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<p>(21) International Application Number: PCT/CA96/00677 (22) International Filing Date: 9 October 1996 (09.10.96) (30) Priority Data: 08/542,341 11 October 1995 (11.10.95) US (71) Applicant: THE UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; University-Industry Liaison Office, Room IRC 331, 2194 Health Sciences Mall, Vancouver, British Co- lumbia V6T 1Z3 (CA). (72) Inventors: LIM, Howard, J.; 2849 West 42nd Avenue, Vancouver, British Columbia V6N 3G7 (CA). MADDEN, Thomas, D.; 1053 Nicola Street #5, Vancouver, British Columbia V6G 2E1 (CA). BALLY, Marcel, B.; SPC 11, Harding Road, Bowen Island, British Columbia V0N 1G0 (CA). BARBER, Lana, W.; 2611 Melfa Lane, Vancouver, British Columbia V6T 2C5 (CA). CHANG, Charmaine, W.; 7651 Sunnymede Crescent, Richmond, British Columbia V6Y 1H3 (CA). (74) Agents: ROBINSON, J., Christopher et al.; Fetherstonhaugh &amp; Co., Vancouver Centre, Suite 2200, 650 West Georgia Street, P.O. Box 11560, Vancouver, British Columbia V6B 4N8 (CA).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: LIPOSOMAL FORMULATIONS OF MITOXANTRONE</p>		
<p>(57) Abstract</p> <p>Compositions and methods which are useful for the treatment of solid tumors in a host and which are also useful for increasing the therapeutic index of mitoxantrone in a host. The pharmaceutical compositions are liposomal formulations of mitoxantrone in which the liposomes comprise a mixture of cholesterol and a diacylphosphatidylcholine, preferably 1,2-<i>sn</i>-dimyristoyl-phosphatidylcholine.</p>		

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## LIPOSOMAL FORMULATIONS OF MITOXANTRONE

### BACKGROUND OF THE INVENTION

Mitoxantrone is an anthracenedione derivative used in chemotherapy. It has demonstrated anti-tumor activity in a wide variety of experimental and human tumor models (Shenkenberg, T.D., *et al.*, *Annals of Internal Medicine*, **105**:67-81 (1986)). Phase III studies indicate activity in non-Hodgkin's lymphoma, myeloma, advanced  
5 breast cancer, bladder, ovarian and hepatocellular carcinomas in combination with other agents or as a single agent (Shenkenberg, T.D. *et al.*, *Annals of Internal Medicine*, **105**:67-81 (1986)). The toxic side effect of mitoxantrone is myelosuppression versus another related anthracycline such as doxorubicin which has a dose limiting cardiotoxicity (Saleton, S., *Cancer Treatment Reviews*, **14**:297-303 (1987)). In addition to this,  
10 mitoxantrone proved to have a lower short and long term toxicity than doxorubicin. (Ehninger, G., *et al.*, *Clin. Pharmacokinet*, **18**:365-380 (1990)). The precise mechanism of action for mitoxantrone is still under investigation, but it has been shown to be a DNA interchelator. As a result, it is thought that the main antitumor activity is conferred by inducing DNA strand breaks and by forming a complex with topoisomerase II (Ehninger,  
15 G., *et al.*, *Clin. Pharmacokinet*, **18**:365-380 (1990)).

It is well established in preclinical animal models that the therapeutic activity of anticancer agents can be improved through the use of liposome formulations. In general terms, liposomes engender pharmacokinetic and pharmacodistribution characteristics that give rise to increased therapeutic activity or reduced drug-related  
20 toxicity. Although the mechanism of therapeutic activity for liposomal anticancer agents is not well understood, studies have suggested that increased drug exposure at the site of tumor growth is an important attribute. Increases of tumor drug levels are a consequence of preferential accumulation of the liposome carrier within tumors. It is important to note that there is no evidence suggesting that the encapsulated form of the  
25 drug is therapeutically active, though it is postulated that antitumor activity is mediated by drug release from regionally localized liposomes.

The emphasis of investigators developing liposomal anticancer agents has, for the reasons cited above, been on using liposomal lipid compositions that: i) are less

permeable to the encapsulated agent and ii) exhibit increased circulation lifetimes. Liposomes that are retained in the plasma compartment for extended periods exhibit a greater tendency to accumulate in regions of tumor growth. However, the kinetics of this extravasation process, where liposomes leave the blood compartment and enter an extravascular site, is slow. For this reason, efficient drug delivery can only be achieved with liposomes that effectively retain the drug.

Studies with liposomal formulation of the anthracycline doxorubicin suggest that properties which promote efficient delivery of drug to tumors also compromise therapeutic activity. A balance between doxorubicin retention (to maximize drug accumulation in a site of tumor growth) and release (to effect therapy) has not been achieved. Additionally, liposomal formulation of doxorubicin that release the drug following intravenous administration exhibited enhanced toxicity and increased doxorubicin accumulation in cardiac tissue.

What is needed in the art are new liposomal formulations of chemotherapeutic agents which do not suffer the drawbacks noted above. Surprisingly, the present invention provides such compositions.

### SUMMARY OF THE INVENTION

The present invention provides compositions and methods which are useful for the treatment of solid tumors in a host and which are also useful for increasing the therapeutic index of mitoxantrone in a host. The pharmaceutical compositions are liposomal formulations of mitoxantrone in which the liposomes comprise a mixture of cholesterol and a diacylphosphatidylcholine, preferably 1,2-*sn*-dimyristoyl-phosphatidylcholine.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of temperature on the pH gradient loading of mitoxantrone into DSPC/Chol (A) and DMPC/Chol (B) liposomes: 37°C (●); 50°C (▲); 65°C (▼).

Figure 2 shows the release of Mitoxantrone from DSPC/Chol (●) and DMPC/Chol (■) liposomes in HBS at 37°C.

Figure 3 shows the *in vivo* release of mitoxantrone from DSPC/Chol (●) and DMPC/Chol (■) liposomes.

Figure 4 shows the percentage change in body weight of BDF1 mice during efficacy studies as a means of determining maximum tolerated doses (MTD). Data shown here is the data from the determined MTD'S: DSPC/Chol at 20 mg/kg (●), DMPC/Chol at 20 mg/kg (■), and free mitoxantrone at 10 mg/kg (▲).

5 Figure 5 shows the percentage survival of BDF1 mice injected with 10<sup>4</sup> L1210 cells, i.v. via the lateral tail vein 24 hours before being treated with 10 mg/kg doses of free mitoxantrone (▲), DSPC/Chol (●), and DMPC/Chol (■) liposomal formulations. Untreated animals served as controls (▼).

10 Figure 6 shows the amount of lipid and drug which accumulate in the liver of CD1 mice over a period of 48 days for DMPC/Chol liposomal mitoxantrone (■) and for DSPC/Chol liposomal mitoxantrone (●).

Figure 7 shows the changes in distribution and efficacy due to incorporation of 5 mole % DSPE-PEG into the DMPC/Chol liposome formulations of mitoxantrone (A is without added DSPE-PEG and B is with the added DSPE-PEG).

15 Figure 7A shows the difference in drug levels in CD1 mice. Figure 7B shows the difference in mean survival times of BDF1 mice with a L1210 tumor burden. The dose for both experiments was 10 mg/kg.

## DETAILED DESCRIPTION OF THE INVENTION

### Abbreviations and Definitions

20 Abbreviations used herein have the following meanings: Chol, cholesterol; DMPC, 1,2-*sn*-dimyristoylphosphatidylcholine; DSPC, 1,2-*sn*-distearoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; ILS, increase in life-span; HEPES, N-(2-hydroxyethyl)piperidine-N'-2-ethanesulphonic acid; HBS, HEPES buffered saline; MLV, multilammellar vesicles; PEG-DSPE, poly(ethylene glycol)-modified distearoylphosphatidylethanolamine; RES, reticulo-endothelial system; 25 LUV, large unilamellar vesicles; PEG, poly(ethylene glycol); <sup>3</sup>H-CDE, <sup>3</sup>H-Cholesteryl hexadecyl ether.

As used herein, the term "mitoxantrone" refers to the free base as well as any pharmaceutically acceptable salts thereof.

### Description of the Embodiments

The therapeutic index of most anticancer drugs is narrow, with severe toxic side effects occurring within the same dose range required to mediate effective therapy. A variety of experimental strategies have been developed in order to improve the therapeutic index of anticancer drugs. These strategies have a common aim: to improve drug specificity. The principal benefit postulated for use of liposomes as carriers of anticancer drugs is liposome mediated increases in drug delivery to the disease site and decreases in drug delivery to sites of target organ toxicity (Sugarmann, S.M., *et al.*, *Critical Reviews in Oncology/Hematology*, **12**:231-242 (1992)). As a result, it is important to design liposomes that (i) have a greater propensity to accumulate within disease sites (Bally, M.B., *et al.*, *Cancer Chemother. Pharmacol.*, **34**:137-146 (1994); Gabizon, A., *et al.*, *Proc. Natl. Acad. Sci.*, **85**:6949-6953 (1988); Gabizon, A.A., *Cancer Res.*, **52**:891-896 (1992); Ojihara-Umeda, I., *et al.*, *Cancer Res.*, **54**:463-467 (1994); Uchiyama, K., *et al.*, *Intl. J. of Pharm.*, **121**:195-203 (1995); Wu, N.Z., *Cancer Res.*, **53**:3765-3770 (1993)); (ii) have been optimized with respect to maximizing the amount of drug contained per liposome (Mayer, L.D., *et al.*, *Biochim. Biophys. Acta*, **1025**:143-151 (1990); Bally, M.B., *et al.*, *Liposomes as Drug Carriers*, G. Gregoriadis (ed.), pp. 841-853, John Wilery and Sons, Ltd., Chichester (1988)); (iii) increase drug retention characteristics (Mayer, L.D., *et al.*, *Cancer Res.*, **49**:5922-5930 (1989); Boman, N.L., *et al.*, *Biochim. Biophys. Acta*, **1152**:253-258 (1993)) and (iv) augment the circulation lifetime of the drug loaded carrier (Gabizon, A., *et al.*, *Proc. Natl. Acad. Sci.*, **85**:6949-6953 (1988); Gabizon, A.A., *Cancer Res.*, **52**:891-896 (1992)). The dilemma associated with this reasoning, however, concerns the assumption and evidence that the therapeutically active component of a liposomal anticancer drug formulation is free drug. The present invention provides compositions which establish a balance between maximizing liposome delivery to the disease site and which exhibit controlled drug release characteristics. Controlled drug release can be achieved for certain drugs by relatively simple changes in liposomal lipid composition (Boman, N.L., *et al.*, *Biochim. Biophys. Acta*, **1152**:253-258 (1993)). As demonstrated herein, using the anticancer drug mitoxantrone, controlled drug release can engender significant improvements in therapeutic activity.

Thus, in one aspect, the present invention provides a pharmaceutical composition comprising a liposomal formulation of mitoxantrone. The liposomes are

prepared from a mixture of cholesterol and 1,2-*sn*-dimyristoylphosphatidylcholine. Preferably, the liposomes comprise of from about 50% to about 70% 1,2-*sn*-dimyristoylphosphatidylcholine and from about 30% to about 50% cholesterol. More preferably, the liposomes comprise about 55% 1,2-*sn*-dimyristoylphosphatidylcholine and about 45% cholesterol.

In one group of preferred embodiments, the pharmaceutical composition further comprises a PEG-lipid conjugate, preferably PEG-Cer-C<sub>14</sub>, PEG-Cer-C<sub>20</sub> or MePEGS-2000-DSPE which is present in an amount of from about 1% to about 8% by weight.

In another group of preferred embodiments, the compositions will further comprise a targeting moiety, such as those described in co-pending USSN 08/316,394, incorporated herein by reference. Methods of attaching a targeting species such as a protein or antibody are also described therein.

For the compositions of the present invention, it was surprising that differences in drug accumulation and leakage rates for DMPC/Chol and DSPC/Chol liposomes were not detected *in vitro*, even when the liposomes were incubated with a serum containing buffer. The phase transition temperatures (T<sub>c</sub>) for DSPC and DMPC are 55.3°C and 23.9°C respectively, and it was anticipated that the permeability characteristics would reflect differences in the gel to liquid crystalline phase transition of these phospholipids. Previous studies with vincristine demonstrated a good correlation between phospholipid T<sub>c</sub> and drug leakage (Boman, N.L., *et al.*, *Biochim. Biophys. Acta*, **1152**:253-258 (1993)). It was also surprising that collapse of the transmembrane pH gradient had little impact on the drug retention characteristics for either liposomal formulation. These data contrast previous results with vincristine (Boman, N.L., *et al.*, *ibid.*) and doxorubicin (Mayer, L.D., *et al.*, *Biochim. Biophys. Acta* **857**:123-126 (1986)). Moreover, it has been suggested that following pH gradient mediated uptake, drugs such as mitoxantrone, can form insoluble precipitates within the liposome (Mayer, L.D., *et al.*, *Biochim. Biophys. Acta*, **816**:294-302 (1985)). Accordingly, the permeability characteristics of the drug in a precipitated form may be less dependent on membrane characteristics or the presence of a residual transmembrane pH gradient. It is not understood, however, why differences in drug permeability become apparent *in vivo*.

The compositions of the present invention can be prepared by methods which are known to those of skill in the art. Typically, the liposomes are formed from the desired lipids, then loaded with mitoxantrone.

A variety of methods are available for preparing liposomes as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,946,787, PCT Publication No. WO 91/17424, Deamer and Bangham, *Biochim. Biophys. Acta*, 443:629-634 (1976); Fraley, *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:3348-3352 (1979); Hope, *et al.*, *Biochim. Biophys. Acta*, 812:55-65 (1985); Mayer, *et al.*, *Biochim. Biophys. Acta*, 858:161-168 (1986); Williams, *et al.*, *Proc. Natl. Acad. Sci.*, 85:242-246 (1988), the text *Liposomes*, Marc J. Ostro, ed., Marcel Dekker, Inc., New York, 1983, Chapter 1, and Hope, *et al.*, *Chem. Phys. Lip.* 40:89 (1986), all of which are incorporated herein by reference. Suitable methods include, *e.g.*, sonication, extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-induced fusion of small liposome vesicles, and ether-infusion methods, all well known in the art. One method produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle-forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with an aqueous buffered solution and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

Unilamellar vesicles are generally prepared by sonication or extrusion. Sonication is generally performed with a tip sonifier, such as a Branson tip sonifier, in an ice bath. Typically, the suspension is subjected to several sonication cycles. Extrusion may be carried out by biomembrane extruders, such as the LIPEX BIOMEMBRANE EXTRUDER<sup>®</sup>. Defined pore size in the extrusion filters may generate unilamellar liposomal vesicles of specific sizes. The liposomes may also be formed by extrusion through an asymmetric ceramic filter, such as a CERAFLOW MICROFILTER<sup>®</sup>, commercially available from the Norton Company, Worcester MA.



Following liposome preparation, the liposomes which have not been sized during formation may be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. A size range of about 0.2-0.4 microns allows the liposome suspension to be sterilized by filtration through a conventional filter, typically a 0.22 micron filter. The filter sterilization method can be carried out on a high throughput basis if the liposomes have been sized down to about 0.2-0.4 microns.

Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. The size of the liposomal vesicles may be determined by quasi-electric light scattering (QELS) as described in Bloomfield, *Ann. Rev. Biophys. Bioeng.*, **10**:421-450 (1981), incorporated herein by reference. Average liposome diameter may be reduced by sonication of formed liposomes. Intermittent sonication cycles may be alternated with QELS assessment to guide efficient liposome synthesis.

Extrusion of liposome through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. For use in the present invention, liposomes having a size of from about 0.05 microns to about 0.20 microns are preferred.

Methods of loading mitoxantrone or other conventional drugs into liposomes include an encapsulation technique and the transmembrane potential loading method.

In one encapsulation technique, mitoxantrone and the liposome components are dissolved in an organic solvent in which all species are miscible and concentrated to a dry film. A buffer is then added to the dried film and liposomes are formed having the drug incorporated into the vesicle walls. Alternatively, mitoxantrone can be placed into

a buffer and added to a dried film of only lipid components. In this manner, the drug will become encapsulated in the aqueous interior of the liposome. The buffer which is used in the formation of the liposomes can be any biologically compatible buffer solution of, for example, isotonic saline, phosphate buffered saline, or other low ionic strength buffers. Generally, the drug will be present in an amount of from about 0.01 ng/mL to about 50 mg/mL. The resulting liposomes with the drug incorporated in the aqueous interior or in the membrane are then optionally sized as described above.

Transmembrane potential loading has been described in detail in U.S. Patent No. 4,885,172, U.S. Patent No. 5,059,421, and U.S. Patent No. 5,171,578, the contents of which are incorporated herein by reference. Briefly, the transmembrane potential loading method can be used with essentially any conventional drug which can exist in a charged state when dissolved in an appropriate aqueous medium. Preferably, the drug will be relatively lipophilic so that it will partition into the liposome membranes. A transmembrane potential is created across the bilayers of the liposomes or protein(targeting species)-liposome complexes and the drug is loaded into the liposome by means of the transmembrane potential. The transmembrane potential is generated by creating a concentration gradient for one or more charged species (*e.g.*, Na<sup>+</sup>, K<sup>+</sup> and/or H<sup>+</sup>) across the membranes. This concentration gradient is generated by producing liposomes having different internal and external media. Thus, for a drug which is negatively charged when ionized, a transmembrane potential is created across the membranes which has an inside potential which is positive relative to the outside potential. For a drug, such as mitoxantrone, which is positively charged in acidic media, the opposite transmembrane potential would be used.

In another aspect, the present invention provides methods of treating solid tumors in a host by administering to the host one of the above liposomal compositions of mitoxantrone. To effect delivery of mitoxantrone, the liposomes can be loaded with mitoxantrone, or optionally, mitoxantrone in combination with another therapeutic agent and administered to the subject requiring treatment. The therapeutic agents which are administered using the present invention can be any of a variety of drugs which are selected to be an appropriate treatment for the disease to be treated in the tissue. Often the drug will be an additional antineoplastic agent, such as vincristine, doxorubicin, camptothecin, cisplatin, bleomycin, cyclophosphamide, methotrexate, streptozotocin, and the like. It may also be desirable to deliver anti-infective agents to specific tissues by the

present methods. The compositions of the present invention can also be used for the selective delivery of other drugs including, but not limited to local anesthetics, *e.g.*, dibucaine and chlorpronazine; beta-adrenergic blockers, *e.g.*, propranolol, timolol and labetolol; antihypertensive agents, *e.g.*, clonidine and hydralazine; anti-depressants, *e.g.*, imipramine, amitriptyline and doxepim; anti-convulsants, *e.g.*, phenytoin; antihistamines, *e.g.*, diphenhydramine, chlorpheniramine and promethazine; antibiotic/antibacterial agents, *e.g.*, gentamycin, ciprofloxacin, and cefoxitin; antifungal agents, *e.g.*, miconazole, terconazole, econazole, isoconazole, butaconazole, clotrimazole, itraconazole, nystatin, naftifine and amphotericin B; antiparasitic agents, hormones, hormone antagonists, immunomodulators, neurotransmitter antagonists, antiglaucoma agents, vitamins, narcotics, and imaging agents. Other particular drugs which can be selectively administered by the compositions of the present invention will be well known to those of skill in the art. Additionally, two or more therapeutic agents may be administered simultaneously if desired, where such agents produce complementary or synergistic effects.

The mitoxantrone-liposome complexes of the present invention can be administered to a subject according to standard techniques. Preferably, pharmaceutical compositions of the mitoxantrone-liposome complexes are administered parenterally, *i.e.*, intraarticularly, intravenously, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously by a bolus injection. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985).

Preferably, the pharmaceutical compositions are administered intravenously. Thus, this invention provides compositions for intravenous administration which comprise a solution of the liposomes suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.9% isotonic saline, and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium

lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of mitoxantrone-liposome complexes, in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually  
5 at or at least about 2-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected.

Liposome charge is an important determinant in liposome clearance from the blood, with negatively charged liposomes being taken up more rapidly by the  
10 reticuloendothelial system (Juliano, *Biochem. Biophys. Res. Commun.* **63**:651 (1975)) and thus having shorter half-lives in the bloodstream. Liposomes with prolonged circulation half-lives are typically desirable for most therapeutic and diagnostic uses. One means of increasing circulation half-life is the incorporation of GM<sub>1</sub> or phospholipid-polyoxyethylene conjugates in the liposome formulation. Additionally, the phospholipid-polyoxyethylene conjugates and related lipid derivatives can be added to the compositions  
15 of the present invention as steric barrier components. The purpose of steric barrier components is to inhibit liposome-liposome interactions and thereby reduce aggregation of the liposome systems formed. Suitable steric barrier components are modified derivatives of lipids and cholesterol, such as polyethylene glycol derivatives of  
20 cholesterol (PEG-cholesterols) and PEG-lipids (*e.g.*, phosphatidylethanolamine-polyoxyethylene conjugates and phosphatidic acid-polyoxyethylene conjugates). The polyoxyethylene conjugates which are used in the compositions of the present invention can be prepared by combining the conjugating group (*i.e.* phosphatidic acid or phosphatidylethanolamine) with an appropriately functionalized polyoxyethylene  
25 derivative. For example, phosphatidylethanolamine can be combined with  $\omega$ -methoxypolyethyleneglycol succinate to provide a phosphatidylethanolamine-polyoxyethylene conjugate. See, Parr, *et al.*, *Biochim. Biophys. Acta* **1195**:21-30 (1994), incorporated herein by reference. Where present, the PEG-cholesterol or PEG-lipids will comprise from 0.5 to about 5.0 mole % of the total lipid composition. Other  
30 PEG-modified lipids are described in co-pending application Ser. No. 08/486,214 (*e.g.* MePEGS-2000-Cer). While PEG-lipid conjugates are preferred in many liposomal compositions, those compositions which are used for treatment of liver tumors will preferably not contain a PEG conjugate.

Additionally, the liposome suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphanatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

5 In yet another aspect, the present invention provides methods for increasing the therapeutic index of mitoxantrone in a patient. These methods comprise

(i) encapsulating mitoxantrone in liposomes comprising a mixture of cholesterol and 1,2-*sn*-dimyristoylphosphatidylcholine to provide a liposomal formulation; and

10 (ii) administering the liposomal formulation to the patient, whereby the therapeutic index of mitoxantrone is improved relative to non-liposomal formulations of mitoxantrone.

Preferred compositions and methods of administration have been discussed above.

15 The following examples are offered solely for the purposes of illustration, and are intended neither to limit nor to define the invention.

## EXAMPLES

### Materials

20 Novatrone (mitoxantrone hydrochloride) was obtained from the British Columbia Cancer Agency and is a product of Cyanamid (Montreal, Quebec, Canada). 1,2-*sn*-distearoylphosphatidylcholine (DSPC) and 1,2-*sn*-dimyristoylphosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). HEPES, citric acid, cholesterol, nigericin, and SEPHADEX<sup>®</sup> G50 (medium) was purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Sodium phosphate dibasic  
25 was obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). The [<sup>14</sup>C] mitoxantrone, used as a tracer, was generously donated by American Cyanamid. [<sup>3</sup>H] Cholesteryl hexadecyl ether, a lipid marker that is not exchanged or metabolized *in vivo* (Stein, Y., *et al.*, *FEBS Lett.*, 111:104-106 (1980)), was purchased from Amersham (Oakville, Ontario, Canada). Aquacide 11 was purchased from Terochem Laboratories  
30 Ltd. (Edmonton, Alberta, Canada). Fetal bovine serum was obtained from Gibco Laboratories (Grand Island, New York, USA). Female CD1 and BDF1 mice were purchased from Charles River Laboratories (Ontario, Canada)

### EXAMPLE 1

This example illustrates the preparation of liposomal formulations of mitoxantrone.

#### *1.1 Preparation of Liposomes*

5 DSPC/Chol (55:45; mol:mol) and DMPC/Chol (55:45; mol:mol) liposomes were prepared, each with a small amount of [<sup>3</sup>H]-cholesterol hexadecyl ether using well established extrusion technology (see Hope, *et al.*, *Biochim. Biophys. Acta* 812:55 (1985), incorporated herein by reference). Briefly, lipid and cholesterol were dissolved in chloroform and then dried down to a homogenous lipid film under a stream  
10 of nitrogen gas. This film was then hydrated in a 300 mM citric acid buffer pH 4.0. The resulting multilamellar vesicle mixture was frozen and thawed five times before being extruded through stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, California) using an extrusion device (Lipex Biomembranes Inc. Vancouver, British Columbia) as described by (Hope, M.J., *et al.*, *Biochim. Biophys. Acta*, 812:55-65  
15 (1985)). The large unilamellar vesicles (LUV) produced were sized by quasielastic light scattering using a Nicomp 270 submicron particle sizer operating at 632.8 nm.

#### *1.2 Encapsulation of Mitoxantrone via pH Gradient*

Initial experiments were performed using mitoxantrone (with trace amounts of [<sup>14</sup>C]-mitoxantrone) in order to determine the loading characteristics of DSPC/Chol  
20 and DMPC/Chol liposomes using the pH gradient method at various temperatures. Drug to lipid ratio was measured by applying 100  $\mu$ L to SEPHADEX<sup>®</sup> G-50 mini spin columns in duplicate and spinning at 500g for 2 minutes. Duplicate samples were taken from the resulting solution and [<sup>3</sup>H] and [<sup>14</sup>C] were measured using a scintillation counter. Data in  
25 Figure 1 represents the average values  $\pm$  SD of four measurements of the drug to lipid ratio and illustrates the temperature dependence of mitoxantrone loading via the pH gradient. At 37°C, 10% of maximum uptake is achieved after 120 minutes. As the temperature is increased to 50°C, greater than 90% of the maximum uptake occurs after 45 minutes. At 65°C, maximum uptake occurs in 15 minutes. Also illustrated in Figure  
30 1 is the difference in uptake due to the differences in lipid composition. The results show that at 37°C and 65°C, there is no significant difference between the loading characteristics of the two formulations. However, at 50°C, 97% of the mitoxantrone is encapsulated within the DMPC/Chol liposomes after 30 minutes whereas only 75% of

the mitoxantrone is encapsulated within the DSPC/Chol liposomes. Data for the percent of liposome loading is presented in Table 1.

**Table 1**

Comparison of Mitoxantrone Uptake by DSPC/Chol and DMPC/Chol Liposomes

Temperature (C°)	Time Points (minutes)	Percentage of Maximum Uptake	
		DSPC/Chol	DMPC/Chol
37	15	4.16	7.99
	30	5.09	7.46
	45	4.99	9.83
	60	5.74	9.72
	90	6.32	11.36
	120	7.43	12.95
50	15	46.69	63.13
	30	74.62	89.91
	45	91.60	96.31
	60	93.53	98.14
	90	96.42	99.37
	120	94.45	100.68
65	15	98.85	102.69
	30	98.31	100.15
	45	97.59	100.56
	60	98.38	103.07
	90	98.83	102.69
	120	100.90	104.28

Mitoxantrone was encapsulated using the transmembrane pH gradient driven loading procedure (see, Madden, *et al.*, *Chem. Phys. Lipids* **53**:37-46 (1990)). The liposomes were heated at 65°C for 10 minutes and then added to mitoxantrone in a drug to lipid weight ratio of 0.1. Subsequently, 350  $\mu$ L of 0.5 M Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.5) was added to increase the pH of the mixture to between 7.0 to 7.2. The resulting solution was then incubated at 65°C for 15 minutes. Encapsulation efficiency of mitoxantrone was determined using size exclusion chromatography on mini-spin columns made of SEPHADEX® G50 (Chonn, A., *et al.*, *Biochem. Biophys. Acta*, **1070**:215-222 (1991)). Drug and lipid concentration in the samples collected in the void volume of these columns was determined by measuring [<sup>3</sup>H]-lipid and [<sup>14</sup>C]-drug. An aliquot of the sample was mixed with Pico-Flor 40 scintillation cocktail prior to assessing radioactivity with a Packard 1900 scintillation counter.

## EXAMPLE 2

This example illustrates the rates of release of mitoxantrone from two liposome formulations, both *in vitro* and *in vivo*.

### *2.1 In Vitro Characteristics of Liposome-Encapsulated Mitoxantrone*

For release studies, liposomal mitoxantrone was prepared as outlined above. The resulting drug-loaded DSPC/Chol and DMPC/Chol liposomal formulations were transferred into 25 mm diameter Spectrapor dialysis tubing (10,000-12,000 MWCO, Spectrum Medical Industries, Los Angeles), and the samples (3 mL) were dialyzed against one liter of HBS at 37°C. At the indicated time periods, 100  $\mu$ L samples were taken 0, 1, 2, 4, 8, 24, 48, 72, 102 hours and assayed for drug and lipid using the mini-spin columns as described above. These studies were completed in the presence and absence of Nigericin, an ionophore that collapses the pH gradient.

Figure 2 shows the results obtained for the release of mitoxantrone from DSPC/Chol (●) and DMPC/Chol (■) liposomes in HBS at 37°C. Solid lines indicate the absence of Nigericin. Dashed lines indicate the addition of Nigericin at time zero. Samples were taken from the dialysis bags and applied to SEPHADEX® G50 mini spin columns in duplicate and spun at 500g for 2 minutes. Duplicate samples were taken from the resulting solution and [<sup>3</sup>H] and [<sup>14</sup>C] were measured using a scintillation counter. Data represents the average values  $\pm$  SD of four measurements of the drug to lipid ratio. With pH gradient intact, there is not a significant decrease in the drug to



lipid ratio of both the DSPC/Chol and DMPC/Chol liposome. At 37°C, virtually all of the mitoxantrone is associated with the liposomes after 67.5 hours. However, with the addition of Nigericin, there is some release of mitoxantrone from the liposomes. As Figure 2 demonstrates at 102 hours, 70% of the mitoxantrone is associated with the DSPC/Chol liposomes whereas 50% of the mitoxantrone is associated with DMPC/Chol liposomes.

## 2.2 *In Vivo Release of Mitoxantrone*

For plasma clearance studies, female CD1 mice (20-25 g, four per group) were injected with a 10 mg/kg drug dose i.v via the lateral tail vein. At 1, 4, 24, and 48 hours, the animals were terminated by CO<sub>2</sub> asphyxiation and whole blood was collected via cardiac puncture. The collected blood was immediately placed into EDTA coated tubes (MICROTAINERS® from Becton Dickinson). Subsequently, plasma was prepared by centrifuging the EDTA treated whole blood at 500 × g for 10 min. Lipid and drug was assayed via [<sup>3</sup>H]-Cholesteryl hexadecyl ether and I<sup>14</sup>C]-mitoxantrone tracer using a scintillation counter as described above.

Results shown in Figure 3 demonstrate that plasma elimination of mitoxantrone loaded DMPC/Chol and DSPC/Chol liposomes are similar following i.v. administration in female CD1 mice (see Figure 3A). An estimation of the amount of mitoxantrone retained in the liposomes that are in the circulation can be made by determining the ratio of mitoxantrone-to-lipid at the indicated time points. This estimation assumes that the level of free drug in the plasma of animals given liposomal mitoxantrone will be negligible. The results of such an analysis, shown in Figure 3B, demonstrated that there is greater release of mitoxantrone from the DMPC/Chol liposomes than the DSPC/Chol liposomes. For DMPC/Chol liposomes, 73% of the mitoxantrone originally associated with the carrier has been released within 48 hr. In contrast, less than 5% of the drug was released from DSPC/Chol liposomes. Between the 4h and 48h time points the rate of mitoxantrone release was estimated to be 1.7 and < 0.025 μg mg-lipid<sup>-1</sup> hr<sup>-1</sup> for DMPC/Chol and DSPC/Chol liposomes, respectively. These results are consistent with results obtained with doxorubicin (Mayer, L.D., *et al.*, *J. Liposome Res.*, 4:529-553 (1994)) and clearly demonstrate that control of *in vivo* mitoxantrone release rates can be achieved through simple changes in liposomal lipid composition. It should be noted that plasma drug levels obtained following administration of free drug are significantly less than those obtained with the liposomal

formulations. Trapezoidal area-under-the-curve (AUC) analysis of plasma drug levels indicate plasma AUCs of 0.01145, 167.8615 and 229.8615  $\mu\text{g mL}^{-1} \text{hr}^{-1}$  following administration of free mitoxantrone, DMPC/Chol mitoxantrone and DSPC/Chol mitoxantrone, respectively.

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### EXAMPLE 3

This example illustrates the *in vivo* efficacy of the liposomal-mitoxantrone compositions of the present invention.

#### *3.1 In Vivo Efficacy of Liposome-Encapsulated Mitoxantrone*

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BDF1 mice were injected with  $10^4$  L1210 cells i.v. twenty four hours before injection with a single treatment of 5 mg/kg, 10 mg/kg 20 mg/kg doses of free mitoxantrone; 5 mg/kg, 10 mg/kg, 20 mg/kg doses of DMPC/Chol mitoxantrone; and 10 mg/kg and 20 mg/kg doses of DSPC/Chol mitoxantrone. Injections were administered in a volume of 200  $\mu\text{L}$ . In order to achieve this volume for the 20 mg/kg dose, the stock mixture was concentrated using Aquacide II. Survival times of the mice were then monitored over a sixty day time course. Increase in life span (ILS%) was calculated as follows:

15

$$\text{ILS}\% = \frac{\text{Mean survival time of treatment group} - \text{Mean survival time of control}}{\text{Mean survival time of control}}$$

20

Maximum tolerated dose was determined by monitoring the weight loss of the mice over a two week period. If the mice lost greater than 30% of the original weight then the mice are terminated.

25

The antitumor effects of free mitoxantrone and liposomal mitoxantrone were compared using the L1210 tumor model. L1210 is a murine lymphocytic leukemia. Typically this cell line is used as ascitic tumor model; however, we have found that upon i.v. injection these cells seed in the liver and the spleen. This provides identical tumor models for the two organs.

30

Toxic dose range finding studies in tumor-free female BDF1 mice found that the maximum tolerated dose (MTD) of free drug was 10 mg/kg. When the drug was encapsulated in DMPC/Chol or DSPC/Chol the MTD of mitoxantrone increased to 30 mg/kg. At this dose 100% of the animals treated survived for greater than 30 days. Necropsies suggested no gross abnormalities in any of the tissues examined. An evaluation of drug induced weight loss, however, suggested that the DMPC/Chol

liposomal formulation was slightly more toxic than the DSPC/Chol system. This result was confirmed in the efficacy experiments, where changes in weight were measured over 14 days following initiation of treatment (Figure 4). In animals that had been given (i.v)  $10^4$  L1210 cells and treated 24 h later with mitoxantrone, the maximum therapeutic dose of free and liposomal mitoxantrone was 10 and 20 mg/kg, respectively. Therefore the results in Figure 4 show drug induced changes in weight loss at these doses. The nadir in weight loss occurred between day 12 and 13 and at this time point animals treated with free drug lost almost 30% of their original body weight. In contrast, animals treated with DMPC/Chol mitoxantrone and DSPC/Chol mitoxantrone exhibited a body weight loss of 20 and 8%, respectively.

The L1210 antitumor studies summarized in Table 2 and Figure 5 clearly demonstrate that the DMPC/Chol liposomal formulation is therapeutically more active than free drug and drug encapsulated in DSPC/Chol liposomes. As shown in Table 2 the maximum % increase in life span achieved with free drug is 93%. Enhanced therapy is observed for drug encapsulated in DSPC/Chol liposomes, where a maximum %ILS value of 180 is obtained at a dose of 20 mg/kg. Improved therapy achieved with DSPC/Chol liposomal drug is primarily a consequence of liposome mediated reductions in drug toxicity. At 10 mg/kg, for example, the L1210 antitumor activity of this liposomal formulation is equivalent to free drug. Remarkably, treatment with DMPC/Chol liposomal mitoxantrone effects 100% long term (> 60 day) survival even at a dose of 10 mg/kg. The survival curves obtained for animals treated at a dose of 10 mg/kg (Figure 5) clearly show that the therapeutic activity of mitoxantrone is enhanced significantly when encapsulated in DMPC/Chol liposomes.

Table 2

**L1210 Anti-Tumor Activity of Free and Liposomal Mitoxantrone in BDF1 Mice**

Sample	Drug Dose (mg/kg)	Lipid Dose (mg/kg)	60 Day Survival	Mean Survival (days)	%ILS <sup>a</sup>	L/F <sup>c</sup>
Control			0/10	8.6		
Free Mitoxantrone	5		0/5	13.6	58	
	10		0/5	16.6	93	
	20		0/5	12.6	47	
DSPC/Chol	10	100	0/10	14.7	71	0.89
	20	200	0/10	24.1	180	1.91
DMPC/Chol	5	50	0/5	17	98	1.25
	10	100	10/10	> 60	ND <sup>b</sup>	ND
	20	200	10/10	> 60	ND	ND

<sup>a</sup> Percentage ILS (Increase in Life Span) Values were determined from mean survival times of treated and untreated control groups. If the animals survived more than 60 days the ILS% was not determined.

<sup>b</sup> ND not determined.

<sup>c</sup> L/F (Liposomal/Free) values were calculated by dividing the mean survival time of the liposomal formulation by the mean survival time of the free drug at the equivalent dose.

The results summarized thus far demonstrate that the therapeutic index of mitoxantrone can be increased dramatically by encapsulating the drug in DMPC/Chol liposomes. The rate of drug release is at least 68-fold faster from DMPC/Chol liposomes in comparison with DSPC/Chol liposome. In addition to assessing drug release from liposomes in the plasma compartment it is important to correlate antitumor activity with drug exposure at the site of tumor growth. For this reason drug delivery to the liver, a primary site of disease progression for the tumor model employed here, was also evaluated. The results, shown in Figure 6, were obtained in tumor-free CD1 mice, however it should be noted that drug/liposome plasma elimination and biodistribution data were similar in tumor-free CD1 and tumor-bearing (L1210 cells given 24 hr prior to drug injection) BDF1 mice. As shown in Figure 6A liposomal lipid accumulation in the

liver is similar for both DSPC/Chol and DMPC/Chol liposomal mitoxantrone formulations. Unlike doxorubicin (Mayer, L.D., *et al.*, *Cancer Res.*, **49**:5922-5930 (1989)), the presence of entrapped mitoxantrone did effect reductions in liposomal lipid accumulation in the liver. The level of mitoxantrone achieved in the liver 24 and 48 hours after i.v. administration of DMPC/Chol liposomal mitoxantrone is, however, significantly less than that observed for DSPC/Chol mitoxantrone.

The above data shows that maximizing drug delivery to the site of tumor growth is can result in little therapeutic value unless the drug can escape from the liposomal carrier. Provided that the drug is permeable across the liposome membrane, the importance of maximizing drug delivery can be established. It would, for example, be predicted that factors that reduce accumulation of DMPC/Chol liposomes in the liver would mediate reduction in i.v. L1210 antitumor activity. The results summarized in Figure 7 clearly support this prediction. In these studies the tendency for DMPC/Chol liposomal mitoxantrone to accumulate in the liver was reduced by incorporation of 5 mole % poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE). PEG-PE/DMPC/Chol liposomes exhibited comparable drug release characteristics *in vivo* (results not shown), however the amount of drug delivered to the liver was reduced by a factor of 2 (Figure 7A). Reduction in liver accumulation was associated with increased levels of drug and lipid in the plasma compartment (results not shown). As shown in Figure 7B there was a surprising reduction in the antitumor activity of mitoxantrone encapsulated in the PEG-PE/DMPC/Chol liposomes.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

**WHAT IS CLAIMED IS:**

- 1           1. A pharmaceutical composition comprising a liposomal formulation of  
2 mitoxantrone, said liposomes comprising a mixture of cholesterol and 1,2-*sn*-  
3 dimyristoylphosphatidylcholine.
- 1           2. A pharmaceutical composition in accordance with claim 1, wherein said  
2 mixture comprises of from about 50% to about 70% 1,2-*sn*-  
3 dimyristoylphosphatidylcholine and from about 30% to about 50% cholesterol.
- 1           3. A pharmaceutical composition in accordance with claim 2, further  
2 comprising a PEG-lipid conjugate.
- 1           4. A pharmaceutical composition in accordance with claim 3, wherein said  
2 PEG-lipid conjugate is selected from the group consisting of PEG-Cer-C<sub>14</sub>, PEG-Cer-C<sub>20</sub>  
3 and MePEGS-2000-DSPE.
- 1           5. A pharmaceutical composition in accordance with claim 3, wherein said  
2 PEG-lipid conjugate is present in an amount of from about 1% to about 8% by weight.
- 1           6. A method of treating solid tumors in a host, said method comprising  
2 administering to said host a pharmaceutical composition in accordance with claim 1.
- 1           7. A method for increasing the therapeutic index of mitoxantrone in a  
2 patient, said method comprising,  
3           (i) encapsulating mitoxantrone in liposomes comprising a mixture of  
4 cholesterol and 1,2-*sn*-dimyristoylphosphatidylcholine to provide a liposomal formulation;  
5 and  
6           (ii) administering said liposomal formulation to said patient, whereby said  
7 therapeutic index of mitoxantrone is improved relative to non-liposomal formulations of  
8 mitoxantrone.

1           8. A method in accordance with claim 7, wherein said liposomes comprise  
2 a mixture of from about 50% to about 60% 1,2-*sn*-dimyristoylphosphatidylcholine and  
3 from about 40% to about 50% cholesterol.

1           9. A method in accordance with claim 7, wherein said mixture further  
2 comprises a PEG-lipid conjugate.

1           10. A method in accordance with claim 9, wherein said PEG-lipid conjugate  
2 is selected from the group consisting of PEG-Cer-C<sub>14</sub>, PEG-Cer-C<sub>20</sub> and MePEGS-2000-  
3 DSPE.

1           11. A method in accordance with claim 9, wherein said PEG-lipid conjugate  
2 is present in an amount of from about 1% to about 8% by weight.

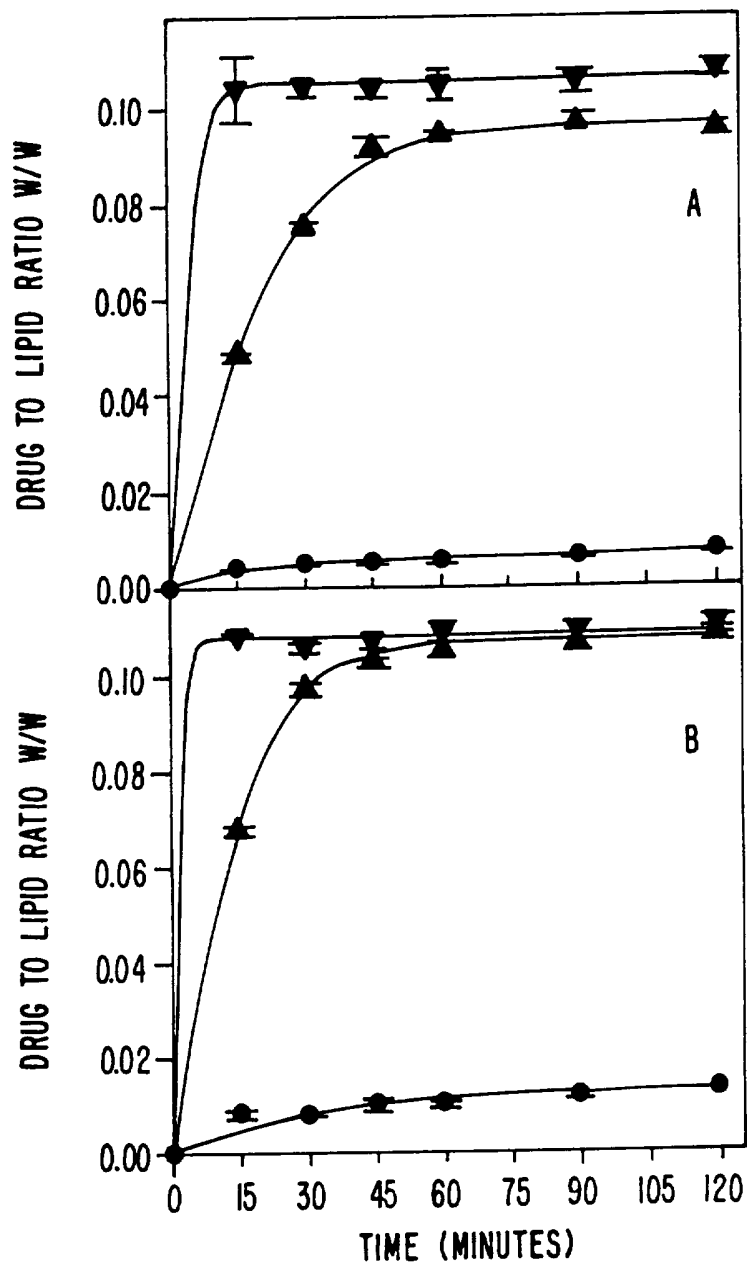


FIG. 1.



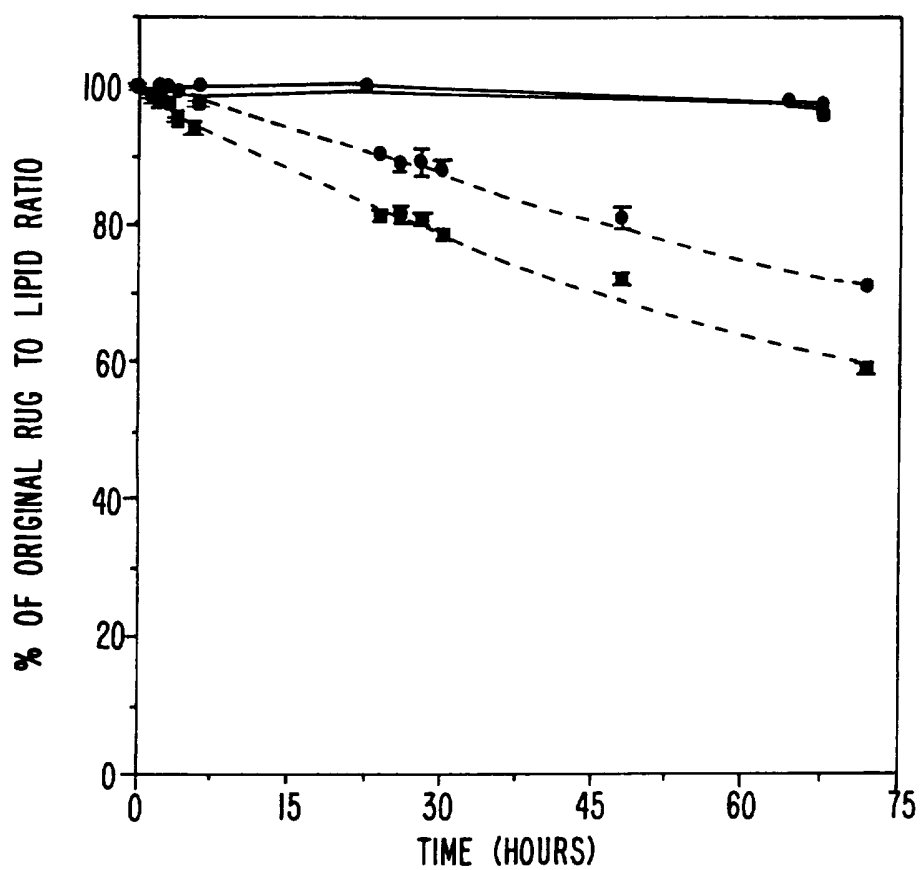


FIG. 2.

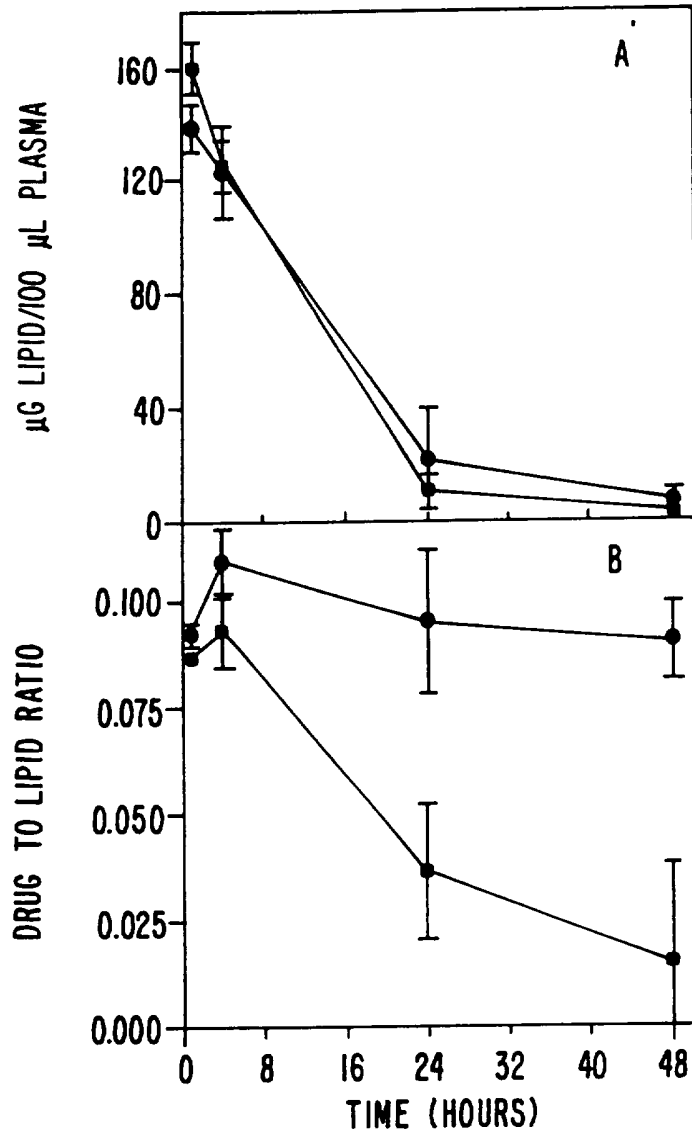


FIG. 3.

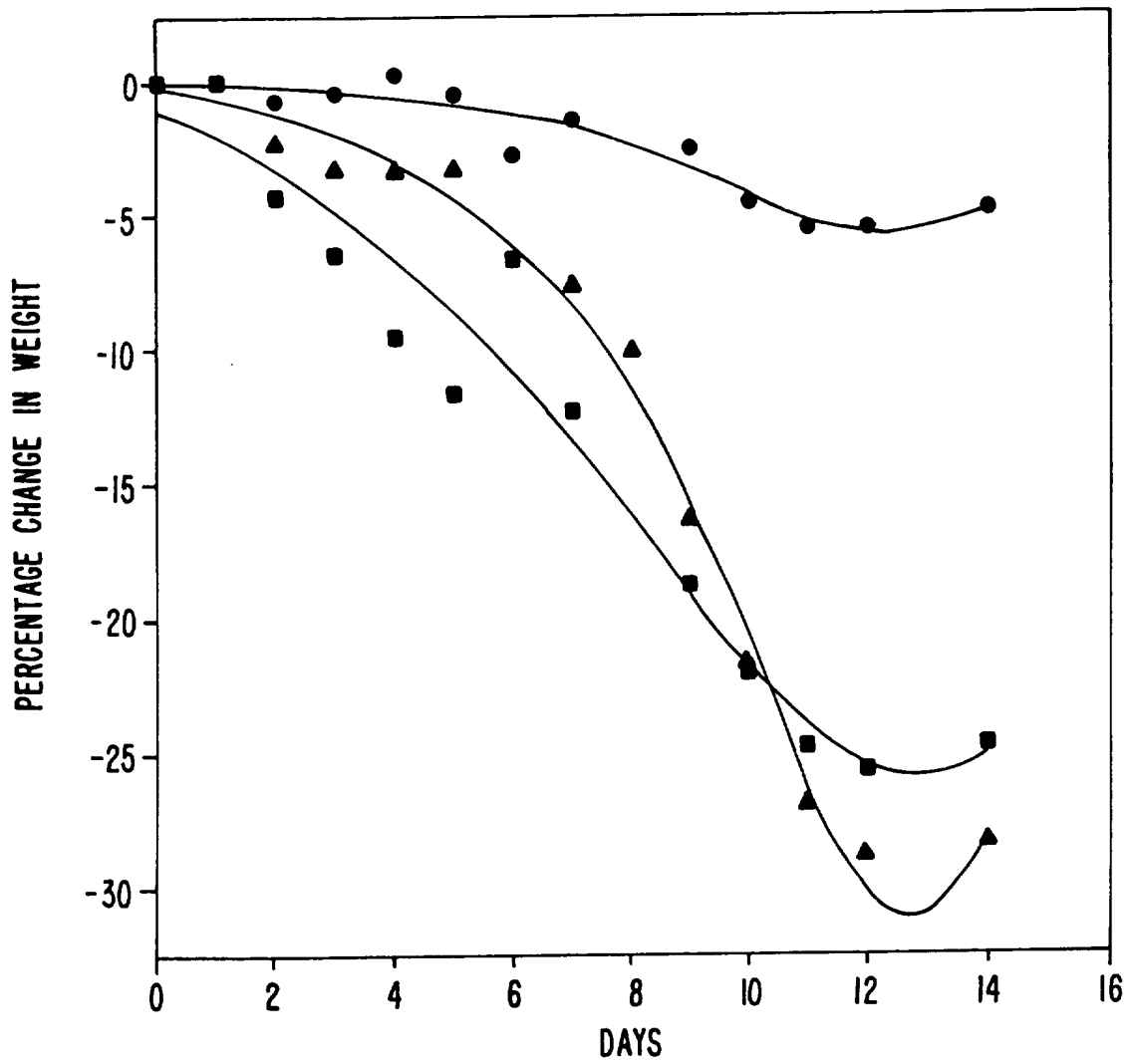


FIG. 4.

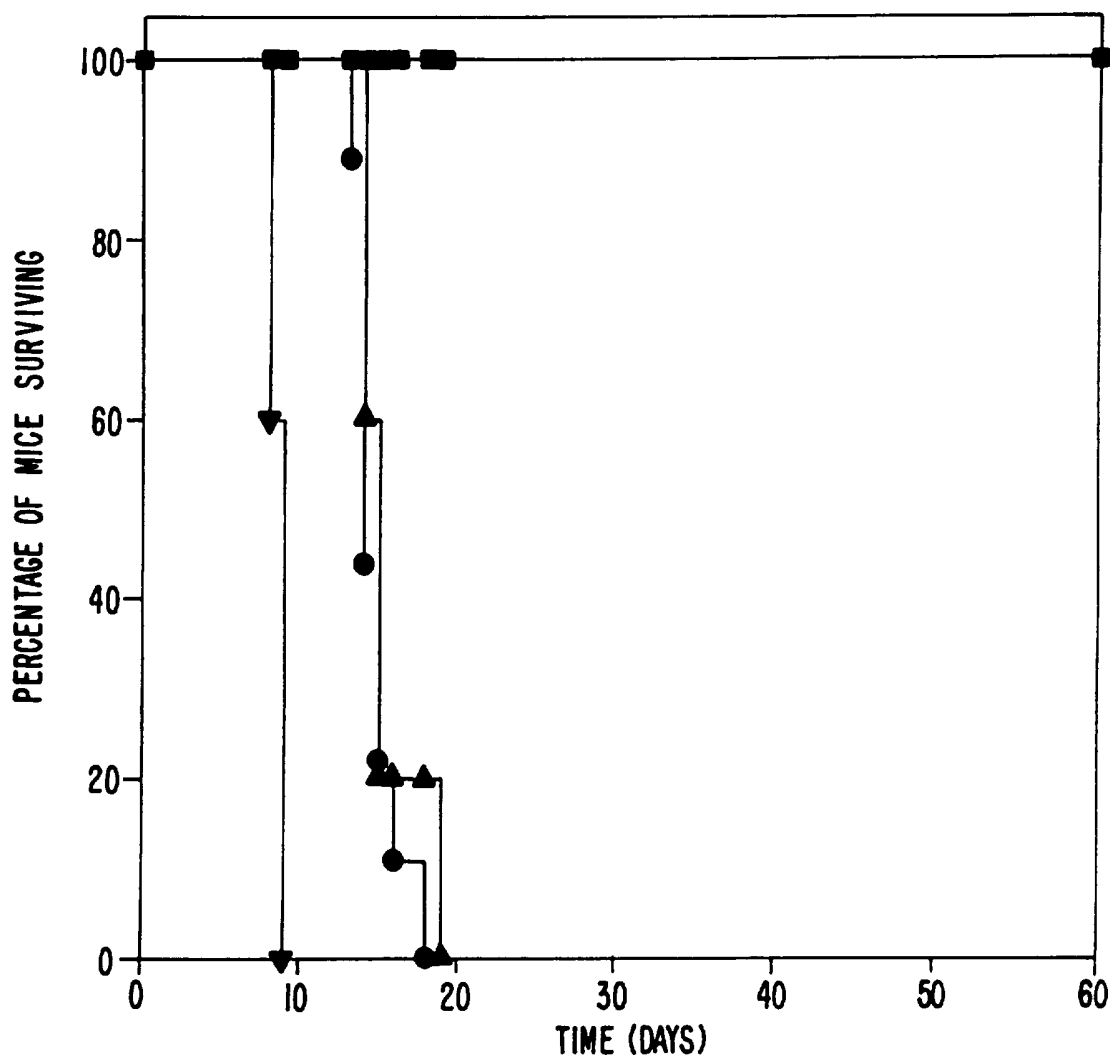


FIG. 5.

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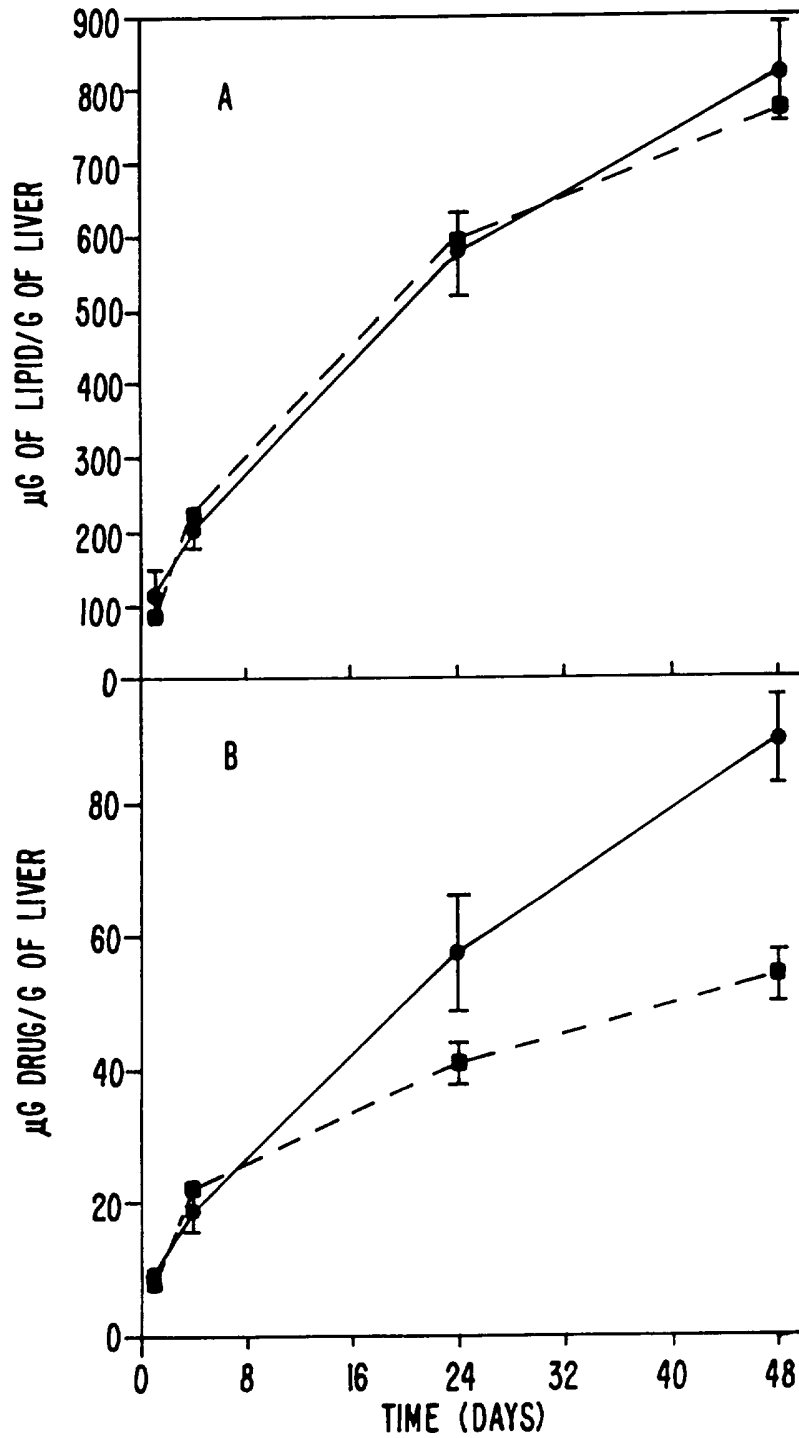


FIG. 6.

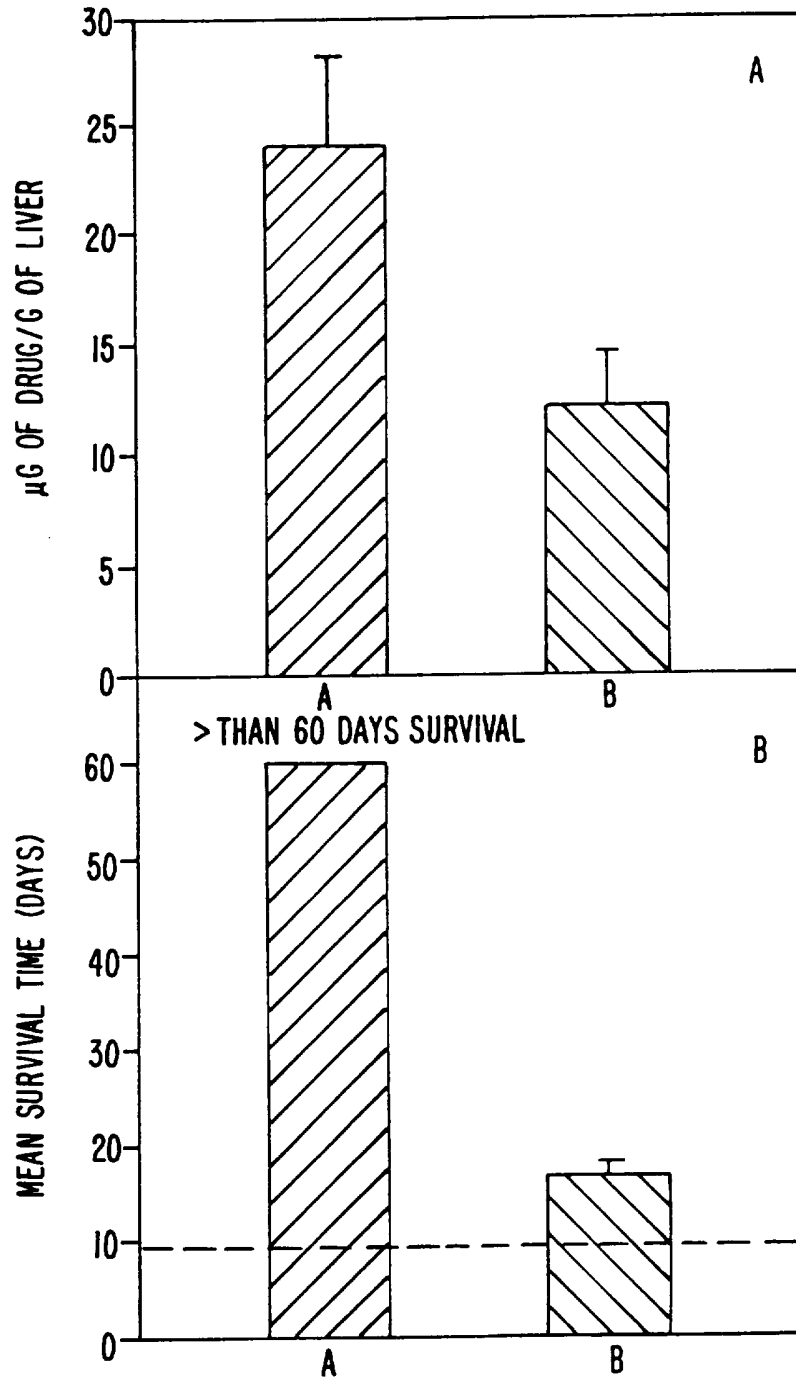


FIG. 7.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 96/00677

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K9/127 A61K31/135

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

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

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

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 88 06442 A (THE LIPOSOME COMPANY, INC.) 7 September 1988 see page 56; example 32 see claims 6,10-12 ---	1,2,6-8
Y	WO 95 15746 A (THE SCHOOL OF PHARMACY) 15 June 1995 see page 8, line 1 - line 13 see page 16, line 20 - line 21 see page 18; example 1.10 ---	1,2,6-8
A	WO 89 06977 A (BOARD OF THE REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 10 August 1989 see page 15, line 21 - page 16, line 15 -----	1-11

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

17 March 1997

Date of mailing of the international search report

21.03.97

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 96/00677

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

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

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark: Although claim(s) 6-11 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.**
2.  Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 96/00677

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8806442 A	07-09-88	AT 137670 T	15-05-96
		AU 620545 B	20-02-92
		AU 1481688 A	26-09-88
		AU 635435 B	18-03-93
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		DE 3855265 T	12-12-96
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		ES 2088385 T	16-08-96
		JP 2502458 T	09-08-90
KR 9613703 B	10-10-96		
WO 9515746 A	15-06-95	NONE	
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		EP 0397774 A	22-11-90