least 5 min until a clear solution is obtained. The prepared solution is filtered through a 0.22 µm PVDF (Millipak) flat filter membrane at ambient temperature. Alternatively, the trehalose solution maybe filtered through a 0.22 µm Celluloseacetate membrane (Sartobran® P) at ambient temperature or through a sterilizing grade sterile filtration membrane (0.22 µm) at ambient temperature. Before starting the ethanol injection pH and temperature are adjusted to pH 3-7 and 2-8° C. respectively and maintained at this temperature.

[0144] 1.3 Ethanol Injection

[0145] Ethanol lipid solution is injected into stirred trehalose solution with a speed of at least 0.433 ml/min but can be enhanced accordingly. Trehalose solution is stirred with a speed of at least 280 rpm but can be enhanced accordingly. Injection is performed with a drop funnel or through a capillary using a piston pump. The obtained raw dispersion is stirred for at least 5 min.

[0146] 1.4 Extrusion

[0147] The raw dispersion is extruded fivefold through a 200 nm polycarbonate membrane. The liposomal dispersion is forced five times through the membrane applying a pressure of at least 2 bar. During the extrusion, temperature is maintained at 2-8° C.

[0148] 1.5 Sterile Filtration

[0149] After extrusion the liposomal dispersion is filtered through a sterilizing grade filter (Millipak 200, 0.22 µm). A pressure of at least 2.5 bar is applied at once. Sterile filtration is performed at 2-8° C. A second sterile filtration step maybe performed to ensure complete removal of bacteria.

[0150] 1.6 Freeze-Drying

[0151] Freeze-drying has to be adjusted to the size of the certain preparation scale resulting in similar preparations.

[0152] Protocol 1 for a 4 l Scale, 6 R-Vials with a Filling Volume of 2.1 ml/Vial:

[0153] Freeze-drying is performed using a Christ freeze-dryer (Epsilon 2-12D). Briefly, samples are frozen at -40° C. for 3 hours. Primary drying was performed at -40° C., -30° C. and -16° C. Pressure was set to 0.1 mbar. Secondary drying was performed at +20° C. and a vacuum was applied (0.01 mbar). Vials are closed at approx. 800 mbar of pressure under nitrogen.

[0154] Protocol 2 for a 12 l Scale, 50 H-Vials with a Filling Volume of 14 ml/Vial:

[0155] Freeze-drying is performed using a Christ freeze-dryer. Briefly, samples are frozen at -30° C. for 3 h. After freezing, temperature and pressure are adjusted to -16° C. and 0.1 mbar. After 60 h of primary drying, temperature is increased to +20° C. and pressure is decreased to 0.001 mbar within 3 h. Secondary drying is performed for 12 h at +20° C. and 0.001 mbar. Vials are closed at approx. 800 mbar of pressure under nitrogen.

[0156] Protocol 3 for a 66 l Scale, 100 H-Vials with a Filling Volume of 25 ml/Vial:

[0157] Freeze-drying is performed using a Kniese EK-10 freeze-dryer. Briefly, samples are frozen at -40° C. for at least

3 h. After freezing, temperature of product is increased to -16° C. Pressure is adjusted to 0.1 mbar. After 12 h of primary drying, temperature is increased to 0° C. within 59 h. Secondary drying is performed for 12 h at +20° C. and 0.01 mbar using a ramp of 3 h to adjust pressure and temperature. Vials are closed at approx. 800 mbar of pressure under nitrogen.

[0158] Protocol 4 for a 66 l Scale, 100 H-Vials with a Filling Volume of 25 ml/Vial:

[0159] Freeze-drying is performed using a Kniese EK-10 freeze-dryer. Briefly, samples are frozen at -40° C. for at least 3 h. After freezing, temperature of product is increased to -16° C. Pressure is adjusted to 0.1 mbar. Temperature and pressure are kept constant for a time period of 60 to 100 h. Secondary drying is performed for 12 h at +20° C. and 0.01 mbar using a ramp of 3 h to adjust pressure and temperature. Vials are closed at approx. 800 mbar of pressure under nitrogen.

[0160] LipoPac™ was manufactured according to the procedure described above. All preparations were homogenous in size (Zave and PI) after extrusion and sterile filtration (Zave about 220 nm and PI about 0.2-0.3. After lyophilisation. samples were obtained with a PI-index of 0.27 (batch GB100 FIG. 1) resp. 0.56 (GB261 FIG. 2), depending on the lyophilisation protocol which was used.

[0161] GB100 has been manufactured according to a lyophilisation protocol similar to protocol 4 whereas GB261 has been manufactured according to a lyophilisation protocol similar to protocol 3.

[0162] HPLC analysis of different batches were made with focus on paclitaxel degradation as it is observable by the formation of 7-epitaxol, one major degradation product. Results are shown in Table 1.

TABLE 1

Formation of 7-epitaxol in different batches.		
Batch	Temperature during manufacturing	7-Epitaxol
1	room temperature	1.5%
2		1.2%
3		0.9%
4	2-8° C.	0.4%
5		0.2%
6		0.3%
7		0.7%
8		0.7%
9		0.5%

[0163] Formation of 7-epitaxol is dependent on temperature of manufacturing of bulk material. Batches 1-3 were manufactured at room temperature in 8-12 l scale. 7-Epitaxol was found to be in the range of 0.9-1.5%. Decreasing manufacturing temperature to 2-8° C. resulted in a decrease of 7-epitaxol-content to 0.2-0.7%. The pH of all liposome suspensions during manufacturing was between 4.7 and 6.

2. Example 2

Influence of the pH Value on the in-Use Stability of Liposomal Paclitaxel after Reconstitution

[0164] 2.1 Summary

[0165] Objective of this study was to determine the influence of temperature and pH-value on the in-use stability of liposomal paclitaxel after reconstitution of lyophilized

preparations. Studies were performed with different samples of liposomal paclitaxel, batch Si 175 at two different temperatures and seven different pH values (pH 5.0 to 8.0).

[0166] Freeze-dried samples of liposomal paclitaxel batch Si175 (prepared as disclosed above in Example 1) were reconstituted in 10 mM BISTRIS or TRIS buffer solutions, which were adjusted to pH values in the range of 5.0 to 8.0 before. The aqueous dispersions were stored either at room temperature or in a refrigerator (2-8° C.) for up to 32 h.

[0167] Room Temperature: The degradation of the liposomal paclitaxel strongly depends on the pH value of the aqueous dispersion. paclitaxel is stable at pH values at and below 6.0 for up to 32 h. Only about 1% of the active substance degraded during 32 h at pH 6.0. At higher pH values the degradation increased dramatically from about 8% at pH 6.5 to about 70% at pH 8.0 within 32 h.

[0168] The main degradation product was 7-epi-taxol. Its amount formed during 32 h increased from about 1% at pH 6.0 to about 25% at pH 8.0. Baccatin III and 10-deacetyltaxol linearly increased to about 12% after 32 h at pH 8.0. An acceptable in-use stability of not more than 2% degradation of liposomal paclitaxel at room temperature could only be achieved if the pH value of the aqueous solution is at or below 6.0. Then, the formation of degradation compounds could nearly be neglected. An in-use stability of 12 h could be achieved without problems in this pH range. Above pH values of 6.0, the in-use stability of 12 h has to be reduced and adjusted according to the amounts of degradation products accepted in the dispersion.

[0169] Refrigerator: The degradation of liposomal paclitaxel could be significantly slowed down at lower temperatures. Paclitaxel is stable at pH values at or below 6.5. That means, that the critical pH value could be increased from 6.0 to 6.5 compared to the experiments conducted at room temperature. At higher pH values degradation increased, but to smaller degrees than at room temperature. At pH 8.0 more than twice the amounts of paclitaxel could be recovered after 32 h.

[0170] Degradation products were formed in the same proportions as in the experiments conducted under room temperature but in significantly lower quantities. Again, 7-epi-taxol was the main degradation product of paclitaxel (10% at pH 8.0). Baccatin III and 10-deacetyltaxol were formed for about 5 to 6% at pH 8.0. Found unknown substances were the same.

[0171] The in-use stability of liposomal paclitaxel could be significantly improved at lower temperatures (2-8° C.). In a pH range of 5.0 to 6.5, reconstituted samples can be stored for up to 32 h without the formation of degradation products. The degradation is much slower compared to room temperature even at higher pH values.

[0172] These experiments demonstrated, that storage of liposomal paclitaxel in an acidic medium (pH below 6.5) in the refrigerator reduced the degradation processes of the active substance paclitaxel. The in-use stability of the dispersions could be extended to more than 12 h under these conditions.

[0173] 2.2 Experimental

[0174] 2.2.1 Test System—Formulation

[0175] A liposomal paclitaxel formulation as shown in Table 2 was used in this study:

TABLE 2

Formulation	Batch	Theoretical Composition [mM]			Volume Lyophilisate per Vial [mL]
		DOTAP	DOPC	Paclitaxel	
Liposomal paclitaxel	Si 175	5.0	4.7	0.3	2.1

[0176] 2.2.2 Instruments

[0177] HPLC-System:

[0178] Autoinjector: SIL-10ADVP with sample rack No. 11

[0179] Isocratic pump: LC-10ADVP

[0180] Degasser: DGU-14A

[0181] Column oven: CTO-10ASVP

[0182] DAD Detector: SPD-M10AVP

[0183] Controller: SCL-10AVP

[0184] Software for evaluation: CLASS VP Version 6.10 Shimadzu Deutschland GmbH; 47269 Duisburg, Germany

[0185] pH-Meter:

[0186] InoLab pH Level 2; WTW GmbH and Co. KG; 82362 Weilheim, Germany Refrigerator and freezer commonly available in the laboratory.

[0187] 2.2.3 HPLC Method

[0188] Columns:

[0189] LiChroCART® 250-4, LiChrospher® 60, RP-select B, length 250 mm,

[0190] ID: 4 mm, particle size: 5 µm; Order No.: 1.50839.0001

[0191] Pre-Column: e.g. 8/4 LiChrospher® 100-5 C18; Order No. 1-50957 Merck KgaA, 64293 Darmstadt, Germany

[0192] Injection Volume: 10 µL

[0193] Oven Temperature: 35° C.

[0194] Mobile Phase: acetonitrile/THF/2 mM ammonium acetate (32/12/56,

[0195] v/v/v; v=Vol %)

[0196] Flow Rate: 1.00 mL/min

[0197] Detector Wavelength: 229 nm

[0198] 2.2.4 Preparation of Samples

[0199] The lyophilized samples (preparation described in Example) were reconstituted in 10 mM BISTRIS or TRIS buffer solutions, which were adjusted to pH values in the range of 5.0 to 8.0 with hydrochloric acid. The solutions were carefully shaken until a homogeneous, slightly turbid dispersion was obtained, which was free of visibles particles. The solutions were used 30 min after preparation earliest.

[0200] Preparation of the 10 mM BISTRIS Buffer Solutions:

[0201] About 1.26 g of BISTRIS were weighed into a 1000 mL beaker and diluted with 600 mL of water (Aqua ad inject., exactly measured with a graduated cylinder). Then, five 100 mL aliquots of this solution were adjusted with 1 M hydrochloric acid (HCl) to pH values of 5.0, 5.5, 6.0, 6.5 and 7.0. About 0.5 mL (pH 7.0) to 4.0 mL (pH 5.0) of the acid were

needed for the adjustment of the pH values. The 1 M HCl was prepared by dilution of about 9 g HCl (37%) with 100 mL of water (Aqua ad inject.).

[0202] The buffer capacity of the BISTRIS buffer at pH values below 5.5 is low and can be neglected at a pH value of 5.0. Nevertheless it was chosen, because most buffers could not be used in combination with cationic liposomes.

[0203] Preparation of the TRIS Buffer Solutions:

[0204] About 1.21 g of TRIS were weighed into a 1000 mL volumetric flask and filled to volume with water (Aqua ad inject.). Then, two 100 mL aliquots of this solution were adjusted with 1 M HCl to pH values of 7.5 and 8.0. About 1 mL (pH 8.0) and 1.5 mL (pH 7.5) of the acid were needed.

[0205] 2.2.5 Storage Conditions and Sampling Schedule

[0206] After preparation, samples were stored as described in Table 3. Sampling was done after 0, 1, 3, 6, 8, 24 and 32 h after reconstitution. At each sampling interval, 200 µL aliquots of the dispersion were taken out of the vial and the parameters purity and content of paclitaxel were determined via HPLC analysis. Additionally, the pH value of the dispersion was determined at each sampling date.

TABLE 3

Storage Conditions			
sample No.	pH value	temperature [° C.]	max. storage time [h]
001	5.0	2-8° C.	34
002	5.5		
003	6.0		
004	6.5		
005	7.0		
006	7.5		
007	8.0		
008	5.0	Room temperature	34
009	5.5		
010	6.0		
011	6.5		
012	7.0		
013	7.5		
014	8.0		

[0207] 2.3 Results and Discussion

[0208] 2.3.1 Degradation of Liposomal Paclitaxel at Room Temperature

[0209] The degradation of liposomal paclitaxel strongly depends on the pH value of the aqueous solution after reconstitution. As can be seen in Table 4, paclitaxel is chemically stable at pH values at and below 6.0 for up to 32 h. Only about 1% of the active substance degraded during 32 h at pH 6.0. Observable degradation of paclitaxel starts at pH values above 6.0 (see Table 6 and Table 7). At pH 6.5 about 10% of the active substance degraded during 32 h. By increasing the pH value from 7.0 to 8.0, the amount of degradation products increased dramatically (see Table 8). At pH 8.0 only about 30% of the original amount of paclitaxel could be recovered. Even in the first sample (0 h) at pH 8.0, 10% of paclitaxel already degraded, because first sampling was done half an hour after reconstitution of the vial.

[0210] An acceptable in-use stability of paclitaxel loaded liposomes at room temperature could only be achieved, if the pH value of the aqueous solution is at or below 6.0. Then, the formation of degradation compounds could nearly be neglected. An in-use stability of 12 h could be achieved without problems in this pH range.

[0211] Above pH values of 6.0, the in-use stability of 12 h has to be reduced and adjusted according to the amounts of degradation products accepted in the dispersion.

[0212] The main degradation product observed in this study was 7-epi-taxol. Its amounts formed during 32 hours

increased from about 1% at pH 6.0 to about 25% at pH 8.0. Baccatin III and 10-deacetyltaxol linearly increased to about 12% after 32 h at pH 8.0 (see Table 6, Table 7, and Table 8).

TABLE 4

pH Value	Influence of storage time and pH on degradation at room temperature.			Absolute Difference
	Total Degradation Product [Area %]	Absolute	Absolute	
	After 0 h	After 8 h	After 32 h	
5.0	3.4	3.4	2.8	-0.6
5.5	3.3	3.3	3.4	+0.1
6.0	3.4	3.8	4.5	+0.1
6.5	3.6	5.4	9.4	+5.8
7.0	4.3	12.9	27.1	+22.8
7.5	5.1	16.7	35.6	+30.5
8.0	9.6	40.2	56.9	+46.5

[0213] 2.3.2 Degradation of Liposomal Paclitaxel at 2-8° C.

[0214] The degradation of liposomal paclitaxel could be slowed down at lower temperatures. As can be seen in Table 5, paclitaxel is stable at pH values \leq 6.5. That means, that the critical pH value could be increased from 6.0 to 6.5 compared to room temperature. In the range of pH 5.0 to 6.5 the aqueous system is stable (see also Table 9 and Table 10).

[0215] Decomposition starts at pH values above 6.5. At pH 7.0 about 7% of the active substance degraded during 32 h. At higher pH values, degradation increased, but to a smaller degree compared to the experiments conducted at room temperature (see Table 10 and Table 11). At pH 8.0 more than twice the amounts of paclitaxel (30% at rt vs. 64% at 2-8° C.) could be recovered after 32 h.

[0216] The in-use stability of liposomal paclitaxel could be significantly improved at lower temperatures (2-8° C.). In a pH range of 5.0 to 6.5, reconstituted samples can be stored for up to 32 h without the formation of degradation products. The degradation is much slower compared to room temperature even at higher pH values. Above pH values of 6.5, the in-use stability has to be adjusted according to the amounts of degradation products accepted in the dispersion.

[0217] Degradation products were formed in the same proportions as in the experiments conducted under room temperature but in significantly lower quantities. Again, 7-epi-taxol was the main degradation product of paclitaxel. Its amounts formed during 32 h increased from about 3% at pH 7.0 to about 10% at pH 8.0 (see Table 11). Baccatin III and 10-deacetyltaxol were formed for about 5 to 6% at pH 8.0.

TABLE 5

pH Value	Influence of storage time and pH on degradation at 2-8° C.			Absolute Difference
	Total Degradation Product [Area %]	Absolute	Absolute	
	After 0 h	After 8 h	After 32 h	
5.0	3.3	3.0	2.9	-0.4
5.5	3.2	3.0	2.8	-0.4
6.0	3.3	3.1	3.1	-0.2
6.5	3.5	3.7	3.9	+0.4
7.0	4.4	6.7	10.0	+5.6
7.5	5.3	10.3	12.8	+7.5
8.0	10.2	25.0	31.8	+21.6

[0218] The same unknown substances were found as in the experiments conducted at room temperature, albeit in much smaller quantities.

[0219] 2.3.3 Raw Data

TABLE 6

Degradation of liposomal paclitaxel and formation of degradation products at pH 5.0, 5.5 and 6.0 at room temperature (data presented in area %)									
	Time [h]	Baccatin	10-Deacetyl-taxol	Paclitaxel	7-Epi-Taxol	unknown 1	unknown 2	unknown 3	Total Degradation Products
Room Temperature pH 5.0	0	0.34	0.59	96.6	2.5	0*	0	0	3.4
	1	0.33	0.56	96.9	2.3	0*	0	0	3.2
	3	0*	0.47	96.9	2.7	0*	0	0	3.2
	6	0.39	0.52	96.7	2.4	0*	0	0	3.3
	8	0.38	0.52	96.7	2.5	0*	0	0	3.4
	24	0*	0.55	95.3	2.4	1.7	0	0	2.9
	32	0.37	0*	97.2	2.4	0*	0	0	2.8
	Room Temperature pH 5.5	0	0.35	0.59	96.7	2.3	0*	0	3.3
	1	0.37	0.59	96.7	2.4	0*	0	0	3.3
	3	0*	0.51	96.9	2.6	0*	0	0	3.1
Room Temperature pH 6.0	6	0.41	0.61	96.6	2.4	0*	0	0	3.4
	8	0.37	0.54	96.7	2.4	0*	0	0	3.3
	24	0*	0.57	94.3	2.6	2.5	0	0	3.2
	32	0.44	0*	96.6	2.9	0*	0	0	3.4
	0	0.29	0.62	96.6	2.5	0*	0	0	3.4
	1	0.38	0.67	96.4	2.5	0*	0	0	3.6
	3	0*	0.52	96.8	2.7	0*	0	0	3.2
	6	0.43	0.69	96.4	2.5	0*	0	0	3.7
	8	0.43	0.65	96.2	2.7	0*	0	0	3.8
	24	0*	0.80	94.0	3.3	1.9	0	0	4.1
	32	0.64	0*	95.5	3.9	0*	0	0	4.5

*the corresponding peaks could not be evaluated due to interferences with the BISTRIS buffer; the area % of the total degradation products were calculated without the compound Unknown 1, as it is most probably not a metabolite of paclitaxel.

TABLE 7

Degradation of liposomal paclitaxel and formation of degradation products at pH 6.5 and 7.0 at room temperature (data presented in area %)									
	Time [h]	Baccatin	10-Deacetyl-taxol	Paclitaxel	7-Epi-Taxol	unknown 1	unknown 2	unknown 3	Total Degradation Products
Room Temperature pH 6.5	0	0.37	0.64	96.4	2.5	0*	0	0	3.6
	1	0.36	0.72	96.1	2.8	0*	0	0	3.9
	3	0*	0.65	96.0	3.4	0*	0	0	4.0
	6	0.59	0.87	95.1	3.5	0*	0	0	4.9
	8	0.64	1.0	94.6	3.8	0*	0	0	5.4
	24	0*	1.6	90.1	6.2	2.1	0	0	7.8
	32	0*	1.8	88.1	7.6	2.5	0	0	9.4
	Room Temperature pH 7.0	0	0.48	0.78	95.7	3.0	0*	0	4.3
	1	0.66	1.0	94.6	3.7	0*	0	0	5.4
	3	0*	1.3	93.1	5.6	0*	0	0	6.9
	6	1.3	2.0	89.1	7.6	0*	0	0	10.9
	8	1.5	2.4	87.1	9.0	0*	0	0	12.9
	24	4.0	4.5	69.7	16.9	4.0	1.0	0	26.4
	32	0*	4.8	67.1	20.8	4.8	1.5	0	27.1

*the corresponding peaks could not be evaluated due to interferences with the BISTRIS buffer; the area % of the total degradation products were calculated without the compound Unknown 1, as it is most probably not a metabolite of paclitaxel.

TABLE 8

Degradation of liposomal paclitaxel and formation of degradation products at pH 7.5 and 8.0 at room temperature (data presented in area %)									
	Time [h]	Baccatin	10-Deacetyl-taxol	Paclitaxel	7-Epi-Taxol	unknown 1	unknown 2	unknown 3	Total Degradation Products
Room Temperature pH 7.5	0	0.61	0.93	94.1	3.5	0.80	0	0	5.1
	1	0.81	1.3	92.1	5.1	0.66	0	0	7.2
	3	0.93	1.8	88.8	7.2	1.4	0	0	9.9
	6	1.9	2.5	84.5	9.7	1.5	0	0	14.1
	8	2.2	3.0	81.2	11.2	2.0	0.46	0	16.7
	24	4.4	5.1	65.7	19.6	3.8	1.4	0	30.4
	32	5.2	6.7	59.6	22.1	4.8	1.7	0	35.6
	Room Temperature pH 8.0	0	0.61	0.93	94.1	3.5	0.80	0	5.1
	1	0.81	1.3	92.1	5.1	0.66	0	0	7.2
	3	0.93	1.8	88.8	7.2	1.4	0	0	9.9

TABLE 8-continued

Degradation of liposomal paclitaxel and formation of degradation products at pH 7.5 and 8.0 at room temperature (data presented in area %)									
	Time [h]	Baccatin	10-Deacetyl-taxol	Paclitaxel	7-Epi-Taxol	unknown 1	unknown 2	unknown 3	Total Degradation Products
Room Temperature pH 8.0	0	1.2	1.8	89.4	6.6	1.1	0	0	9.6
	1	2.0	2.9	83.3	10.7	1.1	0	0	15.6
	3	2.6	4.4	73.0	16.7	2.2	1.2	0	24.9
	6	5.4	6.6	60.8	21.9	3.6	1.8	0	35.6
	8	6.3	7.0	55.1	24.5	4.8	2.4	0	40.2
	24	10.5	10.8	32.5	29.4	12.1	4.3	0.43	55.3
	32	11.1	12.8	27.9	28.4	15.2	4.7	0	56.9

*: the corresponding peaks could not be evaluated due to interferences with the BISTRIS buffer; the area % of the total degradation products were calculated without the compound Unknown 1, as it is most probably not a metabolite of paclitaxel; the compound Unknown 3 is most probably an artefact (e.g. impurity from sample preparation).

TABLE 9

Degradation of liposomal paclitaxel and formation of degradation products at pH 5.0, 5.5 and 6.0 at 2-8° C. (data presented in area %)									
	Time [h]	Baccatin	10-Deacetyl-taxol	Paclitaxel	7-Epi-Taxol	unknown 1	unknown 2	unknown 3	Total Degradation Products
Refrigerator (2-8° C.) pH 5.0	0	0.34	0.59	96.7	2.3	0*	0	0	3.3
	1	0.35	0.43	96.7	2.5	0*	0	0	3.3
	3	0*	0.41	96.8	2.8	0*	0	0	3.2
	6	0.38	0.50	96.7	2.4	0*	0	0	3.3
	8	0*	0.56	97.0	2.4	0*	0	0	3.0
	24	0*	0.53	95.8	2.4	1.3	0	0	2.9
	32	0*	0.53	95.0	2.3	2.1	0	0	2.9
Refrigerator (2-8° C.) pH 5.5	0	0.31	0.59	96.8	2.3	0*	0	0	3.2
	1	0.33	0.43	96.6	2.6	0*	0	0	3.4
	3	0.38	0.51	96.8	2.3	0*	0	0	3.2
	6	0.38	0.55	96.7	2.3	0*	0	0	3.3
	8	0*	0.54	97.0	2.5	0*	0	0	3.0
	24	0*	0.58	95.7	2.3	1.5	0	0	2.9
	32	0*	0.57	95.1	2.2	2.2	0	0	2.8
Refrigerator (2-8° C.) pH 6.0	0	0.34	0.62	96.7	2.3	0*	0	0	3.3
	1	0.32	0.45	96.6	2.6	0*	0	0	3.4
	3	0.40	0.61	96.7	2.3	0*	0	0	3.3
	6	0.40	0.54	96.7	2.4	0*	0	0	3.3
	8	0*	0.56	96.9	2.5	0*	0	0	3.1
	24	0*	0.59	95.8	2.4	1.2	0	0	3.0
	32	0*	0.58	95.1	2.5	1.8	0	0	3.1

*the corresponding peaks could not be evaluated due to interferences with the BISTRIS buffer; the area % of the total degradation products were calculated without the compound Unknown 1, as it is most probably not a metabolite of paclitaxel

TABLE 10

Degradation of liposomal paclitaxel and formation of degradation products at pH 6.5 and 7.0 at 2-8° C. (data presented in area %)									
	Time [h]	Baccatin	10-Deacetyl-taxol	Paclitaxel	7-Epi-Taxol	unknown 1	unknown 2	unknown 3	Total Degradation Products
Refrigerator (2-8° C.) pH 6.5	0	0.40	0.63	96.5	2.4	0*	0	0	3.5
	1	0.30	0.51	96.3	2.9	0*	0	0	3.7
	3	0.43	0.67	96.3	2.6	0*	0	0	3.7
	6	0.45	0.60	96.4	2.6	0*	0	0	3.6
	8	0*	0.74	96.3	3.0	0*	0	0	3.7
	24	0*	0.87	94.8	2.9	1.4	0	0	3.8
	32	0*	0.86	94.0	3.0	2.2	0	0	3.9
Refrigerator (2-8° C.) pH 7.0	0	0.52	0.82	95.6	3.1	0*	0	0	4.4
	1	0.29	0.81	95.5	3.4	0*	0	0	4.5
	3	0.56	0.89	95.2	3.4	0*	0	0	4.8
	6	0.63	1.0	94.9	3.5	0*	0	0	5.2
	8	0*	1.5	92.3	5.2	1.0	0	0	6.7
	24	0*	1.9	90.3	5.7	2.1	0	0	7.6
	32	2.3	1.9	87.8	5.7	2.2	0	0	10.0

*the corresponding peaks could not be evaluated due to interferences with the BISTRIS buffer; the area % of the total degradation products were calculated without the compound Unknown 1, as it is most probably not a metabolite of paclitaxel

TABLE 11

Degradation of liposomal paclitaxel and formation of degradation products at pH 7.5 and 8.0 at 2-8°C. (data presented in area %)									
	Time [h]	Baccatin	10-Deacetyl-taxol	Paclitaxel	7-Epi-Taxol	unknown 1	unknown 2	unknown 3	Total Degradation Products
Refrigerator (2-8°C.) pH 7.5	0	0.63	0.93	93.9	3.8	0.75	0	0	5.3
	1	0.62	1.1	93.0	4.3	1.0	0	0	5.9
	3	0.94	1.3	91.9	4.6	1.2	0	0	6.9
	6	1.1	1.5	91.2	4.7	1.5	0	0	7.3
	8	1.4	2.1	88.0	6.8	1.7	0	0	10.3
	24	1.8	2.8	85.8	7.7	1.9	0	0	12.4
	32	2.0	3.0	85.1	7.8	2.1	0	0	12.8
Refrigerator (2-8°C.) pH 8.0	0	1.3	1.9	89.0	7.1	0.80	0	0	10.2
	1	1.3	2.3	87.2	8.3	0.98	0	0	11.8
	3	2.1	2.9	84.5	9.1	1.4	0	0	14.1
	6	2.5	3.5	82.3	9.7	1.7	0.35	0	16.0
	8	3.8	4.8	72.6	15.2	2.4	1.1	0	25.0
	24	5.1	6.7	66.3	17.2	3.3	1.4	0	30.4
	32	5.5	7.6	63.9	17.1	4.3	1.6	0	31.8

*: the corresponding peaks could not be evaluated due to interferences with the BISTRIS buffer; the area % of the total degradation products were calculated without the compound Unknown 1, as it is most probably not a metabolite of paclitaxel.

3. Example 3

Increase of in-Use Stability of Paclitaxel Loaded into Cationic Liposomes

[0220] In order to investigate the stability of paclitaxel loaded into cationic liposomes under different pH conditions, several additives, preferably additives constituting an acidic pH, are added during preparation. Thereby epimerization at C-7 and the formation of 7-epi-taxol is examined. These compounds may be taken from the group of inorganic and organic acids. Examples for inorganic acids include hydrochloric acid (HCl), phosphoric acid, sulfuric acid, carbonic acid, or other commonly used acids. Examples for organic acids are of the general formula for monobasic acids R—CO₂H with R=CH₃—(CH₂)_n; C₆H₅—(CH₂)_n— and n=0-6 for example acetic acid or benzoic acid. In addition, dibasic acids of the general formula HO₂C—(CH₂)_n—CO₂H with n=0-6 such as succinic acid, adipic acid or unsaturated derivatives, such as maleic acid or fumaric acid, or aromatic acids such as phthaleic acid may be employed. Hydroxy carboxylic acids such as citric acid, lactic acid, tartaric acid are also preferred additives.

[0221] Preparation:

[0222] A liposomal preparation comprising 10 mM DOTAP/DOPC/paclitaxel 50/47/3 is prepared via the ethanol injection method as described earlier. The aqueous solution comprises 10% trehalose (w/v), pH=5.5. The trehalose solution may be adjusted to pH 4.5 through addition of hydrochloric acid, citric acid, or lactic acid. Following the ethanol injection of a solution of both lipid and active compound, the resulting solution is extruded at 4°C. and lyophilized as described earlier. The lyophilisate is analyzed by PCS for its liposomal size distribution and by HPLC for its paclitaxel and 7-epi-taxol content. The in-use stability of the lyophilisates is established as follows: The lyophilisate (prepared as described earlier) is reconstituted with MilliQ quality water and left for 24 hours at room temperature or 4°C. before examination. Both PCS and HPLC analysis is performed.

[0223] Another liposomal preparation has been prepared at room temperature as described earlier with citric acid and lactic acid as additives for the aqueous trehalose solution. Without lyophilizing, these formulations were characterized

by their liposomal size and size distribution (PCS) and by drug concentration and 7-epi-taxol content (area %, HPLC).

[0224] Result:

[0225] When liposomes are prepared at 4°C., lyophilized, and kept in the refrigerator 7-epi-taxol formation is not observed after reconstitution as shown by the data in Table 12. The in-use stability (24 h, rt) of the reconstituted lyophilisates, however, depends on the presence and volatility of the employed additives, as shown in Table 13. Using no additive, 6% 7-epi-taxol is found. This is slightly reduced through the use of volatile hydrochloric acid. After 24 h at room temperature, little or no 7-epi-taxol is found employing solid non-volatile organic acids, such as citric acid or lactic acid (Table 13). Liposomal paclitaxel formulations can be prepared at 25°C. as shown in Table 14 where no degradation of paclitaxel was observed even after storage of 24 h at 25°C. HPLC analysis after 120 h of further storage at 25°C. did not show any 7-epi-taxol (data not shown).

TABLE 12

Paclitaxel-loaded cationic liposomes prepared at 4°C.					
Preparation	pH	Z _{average} [nm]	PI Value	Paclitaxel [%]	7-epi-taxol [%]
10% trehalose	5.5	166	0.20	100	0
10% trehalose/HCl	4.5	164	0.17	100	0
10% trehalose/citric acid	4.5	171	0.182	100	0
10% trehalose/lactic acid	4.5	170	0.17	100	0

TABLE 13

In-Use stability (24 h) of paclitaxel-loaded cationic liposomes prepared at 4°C.					
Preparation	pH	Z _{average} [nm]	PI Value	Paclitaxel [%]	7-epi-taxol [%]
10% trehalose	5.5	152	0.17	93.8	6.2
10% trehalose/HCl	4.5	154	0.17	95.7	4.3
10% trehalose/citric acid	4.5	159	0.16	98.7	1.3

TABLE 13-continued

In-Use stability (24 h) of paclitaxel-loaded cationic liposomes prepared at 4° C.					
Preparation	pH	Z _{average} [nm]	PI Value	Paclitaxel [%]	7-epi-taxol [%]
10% trehalose/lactic acid	4.5	148	0.21	100	0

TABLE 14

In-Use stability (24 h, no lyophilization) of paclitaxel-loaded cationic liposomes prepared at 25° C.					
Preparation	pH	Z _{average} [nm]	PI Value	Paclitaxel [%]	7-epi-taxol [%]
10% trehalose/citric acid	4.5	163.2	0.142	100	0
10% trehalose/lactic acid	4.5	158.4	0.188	100	0

4. Example 4

Preparation of Docetaxel Loaded Cationic Liposomes

[0226] 4.1 Liposome Preparation Via Lipid Film Method
[0227] Liposomal formulations comprising docetaxel were prepared using the lipid film method as follows: Lipids of choice and docetaxel are dissolved in chloroform in a round bottom flask. The flask is then rotated under vacuum (100 to 200 mbar, 40° C.) until a thin lipid film is formed. The lipid film is thoroughly dried at 40° C. under full vacuum (3 to 5 mbar) for approximately 30 minutes. The dry lipid film is cooled in an ice bath and is rehydrated with a cold (4° C.) glucose or trehalose solution (pH 5-7) resulting in a suspension of multilamellar lipid vesicles at a total concentration of about 10 to 20 mM. Once a homogeneous dispersion is formed (after 15-20 min rotating) the liposomal dispersion is extruded (filtration under pressure) preferably at a temperature between 4° C. and 8° C. 1-5 times through polycarbonate membranes of appropriate size, typically between 150 and 250 nm, optionally followed by sterile filtration. The low temperature during manufacturing was found to be critical due to increased chemical stability of docetaxel and lipids and due to the finding that a higher active compound to lipid ratio (higher docetaxel content) can be reached. The formed liposomal dispersion is fully characterized by HPLC, PCS and microscopic analysis.

[0228] 4.2 Liposome Preparation Via Ethanol Injection
[0229] Liposomal formulations comprising docetaxel were also prepared using the ethanol injection method as follows: docetaxel and lipids were dissolved in ethanol (or another suitable organic solvent) usually at total lipid concentration of about 200-400 mM. An aqueous solution of a cryoprotectant, preferably 10% trehalose, was prepared at pH 5-7 and cooled to a temperature between 4 and 8° C. prior injection of the organic solvent. The ethanolic solution was injected (3-300 ml/min injection speed) into the cold, vigorously stirred trehalose solution reaching a final total lipid concentration of 10 mM. Once a homogeneous dispersion is formed the liposomal dispersion is extruded (filtration under pressure) preferably at a temperature between 4° C. and 8° C. 1-5 times

through polycarbonate membranes of appropriate size, typically between 150 and 250 nm, optionally followed by sterile filtration. The low temperature during manufacturing was found to be critical due to increased chemical stability of docetaxel and lipids and due to the finding that a higher active compound to lipid ratio (higher docetaxel content) can be reached. The formed liposomal dispersion is fully characterized by HPLC, PCS and microscopic analysis.

[0230] 4.3 Variation of Docetaxel Content

[0231] Liposomes (10 mM total lipid concentration, 10% trehalose) comprising DOTAP and DOPC are formed with different docetaxel contents. The general composition is defined as 50 mol % DOTAP, (50-X) mol % DOPC and X mol % docetaxel where the docetaxel content is varied from 3 to 13 mol %. Table 15 lists liposomal docetaxel formulations and their typical characteristics, such as average liposomal size, size distribution (PI), active compound and lipid concentration (HPLC), existence of extra-liposomal docetaxel (docetaxel crystals) and their surface charge.

TABLE 15

Liposomal docetaxel formulations			
Docetaxel Content	Liposomal Size (PI)	HPLC, Microscopy	Zeta Potential
3 mol %	175 nm (0.20)	according expectation, no crystals	65 mV
5 mol %	168 nm (0.20)	according expectation, no crystals	64 mV
7 mol %	162 nm (0.24)	according expectation, no crystals	60 mV
9 mol %	166 nm (0.18)	according expectation, no crystals	65 mV
11 mol %	162 nm (0.20)	according expectation, no crystals	62 mV
13 mol %	162 nm (0.14)	according expectation, no crystals	65 mV

[0232] Employing the lipid film method and the DOTAP/DOPC system, liposomes with up to about 13 mol % docetaxel can be prepared. It is notable, that a higher docetaxel content can be loaded into the liposomal membrane when manufacturing takes place at low temperatures (4° C.-8° C.) compared to higher temperatures (room temperature, 40° C.). The average diameter of docetaxel-containing liposomes is between 160 and 170 nm and the low PI value of indicates a favorable small size distribution. Determined concentrations (HPLC) are according theoretical values. According HPLC analysis docetaxel, DOTAP and DOPC were chemically stable during the manufacturing process at 4° C. A temperature higher than 10° C. during manufacturing resulted in the formation of docetaxel degradation product as seen in the HPLC chromatograms. All formulations were checked by microscopy (10 fold magnification) for aggregates/crystals. As docetaxel is only little soluble in trehalose or water (~20 µM) the presence of crystals would indicate a significant fraction of docetaxel which is not embedded (solubilized) in the liposomal membrane. None of the investigated formulations were tested positive for docetaxel crystals. The zeta potential (60-65 mV, Zetasizer 3000, Malvern) did not change with different docetaxel content.

[0233] 4.4 Lyophilization of Docetaxel-Containing Liposomes

[0234] Lyophilization of docetaxel-containing liposomes has been successfully performed applying a procedure as

described earlier. As shown in Table 16 liposomal size is not changed whereas the PI value (size distribution) of the respective reconstituted lyophilisate is slightly lowered compared with those of the non-lyophilized formulation.

TABLE 16

Effect of Lyophilization on Liposomal Size and PI Value		
Docetaxel	Liposomal Size (PI)	
Content	before Lyophilization	after Lyophilization
3 mol %	175 nm (0.20)	171 nm (0.10)
5 mol %	168 nm (0.20)	162 nm (0.08)
7 mol %	162 nm (0.24)	166 nm (0.09)
9 mol %	166 nm (0.18)	160 nm (0.08)
11 mol %	162 nm (0.20)	156 nm (0.09)
13 mol %	162 nm (0.14)	158 nm (0.07)

[0235] Lyophilization has no negative influence on liposomal stability. As checked by HPLC, lipids and docetaxel remain chemically stable.

[0236] 4.5 Determination of Non-Liposomal Docetaxel

[0237] Centrifugation experiments were performed to determine whether there is any free, non-liposomal docetaxel in liposomal docetaxel formulations. This experiment was carried out with Centricon® tubes (centrifugation tubes with a semi-permeable membrane which allows small molecules to pass and retains macromolecules). Liposomal formulations (10 mM, 10% trehalose) based on DOTAP and DOPC with 7, 11 and 13 mol % docetaxel were centrifuged at 4500 g and at 4° C. with Centricon® tubes (membrane specification of 30.000 MWCO). After 30 min centrifugation the supernatant was diluted with trehalose the volume of that had been found as permeate. HPLC analysis are summarized in Table 17.

TABLE 17

Determination of non-liposomal Docetaxel (HPLC)			
Docetaxel Content	Supernatant	Permeate	non-liposomal fraction
7 mol %	0.719 mM	0.026 mM	4%
11 mol %	1.040 mM	0.038 mM	3%
13 mol %	1.305 mM	0.059 mM	4%

[0238] The results show that docetaxel can be incorporated into the liposome membrane at a concentration of at least 13 mol % without increasing the fraction of nonliposomal docetaxel.

[0239] 4.6 Physicochemical Stability of Docetaxel Formulations

[0240] A liposomal formulation containing 5 mol % docetaxel has been used to study the physicochemical stability. A first experiment revealed that the content of docetaxel in the formulation has no influence on the stability. Also no difference has been found for the liquid (non-lyophilized) and the freeze-dried formulation. Storage stability at 4° C., 25° C. and 40° C. has been characterized by PCS (liposomal size and size distribution), light blockage measurements (PAMAS device), microscopy and HPLC. The physical stability is shown in FIG. 3. At all temperatures, the liposomal size and size distribution did not change within 24 h.

[0241] In principle, an increased particle number in the formulation as measured by light blockage measurements

(PAMAS device) indicates a poor physical stability due to ongoing aggregation of liposomes resulting in larger aggregates (larger than 1 µm). Using an adequate experimental setup no such increase has been found. FIG. 4 shows particle numbers that have been found during storage at 25° C. The particle count after 24 h is in the same range compared with the 8 h value.

[0242] HPLC analysis of the formulation at different time point clearly revealed a good chemical stability at 4° C. At 40° C. increase of degradation of up to 20% has been observed after storing of 24 h.

[0243] 4.7 In Vitro Experiments

[0244] The efficacy of the liposomal docetaxel formulation is determined in vitro by analyzing the decrease of cell viability in correlation to the active compound concentration. The active compound concentration at which cell viability is inhibited to 50% (IC_{50}) is used as index for the inhibitory potential.

[0245] C-26 (murine colon carcinoma cell line) and EA.Hy 926 cells (transformed human endothelial cell line) are seeded at a constant density ($2 \times 10^4/cm^2$) in 24-well plates and cultivated over night at conditions of 5-5.5% CO₂, 37° C. and ~90% humidity. At day 1, cell culture medium is replaced by a mixture of fresh medium and a series of 11 consecutive active compound dilutions is added to each well (duplicates) to cover a range between 0.1 and 1000 nM final active compound concentration. After 72 h, the cell viability in each well is determined by measuring the activity of mitochondrial dehydrogenases (MTT assay). In viable cells the MTT substrate is converted to a blue, cell impermeable dye (Formazan). After 1 h the medium is removed, cells are lysed with isopropanol/0.04% HCl and the amount of the blue Formazan given as optical density at a wavelength of 550 nm (OD_{550nm}) is quantitated in an ELISA reader. The experiment is evaluated using the Sigma Plot analysis software by plotting the mean OD_{550nm} value against the respective active compound concentration. A best fit curve is calculated based on a double-sigmoid assumption algorithm and the IC_{50} value is determined according to this best fit curve with results as shown in Table 18.

TABLE 18

IC ₅₀ Values of Taxotere and docetaxel loaded liposomes		
Formulation	IC ₅₀ (C-26)	IC ₅₀ (EA.Hy 926)
Taxotere ®	5 nM	4 nM
Liposomal Docetaxel Formulation	4 nM	7 nM

[0246] IC₅₀ values clearly reveal equal efficacy of docetaxel formulated with Polysorbate 80 (Taxotere®, Aventis) and liposomal docetaxel formulation (composition: DOTAP/DOPC/docetaxel 50:39:11) in both cell lines, C-26 and EA.Hy 926.

[0247] 4.8 In Vivo Experiments (A-375 Melanoma of Nude Mice)

[0248] Materials and Methods:

[0249] NMRI-nude mice were purchased from Elevage Janvier and housed in isolated ventilated cages under save environmental conditions (SPF facility, 22° C., 30-70% humidity, 12 h light/dark cycle) with food and water ad libitum. Experimental design was reviewed and approved by local government.

[0250] Tumor cells (A-375 human melanoma cell line, ATCC Nr.: CRL-1619) were grown as described in the data sheet supplied by ATCC. Tumor cells (5×10^6 in PBS) were inoculated s.c. in the right dorsal flank of mice in a volume of 50 μ l on day 0.

[0251] Mice were assigned to the experimental groups (8 animals per cage), housed and handled (including monitoring of the body weight gain) at least five days before tumor inoculation (=day -6 to 0). Treatment begins after the tumors reached a volume of approximately 100 mm³. Drugs and liposomal preparations were given by five iv injection, every other day at equivalent doses. The liposomal preparations were prepared as described previously. The solutions were administered slowly in a volume of ~5 μ l/g body weight.

[0252] Animals were clinically monitored during the whole experiment and for at least one week after treatment was finished. Monitoring of tumor size was performed three times a week after staging and before application during treatment period (at least one week). The tumor dimensions were measured by calliper and the tumor size was calculated according to the following formula: $V = \pi LW^2/6$ (L=greatest length, W=width of perpendicular axis). The body weight of individual animals was monitored at least twice during handling period (e.g. day -6 and 0), after tumor inoculation and after start of treatment for all groups. EDTA blood was collected from the retrobulbar plexus at four different points: during handling (day -3), tumor staging (day 14) and in the middle of treatment (~day 19) from 4 animals of all treatment groups for haematology. The number of red and white blood cells and platelets were determined using an automated cell counter (Abbott Cell Dyn 3500). The results are shown in FIG. 5.

[0253] Whereas tumors in the control group showed a rapid and progressive tumor growth, LipoDoc™ (DOTAP:DOPC: docetaxel 50:39:11) showed a strong reduction in the tumor growth rate, Taxotere® reduced the tumor growth only to a limited extent.

TABLE 19

Experimental groups and dose			
Group	Formulation	Dose [mg/kg]	N° of mice
0	10% trehalose	—	8
1	LipoDoc™	5	8
2	Taxotere ®	5	8

5. Example 5

Liposomal Paclitaxel (5 Mol %)

[0254] 5.1 Liposome Preparation via Ethanol Injection

[0255] A liposomal formulation comprising 5 mol % paclitaxel was prepared using the ethanol injection method as follows: paclitaxel and lipids of a molar ratio of 50:45:5 DOTAP/DOPC/paclitaxel were dissolved in ethanol (or another suitable organic solvent) usually at total lipid concentration of about 200-400 mM. An aqueous solution of a cryoprotectant, preferably 10% trehalose, pH 5-7, was prepared and cooled to a temperature between 4 and 8°C., preferably 4°C., prior injection of the organic solvent. The ethanolic solution was injected (3-300 ml/min injection speed) into the cold, vigorously stirred trehalose solution. Once a homoge-

neous dispersion is formed the liposomal dispersion is extruded (filtration under pressure) preferably at a temperatures between 4°C. and 8°C., preferably 4°C., 1-5 times through polycarbonate membranes of appropriate size, typically between 150 and 250 nm, optionally followed by sterile filtration. In addition to the manufacturing temperature of about 4°C. temperatures of 25°C. and 40°C. were evaluated for their effect on the product quality. The formed liposomal dispersions were fully characterized by HPLC, PCS and microscopic analysis.

[0256] 5.2 Effect of Temperature on the Preparation Process

[0257] In process control during the preparation procedure was done after ethanol injection, after extrusion and after lyophilization.

[0258] After Ethanol Injection:

[0259] At all three temperatures ethanol injection resulted in liposomes with a liposomal size of about 220 nm and a broad size distribution (PI) of about 0.4-0.6 (according PSC measurements). HPLC analysis revealed paclitaxel degradation when formulated at 40°C. while at 4 and 25°C. chemical stability of each constituent has been proofed. Microscopy showed little amount of paclitaxel crystals at 40°C. but not at 4 and 25°C.

[0260] After Extrusion:

[0261] At 40°C. difficulties occurred during extrusion due to clogged membranes. Replacing the clogged membrane did not solve the problem. Microscopy of the liposomal solution that did not pass the membrane revealed an increased amount of non-liposomal paclitaxel crystals that obviously blocked the membrane. Extrusion at 40°C. was not feasible. This was not the case when extrusion was performed at lower temperatures. In that case HPLC analysis gave concentration according expectations without any loss of material by the extrusion procedure (5 times, 0.2 μ m membrane). PCS data after extrusion at 4 and 25°C. were comparable: Liposomal size of about 170 nm and a small size distribution (PI) of about 0.1-0.2.

[0262] After Lyophilization:

[0263] Lyophilization of the formulation prepared at 4°C. and 25°C. has been successfully performed using a procedure described in example and characterized by HPLC, PCS and microscopic analysis.

[0264] 5.3 In Vivo Experiments

[0265] 5.3.1 Therapeutic Efficacy of LipoPac™ in A-375 Melanoma of Nude Mice

[0266] Materials and Methods:

[0267] NMRI-nude mice were purchased from Elevage Janvier and housed in isolated ventilated cages under save environmental conditions (SPF facility, 22°C., 30-70% humidity, 12 h light/dark cycle) with food and water ad libitum. Experimental design was reviewed and approved by local government.

[0268] Tumor cells (A-375 human melanoma cell line, ATCC Nr.: CRL-1619) were grown as described in the data sheet supplied by ATCC. Tumor cells (5×10^6 in PBS) were inoculated s.c. in the right dorsal flank of mice in a volume of 50 μ l on day 0.

[0269] Mice were assigned to the experimental groups (8 animals per cage), housed and handled (including monitoring of the body weight gain) at least five days before tumor inoculation (=day -6 to 0). Treatment begins after the tumors reached a volume of approximately 100 mm³. Drugs and liposomal preparations were given by iv injection, three times

a week (Mo, Wed, Fri) for the following three weeks at equivalent doses. The liposomal preparations were prepared as described above. The solutions were administered slowly in a volume of ~10 µl/g body weight.

[0270] Animals were clinically monitored during the whole experiment and for at least one week after treatment was finished. Monitoring of tumor size was performed three times a week after staging, before application during treatment period and during recovery period (at least one week). The tumor dimensions were measured by calliper and the tumor size was calculated according to the following formula: $V = \pi LW^2/6$ (L =greatest length, W =width of perpendicular axis). The body weight of individual animals was monitored at least twice during handling period (e.g. day -6 and 0), after tumor inoculation, after start of treatment and during recovery period (at least one week) for all groups. EDTA blood was collected from the retrobulbar plexus at four different points: during handling (day -3), tumor staging (day 7), in the middle of treatment (~day 21), and at the end of the recovery period (day 28) from 4 animals of all treatment groups for haematology. The number of red and white blood cells and platelets were determined using an automated cell counter (Abbott Cell Dyn 3500).

[0271] Whereas tumors in the control group showed a rapid and progressive tumor growth, LipoPac™ (DOTAP:DOPC: paclitaxel 50:45:5) showed a strong reduction in the tumor growth rate, Taxol® reduced the tumor growth only to a limited extent (Table 20, FIG. 6).

TABLE 20

Experimental groups and dose			
Group	Formulation	Dose [mg/kg]	N° of mice
0	10% trehalose	—	8
1	LipoPac™	5	8
2	Taxol ®	5	8

[0272] 5.3.2 Therapeutic Efficacy of LipoPac™ in B-16 Melanoma of C57/BL6 Mice

[0273] Materials and Methods:

[0274] C57/Black6 mice were purchased from Charles River and housed in isolated ventilated cages under save environmental conditions (SPF facility, 22° C., 30-70% humidity, 12 h light/dark cycle) with food and water ad libitum. Experimental design was reviewed and approved by local government.

[0275] Tumor cells (B-16 human melanoma cell line: CRL-6322) were grown as described in the data sheet supplied by ATCC. Tumor cells (5×10^6 in PBS) were inoculated s.c. in the right dorsal flank of mice in a volume of 50 µl on day 0.

[0276] Treatment start on day 6 after tumor cell injection. Three injections per week until end of experiment. End of study was planned to be determined by tumor size of the animals and ethical considerations.

[0277] Mice were assigned to the experimental groups (8 animals per cage), housed and handled (including monitoring of the body weight gain) at least five days before tumor inoculation (=day -6 to 0). The liposomal preparations were prepared as described above. The solutions were administered slowly in a volume of ~10 µl/g body weight.

[0278] Animals were clinically monitored during the whole experiment. Monitoring of tumor size was performed three times a week after staging and before application during

treatment period. The tumor dimensions were measured by calliper and the tumor size was calculated according to the following formula: $V = \pi LW^2/6$ (L =greatest length, W =width of perpendicular axis). The body weight of individual animals was monitored at least twice during handling period (e.g. day -6 and 0), after tumor inoculation and after start of treatment for all groups. EDTA blood was collected from the retrobulbar plexus at four different points: during handling (day -3), tumor staging (day 6) and in the middle of treatment (~day 14) from 4 animals of all treatment groups for haematology. The number of red and white blood cells and platelets were determined using an automated cell counter (Abbott Cell Dyn 3500).

[0279] Whereas tumors in the control group showed a rapid and progressive tumor growth, LipoPac™ (DOTAP:DOPC: paclitaxel 50:45:5) showed a strong reduction in the tumor growth rate, Taxol® reduced the tumor growth only to a limited extent (Table 21, FIG. 7).

TABLE 21

Experimental groups and dose			
Group	Formulation	Dose [mg/kg]	N° of mice
0	10% trehalose	/	8
1	LipoPac™	5	8
2	Taxol ®	5	8

6. Example 6

Preparation of Liposomes Comprising Cationic Lipids and Lipophilic Camptothecin or Camptothecin-Derivatives

[0280] The preparation of liposomes comprising cationic lipids and lipophilic camptothecin (CPT) or CPT-derivatives in the pH range between 3-7 is described. The CPT is loaded into the liposome. Liposomes can be prepared by different methods. All techniques have in common, that a mixture of lipids plus active compound is provided in a suitable organic solvent and then dispersed in an aqueous medium. Subsequently, further processing, like extrusion, sterile filtration or lyophilization may be applied. The active compound/lipid ratio is adjusted by mixing suitable amounts of lipid and active compound in an organic solvent. Typical molar active compound/lipid ratios range from 1:1000 to 1.10.

[0281] Subsequently, two methods are described to more detail for preparations with Camptothecin. The disclosed methods may be applied to any CPT-derivative, which is lipophilic at the desired pH.

[0282] 6.1 Liposome Formation

[0283] 6.1.1 Film Method

[0284] From the organic solution comprising lipid plus active compound, the solvent is evaporated, and a thin film of lipid plus active compound is formed at the inner wall of a flask. The thin molecular film is resuspended in an aqueous phase, which can contain further components such as buffers, ions, cryoprotectants and the like. With this procedure, liposome suspensions are formed in a self-assembly process. A standard preparation is obtained by forming a film of 99.5 µM DOTAP and 0.5 µM Camptothecin from a solution in CHCl₃/MeOH (10:1). The film is then reconstituted with 10 ml of the aqueous phase, in order to achieve a suspension where the

total liposomal concentration (lipid+active compound) is 10 mM. The aqueous solution comprises a cryoprotectant, e.g. glucose or trehalose and (optionally) a buffer, to achieve a desired pH after reconstitution. For camptothecin in the lactone form, a pH of 5-6 is used. For other formulations and CPT derivates, the pH can vary in the range between 3 and 7. [0285] A liposomal preparation with an active compound/lipid ratio of 1:200, and with a total (lipid+active compound) concentration of 10 mM is obtained. Other typical total molarities are 15 mM, 20 mM or 25 mM. If necessary, molarities up to 50 mM or higher can be formulated. The molar percentage of the active compound can be in the range from 0.1 to 10, depending on the experimental necessities (assignment of the liposomes, type of CPT-derivate). The lipid phase can comprise only one cationic lipid, such as DMTAP, DOTAP, DPTAP, DSTAP, DOTMA, or DDAB, or it can comprise up to 60% of charged and/or non-charged colipids. Standard preparations which have been used most frequently comprise DOTAP/DOPC=1:1 or DOTAP/Chol 1:1. Accordingly, other cationic lipids, such as DMTAP, DSTAP, DDAB, DOTMA and the like can be used.

[0286] 6.1.2 Organic Solution Injection

[0287] Liposomal dispersions can be prepared by injection of a solution comprising lipid plus active compound in an organic solvent, into an aqueous solution, pH 3-7, preferably 5-6 for camptothecin lactone. A typical solvent is ethanol ('ethanol injection'). The solution has a (lipid) concentration between 200-400 mM. A suitable volume of the solution is injected under vigorous stirring. All compositions and concentrations as described in the previous section can be prepared by this approach. As an alternative to ethanol, other suitable solvents or mixtures thereof can be taken. Typically, these are alcohols, ethers, chloroform, hydrocarbons, etc. As well solvents in the supercritical state can be applied, such as hydrocarbons, carbon dioxide, perfluorinated compounds, etc. Subsequently to the described preparation procedure, extrusion dialysis, a concentration step or freeze drying can be performed.

[0288] 6.1.3 Extrusion

[0289] The liposomal preparations as prepared by the above-described methods do not have necessarily the desired size distribution. Therefore, an extrusion through a membrane of defined pore size can be performed subsequently. Usually at least one extrusion through a membrane with a pore size of 200 nm (Osmonics Inc., Poretics, polycarbonate 0.2 µm) is performed. Other typical extrusion membranes have a pore size of 100 nm or 400 nm. Size distributions are controlled by quasi-elastic light scattering (Malvern, Herrenberg, Germany).

[0290] Further processing, like sterile filtration or lyophilization can be performed. The liposomal preparation can be lyophilized and reconstituted with water to the original state without changing the sized distribution and the active compound/lipid ratio.

[0291] 6.2 Characterization

[0292] The size distribution of the liposomal preparations is determined by quasi-elastic light scattering (Malvern, Herrenberg, Germany) and the composition is controlled by HPLC. As a further control for successful loading, UV-VIS spectroscopy is applied, enabling the determination of camptothecin in the liposomal preparation in-situ. Different spectra for the active compound in the stock solution, in the liposome, and after dissolving the liposome preparation in organic solvent are found. As an example, data from a lipo-

somal preparation comprising camptothecin with a active compound/lipid ratio of 1:1000 are given. With this active compound/lipid ratio, the spectroscopic measurements could be performed without further dilution of the samples. The preparation comprising 10 mM DOTAP/DOPC 1:1 was produced by the film method in an aqueous solution of trehalose at pH 5.5. The preparation was extruded 5 times through a membrane with a pore size of 200 nm (Osmonics Inc.). By quasi-elastic light scattering a Z_{ave} of 156 nm with a PI of 0.15 was determined. In the figure, the spectra of camptothecin in stock solution ($\text{CHCl}_3/\text{MeOH}$), in the liposomal preparation, and after dissolving the liposomes in $\text{THF}/\text{MeOH}/\text{HCl}$ (1:5) are shown. For the measurement of the liposomal preparation, an empty preparation (pure DOTAP/DOPC) with the same lipid composition was used for the blank measurement. In the same way, for the measurement in $\text{THF}/\text{MeOH}/\text{HCl}$, the empty liposomes were dissolved for the blank measurement. For better comparison, the spectra are vertically shifted and the data from the measurement after dilution of the liposomes in $\text{THF}/\text{MeOH}/\text{HCl}$ are multiplied by 5. As can be seen in FIG. 8, different spectra are obtained for the three cases. A characteristic peak shape for the liposomal camptothecin can be seen (FIG. 8, spectrum b). After dissolving the liposomes in $\text{THF}/\text{MeOH}/\text{HCl}$, a further spectral shift is obtained.

[0293] 6.2.1 Example: Improvement of Chemical Stability of Epothilones in Cationic Liposomes

[0294] Encapsulation of epothilone B is disclosed as specific example. However, other epothilones known in the art such as epothilone A, E or F or derivatives of epothilone A, B, E or F can be encapsulated in the same manner [14, 11, 13, 15].

[0295] Cationic liposomes containing epothilone B were prepared according to the film method. Briefly, for 10 ml of a 10 mM liposome suspension, 95 µmol of DOTAP and 5 µmol of epothilone B were dissolved in 15 ml chloroform in a round bottom flask. The chloroform was evaporated using a rotary evaporator and the resulting thin lipid film was dried for 60 min at 7-10 mbar. Subsequently, the lipid film was dissolved in 10 ml aqueous solution (see Table 22). The suspension was 5 times extruded (Northern Lipids Extruder) through 200 nm polycarbonate membranes (Osmonics Inc.). Liposome composition and size were checked by HPLC and PCS.

TABLE 22

Summary of Epothilone B Liposomes			
Formulation	Components of aqueous solution	Epothilone B concentration	Half life of Epothilone B
DOTAP/epothilone B = 95/5 mol %	10% trehalose, pH 5.5	0.5 mM = 200 mg/l	600 days
DOTAP/epothilone B = 95/5 mol %	10% trehalose and 10 mM Tris/HCl buffer, pH 7.0	0.5 mM = 200 mg/l	285 days

[0296] Stability Assay

[0297] In the formulations, the chemical stability of epothilone B was investigated. The formulations were aliquoted and half of the aliquots was stored at -80° C. (reference formulations). The other half (test formulations) was stored at 4-8° C. At selected time points, epothilone B concentration was determined by HPLC in a respective test formulation and in a reference formulation. The epothilone B concentration found in the test formulation was expressed as % of the epothilone B concentration in the reference formul-

lation (assumed to be 100%). The last column of Table 22 displays the half life of epothilone B, defined as the time point at which the epothilone B concentration in the test formulation amounted to 50% of the epothilone B concentration in the respective reference formulation.

[0298] The data shows that when comparing the epothilone stability in liposomal formulations, at lower pH (5.5) the stability was better than that at higher pH (7.0). This is in strong contrast to the literature where an increasing instability of epothilones at low pH is described.

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- 1.-36. (canceled)
- 37. A dehydrated preparation of a cationic liposomal composition, wherein the cationic liposomal composition has a pH of between about 3 and about 7 and comprises a lipophilic active compound of at least about 0.1%, at least one cationic lipid of at least about 30 mol %, optionally at least one further amphiphile of up to about 69.9 mol %, a lipophilic active compound of at least about 2 mol % and a stabilizing agent of about 0.1% (m/v) to about 20% (m/v).
- 38. The dehydrated preparation of claim 37, wherein the lipophilic active compound is a taxane.
- 39. The dehydrated preparation of claim 38, wherein the taxane is selected from the group consisting of paclitaxel, docetaxel, or a lipophilic derivative thereof.
- 40. The dehydrated preparation of claim 39, wherein the cationic liposomal composition comprises paclitaxel of about 2 mol % to about 5 mol % and a stabilizing agent of about 0.1% (m/v) to about 20% (m/v) and optionally at least one further amphiphile of up to about 65 mol %.
- 41. The dehydrated preparation of claim 39, wherein the cationic liposomal composition comprises docetaxel of at least about 5 mol % and a stabilizing agent of about 0.1% (m/v) to about 20% (m/v) and optionally at least one further amphiphile of up to about 65 mol %.
- 42. The dehydrated preparation of claim 39, wherein the cationic liposomal composition comprises succinyl-paclitaxel of at least about 5 mol % and a stabilizing agent of about 0.1% (m/v) to about 20% (m/v) and optionally at least one further amphiphile of up to about 65 mol %.
- 43. The dehydrated preparation of claim 39, wherein the liposomal composition comprises liposomes having a positive zeta potential in about 0.05 M KCl solution at about pH 7.5 at room temperature.
- 44. The dehydrated preparation of claim 38, wherein the cationic liposomal composition comprises less than 5% degradation product of the taxane.
- 45. The dehydrated preparation of claim 40, wherein the liposomal composition comprises less than 5% degradation product of paclitaxel.
- 46. The dehydrated preparation of claim 45, wherein the liposomal composition comprises less than 5% of 7-Epi-Taxol or Baccatin III.
- 47. The dehydrated preparation of claim 38, wherein the stabilizing agent in the range of about 5% (m/v) to about 15% (m/v).
- 48. The dehydrated preparation of claim 47, wherein the stabilizing agent is a sugar or an alcohol.
- 49. The dehydrated preparation of claim 48, wherein the sugar is trehalose.
- 50. A pharmaceutical composition comprising the dehydrated composition of claim 38 and a pharmaceutically acceptable carrier, diluent, and/or adjuvant.
- 51. A reconstituted cationic liposomal preparation, wherein the reconstituted cationic liposomal preparation comprises the dehydrated preparation of claim 38 reconstituted in an aqueous solution, wherein the aqueous solution has a pH of between about 3 and about 7, and wherein the taxane is physically and chemically stable for at least about 12 hours at about 2° C. to about 8 or at least about 4 hours at ambient temperature.
- 52. The reconstituted cationic liposomal preparation of claim 51, wherein the reconstituted cationic liposomal preparation comprises liposomes with an average particle size of about 50 nm to about 400 nm, or about 100 nm to about 300 nm.

53. The reconstituted cationic liposomal preparation of claim **51**, wherein the reconstituted cationic liposomal preparation comprises less than 5% degradation product of the taxane.

54. A pharmaceutical composition comprising the reconstituted cationic liposomal preparation of claim **51** and a pharmaceutically acceptable carrier, diluent, and/or adjuvant.

55. A reconstituted cationic liposomal preparation, wherein the reconstituted cationic liposomal preparation comprises the dehydrated preparation of claim **40** reconstituted in an aqueous solution, wherein the aqueous solution has a pH of between about 3 and about 7, and wherein the paclitaxel is physically and chemically stable for at least about 12 hours at about 2° C. to about 8° C. or at least about 4 hours at ambient temperature.

56. The reconstituted cationic liposomal preparation of claim **55**, wherein the reconstituted cationic liposomal preparation comprises liposomes with an average particle size of about 50 nm to about 400 nm, or about 100 nm to about 300 nm.

57. The reconstituted cationic liposomal preparation of claim **55**, wherein the reconstituted liposomal composition comprises less than 5% degradation product of paclitaxel.

58. The reconstituted cationic liposomal preparation of claim **57**, wherein the reconstituted liposomal composition comprises less than 5% of 7-Epi-Taxol or Baccatin III.

59. A pharmaceutical composition comprising the reconstituted cationic liposomal preparation of claim **55** and a pharmaceutically acceptable carrier, diluent, and/or adjuvant.

* * * *



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(54) **DUAL LOADED LIPOSOMAL
PHARMACEUTICAL FORMULATIONS**

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(57)

ABSTRACT

A pharmaceutical composition can include a plurality of liposomes comprising docetaxel and doxorubicin. In various embodiments, a liposome can include (i) an active pharmaceutical ingredient (API) comprising docetaxel and doxorubicin; (ii) a lipid layer comprising an unsaturated phospholipid, a cholesterol, a cationic lipid, and preferably a pegylated phospholipid; and (iii) an aqueous interior, wherein the docetaxel is in the lipid layer and the doxorubicin is crystallized in the aqueous interior. The liposomes can be used to treat a subject, for example, a human subject having cancer. The cancer can be, for example, a lung cancer, preferably non-small cell lung cancer (NSCLC), colon cancer, breast cancer, or liver cancer.

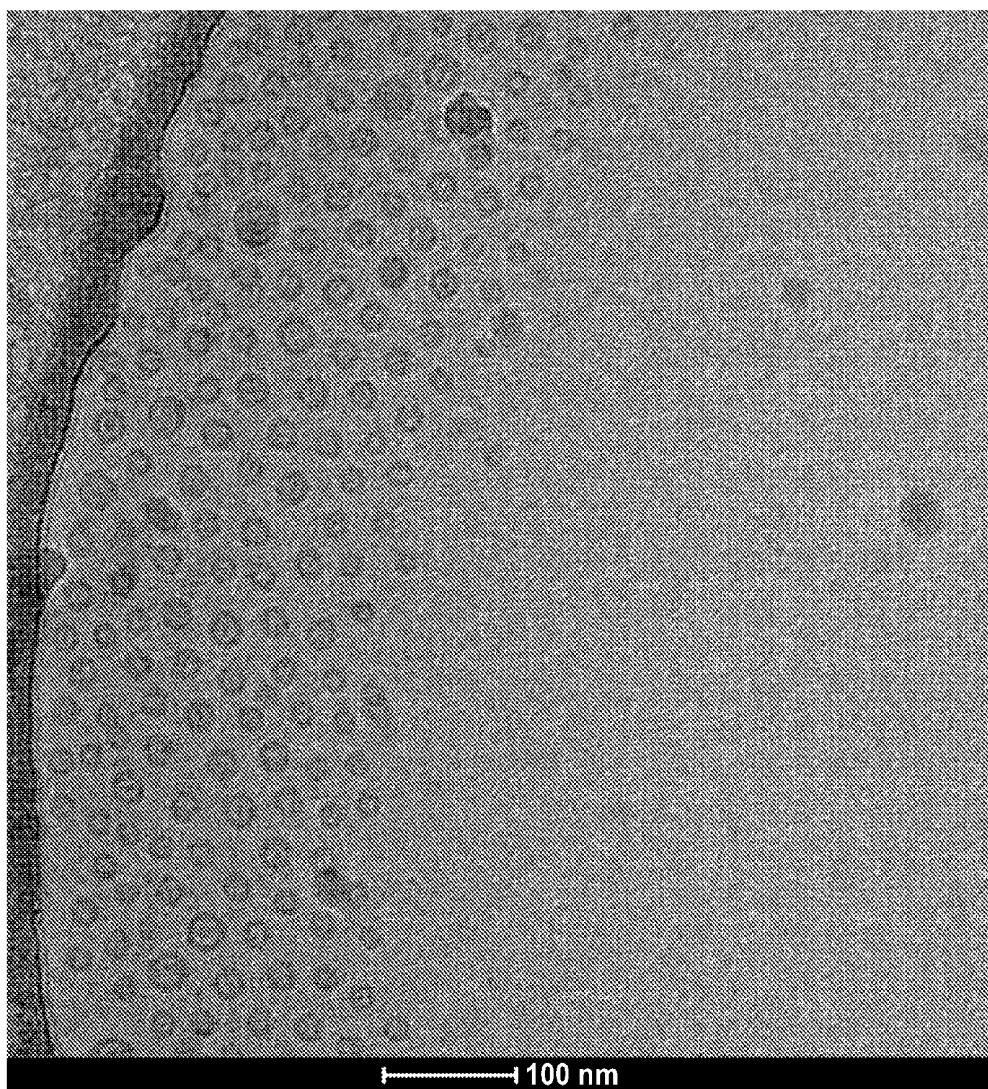


FIG. 1

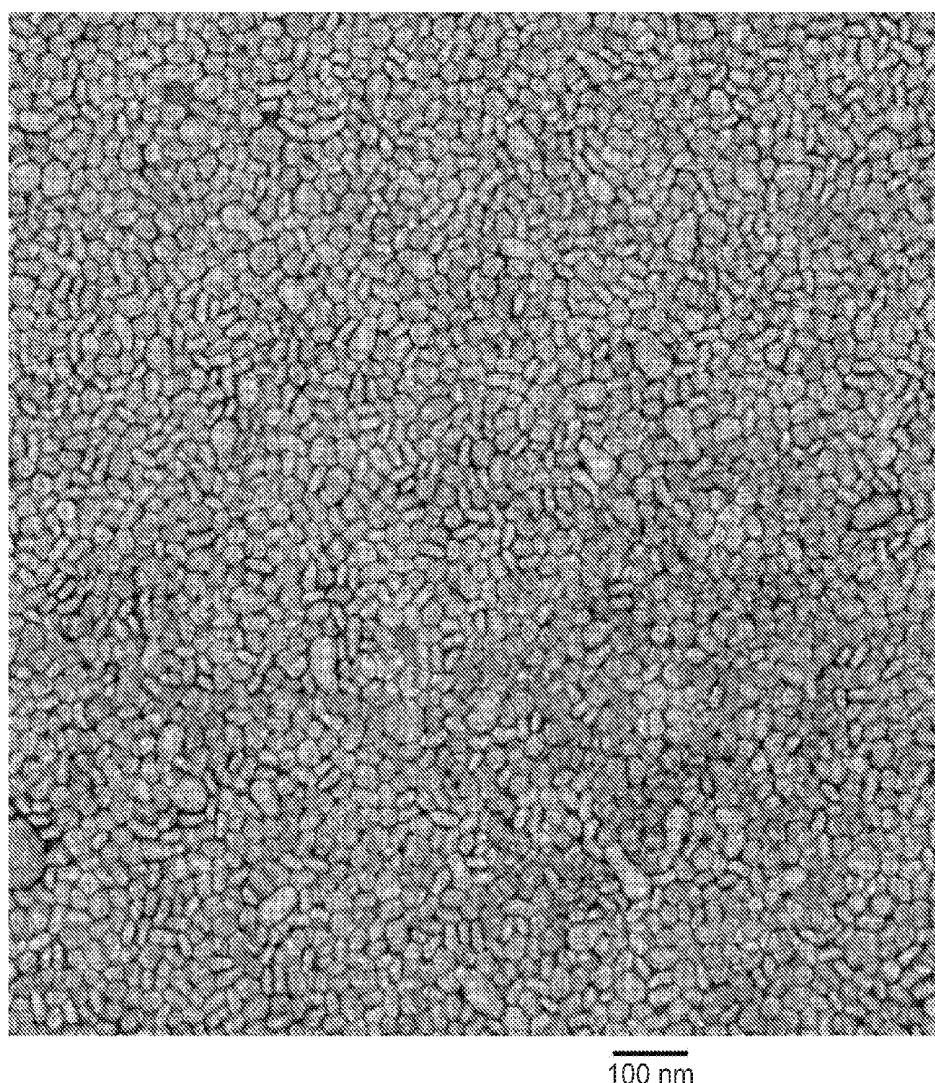


FIG. 2

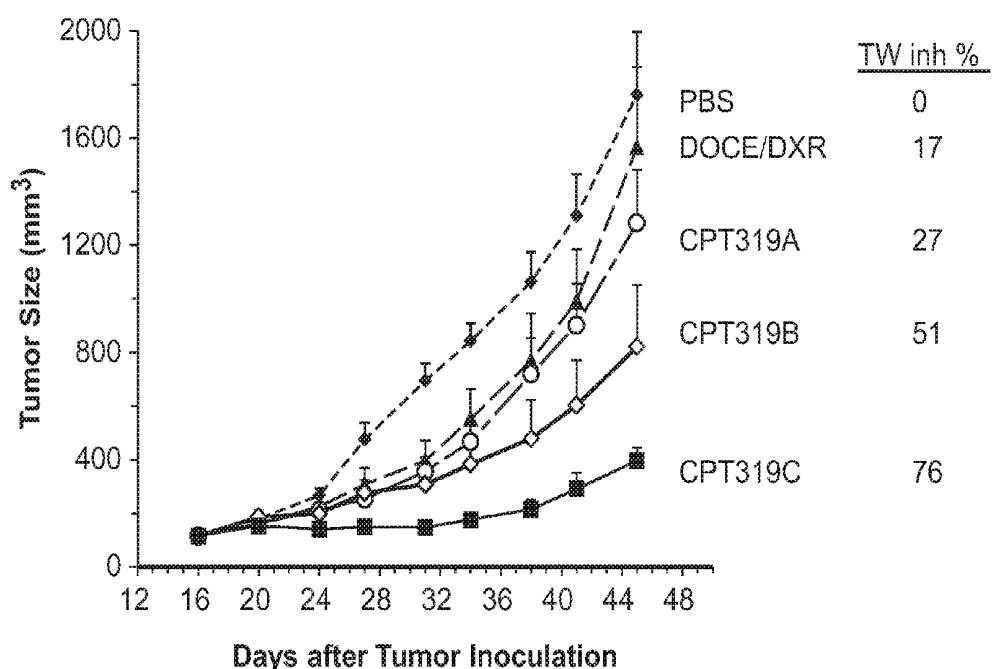


FIG. 3

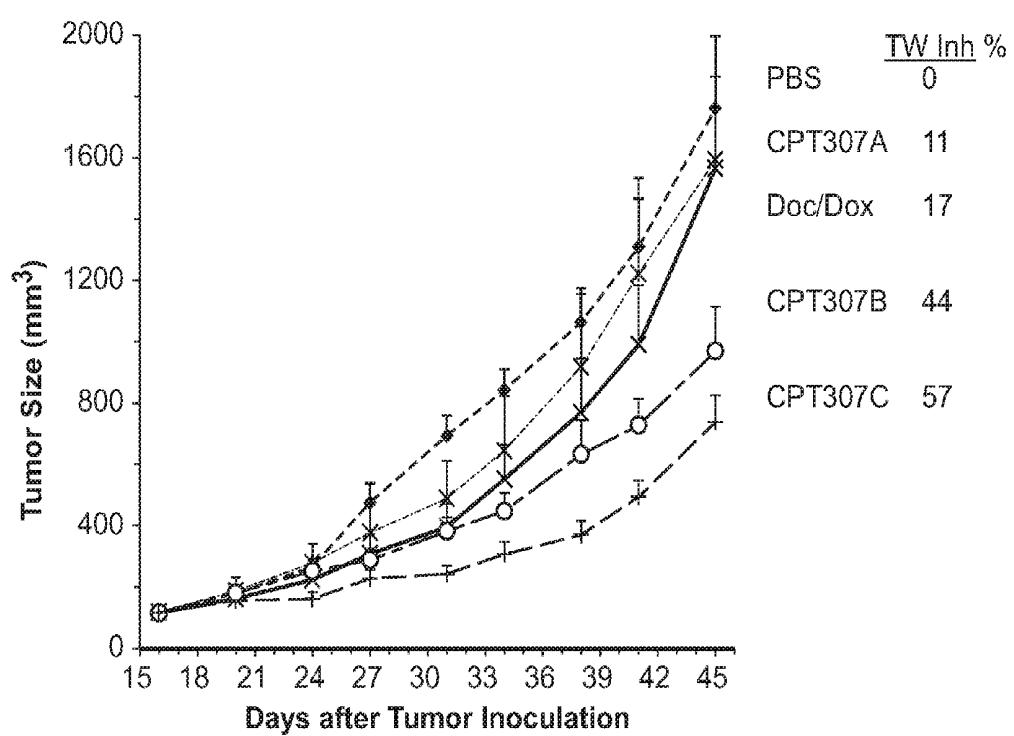


FIG. 4

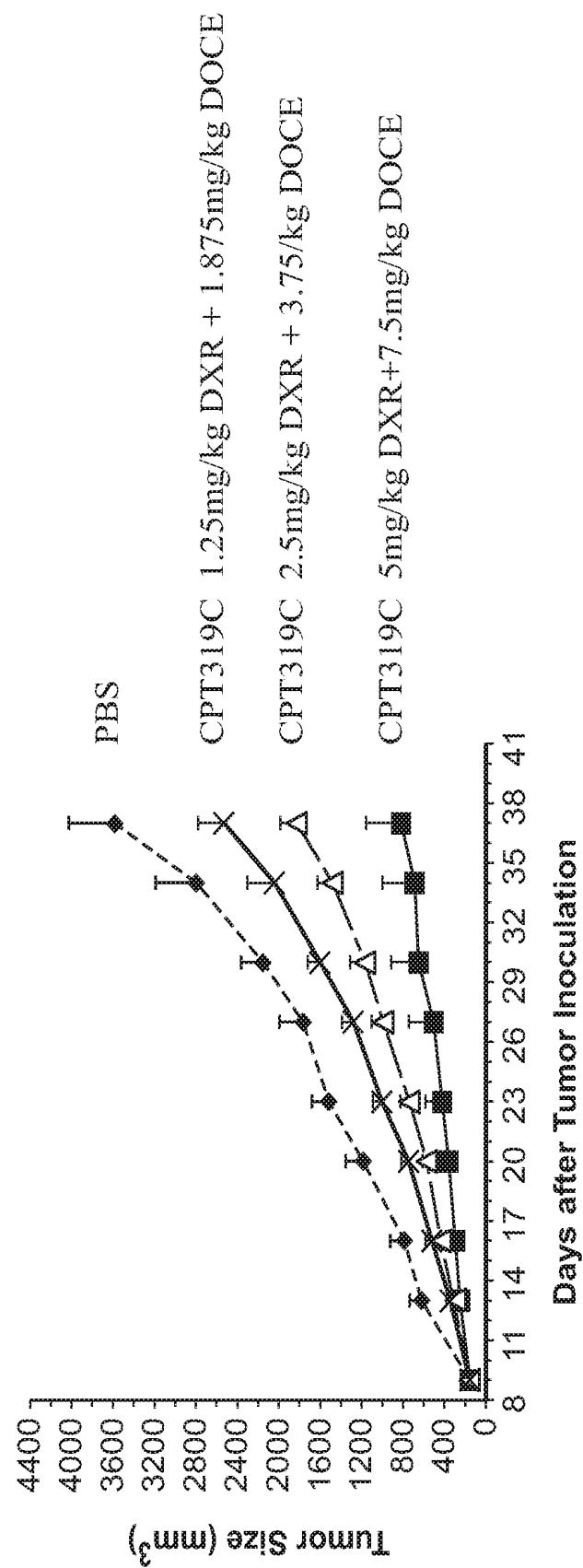


FIG. 5

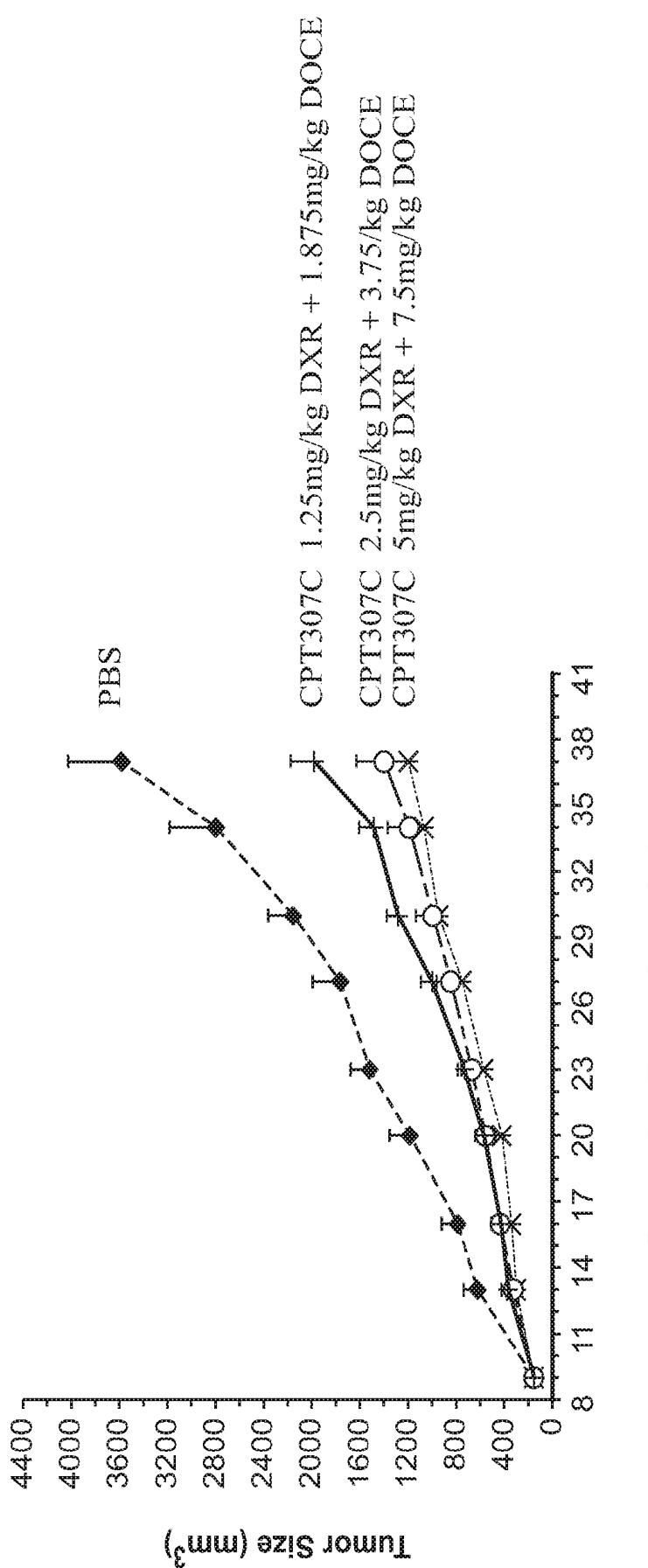


FIG. 6

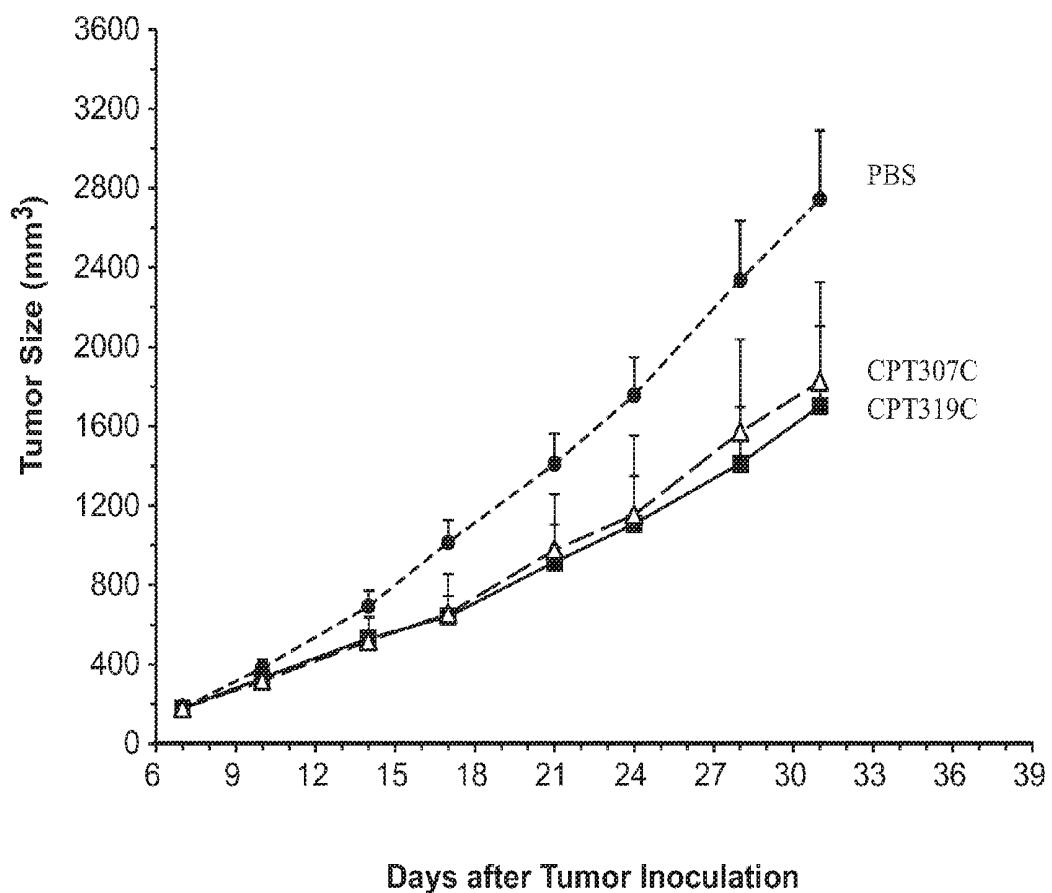


FIG. 7

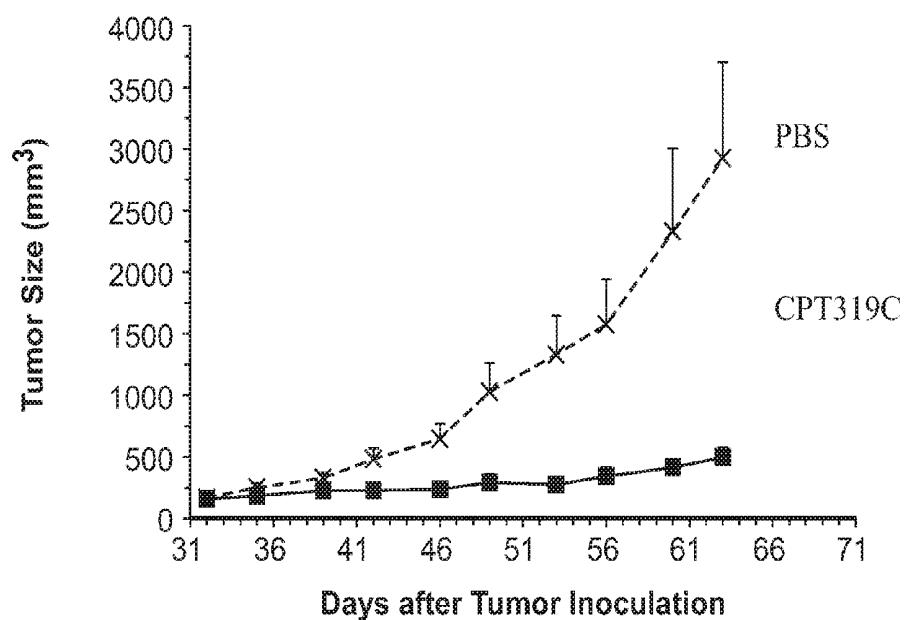


FIG. 8

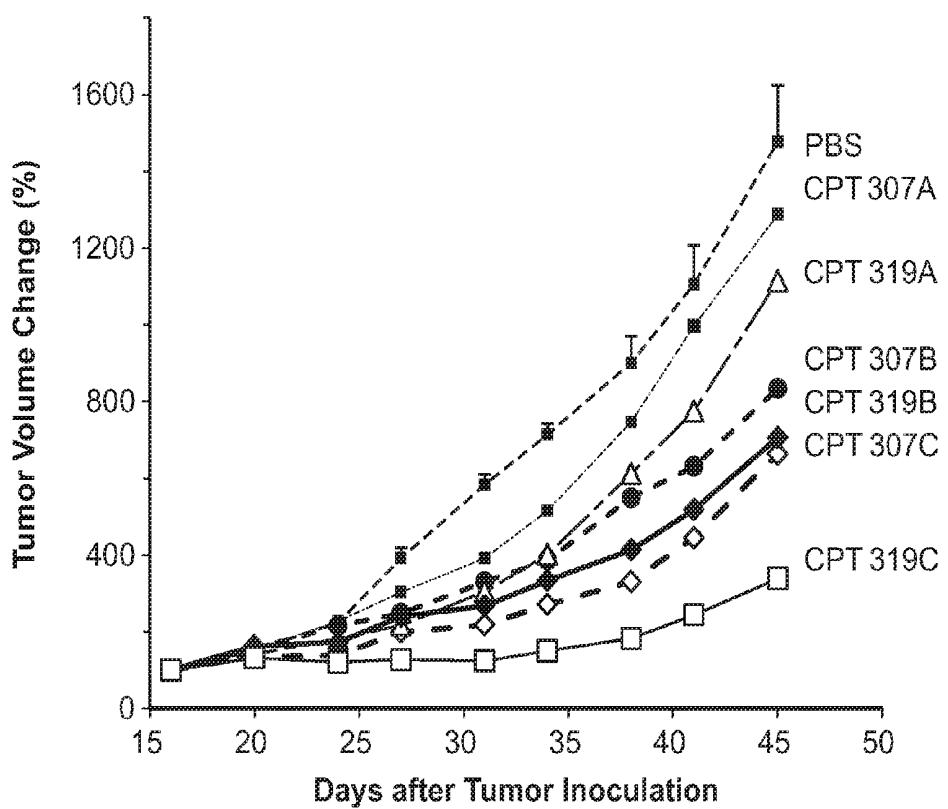


FIG. 9

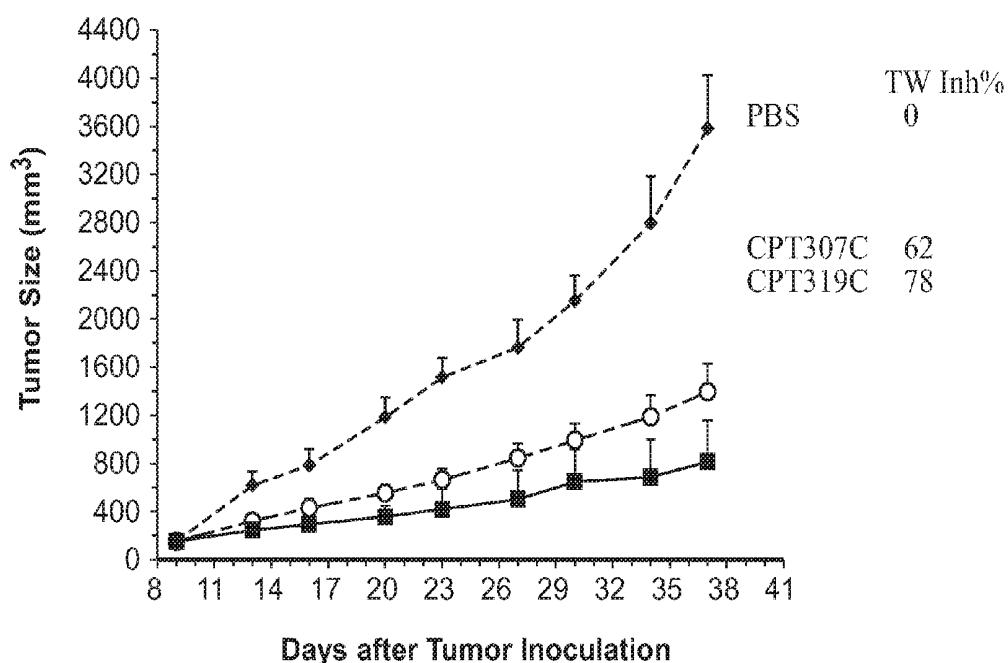
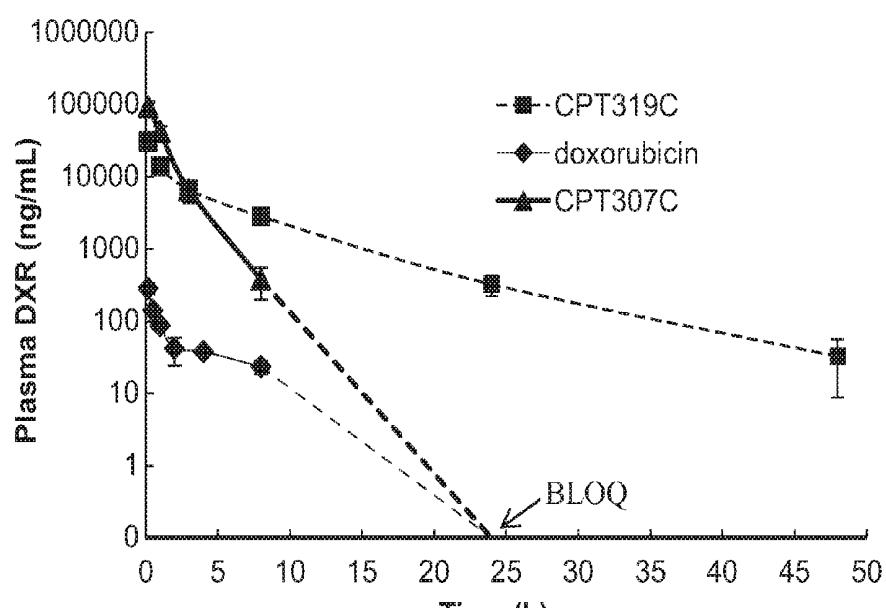


FIG. 10



*BLOQ: Below Limit of Quantitation

FIG. 11

DUAL LOADED LIPOSOMAL PHARMACEUTICAL FORMULATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/127,479, filed Mar. 3, 2015, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention generally relates to liposomal pharmaceutical formulations and, in various embodiments, more specifically to liposomal pharmaceutical formulations including an active pharmaceutical ingredient with two components (e.g., a combination of docetaxel and doxorubicin).

BACKGROUND

[0003] Liposome technology has been utilized for drug delivery in clinical therapy and scientific research. To date, a handful of liposomal pharmaceutical formulations have been approved by the US Food and Drug Administration (“FDA”), and a number of new liposomal formulations are in clinical trials. However, the field of liposomal formulation is still evolving and each active pharmaceutical ingredient (“API”) presents unique challenges.

[0004] One area where liposomal formulations can be applied is in cancer APIs. For example, liposomal formulations of doxorubicin are presently available under the trade names Doxil® and Myocet®. Doxil® is a pegylated (polyethylene glycol coated) liposome-encapsulated form of doxorubicin formerly made by Ben Venue Laboratories in the United States for Janssen Products, LP, a subsidiary of Johnson & Johnson. Myocet® is a non-pegylated liposomal doxorubicin made by Enzon Pharmaceuticals for Cephalon in Europe and for Sopherion Therapeutics in the United States and Canada. Myocet® is approved in Europe and Canada for treatment of metastatic breast cancer in combination with cyclophosphamide, but is not yet approved by the FDA for use in the United States.

[0005] Despite the handful of approved liposomal pharmaceutical formulations, the field is still limited by the unique challenges and unpredictability of each different API, as well as the currently available methods of making liposomal formulations, which present difficult problems associated with scalability, low reproducibility, and product heterogeneity. There exists a need for improved liposomal formulations for use in drug delivery.

SUMMARY OF THE INVENTION

[0006] In various aspects and embodiments, the invention provides a pharmaceutical composition including a plurality of liposomes comprising a first drug (e.g., docetaxel) and a second drug (e.g., doxorubicin). In various embodiments, a liposome can include (i) an active pharmaceutical ingredient (API) comprising a first drug (e.g., docetaxel) and a second drug (e.g., doxorubicin); (ii) a lipid layer comprising an unsaturated phospholipid, a cholesterol, and preferably a pegylated phospholipid; and (iii) an aqueous interior, wherein the first drug (e.g., docetaxel) is in the lipid layer and the second drug (e.g., doxorubicin) is crystallized in the aqueous interior. The liposomes can be used to treat a subject, for example, a human subject having cancer. The cancer can be, for example, a lung cancer, preferably non-small cell lung

cancer (NSCLC); colon cancer; breast cancer; or liver cancer, preferably hepatocellular carcinoma (HCC).

[0007] The invention can provide for increased efficacy and/or decreased toxicity, for example relative to (i) other pharmaceutical compositions where one or both of the first drug (e.g., docetaxel) and the second drug (e.g., doxorubicin) are not in a liposomal formulation and/or (ii) other liposomal formulations. The invention can provide for targeted delivery, for example to the liver or avoiding the liver. The invention can mitigate undesired side effects, for example by providing for increased drug loading, thereby reducing the amount of liposomes needed to deliver a quantity of the first drug (e.g., docetaxel) and the second drug (e.g., doxorubicin).

[0008] The invention provides a liposome comprising: (i) an active pharmaceutical ingredient (API) comprising docetaxel and doxorubicin; (ii) a lipid layer comprising an unsaturated phospholipid, a cholesterol, and preferably a pegylated phospholipid; and (iii) an aqueous interior, wherein the docetaxel is in the lipid layer and the doxorubicin is crystallized in the aqueous interior.

[0009] The invention also provides a pharmaceutical composition comprising a plurality of liposomes according to any of the aspects or embodiments disclosed herein.

[0010] The invention also provides a method comprising administering the liposome according to any of the aspects or embodiments disclosed herein, or the pharmaceutical composition according to any of the aspects or embodiments disclosed herein, to a subject.

[0011] The invention also provides a method of treating a subject comprising administering an effective amount of the liposome according to any of the aspects or embodiments disclosed herein, or the pharmaceutical composition according to any of the aspects or embodiments disclosed herein, to a subject.

[0012] The invention also provides a method of making the liposome according to any of the aspects or embodiments disclosed herein, or the pharmaceutical composition according to any of the aspects or embodiments disclosed herein, comprising: (i) introducing a lipid solution of an unsaturated phospholipid, cholesterol, a first drug (e.g., docetaxel), and preferably a pegylated phospholipid in ethanol through a first or more inlet port of a manifold into a mixing chamber and an aqueous solution through a second or more inlet port of the manifold into the mixing chamber, the liposomes formed exit the mixing chamber through a third or more outlet port of the manifold, thereby making a plurality of liposomes; and (ii) incubating the plurality of liposomes in a second drug (e.g., doxorubicin) solution.

[0013] In various embodiments, the lipid layer consists essentially of the unsaturated phospholipid and cholesterol.

[0014] In various embodiments, the lipid layer consists essentially of the unsaturated phospholipid, cationic lipid, cholesterol, and pegylated phospholipid.

[0015] In various embodiments, the API consists essentially of docetaxel and doxorubicin.

[0016] In various embodiments, the lipid layer comprises: about 20-75%, preferably about 30-60%, (molar) unsaturated phospholipid; about 10-60%, preferably 20-50%, (molar) cholesterol; about 5-75%, preferably about 10-60%, (molar) cationic lipid; and about 0-20%, preferably 1-10%, (molar) pegylated phospholipid.

[0017] In various embodiments, the molar ratio of the lipid layer components:doxorubicin is about 100:1 to about 2:1, preferably about 20:1 to about 5:1; and the molar ratio of the

lipid layer components: docetaxel is about 100:1 to about 2:1, preferably about 20:1 to about 5:1.

[0018] In various embodiments, the molar ratio of doxorubicin:docetaxel is about 10:1 to 1:10, preferably about 5:1 to 1:5, and more preferably about 2:1 to 1:2.

[0019] In various embodiments, the unsaturated phospholipid comprises a polyunsaturated phospholipid or a monounsaturated phospholipid, preferably a phosphatidylcholine, and more preferably and soy phosphatidylcholine or 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC).

[0020] In various embodiments, the cholesterol comprises a cholesterol derivative, preferably a cationic cholesterol derivative, more preferably an amino cholesterol derivative, and still more preferably dimethylaminoethanecarbamoyl-cholesterol (DC-cholesterol).

[0021] In various embodiments, the pegylated phospholipid comprises a phosphoethanolamine, preferably a 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and wherein the pegylation is a PEG 500 to PEG 3000, preferably PEG 2000.

[0022] In various embodiments, the plurality of liposomes are comprised in an intravenous formulation.

[0023] In various embodiments, the Z-average particle size of the liposomes is about 10-200 nm, preferably about 15-150 nm, and more preferably about 20-120 nm.

[0024] In various embodiments, upon intravenous administration to a subject, at least about 10% of the composition is delivered to the liver.

[0025] In various embodiments, the pharmaceutical composition is for use as a medicament.

[0026] In various embodiments, the pharmaceutical composition is for use as a cancer therapeutic.

[0027] In various embodiments, the subject has a cancer. In various embodiments, the cancer is a lung cancer, preferably non-small cell lung cancer (NSCLC); colon cancer; breast cancer; or liver cancer, preferably hepatocellular carcinoma (HCC).

[0028] These and other advantages of the present technology will be apparent when reference is made to the accompanying drawings and the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] FIG. 1 presents a cryo transmission electron microscopy (TEM) image of liposomal formulation CPT319C.

[0030] FIG. 2 presents a negative stained TEM image of CPT319C.

[0031] FIG. 3 illustrates NSCLC tumor growth curves and tumor weight inhibition percentages (TW inh %) after administration of liposomal (CPT319A, CPT319B, or CPT319C) or non-liposomal formulations of docetaxel/doxorubicin, compared to a control group.

[0032] FIG. 4 illustrates NSCLC tumor growth curves and tumor weight inhibition percentages (TW inh %) after administration of liposomal (CPT307A, CPT307B, or CPT307C) or non-liposomal formulations of docetaxel/doxorubicin, compared to the control group.

[0033] FIG. 5 illustrates colon cancer tumor growth curves after administration of three different doses of liposomal formulation (CPT319C), compared to the control group.

[0034] FIG. 6 illustrates colon cancer tumor growth curves after administration of three different doses of liposomal formulation (CPT307C), compared to the control group.

[0035] FIG. 7 illustrates breast cancer tumor growth curves on Day 31 after administration of liposomal formulations (CPT307C or CPT319C), compared to the control group.

[0036] FIG. 8 illustrates hepatocellular carcinoma tumor growth curves after administration of liposomal formulation (CPT319C), compared to the control group.

[0037] FIG. 9 illustrates NSCLC tumor growth curves after administration of liposomal formulations (CPT307A-C or CPT319A-C), compared to the control group.

[0038] FIG. 10 illustrates colon cancer tumor growth curves and tumor weight inhibition percentages (TW Inh %) after administration of liposomal formulations (CPT307C or CPT219C), compared to the control group.

[0039] FIG. 11 illustrates plasma concentration curves of doxorubicin after administration of liposomal formulations (CPT319C or CPT307C), compared to non-liposomal formulation of docetaxel/doxorubicin.

[0040] While the invention comprises embodiments in many different forms, there are shown in the drawings and will herein be described in detail several specific embodiments with the understanding that the present disclosure is to be considered as an exemplification of the principles of the technology and is not intended to limit the invention to the embodiments illustrated.

DETAILED DESCRIPTION

[0041] In various aspects and embodiments, the invention provides a pharmaceutical composition including a plurality of liposomes comprising a first drug (e.g., docetaxel) and a second drug (e.g., doxorubicin). In various embodiments, a liposome can include (i) an active pharmaceutical ingredient (API) comprising a first drug (e.g., docetaxel) and a second drug (e.g., doxorubicin); (ii) a lipid layer comprising an unsaturated phospholipid, a cholesterol, and preferably a pegylated phospholipid; and (iii) an aqueous interior, wherein the first drug (e.g., docetaxel) is in the lipid layer and the second drug (e.g., doxorubicin) is crystallized in the aqueous interior. The liposomes can be used to treat a subject, for example, a human subject having cancer.

[0042] As described and shown in the examples below, the invention can provide for increased efficacy and/or decreased toxicity, for example relative to (i) other pharmaceutical compositions where one or both of the first drug (e.g., docetaxel) and the second drug (e.g., doxorubicin) are not in a liposomal formulation and/or (ii) other liposomal formulations. The invention can provide for targeted delivery, for example to the liver or avoiding the liver. The invention can mitigate undesired side effects, for example by providing for increased drug loading, thereby reducing the amount of liposomes needed to deliver a quantity of the first drug (e.g., docetaxel) and the second drug (e.g., doxorubicin).

[0043] The various features of such liposomes, as well as pharmaceutical compositions including the liposomes and methods of using and making the liposomes are discussed, in turn, below.

[0044] Active Pharmaceutical Ingredient (API)

[0045] In various aspects and embodiments, the API comprises a first drug (e.g., docetaxel) and a second drug (e.g., doxorubicin). While docetaxel and doxorubicin are presented as illustrative examples, other embodiments are possible where the first drug is in the lipid layer of the liposome and the second drug is in (e.g., crystallized in) the aqueous interior of the liposome. In various embodiments, the API can comprise

two (or more) anticancer agents, an anti-inflammatory agents, an anti-diabetic agents, an anti-fungal agents, and/or antibiotic agents.

[0046] Docetaxel (as generic or under the trade name Taxotere® or Docecad®) is a clinically well-established anti-mitotic chemotherapy medication that works by interfering with cell division. Docetaxel is approved by the FDA for treatment of locally advanced or metastatic breast cancer, head and neck cancer, gastric cancer, hormone-refractory prostate cancer and non small-cell lung cancer. Docetaxel can be used as a single agent or in combination with other chemotherapeutic drugs as indicated depending on specific cancer type and stage.

[0047] Docetaxel is a member of the taxane drug class, which also includes the chemotherapeutic medication paclitaxel. Accordingly, in some embodiments, docetaxel can be substituted for another taxane that can be disposed within the lipid layer of the liposome.

[0048] The optimal dose scheduling of taxanes remains unconfirmed, but most studies find significant mortality benefit following either a three-week or a one-week administration schedule. While some research suggests weekly administration as an optimal schedule, the official docetaxel package insert recommends administration every three weeks. Important toxicities to note include neutropenia, febrile neutropenia and neurosensory disturbances. Such toxicities have been well documented in Phase II and Phase III clinical trials and can be anticipated and subsequently managed.

[0049] In various embodiments, the invention can increase the efficacy of, and/or decrease undesired side effects from, the docetaxel.

[0050] Doxorubicin (trade name Adriamycin®; pegylated liposomal form trade name Doxil®; nonpegylated liposomal form trade name Myocet®), also known as hydroxydaunorubicin and hydroxydaunomycin, is a drug used in cancer chemotherapy and derived by chemical semisynthesis from a bacterial species. It is an anthracycline antibiotic (note: in this context, this does not mean it is used to treat bacterial infections) closely related to the natural product daunomycin and like all anthracyclines, it is believed to work by intercalating DNA, with the most serious adverse effect being life-threatening heart damage. It is commonly used in the treatment of a wide range of cancers, including hematological malignancies (blood cancers, like leukaemia and lymphoma), many types of carcinoma (solid tumors) and soft tissue sarcomas. It is often used in combination chemotherapy as a component of various chemotherapy regimens. In some embodiments, doxorubicin can be substituted for another anticancer agent that can be disposed within the aqueous interior of the liposome.

[0051] Common adverse effects of doxorubicin include hair loss (seen in most of those treated with the drug), myelosuppression (a compromised ability of the body's bone marrow to produce new blood cells), nausea and vomiting (which are seen in roughly 30-90% of people treated with the drug), oral mucositis, oesophagitis, diarrhea, skin reactions (including hand-foot syndrome) and localized swelling and redness along the vein in which the drug is delivered. Less common, yet serious reactions include hypersensitivity reactions (including anaphylaxis), radiation recall, heart damage and liver dysfunction.

[0052] The drug is administered intravenously, as the hydrochloride salt. It is sold under a number of different brand

names, including Adriamycin® PFS, Adriamycin® RDF, or Rubex®. Doxorubicin is photosensitive, and containers are often covered by an aluminum bag and/or brown wax paper to prevent light from affecting it. Doxorubicin is also available in liposome-encapsulated forms as Doxil® (pegylated form), Myocet® (nonpegylated form), and Caelyx®, although these forms must also be given by intravenous injection.

[0053] In various embodiments, the invention can increase the efficacy of and/or decrease undesired side effects from, the doxorubicin.

[0054] In some embodiments, the API may be a polynucleotide (including an oligonucleotide) a protein or a small molecule.

[0055] In one embodiment the API is a polynucleotide. The polynucleotide may be a genomic DNA fragment, cDNA, mRNA, ssRNA, dsRNA, microRNA, siRNA, shRNA, sdRNA, DsiRNA, LNA, and antisense DNA or RNA.

[0056] Alternatively, the API may be a small molecule drug. Preferably, the molecule has a molecular weight from about 1500 g/mole to about 50 g/mole.

[0057] An API can include, for example, two or more of the following: an anticancer agent, an antibiotic agent, an antiviral agent, an anti-fungal agent, or an analgesic.

[0058] Exemplary anticancer agents may include but are not limited acivicin, aclarubicin, acodazole, ametantrone, aminoglutethimide, anthramycin, asparaginase, azacitidine, azetepa, bisantrene, bleomycin, busulfan, cactinomycin, calusterone, caracemide, carboplatin, carfilzomib, carmustine, carubicin, chlorambucil, cisplatin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, deazaguanine, diaziquone, docetaxel, doxorubicin, epipropidine, erlotinib, etoposide, etoprine, flouxuridine, fludarabine, fluorouracil, fluorocitabine, hydroxyurea, iproplatin, leuprolide acetate, lomustine, mechlorethamine, megestrol acetate, melengestrol acetate, mercaptopurine, methotrexate, metoprine, mitocromin, mitogillin, mitomycin, mitosper, mitoxantrone, mycophenolic acid, nocodazole, nogalamycin, oxisuran, paclitaxel, peliomycin, pentamustine, porfiromycin, prednimustine, procarbazine hydrochloride, puromycin, pyrazofurin, riboprine, semustine, sparsomycin, spirogermanium, spironustine, spirotiplatin, streptozocin, talisomycin, tegafur, teniposide, teroxirone, thiamiprine, thioguanine, tiazofurin, triciribine phosphate, triethylennemelamine, trimetrexate, uracil mustard, uredopa, vinblastine, vincristine, vindesine, vinepidine, vinrosidine, vinzolidine, zinostatin and zorubicin.

[0059] Exemplary antibiotic agents may include but are not limited to aminoglycoside; amikacin; gentamicin; kanamycin; neomycin; netilmicin; steptomycin; tobramycin; ansamycins; geldanamycin; herbimycin; carbacephem; loracarbef; carbacepenem; ertapenem; doripenem; imipenem/cilastatin; meropenem; cephalosporin; cefadroxil; cefazolin; cefalotin or cefalothin; cefalexin; cefaclor; cefamandole; cefoxitin; cefprozil; cefuroxime; cefixime; cefdinir; cefditoren; cefoperazone; cefotaxime; cefpodoxime; ceftazidime; ceftibuten; ceftizoxime; ceftriaxone; cefepime; ceftobiprole; glycopeptide; teicoplanin; vancomycin; macrolides; azithromycin; clarithromycin; dirithromycin; erythromycin; roxithromycin; troleandomycin; telithromycin; spectinomycin; monobactam; aztreonam; penicillins; amoxicillin; ampicillin; azlocillin; carbenicillin; cloxacillin; dicloxacillin; flucloxacillin; mezlocillin; meticillin; nafcillin; oxacillin; penicillin; piperacillin; ticarcillin; bacitracin; colistin; polymyxin B; quinolone; ciprofloxacin; enoxacin; gatifloxacin;

levofloxacin; lomefloxacin; moxifloxacin; norfloxacin; ofloxacin; trovafloxacin; sulfonamide; mafenide; prontosil (archaic); sulfacetamide; sulfamethizole; sulfanilimide (archaic); sulfasalazine; sulfisoxazole; trimethoprim; trimethoprim-sulfamethoxazole (co-trimoxazole) (TMP-SMX); tetracycline; demeclocycline; doxycycline; minocycline; oxytetracycline; tetracycline; arsphenamine; chloramphenicol; clindamycin; lincomycin; ethambutol; fosfomycin; fusidic acid; furazolidone; isoniazid; linezolid; metronidazole; mupirocin; nitrofuantoin; platensimycin; polymyxin, purazinamide; quinupristin/dalfopristin; rifampin or rifampicin; and timidazole.

[0060] In specific embodiments, the anti-cancer agent is chosen from daunorubicin, doxorubicin, paclitaxel, docetaxel, cisplatin, carboplatin, cytarabine, flouxuridine, fludarabine, fluorouracil, iproplatin, leuprolide acetate, carfilzomib, and methotrexate.

[0061] Exemplary antiviral agents may include, but are not limited to thiosemicarbazone; metisazone; nucleoside and/or nucleotide; acyclovir; idoxuridine; vidarabine; ribavirin; ganciclovir; famciclovir; valaciclovir; cidofovir; penciclovir; valganciclovir; brivudine; ribavirin, cyclic amines; rimantadine; tromantadine; phosphonic acid derivative; foscarnet; fosfonet; protease inhibitor; saquinavir; indinavir; ritonavir; nelfinavir; amprenavir; lopinavir; fosamprenavir; atazanavir; tipranavir; nucleoside and nucleotide reverse transcriptase inhibitor; zidovudine; didanosine; zalcitabine; stavudine; lamivudine; abacavir; tenofovir disoproxil; adefovir dipivoxil; emtricitabine; entecavir; non-nucleoside reverse transcriptase inhibitor; nevirapine; delavirdine; efavirenz; neuraminidase inhibitor; zanamivir; oseltamivir; moroxydine; inosine pranobex; pleconaril; and enfuvirtide.

[0062] Exemplary anti-fungal agents may include but are not limited to allylamine; terbinafine; antimetabolite; flucytosine; azole; fluconazole; itraconazole; ketoconazole; ravuconazole; posaconazole; voriconazole; glucan synthesis inhibitor; caspofungin; micafungin; anidulafungin; polyenes; amphotericin B; amphotericin B Colloidal Dispersion (ABCD); and griseofulvin.

[0063] Exemplary analgesics may include, but are not limited to opiate derivative, codeine, meperidine, methadone, and morphine.

[0064] In various embodiments, the API consists essentially of the first drug (e.g., docetaxel) and the second drug (e.g., doxorubicin).

[0065] In various embodiments, the molar ratio of the lipid layer components:second drug (e.g., doxorubicin) is about 100:1 to about 5:1, preferably about 20:1 to about 10:1; and the molar ratio of the lipid layer components:first drug (e.g., docetaxel) is about 100:1 to about 5:1, preferably about 20:1 to about 10:1.

[0066] In various embodiments, the molar ratio of second drug (e.g., doxorubicin):first drug (e.g., docetaxel) is about 10:1 to 1:10, preferably about 5:1 to 1:5, and more preferably about 3:1 to 1:3.

[0067] The Lipid Layer and Aqueous Solutions

[0068] The invention utilizes lipid and aqueous solutions, for example in making liposomes in accordance with the invention. Accordingly, the composition lipid and/or aqueous solutions can affect the final composition of the liposomes.

[0069] In various embodiments, the lipid solution may comprise an organic solvent. The organic solvent may be a water miscible solvent. Preferably, the water miscible solvent

is selected from the group consisting of ethanol, methanol, DMSO and isopropanol. Most preferably, the organic solvent is ethanol.

[0070] As used herein the term "cationic lipid" refers to a lipid or a cholesterol derivative that carries a net positive charge at about pH 3-pH 9.

[0071] As used herein the term "anionic lipid" refers to a lipid or a cholesterol derivative that carries a net negative charge at about pH 3-pH 9.

[0072] As used herein the term "pegylated lipid" refers to a lipid that is conjugated with a polyethylene glycol polymer.

[0073] As used herein the term "neutral lipid" refers to the lipid that does not carry net charge at about pH 3-pH 9.

[0074] The lipid solution may include a mixture of lipids. The mixture of lipids preferably includes cholesterol.

[0075] The mixture of lipids may also include a cationic lipid. The cationic lipid may be, but is not limited to, N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTMA"); N-(2,3-dioleyloxy)propyl)-N,N-dimethylammonium chloride ("DODMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); N-(2,3-dioleyloxy)propyl)-N,N-dimethylammonium chloride ("DODAP"); 3-(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol ("DC-Chol"); N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"); 1,2-dilinoleyl-N,N-dimethyl-3-aminopropane (DLinDMA); 1,2-distearoyloxy-N,N-dimethyl-3-aminopropane (DSDMA); 1,2-dilinoleyl-N,N-dimethyl-3-aminopropane (DLendDMA); 2-[4-[(3b)-cholest-5-en-3-yloxy]butoxy]-N,N-dimethyl-3-[(9Z, 12Z)-octadeca-9,12-dien-1-yloxy]propan-amine (CLinDMA).

[0076] In some embodiments the mixture of lipids may include an anionic lipid. The anionic lipid may be but is not limited to diacylglycerol phosphatidic acid (1,2-distearoyl-sn-glycero-3-phosphate (DSPG); 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA); 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA); 1,2-dilauroyl-sn-glycero-3-phosphate (DLPA); 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA)), diacylglycerol phosphoglycerol (1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG); 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG); 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG); 1,2-dilauroyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DLPG); 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG)), phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, and other anionic modifying groups joined to neutral lipids. The mixture of lipids may also include a neutral lipid. The neutral lipid may be but is not limited to diacylglycerol phosphocholine (L- α -phosphatidylcholine, hydrogenated (Soy) (HSPC); diacylglycerol phosphocholine (L- α -phosphatidylcholine, (Soy) (Soy PC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC); 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC); 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC); 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), diacylglycerol phosphoethanolamine (1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE); 1,2-dimyristoyl-sn-glycero-

3-phosphoethanolamine (DMPE); 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and phosphatidylserine.

[0077] The mixture of lipids may also include a pegylated lipid. The pegylated lipid may be but is not limited to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-2000-DSPE); 1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-2000-DOPE); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-2000-DPPE); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-2000-DMPE); 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-2000-DLPE); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (mPEG-5000-DSPE); 1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (mPEG-5000-DOPE); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (mPEG-5000-DPPE); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (mPEG-5000-DMPE); 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (mPEG-5000-DLPE).

[0078] The mixture of lipid may also include a lipid-like molecule or lipidoid. The mixture of lipid may also include a lipid- or cholesterol-conjugated molecule including a protein, or a peptide, or an oligonucleotide.

[0079] In various embodiments, the lipid layer includes one or more of the lipid components disclosed herein.

[0080] In various embodiments, the lipid layer consists essentially of the unsaturated phospholipid and cholesterol.

[0081] In various embodiments, the lipid layer consists essentially of the unsaturated phospholipid, cholesterol, and pegylated phospholipid.

[0082] In various embodiments, the lipid layer comprises: about 20-75%, preferably about 30-60%, (molar) unsaturated phospholipid; about 10-60%, preferably 20-50%, (molar) cholesterol; and about 0-20%, preferably 1-10%, (molar) pegylated phospholipid.

[0083] In various embodiments, the molar ratio of the lipid layer components:doxorubicin is about 100:1 to about 5:1, preferably about 20:1 to about 10:1; and the molar ratio of the lipid layer components:docetaxel is about 100:1 to about 5:1, preferably about 20:1 to about 10:1.

[0084] In various embodiments, the molar ratio of doxorubicin:docetaxel is about 10:1 to 1:10, preferably about 5:1 to 1:5, and more preferably about 3:1 to 1:3.

[0085] In various embodiments, the unsaturated phospholipid comprises a polyunsaturated phospholipid or a monounsaturated phospholipid, preferably a phosphatidylcholine, and more preferably and soy phosphatidylcholine or 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC).

[0086] In various embodiments, the cholesterol comprises a cholesterol derivative, preferably a cationic cholesterol derivative, more preferably an amino cholesterol derivative, and still more preferably dimethylaminoethanecarbamoyl cholesterol (DC-cholesterol).

[0087] In various embodiments, the pegylated phospholipid comprises a phosphoethanolamine, preferably a 1,2-

distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and wherein the pegylation is a PEG 500 to PEG 5000, preferably PEG 2000.

[0088] In various embodiments, the composition of the lipid layer is tuned to achieve a desired loading of the first drug. Although at least a fraction of the first drug is in the lipid layer, one of ordinary skill will understand that the first drug will have a partition coefficient between the lipid layer and aqueous interior. In some embodiments, essentially all of the first drug will be in the lipid layer.

[0089] The aqueous solution of the process preferably includes water and a buffer. Buffers may be of but are not limited to phosphate, histidine, HEPES, Tris, acetate, carbonate, and citrate. In various embodiments, the composition of the aqueous solution is tuned to achieve a desired loading (and/or crystallization) of the second drug. Although at least a fraction of the second drug is in the aqueous interior of the liposome, one of ordinary skill will understand that the second drug will have a partition coefficient between the lipid layer and aqueous interior. In some embodiments, essentially all of the second drug will be in the aqueous interior.

[0090] Methods for Making Liposomes

[0091] Examples of apparatuses and methods that can be adapted for making the liposomes of the invention can be found, for example, in U.S. patent application Ser. No. 14/209,187 (and published as US20140348900), which is herein incorporated by reference in its entirety. A description of a number of different methods of making liposomes in accordance with the invention are presented in the Examples below.

[0092] The invention provides a method of making the liposome according to any of the aspects or embodiments disclosed herein, or the pharmaceutical composition according to any of the aspects or embodiments disclosed herein, comprising: (i) introducing a lipid solution of an unsaturated phospholipid, cholesterol, a first drug (e.g., docetaxel), and preferably a pegylated phospholipid in ethanol through a first port into a mixing chamber and an aqueous solution through a second port into the mixing chamber, thereby making a plurality of liposomes; and (ii) incubating the plurality of liposomes in a second drug (e.g., doxorubicin) solution.

[0093] In various embodiments, the angle between at least one lipid and at one aqueous solution inlet ports is not 180° or a substantially similar angle. In some aspects, at least one stream of lipid solution and at one stream of aqueous solution collide at an angle less than about 180°. Thus, in some aspects, the method does not include a T-connector.

[0094] In some embodiments, the angle between at least one lipid and at one aqueous solution inlet ports is about 120° or less, e.g., 115° or less, 100° or less, 90° or less, 80° or less, 72° or less, 60° or less, 45° or less, 30° or less, 18° or less,

[0095] In some embodiments, the aqueous solution in step ii) is introduced via at least two inlet ports, e.g., at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more. In some embodiments, the aqueous solution in step ii) is introduced via at least 3 but no more than 11 inlet ports, e.g., at least 3 but not more than 7, at least 3 but no more than 5, at least 4 but no more than 11, at least 5 but no more than 11, at least 6 but no more than 11.

[0096] In some embodiments, at least two (e.g., 3, 4, 5, 6, 7, etc.) aqueous inlet ports and at least one (e.g., 2, 3, 4, 5, etc.) lipid solution inlet port are in the same plane.

[0097] In some embodiments, at least one (e.g., 2) outlet port is substantially perpendicular to the plane of inlet ports.

In other embodiments, at least one (e.g., 2, 3, 4, 5, etc.) outlet port is substantially not perpendicular to the plane of inlet ports.

[0098] In some embodiments, at least two (e.g., 3, 4, 5, 6, 7, etc.) aqueous solution inlet ports and at least one (e.g., 2, 3, 4, 5, etc.) lipid solution inlet port are not in the same plane.

[0099] **Preparing Lipid Solutions**

[0100] The lipid solution may be made from the stock solutions of individual lipids that are mixed together. Lipids are preferably dissolved in an organic solvent to make a lipid solution. The organic solvent used for making the lipid solution may be miscible with water. Preferably the solvent may be ethanol, methanol, DMSO, propanol, DMF, THF, acetone, dioxane, ethylene glycol, polyethylene glycol and isopropanol. More preferably, the solvent is polyethylene glycol, isopropanol, and ethanol. Preferably, the solvent includes less than 10% water. In some cases, the lipid solution may be made from a mixture of lipids, thereupon dissolving the mixture in an organic solvent. The concentration of the total lipids in the solution may be in the range from about 1 mg/mL to about 200 mg/mL, e.g., from about 1 mg/mL to about 100 mg/mL. More preferably, the concentration of the total lipids in the solution may be in the range from about 5 mg/mL to about 100 mg/mL or from about 10 mg/mL to 100 mg/mL. In some embodiments, the organic solvent is ethanol at a concentration of about 70% or more (e.g., 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 100%).

[0101] The mixture of lipids will be optimized as required for optimal delivery of the API and is readily optimized by routine experimentation by one of ordinary skill in the art.

[0102] In certain embodiments, a water-insoluble API may be dissolved in the lipid solution. The concentration of the API in the lipid solution will depend on the efficacy of the agent and may easily be determined by one of ordinary skill in the art. The lipid/API ratio will be determined by the encapsulation power of the liposome to the API.

[0103] **Preparing Aqueous Solutions**

[0104] A water-soluble API component may be dissolved in a first aqueous solution (S1). The pH and salinity of the solution may be optimized to accommodate the requirements for the interaction between the API component and the lipids to form liposome. These conditions may be readily determined by one of ordinary skill in the art. Samples are provided in the Examples below. As will be readily apparent to those of skill in the art, an aqueous solution that lacks an API, referred to as (S2), may be similar to a solution having the agent. Alternatively, S1 and S2 may be different.

[0105] **Liposome Preparation, Mixing the Solutions**

[0106] The lipid solution and the aqueous solution(s) preferably enter the manifold from different ports, each with a flow rate of from about 1 mL/min to about 6000 mL/min. Preferably, the flow rates may be from about 5 mL/min to about 1000 mL/min. More preferably, the rates may be from about 20 mL/min to about 600 mL/min. In some embodiments, the flow rates are adjusted based on the size of inlet ports to obtain the desired liposome size, morphology, PDI, and manufacturing scales.

[0107] In some embodiments, the lipid solution and/or the aqueous solution is introduced via port size of 0.1-5.0 mm at a flow rate about 1 mL/min to about 2,500 mL/min.

[0108] In some embodiments, the flow velocity of the lipid solution and/or the aqueous solution is from about 0.02 m/s to about 40 m/s, e.g., from 0.1 m/s to 30 m/s, from 0.2 m/s to

20m/s. The flow velocity is adjusted based on the size of inlet ports to obtain the desired liposome size, morphology, PDI, and manufacturing scale.

[0109] **Loading of the API Into Liposome**

[0110] In the mixing chamber the lipids are believed to instantaneously assemble into liposome particles. When the drug API is carried by the lipid solution or by aqueous solution, it may be encapsulated in the liposome by either lipophilic or electrostatic interaction, or both, between the API and the lipids.

[0111] The present invention also provides a method of producing liposome that do not contain an API (so-called "empty" liposome). In such embodiments, the API is absent from both the lipid solution and the aqueous solution that are mixed in the manifold. The API may be loaded into the liposomes by the process of diffusion or another process. For example, doxorubicin may be loaded into the liposome with a pH gradient. See U.S. patent application Ser. No. 10/019, 200, PCT Publication No. WO 2001/005373, U.S. Pat. Nos. 5,785,987, 5,380,531, 5,316,771, and 5,192,549, all of which are incorporated herein by reference.

[0112] Preferably, the API is mixed with a liposome solution to upload the API into the liposome by diffusion. In one aspect, the API is dissolved in an aqueous solution, and the solution is mixed with the empty liposome. In another aspect, the API may be readily soluble in the solution of empty liposome, and therefore, the API may be directly mixed with the solution of the empty liposome.

[0113] The volume ratio of the solution of the API to the empty liposome solution of the API is preferably in the range from about 1:50 to about 1:1. A lower volume of the solution is preferred because it avoids a significant dilution to the final liposome solution.

[0114] The drug encapsulation efficiency is preferably greater than 70%. More preferably the efficiency is greater than 80%. Most preferably, the efficiency is greater than 90%.

[0115] **Liposome Concentration Adjustment**

[0116] Tangent flow filtration may be used to concentrate the liposome solution.

[0117] **Buffer Change**

[0118] Residual organic solvent in the liposome solution may be removed by a buffer change. Preferably, the buffer change is performed by tangent flow filtration. In another embodiment, the buffer change may be performed by dialysis.

[0119] **Sterile Filtration**

[0120] The liposome solutions can be sterilized, for example, by passing the solution through a 0.22 micron sterile filter.

[0121] **Liposomes**

[0122] In various embodiments, the Z-average particle size of the liposomes is about 10-200 nm, preferably about 15-150 nm, and more preferably about 20-120 nm.

[0123] Preferably, more than 70% of API is encapsulated in the liposomes. More preferably, more than 80% of API is encapsulated in the liposomes, most preferably, more than 90% of API is encapsulated in the liposomes.

[0124] Optionally, liposomes can be unilamellar. Alternatively, the liposomes can be of multilamellar, or of inverted hexagonal or cubic morphology, or as lipid discs, or hollow liposomes.

[0125] In some embodiments, the mean particle size of the liposomes is from about 10 nm to about 2,000 nm, preferably less than 300 nm, more preferably, the mean particle size may

be about 10 to 300 nm or about 20 to about 300 nm. Most preferably, the mean particle size is about 20 to 120 nm. In some embodiments, the liposomes have a polydispersity index from about 0.005 to about 0.8, e.g., 0.005 to about 0.5, 0.01 to about 0.5, 0.01 to about 0.4, 0.01 to about 0.2.

[0126] Pharmaceutical Compositions

[0127] In various embodiments, the pharmaceutical composition is for use as a medicament. In various embodiments, the pharmaceutical composition is for use as a cancer therapeutic. In various embodiments, the pharmaceutical composition can include one or more antibiotic, antivirus, anti-diabetes, anti-hypertension, anti-fungal, or analgesic.

[0128] In various embodiments, the plurality of liposomes are comprised in an injectable formulation, for example, by subcutaneous, intravenous, intramuscular, intrathecal or intraperitoneal injection. Injectable formulations can be aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. The injectable formulation can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the liposomes can be in a dried or powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[0129] Treatment and Administration

[0130] The invention provides a method comprising administering the liposome according to any of the aspects or embodiments disclosed herein, or the pharmaceutical composition according to any of the aspects or embodiments disclosed herein, to a subject.

[0131] The invention also provides a method of treating a subject comprising administering an effective amount of the liposome according to any of the aspects or embodiments disclosed herein, or the pharmaceutical composition according to any of the aspects or embodiments disclosed herein, to a subject.

[0132] Accordingly, the invention provides methods for treating cancer cells and/or tissue, including cancer cells and/or tissue in a human subject. Cancer can be caused by malignant tumors formed by an abnormal growth of cells and tissue leading to organ failure.

[0133] Solid tumors can be neoplasms (new growth of cells) or lesions (damage of anatomic structures or disturbance of physiological functions) formed by an abnormal growth of body tissue cells other than blood, bone marrow or lymphatic cells. A solid tumor consists of an abnormal mass of cells which may stem from different tissue types such as liver, colon, breast, or lung, and which initially grows in the organ of its cellular origin. However, such cancers may spread to other organs through metastatic tumor growth in advanced stages of the disease.

[0134] The subject being treated may have been diagnosed with cancer. The subject may have locally advanced, unresectable, or metastatic cancer and/or may have failed a prior first-line therapy. In various embodiments, the cancer is liver cancer (e.g., hepatocellular carcinoma, HCC). In various embodiments, the liver cancer (e.g., HCC) can be intermediate, advanced, or terminal stage. The liver cancer (e.g., HCC) can be metastatic or non-metastatic. Liver cancer can include a liver tumor resulting from the metastasis of a non-liver cancer, to the liver. The liver cancer (e.g., HCC) can be resectable or unresectable. The liver cancer (e.g., HCC) can comprise a single tumor, multiple tumors, or a poorly defined tumor with an infiltrative growth pattern (into portal veins or hepatic veins). The liver cancer (e.g., HCC) can comprise a

fibrolamellar, pseudoglandular (adenoid), pleomorphic (giant cell), or clear cell pattern. The liver cancer (e.g., HCC) can comprise a well differentiated form, and tumor cells resemble hepatocytes, form trabeculae, cords, and nests, and/or contain bile pigment in cytoplasm. The liver cancer (e.g., HCC) can comprise a poorly differentiated form, and malignant epithelial cells are discohesive, pleomorphic, anaplastic, and/or giant. In some embodiments, the liver cancer (e.g., HCC) is associated with hepatitis B, hepatitis C, cirrhosis, or type 2 diabetes.

[0135] In various embodiments, the cancer is a lung cancer, preferably non-small cell lung cancer (NSCLC); colon cancer; breast cancer; or liver cancer, preferably hepatocellular carcinoma (HCC).

[0136] In various embodiments, the docetaxel can be in a concentration of 10, 20, 30, 40, 50, 75, 80, 100, 125, 150, or 160 mg/mL. A dose can be about 10 mg/m² to 150 mg/m² (e.g., 10, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90, 100, 110, 120, 125, 130, 140, or 150 mg/m²). For example, a dose can be 75 mg/m². A dose can be administered every 3 weeks for 1, 2, 3, 5, 5, or 6 cycles. One skilled in the art will appreciate that dosing guidelines for docetaxel are known in the art, and can be adapted based upon factors including, but not limited to the cancer type, the cancer stage, the dosing regimen, the dose of doxorubicin, and/or the efficacy of the pharmaceutical formulations of the invention.

[0137] In various embodiments, the doxorubicin can be in a concentration of 0.1, 0.5, 1, 1.5, 2, 3, 4, or 5 mg/mL. A dose can be about 1 mg/m² to 100 mg/m² (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90, or 100 mg/m²). For example, a dose can be 30 mg/m². A dose can be administered every 3 weeks for 1, 2, 3, 5, 5, or 6 cycles. One skilled in the art will appreciate that dosing guidelines for docetaxel are known in the art, and can be adapted based upon factors including, but not limited to the cancer type, the cancer stage, the dosing regimen, the dose of doxorubicin, and/or the efficacy of the pharmaceutical formulations of the invention.

[0138] The following examples are illustrative and not restrictive. Many variations of the technology will become apparent to those of skill in the art upon review of this disclosure. The scope of the technology should, therefore, be determined not with reference to the examples, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

EXAMPLES

Example 1

Preparation of Liposomal Formulation CPT307C

[0139] CPT307 comprises of a nonsaturated lipid 1,2-Dioleoyl-sn-glycero-3-Phosphatidylcholine (DOPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy9polyethyleneglycol]-2000] (mPEG2000-DSPE). It was found that compared the saturated lipid, the nonsaturated lipid has a greater capacity to encapsulate docetaxel. Liposomal formulation CPT307B was prepared by first dissolving 2100 mg of DOPC, 280 mg of cholesterol, 700 mg of mPEG2000-DSPE, and 175 mg of docetaxel (DOCE) in 70 mL of anhydrous ethanol. The composition (% molar) of the CPT307B lipid solution is illustrated in Table 1. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Twenty milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was

connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass vial. The liposome was concentrated by tangent flow filtration. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by tangent flow filtration. The formulation was then sterilized by filtration through a 0.22 µm filter. The Z-average particle size was 32.9 nm.

[0140] CPT307C was prepared by loading doxorubicin (DXR) into CPT307B. Fourteen milliliters of CPT307B containing 36 mg/mL of DOCE was mixed with 24 mg of DXR that had been pre-dissolved in the histidine/sucrose buffer, and incubated at 42° C. for 3 hours. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 µm filter. The composition (% molar) of the CPT307C lipid solution is illustrated in the Table 1, 99.6% of DXR was encapsulated. The molar ratio of DOCE:DXR was 1:1.

TABLE 1

Lipid Compositions of Example 1.		
Component	CPT307B % (molar)*	CPT307C % (molar)*
DOPC	73.6	73.6
Cholesterol	20	20
mPEG2000-DSPE	6.4	6.4
DOCE	6.0	6.0
DXR	0	6.0

*The value represents the molar % of each component vs. total lipids.

Example 2

Preparation of Liposomal Formulation CPT308C

[0141] Different from CPT307C in Example 1, CPT308C contains a polyunsaturated lipid L- α -phosphatidylcholine (Soy PC) that has a high capacity to encapsulate DOCE. Two milliliters of lipids/DOCE solution was prepared by dissolving 30 mg of Soy PC, 4 mg of cholesterol, 10 mg of mPEG2000-DSPE, and 6 mg of DOCE in anhydrous ethanol. The composition (% molar) of the liposomal formulation CPT308C lipid solution is illustrated in Table 2. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Two milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass vial. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by dialysis. The formulation was then sterilized by filtration through a 0.22 µm filter.

[0142] The DOCE loaded liposome was then mixed with DXR that had been pre-dissolved in the histidine/sucrose buffer at a doxorubicin/lipid ratio (w/w) of 1:10, and incubated at 42° C. for 2 hours, 97% of DXR was encapsulated. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 µm filter. The Z-average particle size of the dual-loaded liposome was 38.2 nm for CPT308C.

TABLE 2

Lipid Composition of Example 2.	
Component	CPT308C % (molar) *
Soy PC	56.8
Cholesterol	38.0
mPEG2000-DSPE	5.2
DOCE	13.5
DXR	10.9*

* The value represents the molar % of each component vs. total lipids.

Example 3

Preparation of Liposomal Formulation CPT309C

[0143] CPT309C contains the polyunsaturated lipid Soy PC at a higher molar ratio than CPT308C in Example 2 and thus showed a greater capacity to encapsulate DOCE. Two milliliters of lipids/DOCE solution was prepared by dissolving 30 mg of L- α -phosphatidylcholine (Soy PC), 4 mg of cholesterol, 10 mg of mPEG2000-DSPE, and 6 mg of DOCE in anhydrous ethanol. The composition (% molar) of the liposomal formulation CPT309C lipid solution is illustrated in Table 3. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Two milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass vial. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by dialysis. The formulation was then sterilized by filtration through a 0.22 µm filter.

[0144] The DOCE loaded liposome was then mixed with DXR that had been pre-dissolved in the histidine/sucrose buffer at a doxorubicin/lipid ratio (w/w) of 1:10, and incubated at 42° C. for 2 hours, 98.8% of DXR was encapsulated. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 µm filter. The Z-average particle size of the dual-loaded liposome was 38.6 nm for CPT309C.

TABLE 3

Lipid Composition of Example 3.	
Component	CPT309C % (molar)*
Soy PC	73.6
Cholesterol	19.7
mPEG2000-DSPE	6.8
DOCE:Lipids	17.5
DXR:Lipids	14.1

* The value represents the molar % of each component vs. total lipids.

Example 4

Preparation of Liposomal Formulation CPT311C

[0145] CPT311C is a cationic liposome as it contains a cationic derivative of cholesterol (DC-cholesterol). It was found that cationic lipids enhances liver-targeting delivery and anti-tumor efficacy of the therapeutic agent in the liposome. Two milliliters of lipids/DOCE solution (liposomal

formulation CPT311B) was prepared in anhydrous ethanol to give concentrations of 15 mg/mL DOPC, 9.3 mg/mL of 3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Cholesterol), 5 mg/mL of mPEG2000-DSPE, and 2.0 mg/mL of DOCE. The composition (% molar) of the CPT311B lipid solution is illustrated in Table 4. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Two milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass vial. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by dialysis. The formulation was then sterilized by filtration through a 0.22 μ m filter. The Z-average particle size was 34.5 nm.

[0146] Liposomal formulation CPT311C was prepared by loading doxorubicin (DXR) into CPT311B. Two milliliters of CPT311B was mixed with 0.5 mg of DXR that had been pre-dissolved in the histidine/sucrose buffer at 10 mg/mL, and incubated at 42° C. for 2 hours, 94.5% of DXR was encapsulated. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 μ m filter. The composition (% molar) of the CPT311C is illustrated in Table 4. The Z-average particle size of the dual-loaded liposome was 34.9 nm for CPT311C.

TABLE 4

Lipid Compositions of Example 4.		
Component	CPT311B % (molar)*	CPT311C % (molar)*
DOPC	50	50
DC-Cholesterol	45	45
mPEG2000-DSPE	5	5
DOCE	6.5	6.5
DXR	0	4.8

*The value represents the molar % of each component vs. total lipids.

[0147] It was found that the cationic surface charge of liposome promotes liposome delivery to the liver. The alternation of the molar ratio of the cationic lipid, for example, DC-cholesterol in the liposome, controls the liver delivery of liposome and the clearance rate from the blood. The following examples (from Examples 5 to Example 7) comprise of DOCE, cholesterol, DC-cholesterol, and mPEG2000-DSPE with increased molar ratio of DC-cholesterol (from 3.9% increased to 15.4%) and decreased cholesterol molar ratio (from 34.4% reduced to 17.8%) while the molar ratio of DOPC and mPEG2000-DSPE remain unchanged or a minorly changed.

Example 5

Preparation of Liposomal Formulation CPT315C

[0148] Two and one half (2.5) milliliters of lipids/DOCE solution was prepared by dissolving 37.5 mg DOPC, 11.3 mg cholesterol, 1.4 mg of 3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Cholesterol), 12.5 mg mPEG2000-DSPE, and 4 mg DOCE in 2.5 mL anhydrous ethanol. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Two and one half (2.5) milliliter of each of the above four solutions was loaded into

a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass vial. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by dialysis.

[0149] The DOCE loaded liposome was then mixed with DXR that had been pre-dissolved in the histidine/sucrose buffer at a doxorubicin/lipid ratio (w/w) of 1:16, and incubated at 42° C. for 2 hours, 96.9% of DXR was encapsulated. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 μ m filter. The Z-average particle size of the dual-loaded CPT315C was 35.1 nm.

TABLE 5

Lipid Composition of Example 5.	
Component	CPT315C % (molar)*
DOPC	56.4
Cholesterol	34.4
DC-Cholesterol	3.9
mPEG2000-DSPE	5.3
DOCE	7.2
DXR	7.3

*The value represents the molar % of each component vs. total lipids.

Example 6

Preparation of Liposomal Formulation CPT317C

[0150] Twenty milliliters of lipids/DOCE solution was prepared by dissolving 600 mg of DOPC, 140 mg of cholesterol, 84 mg of DC-Cholesterol, 200 mg of mPEG2000-DSPE, and 50 mg of DOCE in anhydrous ethanol. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Twenty milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass bottle and then was concentrated by tangent flow filtration. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by tangent flow filtration. The formulation was then sterilized by filtration through a 0.22 μ m filter. Liposomal formulation CPT317B loaded with DOCE was obtained. The Z-average particle size of CPT317B was 37.5 nm.

[0151] Liposomal formulation CPT317C was prepared by loading doxorubicin (DXR) into CPT317B. Five milliliters of CPT317B was mixed with 5.2 mg of DXR, and incubated at 42° C. for 3 hours. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 μ m filter. The composition (% molar) of the CPT317C lipid solution is illustrated in Table 6. 99.8% of DXR was encapsulated.

TABLE 6

Lipid Composition of Example 6.	
Component	CPT317C % (molar)*
DOPC	56.4
Cholesterol	26.7

TABLE 6-continued

Lipid Composition of Example 6.	
Component	CPT317C % (molar)*
DC-Cholesterol	11.6
mPEG2000-DSPE	5.3
DOCE	4.5
DXR	4.5

*The value represents the molar % of each component vs. total lipids.

Example 7

Preparation of Liposomal Formulation CPT319C

[0152] The lipids/DOCE solution was prepared by dissolving 1848 mg of DOPC, 303 mg of cholesterol, 423 mg of DC-Cholesterol, 605 mg of mPEG2000-DSPE, and 154 mg of DOCE in 61.5 mL of anhydrous ethanol. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Twenty milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass bottle and was then concentrated by tangent flow filtration. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by tangent flow filtration. The formulation was then sterilized by filtration through a 0.22 μ m filter to obtain liposomal formulation CPT319B loaded with DOCE.

[0153] Liposomal formulation CPT319C was prepared by loading doxorubicin (DXR) into CPT319B. In a glass bottle 31.4 mg of DXR was dissolved in 30mL of CPT319B. The mixture was incubated at 42° C. for 4 hours, 99.8% of doxorubicin was encapsulated. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 μ m filter. The final composition (% molar) of the CPT317C lipid solution is illustrated in Table 7. The Z-average particle size was 40.7 nm and the molar ratio of DOCE:DXR was 1:1 for CPT319C. The Cryo-TEM images of CPT319C are shown in FIG. 1. The DXR crystals formed inside the liposome can be seen from these images. The negative stained TEM image of CPT319C is shown in FIG. 2, which indicates particle size and homogeneity.

TABLE 7

Lipid Composition of Example 7.	
Component	CPT319C % (molar)*
DOPC	62.8
Cholesterol	17.8
DC-Cholesterol	15.4
mPEG2000-DSPE	4.6
DOCE	3.5
DXR	3.5

*The value represents the molar % of each component vs. total lipids.

Example 8

Preparation of Liposomal Formulation CPT323C

[0154] CPT323C was prepared in the absence of pegylated lipid, thus the pegylated lipid is optional to the formulations.

The lipids/DOCE solution was prepared by dissolving 300 mg of DOPC, 50 mg of cholesterol, 70 mg of DC-Cholesterol, and 25 mg of DOCE in 10 mL of anhydrous ethanol. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Ten milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass bottle and was then concentrated by tangent flow filtration. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by tangent flow filtration. The formulation was then sterilized by filtration through a 0.22 μ m filter.

[0155] Three milliliters of the DOCE loaded liposome was then mixed with 3 mg of DXR that had been pre-dissolved in the histidine/sucrose buffer at 7 mg/mL, incubated at 42° C. for 6 hours, 96.3% of DXR was encapsulated. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 μ m filter to obtain CPT323C. The composition (% molar) of the liposomal formulation CPT323C lipid solution is illustrated in Table 8. The Z-average particle size of the dual-loaded liposome was 49.0 nm for CPT323C.

TABLE 8

Lipid Composition of Example 8.	
Component	CPT323C % (molar)*
DOPC	59.5
Cholesterol	20.1
DC-Cholesterol	20.3
DOCE	4.8
DXR	4.8

*The value represents the molar % of each component vs. total lipids.

Example 9

Preparation of Liposomal Formulation CPT324C

[0156] Different from other exemplary formulations, CPT324C contains a polyunsaturated lipid-Soy PC and a cationic lipid DOTAP other than DC-cholesterol. The lipids/DOCE solution was prepared by dissolving 60 mg of Soy PC, 40 mg of cholesterol, 60 mg of 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 40 mg of mPEG2000-DSPE, and 25 mg of DOCE in 10 mL of anhydrous ethanol. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. Ten milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass bottle and was then concentrated by tangent flow filtration. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by tangent flow filtration. The formulation was then sterilized by filtration through a 0.22 μ m filter.

[0157] Three milliliters of the DOCE loaded liposome was then mixed with 0.95 mg of DXR that had been pre-dissolved in the histidine/sucrose buffer at 7 mg/mL, and incubated at 42° C. for 6 hours. The DOCE and DXR dual-loaded liposome was then sterilized by filtration through a 0.22 μ m filter.

to obtain liposomal formulation CPT324C. The composition (% molar) of the CPT324C lipid solution is illustrated in Table 9. The Z-average particle size of the dual-loaded liposome was 56 nm for CPT324C.

TABLE 9

Lipid Composition of Example 9.	
Component	CPT324C % (molar)*
Soy PC	27.6
Cholesterol	36.9
mPEG2000-DSPE	4.8
DOTAP	30.7
DOCE	11.0
DXR	11.0

*The value represents the molar % of each component vs. total lipids.

Example 10

Preparation of Liposomal Formulation CPT313C

[0158] CPT313C was prepared in the presence of DC-cholesterol and the absence of cholesterol. The lipids/DOCE solution was prepared by dissolving 33 mg of Soy PC, 20.5 mg of DC-Cholesterol, 11 mg of mPEG2000-DSPE, and 4.4 mg of DOCE in 2.2 mL of anhydrous ethanol. In addition, three aqueous solutions of 250 mM ammonium sulfate, pH 6.5 were used. 2.2 milliliter of each of the above four solutions was loaded into a 20 mL syringe. Each syringe was connected to an inlet port of a five-port manifold by tubing. Through the tubing, the solutions in the syringes were pumped into the mixing chamber of the manifold by a syringe pump. The liposome solution exited through an outlet port and was collected in a glass vial. The buffer was changed into a histidine/sucrose buffer (10 mM histidine, 9.2% sucrose, pH 6.5) by dialysis. The formulation was then sterilized by filtration through a 0.22 μ m filter.

[0159] Two milliliters of the DOCE loaded liposome was then mixed with 0.5 mg of DXR that had been pre-dissolved in the histidine/sucrose buffer at 10 mg/mL, and incubated at 42° C. for 2 hours, 90.6% of DXR was encapsulated. The encapsulated liposome was then sterilized by filtration through a 0.22 μ m filter to obtain liposomal formulation CPT313C. The composition (% molar) of the CPT313C lipid solution is illustrated in Table 10. The Z-average particle size of the dual-loaded liposome was 38.7 nm for CPT313C.

TABLE 10

Lipid Composition of Example 10.	
Component	CPT313C % (molar)*
Soy PC	50.4
mPEG2000-DSPE	4.4
DC-Cholesterol	45.2
DOCE	6.5
DXR	6.5

*The value represents the molar % of each component vs. total lipids.

Example 11

Dual-Loaded Liposome CPT319C Augments Efficacy Against Non-Small Cell Lung Cancer (NSCLC)

[0160] Female Balb/c nude mice ranging from 6-8 weeks of age were inoculated subcutaneously on the right flank with

NSCLC cell line A549 tumor cells (1×10^7 cells/mouse) in 0.1 mL phosphate buffered saline (PBS) buffer for tumor development. On Day 16 following tumor cell inoculation (tumor size was approximately 117 mm³), treatments were started with formulations of CPT319A at 5 mg/kg doxorubicin, CPT319B at 7.5 mg/kg docetaxel, CPT319C at 5 mg/kg doxorubicin/7.5 mg/kg docetaxel, or the non-liposomal combination formulation of 5 mg/kg doxorubicin/7.5 mg/kg docetaxel by intravenous (IV) injection through the tail vein. Three additional treatments were administered on Day 20, Day 27, and Day 34. The study was terminated on Day 45. The tumor growth curves and tumor weight inhibition percentages (TW inh %) on Day 45 of the formulations compared to the PBS control group are shown in FIG. 3.

[0161] To summarize, FIG. 3 shows that dual-loaded Liposome CPT319C enhances the antitumor activity of the formulation in NSCLC Xenograft mouse model. All of the liposomal formulations were more efficacious than the non-liposomal combination of DOCE/DXR. In addition, the dual-loaded liposome, CPT319C was the most efficacious formulation in this example. Compared to the PBS control group, CPT319C reduced 76% of the tumor weight that was significantly more efficacious than the 51% of DOCE liposome CPT319B, 27% of DXR liposome CPT319A, and 17% of the non-liposomal combination of DOCE/DXR.

Example 12

Dual-Loaded Liposome CPT307C Augments Efficacy Against Non-Small Cell Lung Cancer (NSCLC)

[0162] Female Balb/c nude mice ranging from 6-8 weeks of age were inoculated subcutaneously at the right flank with NSCLC cell line A549 tumor cells (1×10^7 cells/mouse) in 0.1 mL PBS buffer for tumor development. On Day 16 following tumor cell inoculation (tumor size was approximately 117 mm³), treatments were started with formulations of CPT307A at 5 mg/kg doxorubicin, CPT307B at 7.5 mg/kg docetaxel, CPT307C at 5 mg/kg doxorubicin/7.5 mg/kg docetaxel, or the non-liposomal combination formulation of 5 mg/kg doxorubicin/7.5 mg/kg docetaxel by intravenous (IV) injection through the tail vein. Three additional treatments were administered on Day 20, Day 27, and Day 34. The study was terminated on Day 45. The tumor growth curves and tumor weight inhibition percentages (TW inh %) on Day 45 of the formulations compared to the PBS control group are shown in FIG. 4.

[0163] To summarize, FIG. 4 shows that dual-loaded liposome CPT307C augments efficacy against non-small cell lung cancer (NSCLC). All of the liposomal formulations were more efficacious than the non-liposomal combination of DOCE/DXR. In addition, the dual-loaded liposome, CPT307C was the most efficacious in this example. Compared to the PBS control group, the dual-loaded liposome CPT307C 57% of the tumor weight that is significant more efficacious than the 44% of DOCE liposome CPT307B, 11% of DXR liposome CPT307A, and 17% of the non-liposomal combination of DOCE/DXR.

Example 13

Antitumor Activity of Dual-Loaded Liposome CPT319C Against Human Colon Cancer in Xenograft Mouse Model

[0164] Female Balb/c nude mice ranging from 6-8 weeks of age were inoculated subcutaneously at the right flank with

human colon cancer cell line HCT-116 tumor cells (5×10^6 cells/mouse) in 0.1 mL PBS buffer for tumor development. On Day 9 following tumor cell inoculation (tumor size was approximately 141 mm^3), treatments were started with formulations of CPT319C at 3 different doses: 5 mg/kg doxorubicin/7.5 mg/kg docetaxel, 2.5 mg/kg doxorubicin/3.75 mg/kg docetaxel, or 1.25 mg/kg doxorubicin/1.875 mg/kg docetaxel by intravenous (IV) injection through the tail vein. Two additional treatments were administered on Day 16 and Day 23. The study was terminated on Day 37. The tumor growth curves shown in FIG. 5, which illustrates dose responses of the liposomal formulations in a HCT-116 human colon cancer xenograft model. Compared to the PBS control group, the dual-loaded CPT319C reduced 77% of the HCT-116 tumor size on Day 37 in the 5 mg/kg doxorubicin/7.5 mg/kg docetaxel group, 49% in the group treated with 2.5 mg/kg doxorubicin/3.75 mg/kg docetaxel, and 29% in the group treated with 1.25 mg/kg doxorubicin/1.875 mg/kg docetaxel.

Example 14

Antitumor Activity of Dual-Loaded Liposome CPT307C Against Human Colon Cancer in Xenograft Mouse Model

[0165] Female Balb/c nude mice ranging from 6-8 weeks of age were inoculated subcutaneously at the right flank with human colon cancer cell line HCT-116 tumor cells (5×10^6 cells/mouse) in 0.1 mL PBS buffer for tumor development. On Day 9 following tumor cell inoculation (tumor size was approximately 141 mm^3), treatments were started with formulations of CPT307C at 3 different doses: 5 mg/kg doxorubicin/7.5 mg/kg docetaxel, 2.5 mg/kg doxorubicin/3.75 mg/kg docetaxel, or 1.25 mg/kg doxorubicin/1.875 mg/kg docetaxel by intravenous (IV) injection through the tail vein. Two additional treatments were administered on Day 16 and Day 23. The study was terminated on Day 37. The tumor growth curves shown in FIG. 6, which illustrates dose responses of the liposomal formulations in a HCT-116 human colon cancer xenograft model. Compared to the PBS control group, the dual-loaded CPT307C reduced 67% of the HCT-116 tumor size on Day 37 in the 5 mg/kg doxorubicin/7.5 mg/kg docetaxel group, 61% in the group treated with 2.5 mg/kg doxorubicin/3.75 mg/kg docetaxel, and 45% in the group treated with 1.25 mg/kg doxorubicin/1.875 mg/kg docetaxel.

Example 15

Antitumor Activity of Dual-Loaded Liposome CPT319C and CPT307C Against Human Breast Cancer in Xenograft Mouse Model

[0166] Female Balb/c nude mice ranging from 6-8 weeks of age were inoculated subcutaneously at the right flank with human breast cancer cell line MDA-MB-231 tumor cells (7×10^6 cells/mouse) in 0.1 mL PBS buffer for tumor development. On Day 7 after tumor cell inoculation (tumor size was approximately 174 mm^3), treatments were started with formulations of CPT319C or CPT307C at 5 mg/kg doxorubicin/7.5 mg/kg docetaxel by intravenous (IV) injection through the tail vein. Two additional treatments were made on Day 14 and Day 21. The study was terminated on Day 31. The tumor growth curves were shown in FIG. 7.

[0167] To summarize, FIG. 7 shows CPT319C and CPT307C antitumor activity in a human breast cancer xenograft model. Compared to the PBS control group, the tumor size was reduced 38% and 32% in the group treated by CPT319C and CPT307C, respectively.

Example 16

Antitumor Activity of Dual-Loaded Liposome CPT319C Against Human Primary Hepatocellular Carcinoma in Xenograft Mouse Model

[0168] Female Balb/c nude mice ranging from 6-8 weeks were split up into groups of three. Each mouse was inoculated subcutaneously at the right flank with fragments of human primary hepatocellular carcinoma tumor cells (P3 WP HCC) for tumor development. On Day 32 after tumor inoculation (tumor size was approximately 143 mm^3), treatments were started with a formulation of CPT319C at 5 mg/kg doxorubicin/7.5 mg/kg docetaxel by intravenous (IV) injection through the tail vein. Two additional treatments were made on Day 39 and Day 46. The study was terminated on Day 63. The tumor growth curves are shown in FIG. 8.

[0169] To summarize, FIG. 8 shows CPT319C antitumor activity in a human primary HCC xenograft model. Tumor growth was almost completely inhibited by CPT319C. Specifically, CPT319C inhibited 88% of tumor growth when the study was terminated on Day 63 compared to the vehicle control group.

Example 17

Cationic Lipid DC-Cholesterol Enhances the Antitumor Activity of Liposomes Against NSCLC

[0170] Female Balb/c nude mice ranging from 6-8 weeks of age were inoculated subcutaneously at the right flank with NSCLC cell line A549 tumor cells (1×10^7 cells/mouse) in 0.1 mL PBS buffer for tumor development. On Day 16 following tumor cell inoculation (tumor size was approximately 117 mm^3), treatments were started with formulations of CPT307A or CPT319A at 5 mg/kg doxorubicin, CPT307B or CPT319B at 7.5 mg/kg docetaxel, CPT307C or CPT319C at 5 mg/kg doxorubicin/7.5 mg/kg docetaxel, or the non-liposomal combination formulation of 5 mg/kg doxorubicin/7.5 mg/kg docetaxel by intravenous (IV) injection through the tail vein. Three additional treatments were made on Day 20, Day 27, and Day 34. The study was terminated on Day 45. The lipid compositions of the CPT307 (without DC-Cholesterol) and CPT319 (with DC-Cholesterol) formulations are shown in Table 11. The tumor growth curves are shown in FIG. 9.

[0171] To summarize, FIG. 9 shows that a cationic lipid DC-cholesterol can enhance the antitumor activity of the liposomes against NSCLC. The tumor inhibition rank order (from low to high) was: PBS < CPT307A < CPT319A < CPT307B < CPT319B < CPT307C < CPT319C. For each instance, CPT319 (with DC-Cholesterol) was no exceptionally more efficacious than CPT307 (without DC-Cholesterol) indicating that the incorporation of the cationic lipid DC-cholesterol enhances the anti-tumor efficiency of the liposomal formulations.

TABLE 11

Lipid Compositions of Example 17.				
Formulation	DC-cholesterol	cholesterol	mPEG-DSPE	DOPC
CPT319	15	18	5	62
CPT307	0	24	6	70

Example 18

Cationic Lipid DC-Cholesterol Enhances the Antitumor Activity of Liposomes Against Colon Cancer

[0172] Female Balb/c nude mice ranging from 6-8 weeks of age were inoculated subcutaneously at the right flank with human colon cancer cell line HCT-116 tumor cells (5×10^6 cells/mouse) in 0.1 mL PBS buffer for tumor development. On day 9 following tumor cell inoculation (tumor size was approximately 141 mm^3), treatments were started with formulations of CPT319C (containing DC-Cholesterol) or CPT307C (without DC-Cholesterol) at 5 mg/kg DXR/7.5 mg/kg DOCE by intravenous (IV) injection through the tail vein. Two additional treatments were made on Day 16 and Day 23. The study was terminated on Day 37. The tumor growth curves and tumor weight inhibition percentages (TW Inh %) on Day 37 of the formulations compared to the vehicle control group are shown in FIG. 10.

[0173] To summarize, FIG. 10 shows that a cationic lipid DC-cholesterol can enhance the antitumor activity of the liposomes against colon cancer. CPT319C (with DC-Cholesterol) was more efficacious than CPT307C (without DC-Cholesterol), indicating that the incorporation of the cationic lipid DC-cholesterol enhances the anti-tumor efficiency of the liposomal formulations.

Example 19

Liposome Improves Pharmacokinetics (PK) and Cationic Lipid DC-Cholesterol Increases the Half-Life ($t_{1/2}$) of DXR

[0174] Male CD-1 mice ranging from 20-25 g body weight were split up into groups of three. Each mouse was administered with a single dose of CPT319C or CPT307C at 5 mg/kg DXR/7.5 mg/kg DOCE by intravenous (IV) injection through the tail vein. A non-liposomal combination of DXR/DOCE was used as the control. Blood samples were collected at 0.167, 1, 3, 8, 24, and 48h after the injection. DXR plasma concentration was determined by liquid chromatography-tandem mass spectrometry. The plasma concentration curves of DXR are shown in FIG. 11.

[0175] To summarize, FIG. 11 shows that a liposome can improve pharmacokinetics and that a cationic lipid DC-cholesterol increases the half-life ($t_{1/2}$) of DXR. BLOQ=Below Limit of Quantitation. The $t_{1/2}$ and area under the plasma concentration time curve (AUC) are provided in the table below. The non-liposomal DXR was cleared quickly from the blood and resulted in a very low AUC (688 $\text{h} \times \text{ng}/\text{mL}$), whereas CPT319C and CPT307C increased AUC by 143 and 204 fold, respectively. Moreover, CPT319C exhibited a 5.9 h $t_{1/2}$ compared to the 1 h $t_{1/2}$ of CPT307C, indicating that the cationic lipid DC-Cholesterol in CPT319C improves PK of the formulation by increasing circulation time in the blood.

TABLE 12

$t_{1/2}$ and AUC of DXR in CD-1 mice		
Formulation	$t_{1/2}$ (h)	AUC ($\text{h} \times \text{ng}/\text{mL}$)
CPT309C	5.9	98628
CPT307C	1.0	140398
Naked Dox		688

1. A liposome comprising:
an active pharmaceutical ingredient (API) comprising docetaxel and doxorubicin;
a lipid layer comprising an unsaturated phospholipid and a cholesterol; and
an aqueous interior,
wherein the docetaxel is in the lipid layer and the doxorubicin is crystallized in the aqueous interior.
2. The liposome of claim 1, wherein the lipid layer consists essentially of the unsaturated phospholipid and cholesterol.
3. The liposome of claim 1, wherein the lipid layer consists essentially of the unsaturated phospholipid, cholesterol, and a pegylated phospholipid.
4. The liposome of claim 1, wherein the API consists essentially of docetaxel and doxorubicin.
5. The liposome of claim 1, wherein the lipid layer comprises:
about 20-75% (molar) unsaturated phospholipid;
about 10-60% (molar) cholesterol; and
about 0-20% (molar) pegylated phospholipid.
6. The liposome of claim 1, wherein:
the molar ratio of the lipid layer components:doxorubicin is about 100:1 to about 2:1; and
the molar ratio of the lipid layer components:docetaxel is about 100:1 to about 2:1.
7. The liposome of claim 1, wherein the molar ratio of doxorubicin:docetaxel is about 10:1 to 1:10.
8. The liposome of claim 1, wherein the unsaturated phospholipid comprises a polyunsaturated phospholipid or a monounsaturated phospholipid.
9. The liposome of claim 1, wherein the cholesterol comprises a cationic cholesterol derivative.
10. The liposome of claim 1, wherein the lipid layer further comprises a pegylated phospholipid.
11. The liposome of claim 1, comprised in a pharmaceutical composition comprising a plurality of liposomes according to claim 1.
12. The liposome of claim 11, wherein the plurality of liposomes are comprised in an intravenous formulation.
13. The liposome of claim 11, wherein the Z-average particle size of the plurality of liposomes is about 10-200 nm.
14. The liposome of claim 11, wherein, upon intravenous administration to a subject, at least about 10% of the composition is delivered to the liver.
15. (canceled)
16. (canceled)
17. A method comprising administering the liposome of claim 1 to a subject.
18. The method of claim 17, wherein the subject has a cancer.
19. The method of claim 17, wherein the cancer is a lung cancer; colon cancer; breast cancer; stomach cancer, esophagus cancer, prostate cancer, leukemia, head and neck cancer, pancreatic cancer, multiple myeloma, or liver cancer.

20. A method of making the liposome of any of claim **1**, comprising:

concurrently introducing a lipid solution of an unsaturated phospholipid, cholesterol, and docetaxel in ethanol through a first or plural inlet port of a manifold into a mixing chamber of the manifold and an aqueous solution through a second or plural inlet port of the manifold into the mixing chamber of the manifold into the mixing chamber, and the liposomes formed exit the mixing chamber through one or plural outlet chambers of the manifold, thereby making a plurality of liposomes; and incubating the plurality of liposomes in a doxorubicin solution.

21. The liposome of claim **9**, wherein the pegylated phospholipid comprises a 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and wherein the pegylation is PEG 2000.

22. The method of claim **20**, wherein the first lipid solution further comprises a pegylated phospholipid.

* * * *



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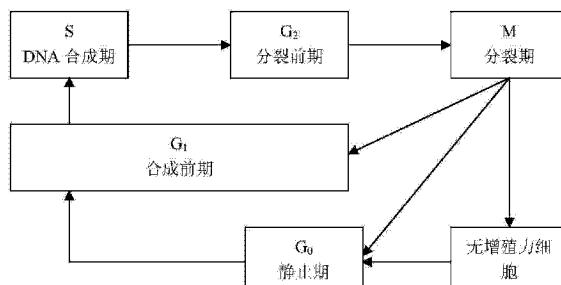
权利要求书1页 说明书13页 附图1页

(54) 发明名称

盐酸多柔比星 - 多西他赛或紫杉醇脂质体制
剂及其制备方法

(57) 摘要

本发明所采用的技术方案为，一种盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂，所述的脂质体同时包封水溶性药物和脂溶性药物，水溶性药物包裹在脂质体内环境中，脂溶性药物包裹在脂质体的双层磷脂膜之间；其中所述的水溶性药物为盐酸多柔比星，所述的脂溶性药物为多西他赛或紫杉醇。本发明的有益效果是：通过实验证明本发明盐酸多柔比星脂质体 - 多西他赛或紫杉醇脂质体毒副作用明显减，并增强其抗肿瘤的治疗功能；本发明的脂质体药物包裹率可达 90% 以上，本发明冻干粉在 2℃ -8℃ 下的两年稳定性实验结果表明其平均粒径变化率小于 6%，药物包裹率的变化率小于 5%，显示了本发明盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂优异的稳定性。



1. 一种盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂, 其特征在于 : 所述的脂质体同时包封水溶性药物和脂溶性药物, 具体为 : 水溶性药物包裹在脂质体内环境中, 脂溶性药物包裹在脂质体的双层磷脂膜之间 ; 其中所述的水溶性药物为盐酸多柔比星, 所述的脂溶性药物为多西他赛或紫杉醇。

2. 根据权利要求 1 所述的盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂, 其特征在于 : 所述的脂质体配方如下 : 盐酸多柔比星、多西他赛或紫杉醇、中性磷脂、带电荷磷脂、胆固醇、抗氧剂、糖类和缓冲剂 ;

其中所述的盐酸多柔比星与紫杉醇的质量比为 1:4 ~ 1:8 ; 盐酸多柔比星与多西他赛的质量比为 1:2 ~ 1:3 。

3. 根据权利要求 2 所述的盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂, 其特征在于 : 所述的盐酸多柔比星和中性磷脂的质量比为 0.01:1 ~ 0.1:1 , 带电荷磷脂和中性磷脂的质量比为 0.01 ~ 0.2:1 ; 胆固醇与中性磷脂质量比为 0 ~ 0.3:1 ; 抗氧剂与中性磷脂质量比为 0 ~ 0.02:1 。

4. 根据权利要求 2 所述的盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂, 其特征在于 : 所述的糖为乳糖、麦芽糖、蔗糖、葡萄糖、海藻糖或蔗糖中一种或多种。

5. 根据权利要求 2 所述的盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂, 其特征在于 : 所述的缓冲剂为氨基酸、无机盐或有机酸碱中一种或多种。

6. 根据权利要求 2 所述的盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂, 其特征在于 : 所述的中性磷脂为蛋黄卵磷脂、氢化蛋黄卵磷脂、双硬脂酸卵磷脂、大豆卵磷脂、氢化大豆卵磷脂、双软脂酸卵磷脂或双肉豆蔻酸卵磷脂 ;

所述的带电荷磷脂为带负电荷磷脂或带正电荷磷脂 ; 其中负电荷磷脂选自双肉豆蔻酸磷脂酰甘油、双月桂酸磷脂酰甘油、双软脂酸磷脂酰甘油、双硬脂酸磷脂酰甘油、双肉豆蔻酸磷脂酸、双硬脂酸磷脂酸、双月桂酸磷脂酸、双软脂酸磷脂酸、双油酸磷脂酰丝氨酸或双亚油酸磷脂酰肌醇其中一种或多种 ;

所述带正电荷磷脂选自二棕榈酰基磷脂酰基乙基氨基-L-赖氨酸、二油酸磷脂酰丝氨酸、二硬脂酰磷脂酸或十八胺中一种或多种。

7. 根据权利要求 1 ~ 6 任一项所述的盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂的制备方法, 其特征在于 : 步骤如下 :

1) 制备多西他赛或紫杉醇脂质体 : 根据配方选用多西他赛或紫杉醇、中性磷脂、带电荷磷脂、胆固醇和抗氧剂溶于氯仿或氯仿 - 甲醇溶剂中, 混合均匀 ; 用旋转蒸发仪将溶液中溶剂减压除去, 形成脂质薄膜 ; 加入缓冲体系水化, 水化温度一般在 40°C ~ 70°C 之间, 得多西他赛或紫杉醇脂质体悬浮液 ;

2) 均化脂质体 : 水化完全后用高压均粒机制备脂质体至所需粒径和均匀度或通过挤压设备把空白脂质体悬浮液在压力下挤过相应孔径的微孔膜来达到, 脂质体的粒径控制在 50~300nm, 脂质体粒径和均匀度用多角度纳米粒子分析器来检测 ;

3) 将盐酸多柔比星溶于糖类水溶液中加热至 40 ~ 50°C , 采用硫酸铵梯度方法, 对盐酸多柔比星进行包裹 ;

4) 定容、除菌、分装、保存 : 用注射用水定容 ; 将盐酸多柔比星脂质体悬浮液用微孔膜过滤除菌, 分装即得成品, 成品可在 2°C ~ 8°C 下保存或冻干保存至使用。

盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂及其制备方法

技术领域

[0001] 本发明涉及一种药物脂质体，尤其是一种盐酸多柔比星 - 多西他赛或紫杉醇脂质体。

背景技术

[0002] 盐酸多柔比星(DOX-HCL)为蒽环类广谱抗肿瘤抗生素盐酸多柔比星的盐酸盐，其作用机制主要是盐酸多柔比星分子进入细胞核与DNA结合，从而抑制核酸的合成和有丝分裂。盐酸多柔比星具有广谱的抗实验性肿瘤的作用，对拓扑异构酶也有抑制作用，可适用于急性白血病，恶性淋巴瘤，多发性骨髓瘤，肺癌，乳腺癌，膀胱癌，睾丸癌，甲状腺癌，软组织肿瘤，骨肉瘤，神经母细胞癌等，也可用于胃癌，结肠直肠癌，肝癌，食管癌，卵巢癌，宫颈癌等。虽然盐酸多柔比星抗癌谱广，疗效好，但是由静脉注射后毒副作用较大，主要包括恶心，呕吐，骨髓抑制，严重脱发等，临幊上出现的毒副作用还有厌食、胃炎甚至溃疡、口腔粘膜炎症等。而且，当累积剂量大时会导致严重的心脏损害，表现为心动过速、心律失常及充血性心力衰竭等。上述毒副作用严重限制了盐酸多柔比星的临床应用。

[0003] 多西他赛为半合成的紫杉醇类抗肿瘤药，通过干扰细胞有丝分裂和分裂间期细胞功能所必需的微管网络而起抗肿瘤作用。多西他赛可与游离的微管蛋白结合，促进微管蛋白装配成稳定的微管，同时抑制其解聚，导致丧失了正常功能的微管束的产生和微管的固定，从而抑制细胞的有丝分裂。多西他赛与微管的结合不改变原丝的数目。这一点与目前临幊应用的大多数纺锤体毒性药物不同。多西他赛的抗瘤活性强于紫杉醇，并和紫杉醇没有交叉耐药。

[0004] 多西他赛($\leqslant 10 \mu\text{mol/L}$)诱导人乳腺癌Bcap37细胞凋亡，使细胞周期阻滞在C2和M期，从而抑制癌细胞的有丝分裂和增殖。本品在细胞内浓度比紫杉醇高3倍，并在细胞内滞留时间长，这是本品在体外试验中比紫杉醇抗肿瘤活性大的重要原因。在体内试验中，对小鼠的结肠癌、乳腺癌、肺癌、卵巢肿瘤移植植物等有效。对顺铂、足叶乙苷、5Fu、或紫杉醇耐药的细胞株，本品不产生交叉耐药。

[0005] 紫杉醇是一种新型的抗微管药物，具有广谱抗癌作用。当它在载体脂质体的靶向下，离开血液循环到达肿瘤组织后，通过与肿瘤细胞中 β -微管蛋白N端第31位氨基酸和第217-231位氨基酸结合，促进微管蛋白的聚合，使微管蛋白聚合形成对热和钙稳定的微管聚合物，再以非共价键化学计量地与聚合的微管的亚基结合，阻碍微管束的正常动态再生，并抑制聚合物正常的生理性解聚，从而使游离的微管数量显著减少，起到干扰有丝分裂纺锤体装配的作用，抑制纺锤体向两级分离，阻断肿瘤细胞的有丝分裂(微管是真核细胞的一个组成部分，其功能为构成细胞的网状支架、维持细胞的形态、参与洗白器的位移和胞内物质运输等，纺锤体和纺锤丝的分离需要这些微管的协助)。

[0006] 进行有丝分裂的纺锤体和纺锤丝受到冻结，导致肿瘤细胞的分裂和增殖受抑制，停止于G2分裂期和M期(肿瘤细胞的增殖示意图见图1)，许多研究证实阻滞于M期的细胞

有明显的凋亡倾向。最终快速分裂的肿瘤细胞因出现生长抑制进而死亡。换言之，紫杉醇通过停止肿瘤细胞有事分裂过程，终端肿瘤细胞的生长，促使癌细胞萎缩而发生凋亡，最终抑制了肿瘤的生长，起到了抗癌作用。紫杉醇脂质体抑制肿瘤细胞生长的过程示意图如图2所示。

[0007] 脂质体(Liposome)最初是由英国学者 Bangham 和 Standish 将磷脂分散在水中进行电镜观察时发现的。磷脂分散在水中自然形成多层囊泡，每层均为脂质的双分子层；囊泡中央和各层之间被水相隔开，双分子层厚度约为 4 纳米。后来，将这种具有类似生物膜结构的双分子层小囊称为脂质体。脂质体可分为多室脂质体和单室脂质体。单室脂质体又分为小单室脂质体和大单室脂质体。Bangham 和 Standish 最初发现的就是多室脂质体，它们较易制备，尺寸一般为佳 0.5-5 微米。小单室脂质体为球形，尺寸一般为佳 20-50 纳米；大单室脂质体的尺寸为微米数量级。1971 年英国莱门等人开始将脂质体用于药物载体，主要作用机理是将药物粉末或溶液包裹在脂质体双层脂质膜所封闭的水相中或嵌入脂质体双层脂质膜中，这种微粒具有类细胞结构，进入人体内主要被网状内皮系统吞噬而激活机体的自身免疫功能，并改变被包封药物的体内分布，使药物主要在肝、脾、肺和骨髓等组织器官中积蓄，从而提高药物的治疗指数，减少药物的治疗剂量和降低药物的毒性。目前用作药物载体的脂质体多为大单室脂质体，粒径一般位于 100-1000 纳米之间。70 年代中后期，人们开始研究将脂质体用作蒽环类抗癌药物，包括盐酸多柔比星，的有效载体，80 年代末已有临床实验开始进行，到 90 年代中期欧美已有脂质体药物上市。当盐酸多柔比星被包裹在特定的脂质体再通过静脉注射进入人体后，临床实验结果表明其毒副作用明显减少，药物半衰期显著延长，但其药效却并不减弱反而得到加强。

[0008] 目前国内外在肿瘤治疗领域常用盐酸多柔比星与多西他赛或紫杉醇普通制剂联合用药，用于治疗乳腺癌、非小细胞肺癌并且成为医疗规范。但由于多西他赛或紫杉醇普通制剂中存在表面活性剂和有机溶剂，对与盐酸多柔比星复配时存在不稳定因素，且毒副作用大。

发明内容

[0009] 本发明克服现有技术中盐酸多柔比星与多西他赛或紫杉醇普通制剂联合用药存在毒副作用大的不足，提供一种盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂及其制备方法。

[0010] 为解决上述技术问题，本发明所采用的技术方案为，一种盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂，所述的脂质体同时包封水溶性药物和脂溶性药物，具体为：水溶性药物包裹在脂质体内环境中，脂溶性药物包裹在脂质体的双层磷脂膜之间；其中所述的水溶性药物为盐酸多柔比星，所述的脂溶性药物为多西他赛或紫杉醇。

[0011] 本发明很好利用多西他赛或紫杉醇脂溶性药物包裹在脂质体双层膜之间，水溶性药物盐酸多柔比星类药物包裹在脂质体内环境中，形成一个脂质体球中最里层是盐酸多柔比星类药物的胶体，然后覆盖单层磷脂膜，膜亲水性部分朝向盐酸多柔比星，亲脂性部分朝向多西他赛或紫杉醇层，在多西他赛或紫杉醇层外层再覆盖一层磷脂膜，此处膜亲脂性部分朝向多西他赛或紫杉醇层，亲水性部分朝向外层的“三明治”结构。能够有效降低盐酸多柔比星、多西他赛和紫杉醇的毒副作用，同时使其药效增加。

[0012] 进一步地，盐酸多柔比星、多西他赛或紫杉醇、中性磷脂、带电荷磷脂、胆固醇、抗氧剂、糖类和缓冲剂；

[0013] 其中所述的盐酸多柔比星与紫杉醇的质量比为 1:4 ~ 1:8；盐酸多柔比星与多西他赛的质量比为 1:2 ~ 1:3。

[0014] 作为优选，所述的盐酸多柔比星和中性磷脂的质量比为 0.01:1 ~ 2:1，带电荷磷脂和中性磷脂的质量比为 0.05:5 ~ 2:5；胆固醇与中性磷脂质量比为 0 或 0.1:1 ~ 1:1；抗氧剂与中性磷脂质量比为 0 ~ 1:50；胆固醇可使脂质体双分子层膜固化，从而减少自由基的生成，降低了氧化水平，使脂质体稳定性显著增强。为了进一步降低形成脂质体的磷脂和脂质体所包裹药物的化学降解，抗氧剂通过与磷脂过氧化自由基反应并猝灭单一态的氧分子和对磷脂的双分子层进行排序（如限制类脂层分子的流动性）等分子机制而发挥其抗氧化作用。

[0015] 进一步地，所述的糖为乳糖、麦芽糖、蔗糖、葡萄糖、海藻糖或蔗糖中一种或多种。

[0016] 进一步地，所述的缓冲剂为氨基酸、无机盐或有机酸碱中一种或多种。

[0017] 进一步地，所述的中性磷脂为蛋黄卵磷脂、氢化蛋黄卵磷脂、双硬脂酸卵磷脂、大豆卵磷脂、氢化大豆卵磷脂、双软脂酸卵磷脂或双肉豆蔻酸卵磷脂；

[0018] 所述的带电荷磷脂为带负电荷磷脂或带正电荷磷脂；其中负电荷磷脂选自双肉豆蔻酸磷脂酰甘油、双月桂酸磷脂酰甘油、双软脂酸磷脂酰甘油、双硬脂酸磷脂酰甘油、双肉豆蔻酸磷脂酸、双硬脂酸磷脂酸、双月桂酸磷脂酸、双软脂酸磷脂酸、双油酸磷脂酰丝氨酸或双亚油酸磷脂酰肌醇其中一种或多种；

[0019] 所述带正电荷磷脂选自二棕榈酰基磷脂酰基乙基氨基-L-赖氨酸、二油酸磷脂酰丝氨酸、二硬脂酰磷脂酸或十八胺中一种或多种。目前脂质体制备主要选用中性的卵磷脂，但由于含有卵磷脂的脂质体的粒径在贮存期间会发生改变而影响其药效，本发明在配方中加入带电荷的磷脂可使脂质体粒径在贮存期间的变化减小到最小低程度。

[0020] 上述的盐酸多柔比星 - 多西他赛或紫杉醇脂质体的制备方法，步骤如下：

[0021] 1) 制备多西他赛或紫杉醇脂质体：根据配方选用多西他赛或紫杉醇、中性磷脂、带电荷磷脂、胆固醇和抗氧剂溶于氯仿或氯仿 - 甲醇溶剂中，混合均匀；用旋转蒸发仪将溶液中溶剂减压除去，形成脂质薄膜；加入缓冲体系水化，水化温度一般在 40°C - 70°C 之间，得多西他赛脂质体悬浮液；

[0022] 2) 均化脂质体：水化完全后用高压均粒机制备脂质体至所需粒径和均匀度或通过挤压设备把空白脂质体悬浮液在压力下挤过相应孔径的微孔膜来达到，脂质体的粒径控制在 50-300nm，脂质体粒径和均匀度用多角度纳米粒子分析器来检测；

[0023] 3) 将盐酸多柔比星溶于糖类水溶液中加热至 40 ~ 50°C，采用硫酸铵梯度方法，对盐酸多柔比星进行包裹；

[0024] 4) 定容、除菌、分装、保存：用注射用水定容；将盐酸多柔比星脂质体悬浮液用微孔膜过滤除菌，分装即得成品，成品可在 2°C - 8°C 下保存或冻干保存至使用。

[0025] 本发明的有益效果是：通过实验证明本发明盐酸多柔比星脂质体 - 多西他赛或紫杉醇脂质体毒副作用明显减，并增强其抗肿瘤的治疗功能；

[0026] 本发明的脂质体的粒径位于 50nm-300nm 之间，药物包裹率可达 90% 以上，非包裹盐酸多柔比星、多西他赛或紫杉醇含量少，一般小于 3%。本发明冻干粉在 2°C - 8°C 下的两年

稳定性实验结果表明其平均粒径变化率小于 6%, 药物包裹率的变化率小于 5%, 显示了本发明盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂优异的稳定性。

附图说明

- [0027] 图 1 肿瘤细胞增殖示意图 ;
- [0028] 图 2 紫杉醇脂质体抑制肿瘤细胞增殖图。

具体实施方式

[0029] 以下通过实施例对本发明作进一步阐明, 并不只限于下述保护范围。

[0030] 实施例 1

[0031] 制剂处方 :

[0032]

盐酸多柔比星	30mg
紫杉醇	120mg
双硬脂酸卵磷脂 (DSPC)	300 mg
双硬脂酸磷脂酰甘油 (DSPG)	60 mg
胆固醇	90 mg
维生素 E	8 mg
柠檬酸	适量
无水碳酸钠	适量
蔗糖	适量
甘氨酸	适量
注射用水	定容至所需容量

[0033] 制剂工艺如下 :

[0034] 根据配方选用磷脂、紫杉醇、胆固醇和维生素 E 溶于氯仿中混合均匀 ; 用旋转蒸发仪将溶液中氯仿减压除去, 形成脂质薄膜 ; 配制 0.3M 的柠檬酸溶液, 用柠檬酸溶液来水化脂质薄膜, 水化温度为 55°C, 得紫杉醇脂质体悬浮液 ; 水化完全后用高压均粒机制备脂质体至平均粒径为 120±10nm, 脂质体粒径和均匀度用多角度纳米粒子分析器来检测 ; 将盐酸多柔比星溶于 9% 的蔗糖水溶液, 加热到 50°C; 用 0.5M 的无水碳酸钠水溶液调节空紫杉醇脂质体悬浮液 pH 为 7.5±0.3 ; 将盐酸多柔比星溶液和碱性紫杉醇脂质体悬浮液在 60°C 混合均匀并保温 10-30 分钟 ; 配制 9% 的蔗糖水溶液并加入 1.0% 的甘氨酸, 得脂质体分散液 ; 采用脂质体分散液洗涤含盐酸多柔比星的脂质体, 利用渗析过滤法使盐酸多柔比星脂质体外溶液置换为 pH 为 7.0 的脂质体分散液, 以盐酸多柔比星为剂量单位, 用注射用水定容并调节至盐酸多柔比星的浓度为 2.0mg/ml 溶液, 将盐酸多柔比星脂质体悬浮液用 0.22 微米孔径的微孔膜过滤除菌, 分装即得成品, 成品可在 2°C -8°C 下保存。

[0035] 实施例 2

[0036] 制剂处方：

[0037]

	盐酸多柔比星	60mg
	紫杉醇	360mg
	双软脂酸卵磷脂 (DPPC)	3600mg
	氢化大豆卵磷脂	3600mg
[0038]	二硬脂酰磷脂酸	1000mg
	维生素 E	150mg
	硫酸铵	适量
	乳糖	适量
	甘氨酸	适量
	注射用水	定容至所需容量

[0039] 制备工艺：

[0040] 根据配方选用紫杉醇、磷脂、胆固醇和维生素 E 溶于氯仿中混合均匀；用旋转蒸发仪将溶液中氯仿减压除去，形成脂质薄膜；配制 0.3M 的硫酸铵溶液，用硫酸铵溶液来水化脂质薄膜，水化温度为 40℃，得含紫杉醇脂质体悬浮液；水化完全后用高压均粒机制备脂质体至平均粒径为 $120 \pm 10\text{nm}$ ，脂质体粒径和均匀度用多角度纳米粒子分析器来检测；用交叉流技术除去脂质体外部硫酸铵；将盐酸多柔比星溶于 9% 的乳糖水溶液，加热到 40℃；加入配方量的无水碳酸钠粉末调节含紫杉醇脂质体悬浮液 pH 为 7.5 ± 0.3 ；将盐酸多柔比星溶液和含紫杉醇脂质体悬浮液在 60℃ 混合均匀并保温 20 分钟；加入 1.0% 的甘氨酸，得脂质体分散液；用注射用水定容并调节至以盐酸多柔比星的浓度计 2.0mg/ml 溶液，得 pH 为 7.0 的脂质体分散液，将盐酸多柔比星 - 紫杉醇脂质体悬浮液用 0.22 微米孔径的微孔膜过滤除菌，分装即得成品，成品可在 2℃ - 8℃ 下保存或冻干保存至使用。紫杉醇包封率 $\geq 93\%$ ，盐酸多柔比星包封率 $\geq 98\%$ ；产品放置一年粒径、包封率未有明显变化。

[0041] 实施例 3

[0042] 制剂处方：

[0043]

盐酸多柔比星	60mg
紫杉醇	480mg
双软脂酸卵磷脂 (DPPC)	3600mg
氢化大豆磷脂 (HSPC)	3600mg
二油酸磷脂酰丝氨酸	1000mg
胆固醇	300mg
硫酸铵	适量
乳糖	适量
甘氨酸	适量
注射用水	定容至所需容量

[0044] 制备工艺：

[0045] 根据配方选用紫杉醇、磷脂、胆固醇和维生素 E 溶于氯仿中混合均匀；用旋转蒸发仪将溶液中氯仿减压除去，形成脂质薄膜；配制 0.3M 的硫酸铵溶液，用硫酸铵溶液来水化脂质薄膜，水化温度为 70℃ 之间，得含紫杉醇脂质体悬浮液；水化完全后用高压均粒机制备脂质体至平均粒径为 120±10nm，脂质体粒径和均匀度用多角度纳米粒子分析器来检测；用交叉流技术除去脂质体外部硫酸铵；将盐酸多柔比星溶于 9% 的乳糖水溶液，加热到 40℃；加入配方量的无水碳酸钠粉末调节含紫杉醇脂质体悬浮液 pH 为 7.5±0.3；将盐酸多柔比星溶液和含紫杉醇脂质体悬浮液在 60℃ 混合均匀并保温 20 分钟；加入 1.0% 的甘氨酸，得脂质体分散液；用注射用水定容并调节至以盐酸多柔比星的浓度计 2.0mg/ml 溶液，得 pH 为 7.0 的脂质体分散液，将盐酸多柔比星 - 紫杉醇脂质体悬浮液用 0.22 微米孔径的微孔膜过滤除菌，分装即得成品，成品可在 2℃ -8℃ 下保存或冻干保存至使用。紫杉醇包封率≥93%，盐酸多柔比星包封率≥98%；产品放置一年粒径、包封率未有明显变化。

[0046] 实施例 4

[0047] 制剂处方：

[0048]

盐酸多柔比星	30 mg
多西他赛	60mg
大豆卵磷脂 (SPC)	900mg
双肉豆蔻酸磷脂酰甘油(DMPG)	9mg
胆固醇	100mg
维生素 E	0 mg
琥珀酸	适量
氢氧化钠	适量
乳糖	适量
甘氨酸	适量
注射用水	定容至所需容量

[0049] 制剂工艺如下：

[0050] 根据配方选用多西他赛、磷脂、胆固醇和维生素 E 溶于 2:1 氯仿 - 甲醇中混合均匀；用旋转蒸发仪将溶液中氯仿和甲醇减压除去，形成脂质薄膜；配制 0.25M 的琥珀酸溶液，用琥珀酸溶液来水化脂质薄膜，水化温度为 65℃，得多西他赛脂质体悬浮液；水化完全后用高压均粒机制备脂质体至平均粒径为 180±10nm，脂质体粒径和均匀度用多角度纳米粒子分析器来检测，将盐酸多柔比星溶于 4.5% 的乳糖水溶液，加热到 65℃；用 0.5M 的氢氧化钠水溶液调节多西他赛脂质体悬浮液 pH 为 7.3±0.3；将盐酸多柔比星溶液和碱性多西他赛脂质体悬浮液在 65℃ 混合均匀并保温 30 分钟；配制 4.5% 的乳糖水溶液并加入 1.0% 的甘氨酸，得脂质体分散液；采用脂质体分散液洗涤含盐酸多柔比星的脂质体，利用渗析过滤法使盐酸多柔比星脂质体外溶液置换为 pH 为 7.0 的脂质体分散液，用注射用水定容并调节至盐酸多柔比星的浓度为 2.0mg/ml 溶液，将盐酸多柔比星脂质体悬浮液用 0.22 微米孔径的微孔膜过滤除菌，分装即得成品，冻干保存至使用。

[0051] 实施例 5

[0052] 制剂处方(25ml 容量)：

[0053]

盐酸多柔比星	120 mg
多西他赛	300mg
蛋黄卵磷脂 (EPC)	1000 mg
氢化大豆卵磷脂 (HSPC)	800 mg
双硬脂酸磷脂酰甘油 (DSPG)	80 mg
胆固醇	20 mg
维生素 E	0mg
硫酸铵	适量
蔗糖	适量
组氨酸	适量
注射用水	定容至所需容量

[0054] 制剂工艺如下：

[0055] 根据配方选用多西他赛、磷脂、胆固醇和维生素 E 溶于氯仿中混合均匀；用旋转蒸发仪将溶液中氯仿减压除去，形成脂质薄膜；配制 0.3M 的硫酸铵溶液，用硫酸铵溶液来水化脂质薄膜，水化温度为 65℃，得含多西他赛的脂质体悬浮液；水化完全后用高压均粒机制备脂质体至平均粒径为 120±10nm，脂质体粒径和均匀度用多角度纳米粒子分析器来检测；采用切向流技术除去脂质体外部的硫酸铵；将盐酸多柔比星溶于 9% 的蔗糖水溶液，加热到 60℃；用 1.0M 的氢氧化钠水溶液调节空白脂质体悬浮液 pH 为 6.5±0.3；将盐酸多柔比星溶液和多西他赛脂质体悬浮液在 65℃ 混合均匀并保温 30 分钟；配制 9% 的蔗糖水溶液并加入 1.0% 的组氨酸分散液洗涤含盐酸多柔比星的脂质体，利用渗析过滤法使盐酸多柔比星脂质体外溶液置换为 pH 为 6.0 的脂质体分散液，用注射用水定容并调节至含盐酸多柔比星为 2.0mg/ml 溶液，将盐酸多柔比星 - 多西他赛脂质体悬浮液用 0.22 微米孔径的微孔膜过滤除菌，分装即得成品，成品可在 2℃ -8℃ 下保存。多西他赛包封率≥95%，盐酸多柔比星包封率≥98%；产品放置一年粒径、包封率未有明显变化。

[0056] 实施例 6

[0057] 制剂处方(25ml 容量)：

[0058]

盐酸多柔比星	120 mg
多西他赛	360mg
蛋黄卵磷脂 (EPC)	1000mg
氢化大豆卵磷脂 (HSPC)	800 mg
双月桂酸磷脂酰甘油	80 mg
胆固醇	20 mg
维生素 E	0mg
硫酸铵	适量
蔗糖	适量
组氨酸	适量
注射用水	定容至所需容量

[0059] 制剂工艺如下：

[0060] 根据配方选用多西他赛、磷脂、胆固醇和维生素 E 溶于氯仿中混合均匀；用旋转蒸发仪将溶液中氯仿减压除去，形成脂质薄膜；配制 0.3M 的硫酸铵溶液，用硫酸铵溶液来水化脂质薄膜，水化温度为 65℃，得含多西他赛的脂质体悬浮液；水化完全后用高压均粒机制备脂质体至平均粒径为 120±10nm，脂质体粒径和均匀度用多角度纳米粒子分析器来检测；采用切向流技术除去脂质体外部的硫酸铵；将盐酸多柔比星溶于 9% 的蔗糖水溶液，加热到 60℃；用 1.0M 的氢氧化钠水溶液调节空白脂质体悬浮液 pH 为 6.5±0.3；将盐酸多柔比星溶液和多西他赛脂质体悬浮液在 65℃ 混合均匀并保温 30 分钟；配制 9% 的蔗糖水溶液并加入 1.0% 的组氨酸分散液洗涤含盐酸多柔比星的脂质体，利用渗析过滤法使盐酸多柔比星脂质体外溶液置换为 pH 为 6.0 的脂质体分散液，用注射用水定容并调节至含盐酸多柔比星为 2.0mg/ml 溶液，将盐酸多柔比星 - 多西他赛脂质体悬浮液用 0.22 微米孔径的微孔膜过滤除菌，分装即得成品，成品可在 2℃ - 8℃ 下保存。多西他赛包封率≥95%，盐酸多柔比星包封率≥98%；产品放置一年粒径、包封率未有明显变化。

[0061] 对比实施例 1

[0062] 将实施例 1 配方中紫杉醇量减小至 105mg，使盐酸多柔比星与紫杉醇质量比为 1:3.5，其他组分及含量不变，制备方法同实施例 2。

[0063] 对比实施例 2

[0064] 将实施例 3 中紫杉醇量增加至 510mg，使盐酸多柔比星与紫杉醇质量比为 1:8.5，其他组分及含量不变，制备方法同实施例 2。

[0065] 对比实施例 3

[0066] 将实施例 4 中多西他赛量减少至 45mg，使盐酸多柔比星与多西他赛质量比为 1:1.5，其他组分及含量不变，制备方法同实施例 4。

[0067] 对比实施例 4

[0068] 将实施例 6 中多西他赛量增加至 420mg，使盐酸多柔比星与多西他赛质量比为 1:3.5，其他组分及含量不变，制备方法同实施例 6。

[0069] 毒性试验及抗肿瘤实验：

[0070] 实验组别划分与设计：

[0071] 一、传统盐酸多柔比星脂质体 - 紫杉醇脂质体与本发明盐酸多柔比星 - 紫杉醇脂质体的毒性试验比较：

[0072] (1)传统盐酸多柔比星脂质体 - 紫杉醇脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含紫杉醇 2mg 的溶液(A1 组)；

[0073] 实施例 1 盐酸多柔比星 - 紫杉醇脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含紫杉醇 2mg 的溶液。

[0074] 对比实施例 1 盐酸多柔比星 - 紫杉醇脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含紫杉醇 1.75mg 的溶液。

[0075] (2)传统盐酸多柔比星脂质体 - 紫杉醇脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含紫杉醇 3mg 的溶液(A2 组)。

[0076] 实施例 2 盐酸多柔比星 - 紫杉醇脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含紫杉醇 3mg 的溶液。

[0077] (3)传统盐酸多柔比星脂质体 - 紫杉醇脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含紫杉醇 4mg 的溶液(A3 组)；

[0078] 实施例 3 盐酸多柔比星 - 紫杉醇脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含紫杉醇 4mg 的溶液。

[0079] 对比实施例 2 盐酸多柔比星 - 紫杉醇脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含紫杉醇 4.25mg 的溶液。

[0080] 二、传统盐酸多柔比星脂质体 - 多西他赛脂质体与本发明盐酸多柔比星 - 多西他赛脂质体的毒性试验比较：

[0081] (1)传统盐酸多柔比星脂质体 - 多西他赛脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含多西他赛 1mg 的溶液(B1 组)。

[0082] 实施例 4 盐酸多柔比星 - 多西他赛脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含多西他赛 1mg 的溶液。

[0083] 对比实施例 3 盐酸多柔比星 - 多西他赛脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含多西他赛醇 0.75mg 的溶液。

[0084] (2)传统盐酸多柔比星脂质体 - 多西他赛脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含多西他赛 1.25mg 的溶液(B2 组)。

[0085] 实施例 5 盐酸多柔比星 - 多西他赛脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含多西他赛 1.25mg 的溶液。

[0086] (3)传统盐酸多柔比星脂质体 - 多西他赛脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含多西他赛 1.5mg 的溶液(B3 组)。

[0087] 实施例 6 盐酸多柔比星 - 多西他赛脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含多西他赛 1.5mg 的溶液。

[0088] 对比实施例 4 盐酸多柔比星 - 多西他赛脂质体组 :用 5% 的葡萄糖配制成每 1ml 中含盐酸多柔比星 0.5mg, 含多西他赛 1.75mg 的溶液。

[0089] ①急性毒性实验

[0090] 取健康 BALB/c 小鼠, 雌雄各半。按性别、体重随机分为 A1 组、实施例 1 组、对比实施例 1 组、A2 组、实施例 2 组、A3 组、实施例 3 组、对比实施例 2 组。以单次静脉 $40\text{ml} \cdot \text{kg}^{-1}$ 给药, 药后连续观察 21d。以 21d 累计死亡动物数, 用 Bliss 法计算各组的 LD₅₀。

[0091] 表 1 盐酸多柔比星 - 紫杉醇脂质体毒性试验对比

[0092]

组别	LD ₅₀ ($\text{mg} \cdot \text{kg}^{-1}$)
A1 组	80.25
实施例 1 组	119.84
对比实施例 1 组	100.13
A2 组	78.25
实施例 2 组	131.32
A3 组	83.24
实施例 3 组	125.38
对比实施例 2 组	102.58

[0093]

[0094] 表 2 盐酸多柔比星 - 多西他赛脂质体毒性试验对比

[0095]

组别	LD ₅₀ ($\text{mg} \cdot \text{kg}^{-1}$)
B1 组	130.31
实施例 4	235.17
对比实施例 3	200.82
B2 组	160.68
实施例 5	262.89
B3 组	185.65
实施例 6	295.22
对比实施例 4	201.43

[0096] 表 1 和表 2 中实验结果表明本发明的脂质体毒性最低: 本发明盐酸多柔比星 - 多西他赛或紫杉醇脂质体相比于传统盐酸多柔比星脂质体和多西他赛或紫杉醇脂质体的混

合给药的毒性显著降低。同时发现盐酸多柔比星与多西他赛或紫杉醇的比例与本发明稍有不同时，毒性也会增强，说明在本发明要求保护的范围内的脂质体毒性最低。

[0097] ②盐酸多柔比星 - 多西他赛或紫杉醇脂质体对 S180 肉瘤小鼠模型的药效实验

[0098] 取生长期的肉瘤 S180 细胞株，在无菌条件下后制备成 2mm*2mm 瘤块，接种于裸小鼠右侧腋窝皮下。接种 10 天后，根据瘤体积，随机分组，每组 8 只，给药剂量 0.2ml/10g/ 次，每日给药 2 次，连续治疗 5 周。试验结束时称体重和肿瘤重量，计算抑制率。

[0099] 药物疗效评价方法：给药 5 周后，将裸鼠处死，取肿瘤，称重。药物对肿瘤抑制率计算公式为：抑制率 % = $(W_0 - W) / W_0 \times 100\%$ 。其中， W_0 为阴性对照组肿瘤平均重量， W 为给药组肿瘤平均重量。

[0100] 实验结果如下表

[0101] 表 3 盐酸多柔比星 - 紫杉醇脂质体抑瘤试验对比

[0102]

组 别	动物数(n) 开始/结束	体 重 (mean±SD, g)	瘤 重 (mean±SD, g)	抑 制 率 (%)
空白	8/8	25.91±1.38	0.81±0.12	/
A1	8/8	26.13±2.15	0.49±0.19	39.5
实施例 1	8/8	24.82±1.60	0.35±0.16	56.8
对比实施例 1	8/8	27.67±1.51	0.45±0.14	44.4
A2	8/8	25.31±1.38	0.50±0.39	38.3
实施例 2	8/8	24.14±1.66	0.38±0.16	53.1
A3	8/8	25.31±1.38	0.48±0.39	40.7
实施例 3	8/8	25.58±1.75	0.37±0.14	54.3
对比实施例 2	8/8	24.01±1.65	0.43±0.13	46.9

[0103]

[0104] 表 4 盐酸多柔比星 - 多西他赛脂质体抑瘤试验对比

[0105]

组别	动物数(n) 开始/结束	体 重 (mean±SD, g)	瘤 重 (mean±SD, g)	抑 制 率 (%)
空白	8/8	22.44±1.12	0.67±0.15	/
B1 组	8/8	21.99±1.22	0.36±0.15	46.3%
实施例 4	8/8	22.99±1.13	0.22±0.23	67.2%
对比实施例 3	8/8	21.56±1.51	0.27±0.18	59.7%
B2 组	8/8	22.50±1.49	0.34±0.16	49.3%
实施例 5	8/8	20.84±1.56	0.23±0.15	65.7%
B3 组	8/8	21.11±2.14	0.35±0.19	47.8%
实施例 6	8/8	20.70±1.34	0.21±0.21	68.7%
对比实施例 4	8/8	20.12±1.38	0.28±0.09	58.2%

[0106] 其中空白组的小鼠在同样条件下注射 5% 的葡萄糖溶液。

[0107] 结果发现盐酸多柔比星 - 多西他赛或紫杉醇脂质体对 S180 肉瘤有明显的抑瘤效果 : 在相等剂量条件下抑瘤率与传统单独脂质体混合注射或其他比例的盐酸多柔比星 - 多西他赛或紫杉醇脂质体相比有显著的优势。可见本发明的盐酸多柔比星 - 多西他赛或紫杉醇脂质体制剂能够明显增加药物在病体的摄入量并增强其抗肿瘤的治疗功能。

[0108] 本发明盐酸多柔比星 - 多西他赛或紫杉醇脂质体的用量及用法 :

[0109] ①盐酸多柔比星与紫杉醇的复方制剂

[0110] 只能用于静脉滴注,所有病人在接受治疗期前均必须口服糖皮质激素类;

[0111] 本品应为每 2 ~ 3 周静脉内给药 1 次,盐酸多柔比星 20mg/m²,紫杉醇 40~160mg/m²,给药间隔不宜少于 10 天,因为不能排除药物蓄积和毒性增强的可能。病人应持续治疗 2 ~ 3 个月以产生疗效。为保持一定的疗效,在需要时继续给药。

[0112] ②盐酸多柔比星与多西他赛的复方制剂

[0113] 只能用于静脉滴注,所有病人在接受治疗期前均必须口服糖皮质激素类;

[0114] 推荐剂量为盐酸多柔比星 20mg/m²,多西他赛为 40 ~ 60mg/m²,静脉滴注一小时,每三周一次。病人应持续治疗 2 ~ 3 个月以产生疗效。为保持一定的疗效,在需要时继续给药。

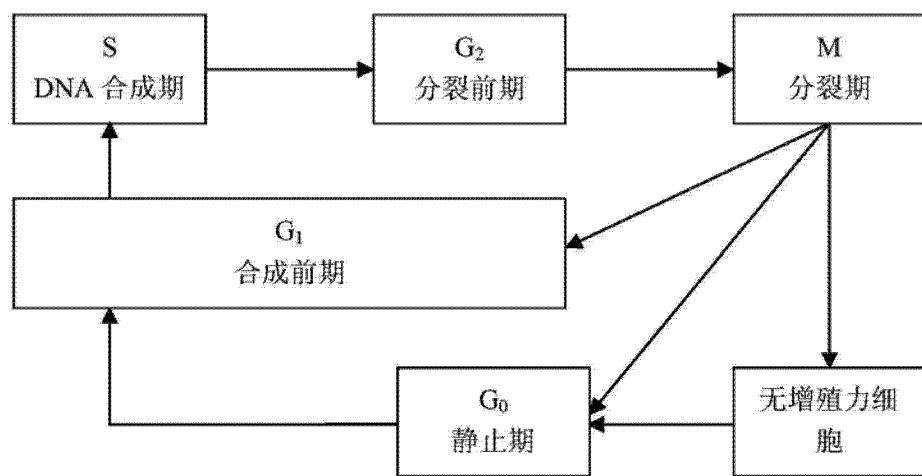


图 1

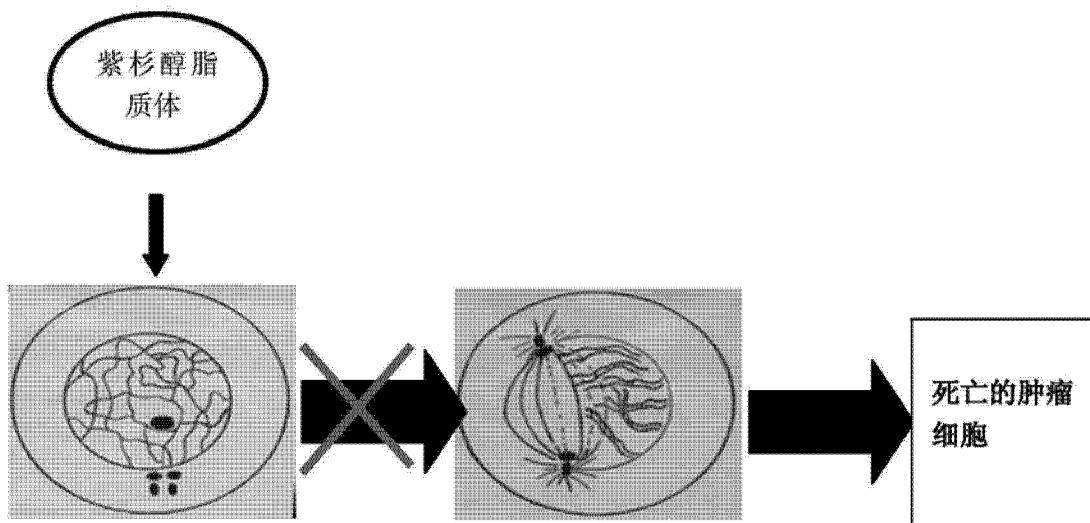


图 2

Codelivery of Doxorubicin and Paclitaxel by Cross-Linked Multilamellar Liposome Enables Synergistic Antitumor Activity

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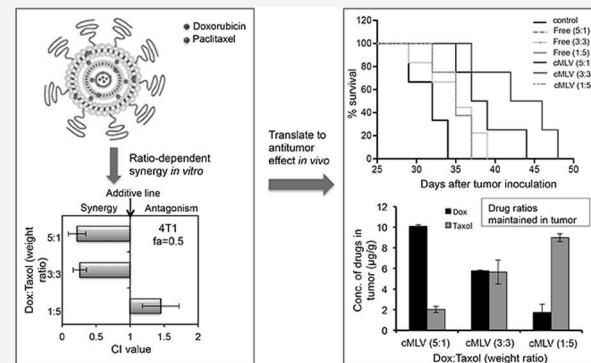
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Supporting Information

ABSTRACT: Combining chemotherapeutics is a promising method of improving cancer treatment; however, the clinical success of combination therapy is limited by the distinct pharmacokinetics of combined drugs, which leads to nonuniform distribution. In this study, we report a new robust approach to load two drugs with different hydrophilicities into a single cross-linked multilamellar liposomal vesicle (cMLV) to precisely control the drug ratio that reaches the tumor *in vivo*. The stability of cMLVs improves the loading efficiency and sustained release of doxorubicin (Dox) and paclitaxel (PTX), maximizing the combined therapeutic effect and minimizing the systemic toxicity. Furthermore, we show that the cMLV formulation maintains specific drug ratios *in vivo* for over 24 h, enabling the ratio-dependent combination synergy seen *in vitro* to translate to *in vivo* antitumor activity and giving us control over another parameter important to combination therapy. This combinatorial delivery system may provide a new strategy for synergistic delivery of multiple chemotherapeutics with a ratiometric control over encapsulated drugs to treat cancer and other diseases.

KEYWORDS: cross-linked multilamellar liposomal vesicle, combination therapy, doxorubicin, paclitaxel, synergy, dose ratios, nanomedicine



INTRODUCTION

Target-based drug design has been successfully used to develop many drugs that can act on novel molecular targets; however, these drugs have shown poor efficacy in clinical trials. This can be attributed to the compensatory mechanism, or drug-mitigating response, enacted by complex diseases such as cancer.^{1,2} Overcoming this drug-mitigating response often requires high drug doses, which can induce drug resistance in target cells or side effects in other tissues,³ thus limiting the efficacy of many potential drugs in cancer therapy. These limitations of monotherapy can be overcome by synergistic combination of two or more agents, which can kill cells at lower drug doses by affecting multiple disease targets.^{4,5} However, current combination methods, through cocktail administration, have shown limited improvement over single drugs in clinical studies due to the distinctive pharmacokinetics of individual drugs, which lead to noncoordinated distribution after systemic administration.^{6,7} Moreover, unexpected adverse effects were reported in clinical trials using these cocktail combinations,

raising concerns about the induction of synergistic systemic toxicities by combination therapies.⁸ For instance, although a combination of doxorubicin (Dox) and paclitaxel (PTX) has been widely used in the treatment of tumors, particularly in metastatic breast cancer, the clinical results were limited by increased cardiotoxicity.^{9–12} Clinical pharmacokinetic studies also revealed a noncoordinated plasma distribution of Dox and PTX when given in combination,^{13,14} rendering *in vitro* data ineffective in predicting *in vivo* therapeutic efficacy of combination therapy. A more effective combination strategy with the ability to coordinate the pharmacokinetics and biodistribution of various drug molecules is highly desirable to maximize the combinatorial effects without significant toxicity.

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The development of nanotechnology has provided a novel combination strategy by enabling the simultaneous delivery of multiple drugs to a site of interest via a single vehicle.⁷ Nanoparticles are considered promising drug delivery vehicles for cancer therapy based on their ability to prolong drug circulation time, reduce systemic toxicity, and increase drug accumulation at tumor sites through the enhanced permeation and retention (EPR) effect.^{15–18} The pharmacokinetic behavior of the coformulated drugs can be determined by the pharmacokinetic behavior of the drug carriers. Thus, nanoparticle delivery systems offer the potential to coordinate the plasma elimination and biodistribution of multiple drugs, enabling dosage optimization to maximize cytotoxicity while minimizing the chances to develop drug resistance. Compared to other nanoparticle delivery systems, liposomes have shown superior ability to codeliver multiple drugs with vastly different hydrophobicities to the same site of action.^{19,20} However, the poor stability and limited loading efficiency of hydrophobic drugs remain the most significant concerns for conventional formulations of liposomes, limiting their clinical benefit in cancer therapy.^{21,22} For example, a number of studies reported that the maximal drug-to-lipid molar ratio of paclitaxel-encapsulated by a conventional liposome formulation was below 4%,^{23–26} thwarting the practical application of liposomal drug carriers. Moreover, fine-tuning of the comparative loading yield and release kinetics of multiple drugs in conventional liposomes remains an unmet need. Thus, a stable liposomal formulation that enables improved drug loading and drug release from the carrier in a controlled and sustained manner is necessary for combinatorial drug delivery.

To address such a need, we have previously reported the development of cross-linked multilamellar liposomal vesicles (cMLVs) and demonstrated their efficacy in achieving sustained delivery of doxorubicin both *in vitro* and *in vivo*.²⁷ Herein, we extend the potential of cMLVs to facilitate synergistic combinatorial delivery of hydrophobic and hydrophilic drugs in a precisely controlled manner. Dox, a model hydrophilic drug, and PTX, a hydrophobic drug, were coencapsulated into the same cMLVs at predefined stoichiometric ratios. We show that the combination effects (antagonistic, additive, or synergistic) could be determined by controlling drug ratios of Dox and PTX in cMLVs. We also demonstrate that the drug ratio-dependent synergistic effect could be achieved via the cMLV codelivery system in a breast tumor model without significant cardiac toxicity. Moreover, cMLV particles are capable of prolonging maintenance of the synergistic ratios of combined drugs *in vivo* and, in turn, providing a significantly enhanced antitumor efficacy compared to free-drug cocktail administration. The results demonstrate the great potential of cMLVs as combinatorial drug delivery vesicles to induce synergy of antitumor therapeutics both *in vitro* and *in vivo*, thus setting a new paradigm in nanomedicine for combination therapies.

■ EXPERIMENTAL SECTION

Cell Lines, Antibodies, Reagents, and Mice. B16-F10 (ATCC number: CRL-6475) and 4T1 tumor cells (ATCC number: CRL-2539) were maintained in a 5% CO₂ environment with Dulbecco's modified Eagle's medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) and 2 mM of L-glutamine (Hyclone Laboratories, Inc., Omaha, NE). Mouse anti- β -Actin and rabbit antibody against phospho-specific

protein p44/42 MAPK (Erk 1/2) were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-Rabbit IR dye680RD and goat anti-mouse IR Dye800CW were obtained from LI-COR BioSciences (Lincoln, Nebraska). Doxorubicin, paclitaxel, daunorubicin, and doxetaxel were purchased from Sigma-Aldrich (St. Louis, MO).

All lipids were obtained from NOF Corporation (Japan): 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(10-rac-glycerol) (DOPG), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(*p*-maleimidophenyl) butyramide (maleimide-headgroup lipid, MPB-PE).

Female 6–10 week-old BALB/c mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). All mice were held under specific pathogen-reduced conditions in the Animal Facility of the University of Southern California (Los Angeles, CA, USA). All experiments were performed in accordance with the guidelines set by the National Institute of Health and the University of Southern California on the Care and Use of Animals.

Synthesis of cMLVs. Liposomes were prepared based on the conventional dehydration–rehydration method. All lipids were obtained from NOF Corporation (Japan). DOPC, DOPG, and MPB-PE were combined in chloroform, at a molar lipid ratio of DOPC–DOPG–MPB = 4:1:5, and the organic solvent in the lipid mixture was evaporated under argon gas. The lipid mixture was further dried under vacuum overnight to form dried thin lipid films. To prepare cMLV (Dox+PTX), paclitaxel in organic solvent was mixed with the lipid mixture before formation of the dried thin lipid films. The resultant dried film was hydrated in 10 mM Bis-Tris propane at pH 7.0 with doxorubicin by vigorous vortexing every 10 min for 1 h and then applied with four cycles of 15 s sonication (Misonix Microson XL2000, Farmingdale, NY) on ice in 1 min intervals for each cycle. To induce divalent-triggered vesicle fusion, MgCl₂ was added at a final concentration of 10 mM. The resulting multilamellar vesicles were further cross-linked by addition of dithiothreitol (DTT, Sigma-Aldrich) at a final concentration of 1.5 mM for 1 h at 37 °C. The resulting vesicles were collected by centrifugation at 14 000 g for 4 min and then washed twice with phosphate-buffered saline (PBS). For pegylation of cMLVs, the particles were incubated with 1 μ mol of 2 kDa PEG-SH (Laysan Bio Inc. Arab, AL) for 1 h at 37 °C. The particles were then centrifuged and washed twice with PBS. The final products were stored in PBS at 4 °C.

Characterization of Physical Properties. The hydrodynamic size and size distribution of cMLVs were measured by dynamic light scattering (Wyatt Technology, Santa Barbara, CA).

In Vitro Drug Encapsulation and Release. To study the loading capacity of Dox, cMLV (Dox) and cMLV (Dox+PTX) were collected and washed twice with PBS, followed by lipid extraction of vesicles with 1% Triton X-100 treatment. Dox fluorescence (excitation 480 nm, emission 590 nm) was then measured by a Shimadzu RF-5301PC spectrophluorometer (Japan). The amount of paclitaxel incorporated in the cMLV(PTX) and cMLV(Dox+PTX) was determined by C-18 reverse-phase high-performance liquid chromatography (RP-HPLC) (Beckman Coulter, Brea, CA). The cMLV(PTX) and cMLV(Dox+PTX) suspensions were diluted by adding water and acetonitrile to a total volume of 0.5 mL. Extraction of paclitaxel was accomplished by adding 5 mL of *tert*-butyl methyl ether and vortex-mixing the sample for 1 min. The mixtures were centrifuged, and the organic layer was transferred

into a glass tube and evaporated to dryness under argon. Buffer A (95% water, 5% acetonitrile) was used to rehydrate the glass tube. To test PTX concentration, 1 mL of the solution was injected into a C18 column, and the paclitaxel was detected at 227 nm (flow rate 1 mL/min). To obtain the release kinetics of Dox and PTX from liposomes, the releasing media was removed from cMLVs incubated in 10% FBS-containing media at 37 °C and replaced with fresh media daily. The removed media was quantified for Dox fluorescence (by spectrofluorometer) and PTX fluorescence (by HPLC) every day.

In Vitro Drug Loading Efficiency. Loading efficiency was determined by the ratio of encapsulated drug to total phospholipid mass. A phospholipid phosphate assay was carried out to calculate the phospholipid mass. cMLVs were centrifuged, and 100 μL chloroform was added to the pellets to break down the lipid bilayers. The samples were transferred to glass tubes and evaporated to dryness. After adding 100 μL perchloric acid, the samples were boiled at 190 °C for 25 min. Samples will turn brown then clear as the lipids are digested. Samples were cooled to room temperature and diluted to 1 mL with distilled water. The amount of phospholipid phosphate was determined by the malachite green phosphate detection kit (R&D systems, Minneapolis, MN).

In Vitro Cytotoxicity and Data Analysis. B16-F10 and 4T1 cells were plated at a density of 5×10^3 cells per well in 10% FBS-containing media in 96-well plates and grown for 6 h. The cells were then exposed to a series of concentrations of cMLV (single drug) or cMLV (drug combinations), at different weight ratios of combined drugs, for 48 h. The cell viability was assessed using the Cell Proliferation Kit II (XTT assay) from Roche Applied Science according to the manufacturer's instructions. The cell viability percentage was determined by subtracting absorbance values obtained from media-only wells from drug-treated wells and then normalizing to the control cells without drugs. The fraction of cells affected (f_a) at each drug concentration was subsequently determined for each well. The data was analyzed by nonlinear regression to get the IC_{50} value. The combination index (CI) values were calculated by the equation: $CI = C_{A,X}/IC_{X,A} + C_{B,X}/IC_{X,B}$.²⁸ Using this analysis method, a CI = 0.9–1.1 reflects additive activity, and a CI >1.1 indicates antagonism, while a CI < 0.9 suggests synergy.

Western Blot Analysis. Cells were collected 24 h after treatment and lysed in lysis buffer supplemented with protease inhibitors, incubated on ice for 15 min, and then cleared by centrifugation at 10 000 × g at 4 °C for 10 min. The protein concentration was determined using Micro BCA Protein Assay Kit (Thermo Scientific). Lysates (20 μg) were separated by reducing 12% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes. Immunodetection of ERK was carried out with antibodies specific to rabbit phospho-specific protein p44/42 MAPK (Erk 1/2) and goat antirabbit IR dye 680RD. Immunodetection of β-actin was carried out with antibodies against β-actin and goat antimouse IR dye 800CW. Membranes were developed using Odyssey infrared fluorescent imager (LI-COR BioSciences, Lincoln, Nebraska).

Determination of Doxorubicin and Paclitaxel Levels in Tumor. BALB/c female mice (6–10 weeks-old) were inoculated subcutaneously with 0.2×10^6 4T1 tumor cells. The tumors were allowed to grow for 20 days to a volume of ~ 500 mm³ before treatment. On day 20, the mice were injected intravenously through the tail vein with 8.33 mg/kg Dox + 1.66

mg/kg PTX, 5 mg/kg Dox + 5 mg/kg PTX, or 1.66 mg/kg Dox + 8.33 mg/kg PTX either in solution or in cMLVs. Three days after injection, tumors were excised and frozen at -20 °C. Docetaxel (10 μL, 100 μg/mL) as an internal standard (IS) for paclitaxel, or 10 μL of daunorubicin (100 μg/mL) as an internal standard for doxorubicin, was added to the weighted tumor tissues. In order to extract paclitaxel and the internal standard (docetaxel), tumor tissue was homogenized in 1 mL ethyl acetate and then centrifuged at 5000 rpm for 10 min. In order to extract doxorubicin and its internal standard (daunorubicin), tumor tissue was homogenized in 1 mL of methanol and then centrifuged at 5000 rpm for 10 min. Then the organic layer was transferred to a clean glass tube and evaporated to dryness under a stream of argon. Buffer A (95% water, 5% acetonitrile) was used to rehydrate the sample in the glass tube. A portion of 1 mL of the solution was injected into C18 column, and the paclitaxel was detected at 227 nm (flow rate 1 mL/min), and doxorubicin was detected at 482 nm (flow rate 1 mL/min). Stock solutions of Dox and PTX (100, 10, and 1 μg/mL) and IS were prepared as calibration samples. Then 500 μL of tumor homogenates were spiked with 500 μL calibration samples with the internal standard at fixed concentration of 1 μg/mL. Calibration curves of doxorubicin and paclitaxel were constructed using the ratio of peak height of doxorubicin or paclitaxel and internal standard by weighted (1/y) linear regression analysis.

In Vivo Antitumor Activity Study. BALB/c female mice (6–10 weeks-old) were inoculated subcutaneously with 0.2×10^6 4T1 breast tumor cells. The tumors were allowed to grow for 8 days to a volume of ~ 50 mm³ before treatment. On day 8, the mice were injected intravenously through the tail vein with 3.33 mg/kg Dox + 0.67 mg/kg PTX, 2 mg/kg Dox + 2 mg/kg PTX, or 0.67 mg/kg Dox + 3.33 mg/kg PTX, either in cMLVs or in solution every 3 days (six mice per group). The tumor growth and body weight were monitored until the end of an experiment. The length and width of the tumor masses were measured with a fine caliper every 3 days after injection. The tumor volume was expressed as $1/2 \times (\text{length} \times \text{width}^2)$. The survival end point was set when the tumor volume reached 1000 mm³. The survival rates are presented as Kaplan–Meier curves. The survival curves of individual groups were compared by a log-rank test.

Immunohistochemistry of Tumors, Cardiac Toxicity, and Confocal Imaging. BALB/c female mice (6–10 weeks-old) were inoculated subcutaneously with 0.2×10^6 4T1 tumor cells. The tumors were allowed to grow for 20 days to a volume of ~ 500 mm³ before treatment. On day 20, the mice were injected intravenously through tail vein with 8.33 mg/kg Dox + 1.66 mg/kg PTX, 5 mg/kg Dox + 5 mg/kg PTX, or 1.66 mg/kg Dox + 8.33 mg/kg PTX in solution or cMLVs. Three days after injection, tumors were excised, fixed, frozen, cryo-sectioned, and mounted onto glass slides. Frozen sections were fixed and rinsed with cold PBS. After blocking and permeabilization, the slides were washed by PBS and incubated with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture (Roche, Indianapolis, Indiana) for 1 h and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). Fluorescence images were acquired by a Yokogawa spinning-disk confocal scanner system (Solamere Technology Group, Salt Lake City, UT) using a Nikon Eclipse Ti-E microscope. Illumination powers at 405, 491, 561, and 640 nm solid-state laser lines were provided by an AOTF (acousto-optical tunable filter)-controlled laser-merge

system with 50 mW for each laser. All images were analyzed using Nikon NIS-Elements software. For quantifying TUNEL positive cells, four regions of interest (ROI) were randomly chosen per image at $\times 2$ magnification. Within one region, the area of TUNEL-positive nuclei and the area of nuclear staining were counted by Nikon NIS-Element software, with data expressed as % total nuclear area stained by TUNEL in the region.

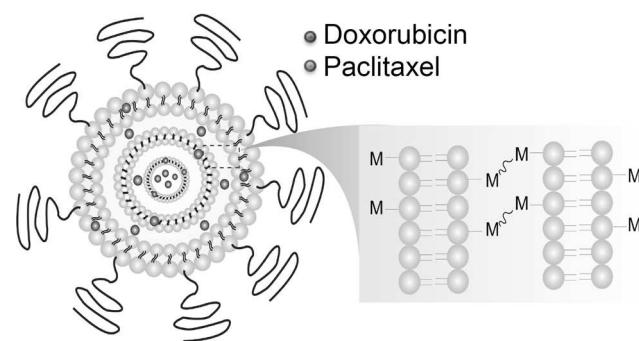
For cardiac toxicity, heart tissues were harvested 3 days after injection and were fixed in 4% formaldehyde. The tissues were frozen and then cut into sections and mounted onto glass slides. The frozen sections were stained with hematoxylin and eosin. Histopathologic specimens were examined by light microscopy.

Statistics. The differences between two groups were determined with Student's *t* test. The differences among three or more groups were determined with a one-way analysis of variance (ANOVA).

■ RESULTS AND DISCUSSION

Characteristics of Combinatorial Drug Delivery via cMLVs. Our strategy of combination drug delivery via cross-linked multilayer liposomal vesicles was to incorporate the hydrophobic drug paclitaxel (PTX) into the lipid membranes and encapsulate the hydrophilic drug doxorubicin (Dox) in the aqueous core of liposomal vesicles, shown in Scheme 1. The

Scheme 1. Schematic Illustration of the Codelivery of Hydrophobic Drug Paclitaxel (Green) and Hydrophilic Drug Doxorubicin (Red) via cMLVs



cross-linked multilamellar liposomal vesicles (cMLVs) were formed by adding $MgCl_2$ to trigger vesicle fusion and then stabilized by dithiothreitol (DTT) to form cross-linkers between adjacent liposomal vesicles.^{27,29} The surface of the cross-linked multilayer liposomes was further PEGylated with thiol-termineated PEG, which is known to enhance vesicle stability and elongate the blood circulation half-life.^{30,31} First, we characterized the physical properties of dual drug-loaded cMLVs compared to single drug-loaded cMLVs to determine whether drug combinations could change the physical properties of liposomal formulation. Dynamic light scattering (DLS) measurements showed that the resulting dual drug-loaded cMLVs had a similar average hydrodynamic diameter as single drug-loaded cMLVs (Figure 1A–C). We found no significant aggregation of particles during the cross-linking process in all three liposomal formulations, as evident by the narrow size distribution and similar polydispersity observed in both dual drug-loaded and single drug-loaded cMLVs. This suggests that

the combination of Dox and PTX in a single nanoparticle has a negligible effect on the formation of cMLV particles.

We next determined whether the encapsulation efficiency or loading yield of cMLVs were affected by loading multiple therapeutics. Single drug-loaded and dual drug-loaded cMLVs were dissolved in organic solvents to free all encapsulated drugs (Dox and/or PTX). Dox and PTX concentrations were quantified by spectrofluorometer and/or HPLC, respectively. As shown in Figure 1D, the drug encapsulation efficiency of Dox and PTX in cMLV (Dox+PTX) was not significantly different from that in either cMLV (Dox) or cMLV (PTX). It was also shown that cMLV (Dox+PTX) had a comparable drug loading yield (~ 270 mg drug per g of phospholipids) compared to single drug-loaded cMLVs (Figure 1E). The drug release profiles of Dox and PTX were also evaluated in dual drug-loaded cMLVs to investigate whether the cMLVs are able to release the individual drugs in a controlled manner. The results of *in vitro* drug release assay showed that cMLV (Dox+PTX) has slow and linearly sustained release kinetics of both Dox and PTX (up to 2 weeks), similar to that of single drug-loaded cMLVs (Figure 1F–H). These results confirm that this approach enables the loading of drugs with different hydrophobicity into the same nanoparticles with an efficient drug loading yield and sustained drug release profiles.

In Vitro Analysis of Doxorubicin: Paclitaxel for Drug Ratio-Dependent Synergy. Certain cases of combinatorial drug delivery are able to induce synergistic effects, and it has been reported that the combination effect, synergy, additivity, or antagonism can be affected by the dose ratio.^{19,32} To test this hypothesis, the cytotoxicities of cMLV (Dox+PTX) encapsulating three different drug weight ratios (5:1, 3:3, and 1:5) were examined in B16 and 4T1 cell lines. The cytotoxicities of cMLVs were compared to the cytotoxicities of the same three ratio combinations in cocktail solutions. Figure 2A summarizes the results of IC_{50} measurements of the dual drug-loaded cMLVs with the three different dose ratios after 48 h of incubation with B16 and 4T1 cells. The IC_{50} values of cMLV (Dox+PTX) at Dox–PTX ratios of 3:3 and 5:1 were significantly smaller than that of the 1:5 ratio in the cell lines studied. A similar trend of IC_{50} values at the different dose ratios was observed for free Dox and PTX combinations (Figure 2B).

Moreover, combination index (CI) values were analyzed from *in vitro* cytotoxicity curves for Dox and PTX combinations either in cMLVs or cocktail solutions to assess the effects of combination. The IC_{50} values of individual drugs either in cMLVs or in solution are shown in Figure S1 of the Supporting Information. A CI of less than, equal to, and greater than 1 is known to indicate synergy, additivity, and antagonism, respectively.^{19,28,33,34} Although combination indexes are only shown for a 0.5 fraction of affected cells (f_a) (50% cell growth inhibition relative to control cells) in Figure 2, the profile of synergy/antagonism was similar for other f_a values. As shown in Figure 2C, at $f_a = 0.5$, synergistic effects were observed in both B16 and 4T1 tumor cells for coloaded cMLVs at Dox–PTX ratios of 5:1 and 3:3 (Dox–PTX), while the combination at a 1:5 ratio was additive or antagonistic in B16 and 4T1 cells. In contrast, no synergistic effect was observed in B16 or 4T1 cells treated with three ratios of Dox and PTX in cocktail, as shown in Figure 2D, further confirming the potential of cMLVs to induce synergy by controlling dose ratios.

Our data indicated that combinatorial delivery via cMLVs with high ratio of PTX induced additivity or antagonism. In

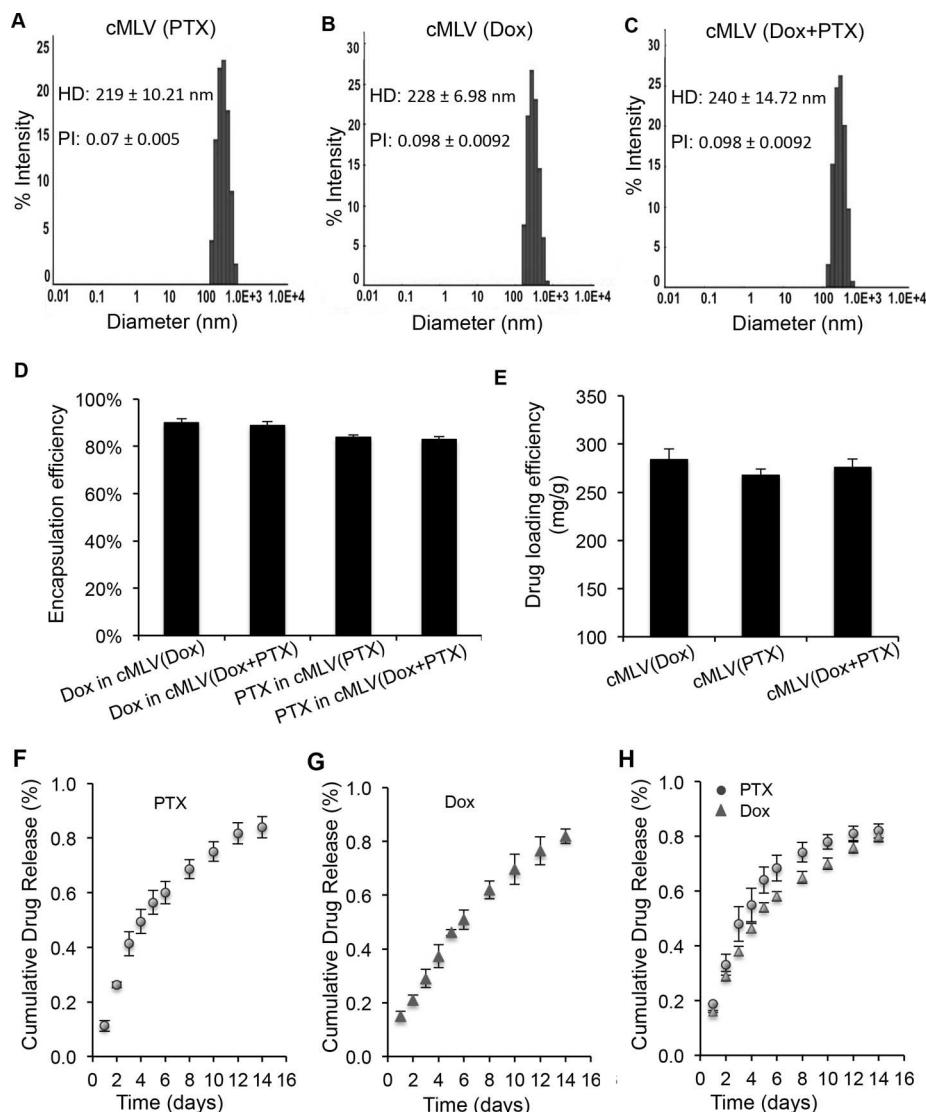


Figure 1. Characteristics of cMLV (Dox+PTX). (A–C) The hydrodynamic size distribution of cMLV(Dox), cMLV(PTX), and cMLV(Dox+PTX) measured by dynamic light scattering. The mean hydrodynamic diameter (HD) and polydispersity index (PI) of cMLV(Dox), cMLV(PTX), and cMLV(Dox+PTX) are indicated on the graph. (D, E) Effects of coencapsulation of Dox and PTX on loading capability and drug release kinetic profiles of cMLVs. The encapsulation efficiency (D) and loading efficacy (E) of drugs in cMLV(combined drugs) and cMLV (single drug). (F–H) *In vitro* release kinetics of doxorubicin and paclitaxel from dual-drug loaded cMLVs and single-drug loaded cMLVs. Error bars represent the standard deviation of the mean from triplicate experiments.

fact, some studies have shown that low concentrations of PTX can induce cell apoptosis more effectively than high concentrations, but the mechanism remains elusive.^{35,36} Further studies suggested that PTX could activate the extracellular signal regulated kinase (ERK), leading to cell proliferation and building drug resistance.^{37–39} It was also shown that inhibiting the ERK pathway dramatically enhanced cell apoptosis induced by PTX.^{37,39} These studies indicate that the high PTX concentration could be responsible for the antagonism seen between Dox and PTX at a 1:5 dose ratio. To investigate whether there is a difference in activation of ERK in melanoma cells treated by cMLV(Dox+PTX) at the three different dose ratios, phosphorylated ERK expression was detected by Western blot. As shown in Figure 2E, the combination of Dox and PTX at a 1:5 ratio showed significantly increased expression of phosphorylated ERK compared to the 3:3 and 5:1 ratios. Quantification of ERK phosphorylation (Figure 2F) showed a 30-fold enhancement in phosphorylated ERK in cells

treated by the cMLV(Dox+PTX) 1:5 ratio. These data suggest that ratio-dependent combination effects are likely linked to the ERK activation caused by high concentrations of PTX.

Drug Ratio-Dependent Efficacy of cMLV(Dox+PTX) in Tumor Treatment. In order to assess whether the drug ratio-dependent *in vitro* cytotoxicity was also manifested *in vivo*, doxorubicin and paclitaxel were coencapsulated in cMLV particles at a weight ratios ranging from 5:1 to 1:5, while keeping the total drug mass encapsulated in cMLVs constant. This panel of fixed ratio cMLV formulations and the same fixed ratio combination in cocktail solutions were evaluated for their antitumor efficacy in an *in vivo* 4T1 breast tumor model. As shown in Figure 3A, tumor volume in the groups treated with drug combinations in solution decreased significantly compared to that in the control group ($p < 0.01$). The tumor volume between the groups treated with different ratios of drug combinations in solution did not show a significant difference ($p > 0.05$), consistent with the *in vitro* finding that free drug

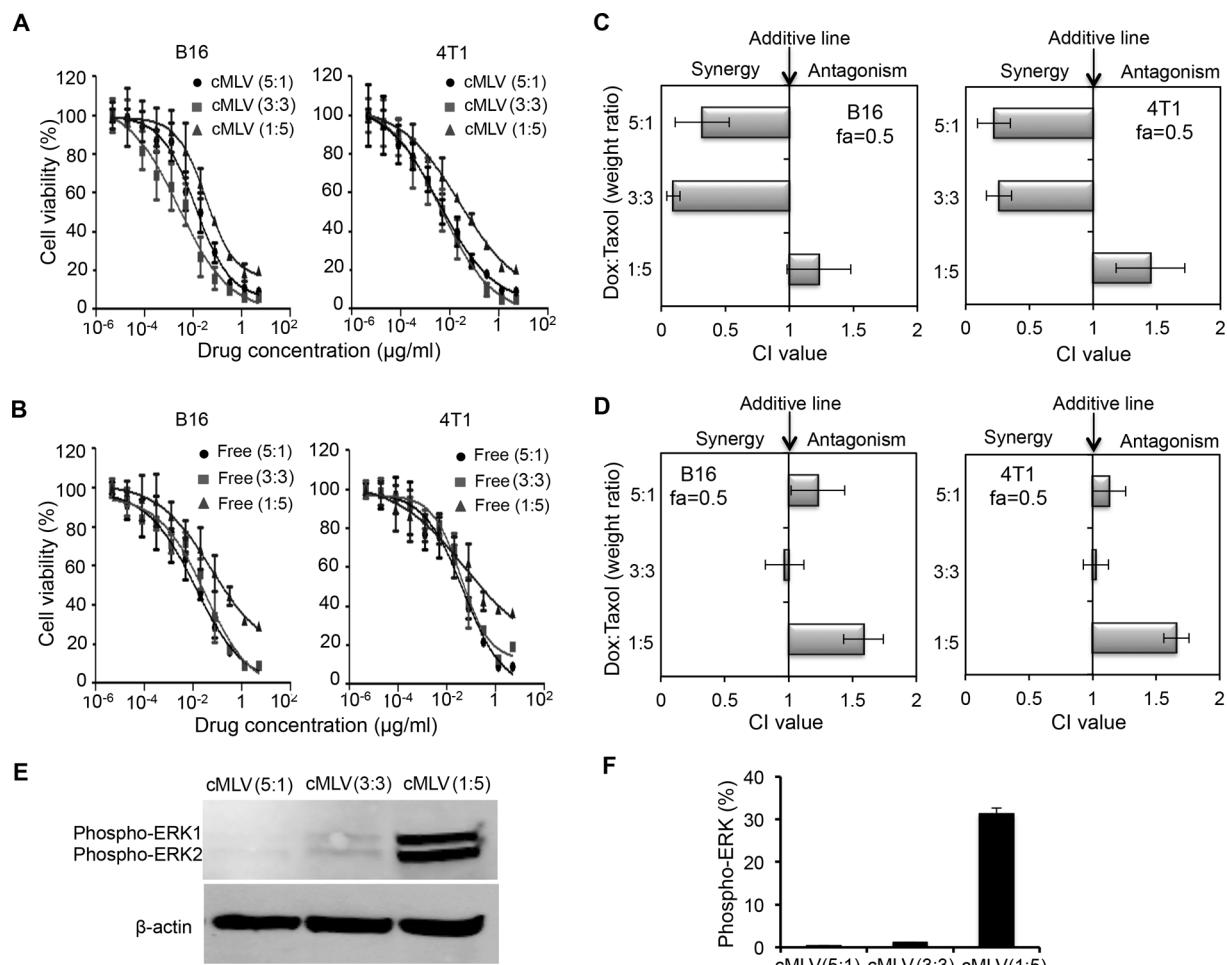


Figure 2. Determination of the ratio of drug combinations to induce synergy. (A, B) *In vitro* cytotoxicity of three weight ratios (5:1, 3:3, and 1:5) of Dox and PTX in cMLV formulations (A) or solution (B) in B16 melanoma tumor or 4T1 breast tumor cell lines. The cytotoxicity was measured by a standard XTT assay. (C) Combination index (CI) histogram for cMLV (different drug combinations) exposed to cultured B16 and 4T1 tumor cells. (D) Combination index histogram for different ratios of drug combination in solution exposed to culture B16 and 4T1 tumor cells. The surviving cell fraction from three replicates was averaged and analyzed by nonlinear regression. The histogram presents the CI values obtained at a fraction of 0.5. Error bars represent the standard deviation of the mean from triplicate experiments. (E) Immunoblot analysis of phosphorylated ERK in B16 cells treated by cMLV(Dox+PTX) with three dose ratios: 5:1, 3:3, and 1:5. β-actin was used as control. (F) Quantification of phosphorylated ERK shown in (E). Protein amounts were estimated by densitometry of immunoblots. Error bars represent SD.

combinations did not show a synergistic effect. In comparison, administration of the 5:1 and 3:3 weight ratio of Dox to PTX in cMLV resulted in significantly enhanced antitumor activity compared to the 1:5 ratio, indicating the ability of cMLVs to induce a ratio-dependent synergistic effect *in vivo*. Moreover, no weight loss was observed for all treated groups during the experiment (Figure 3B), indicating that there was no significant toxicity from these dose combinations.

The dose-dependent antitumor activity was further confirmed by survival test as shown in Figure 3C. Treatment with three ratios of drug combinations in cocktail solutions resulted in an increased survival time (35 days) compared to PBS treatment (28 days, $p < 0.05$). Administration of the 5:1 and 3:3 weight ratios in cMLV formulations resulted in a significant increased life span compared to 1:5 ratio in cMLVs ($p < 0.05$). These results confirmed a dose-dependent synergy of drug combinations in cMLV formulations and provide a positive correlation linking the combination effects *in vitro* to the degree of antitumor efficacy *in vivo*.

Drug Ratio-Dependent Efficacy of Coencapsulated Dox–PTX on Tumor Apoptosis.

To investigate the ratio-

dependent antitumor mechanism *in vivo*, a TUNEL assay was performed to detect apoptotic cells in 4T1 tumors treated with different ratios of Dox and PTX in cocktail and in cMLV formulations for 3 days. As shown in Figure 4A, 4T1 tumors treated with three different ratios (5:1, 3:3, and 1:5) of Dox and PTX in solution-induced cell apoptosis by a significant amount compared to controls. The apoptosis index was not remarkably different among different ratios of drug combination cocktails ($p > 0.05$), consistent with the similar effect on tumor growth between the cocktail treatments. Moreover, the 5:1 and 3:3 ratios of Dox and PTX in cMLVs promoted tumor cell apoptosis compared to the antagonistic ratio (1:5). The quantified data (Figure 4B) further confirm that drug ratio-dependent antitumor efficacy via cMLVs can contribute to different levels of tumor apoptosis.

In Vivo Cardiac Toxicity Evaluation of Drug Combinations in cMLV Formulations. An unexpected clinical outcome of increased cardiotoxicity after combined treatments of Dox and PTX has been reported, thus limiting their clinical applications.^{40,41} To investigate whether the synergistic therapies could induce synergistic cardiac toxicity, three weight

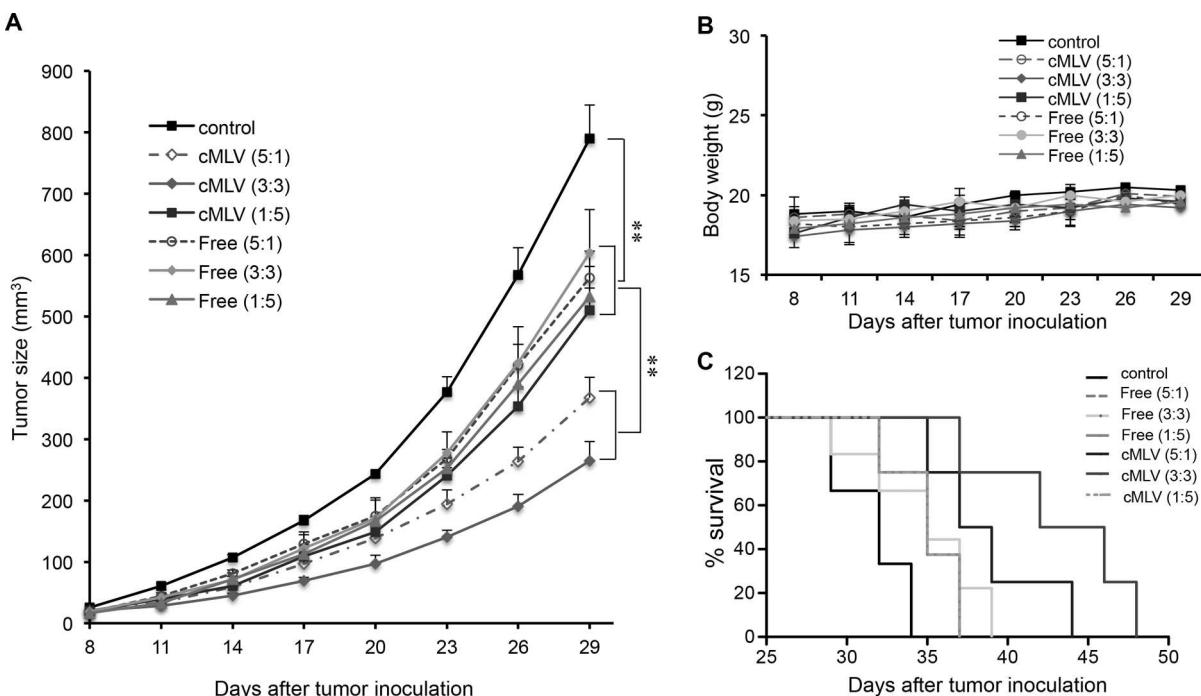


Figure 3. Drug ratio-dependent efficacy of cMLV(Dox+PTX) in tumor treatment. (A) Tumor growth was measured after treatment with PBS, 3.33 mg/kg Dox + 0.67 mg/kg PTX, 2 mg/kg Dox + 2 mg/kg PTX, 0.67 mg/kg Dox + 3.33 mg/kg PTX, either in cMLVs or in solution every 3 days. Tumor growth and body weights were monitored until the end of the experiment. Error bars represent standard error of the mean, $n = 6$ for each treatment group (* $p < 0.05$, ** $p < 0.01$). (B) Average mouse weight loss over the duration of the experiment. (C) Survival curves for 4T1 bearing mice treated with PBS, 3.33 mg/kg Dox + 0.67 mg/kg PTX, 2 mg/kg Dox + 2 mg/kg PTX, 0.67 mg/kg Dox + 3.33 mg/kg PTX either in cMLVs or in solution every 3 days. The survival rates are presented as Kaplan–Meier curves. The survival curves of individual groups were compared by a log-rank test.

ratios of doxorubicin and paclitaxel in both cMLV formulations and cocktail solutions were evaluated for cardiac effects. Mice-bearing 4T1 tumors were injected intravenously through tail vein with 8.33 mg/kg Dox + 1.66 mg/kg PTX, 5 mg/kg Dox + 5 mg/kg PTX, or 1.66 mg/kg Dox + 8.33 mg/kg PTX in solution or in cMLVs. Hematoxylin and eosin staining of cardiac tissue sections from each treatment group were examined. As shown in Figure 5, all three dose ratios of Dox and PTX in cocktail solutions caused damage to cardiac tissue indicated by myofibrillary loss, disarray, and cytoplasmic vacuolization. No significant histopathologic changes in cardiac tissue were observed in three dose ratios of Dox and PTX in cMLV formulations compared to the control group, indicating that a reduction in systemic toxicity can be achieved when drugs are coencapsulated in cMLVs. Moreover, no synergistic toxicity was observed in the synergistic ratios (5:1 and 3:3) of Dox and PTX in cMLVs.

In Vivo Maintenance of Drug Ratios in cMLV Formulations. In order to determine if dose ratios of drugs delivered via cMLVs were well-maintained *in vivo* and to correlate the *in vivo* effects to the *in vitro* combination effect, the drug concentrations in tumor tissues were measured. Doxorubicin and paclitaxel were coencapsulated at the 5:1, 3:3, and 1:5 weight ratios inside cMLVs and administered i.v. to mice, while the same ratios of drug combinations in cocktail solutions were administrated as controls. Twenty-four hours after injection, tumors were excised and homogenized, and Dox and PTX were extracted and detected by HPLC analysis, as illustrated in Figure 6A. The HPLC results show that cMLVs maintain the doxorubicin–paclitaxel weight ratios at 5:1, 3:3, and 1:5, respectively, in tumors for over 24 h (Figure 6B). In

comparison, the free-drug cocktail Dox–PTX weight ratio changed dramatically after administration, shown in Figure 6C. In addition, remarkably more doxorubicin and paclitaxel accumulated in tumors when administered via cMLV formulations compared to free-drug cocktails with equivalent amounts of Dox and PTX, thus maximizing their combinatorial effect. These results indicate that cMLVs can efficiently maintain dose ratio *in vivo*, thus translating the combination effects (synergy, additivity, and antagonism) from *in vitro* to *in vivo*.

To summarize, a robust approach for combinatorial chemotherapy was presented by encapsulating two different types of antitumor therapeutics, with ratiometric control over drug loading, into a cross-linked multilamellar liposomal formulation. Previously, we have demonstrated the superior ability of cMLVs as drug carriers to offer controllable and sustainable drug release profiles of doxorubicin with increased vesicle stability, enabling improved antitumor activity. In the present study, we explore the potential of cMLVs in combinatorial delivery of Dox and PTX, which have been widely used as a combined anthracycline–taxane regimen in metastatic breast cancer,⁴² to achieve synergistic antitumor activity. A number of studies suggest the noncoordinated biodistribution profiles of this combination when administered in cocktail solutions limit the efficacy of the combination.^{13,14} However, the versatile cross-linked multilamellar liposomes enabled codelivery of Dox and PTX via a single vesicle to the cancer site, thus coordinating the plasma elimination and tissue distribution of the combined drugs.

Recent studies revealed that the activity of antitumor drug combinations is determined by the ratio of the combined drugs

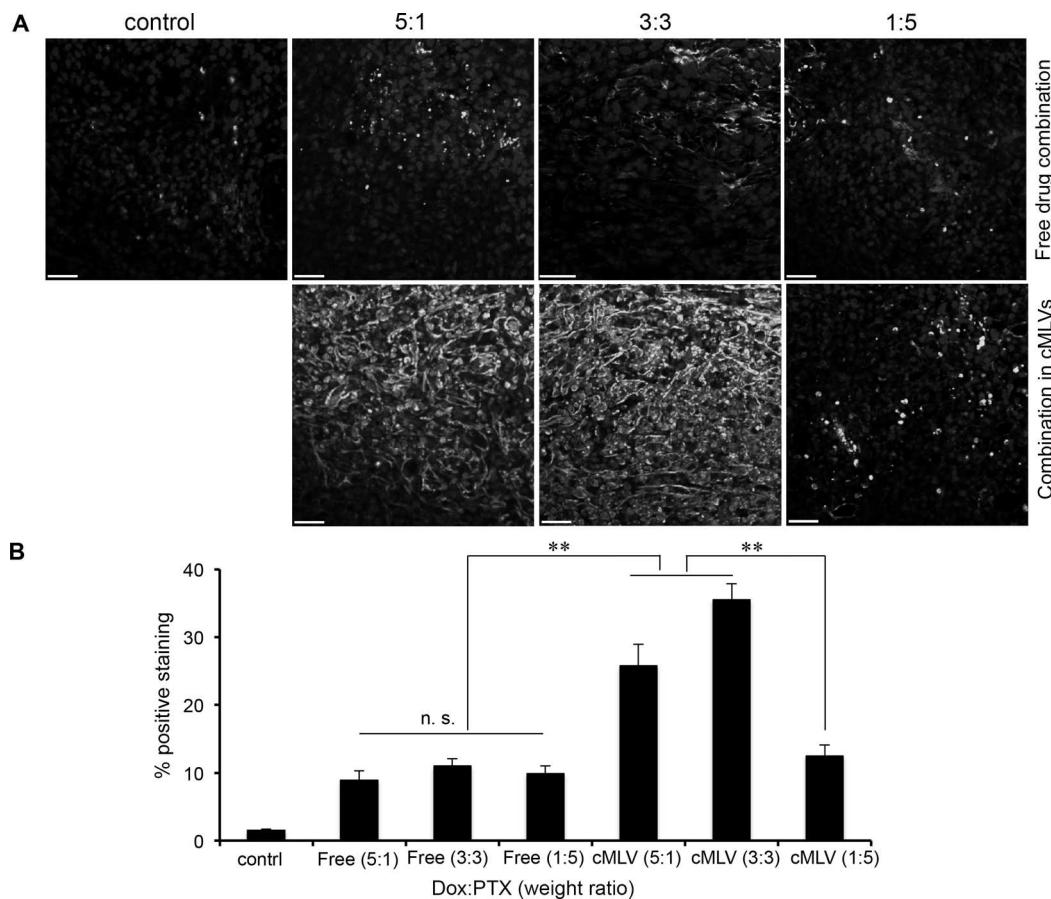


Figure 4. Drug ratio-dependent efficacy of coencapsulated Dox–PTX on tumor cell apoptosis. (A) 4T1 tumor-bearing mice were treated with PBS, 8.333 mg/kg Dox + 1.667 mg/kg PTX, 5 mg/kg Dox + 5 mg/kg PTX, or 1.667 mg/kg Dox + 8.33 mg/kg PTX, either in cMLVs or in solution. Three days after injection, tumors were excised. Apoptotic cells were detected by a TUNEL assay (green) and costained by nuclear staining DAPI (blue). The scale bar represents 50 μ m. (B) Quantification of apoptotic positive cells in the 4T1 tumor. To quantify TUNEL positive cells, four regions of interest (ROI) were randomly chosen per image at $\times 2$ magnification. Within one region, the area of TUNEL positive nuclei and the area of nuclear staining were counted by software. The data are expressed as % total nuclear area stained by TUNEL in the region. Data represented as mean \pm SD ($n = 3$).

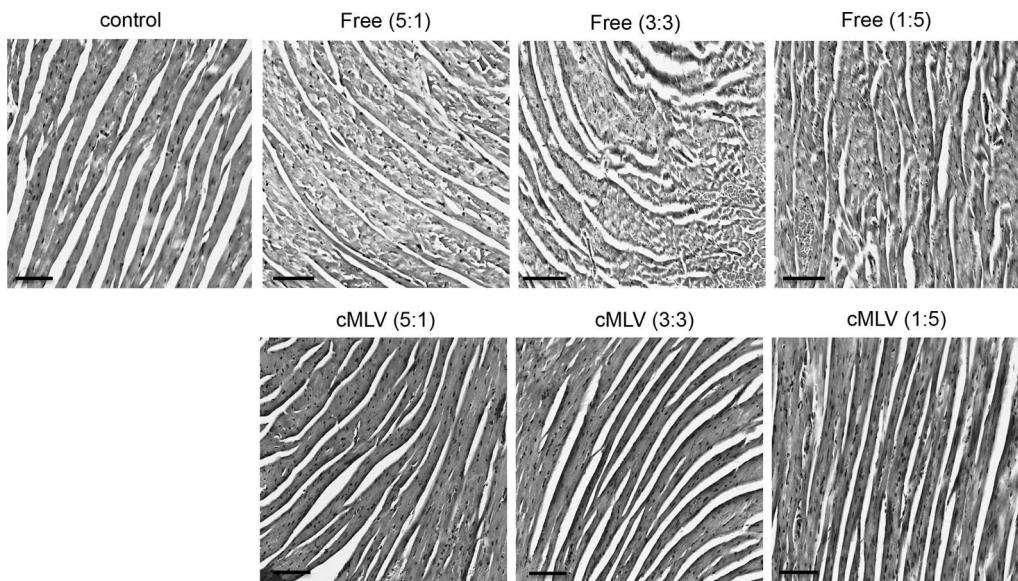


Figure 5. *In vivo* toxicity. Histologic appearance of cardiac tissues obtained from C57/BL6 mice with no drug treatment or administered a single intravenous injection with three dose ratios of Dox and PTX (5:1, 3:3, and 1:5) in solutions or cMLV formulations at 10 mg/kg total drug equivalent. The scale bar represents 100 μ m.

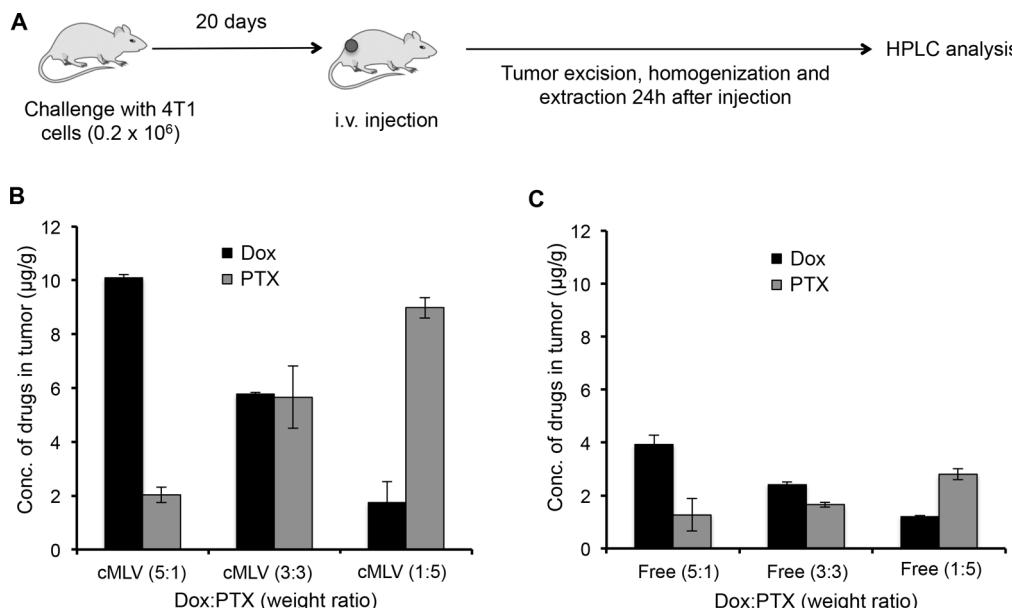


Figure 6. *In vivo* maintenance of Dox–PTX ratios in cMLV formulations. (A, B) Tumor-bearing mice were treated with PBS, 8.333 mg/kg Dox + 1.667 mg/kg PTX, 5 mg/kg Dox + 5 mg/kg PTX, or 1.667 mg/kg Dox + 8.33 mg/kg PTX, either in cMLVs (A) or in solution (B). Twenty-four h after injection, tumors were excised, and drug concentrations of Dox and PTX were measured by HPLC. All data are shown as the means of triplicate experiments.

exposed to cells.^{32,43–45} Therefore, it is highly desirable to maintain a synergistic ratio of combined drugs *in vivo*. Here, we demonstrate that the stability of cMLVs enables us to coload Dox and PTX with predefined ratios and induce a ratio-dependent synergy in tumor cells. It was previously reported by a number of studies that paclitaxel-containing liposomes could not maintain stability over a drug-to-lipid molar ratio of 3–4%. For example, one study showed that more than 8% PTX-to-lipid formulations (PG–PC 3:7 molar ratio) were not stable for 1 day.²⁴ cMLVs can maintain a high stability up to 30% paclitaxel-to-lipid molar ratio. This is most likely due to the cross-linked multilamellar structure of cMLVs, which allows codelivery of Dox and PTX with high loading efficiency. In addition, enhanced vesicle stability of cMLVs enables these nanoparticles to maintain the dose ratios of Dox and PTX at tumor sites, translating the ratio-dependent synergy from *in vitro* to *in vivo*. This would be beneficial for predicting the efficacy of treatment in clinical trials and the optimal design of combination therapy based on *in vitro* cellular experiments. Our *in vivo* results also reveal that the enhanced combinatorial efficacy of cMLVs compared to cocktail combination is due to the augmented accumulation of drugs at tumor sites.

In clinical studies, Dox and PTX exhibit an increased cardiac toxicity when combined in cocktail,^{40,41} raising the concern that a significant side effects could be associated with the synergistic therapeutic efficacy. However, we previously demonstrated that the robust cMLV formulation greatly reduced systemic toxicity of Dox, most likely due to the sustained drug release profile of Dox. Here, we show that cMLVs can induce synergistic effects on tumor growth without causing cardiac toxicity, further demonstrating their potential in combinatorial drug delivery. These results, taken together, indicated that the superior ability of cMLVs in combination therapy is not only attributed to the prolonged exposure of drugs to tumor cells, but also to the maintenance of synergistic dose ratios at the site of action with no significant systemic toxicity.

CONCLUSIONS

In conclusion, we have demonstrated that the ratio-dependent synergy of drug combinations shown *in vitro* can be translated into the synergistic antitumor efficacy *in vivo* by coloading two types of drugs into cross-linked multilamellar liposomal formulations. Unlike the free-drug cocktail, cMLVs maintain dose ratios for prolonged times after administration *in vivo* due to the ability of cMLVs to coencapsulate and retain the combined drugs in a manner that coordinates their pharmacokinetics. In the present study two drugs (Dox and PTX) were chosen to demonstrate the advantage of this combination drug delivery system by cMLVs. In this regard, we believe this delivery system can offer the clinical possibility for improved synergistic delivery of multiple therapeutics with a ratiometric control over drug encapsulation for combination cancer treatment.

ASSOCIATED CONTENT

Supporting Information

IC₅₀ values of Dox and PTX in cMLV formulation or free drug solution in B16 melanoma or 4T1 breast tumor cells. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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