Anti-Cancer Drugs 1995, **6**, pp. 83–93

Antitumor effect of liposome-incorporated camptothecin in human malignant xenografts

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The nuclear enzyme topoisomerase I (topo I) has been recently recognized as the target for the anticancer drug camptothecin (CPT; NSC 94600) and its derivatives. This drug has been reported to display effective antitumor effects on a variety of human tumor models xenografted in nude mice. However clinical studies of sodium CPT have revealed that the open-ring form of the drug is a poor inhibitor of topo I and much less potent antitumor agent than CPT lactone. However, the insolubility of CPT lactone makes it difficult to devise a suitable formulation for further clinical testing. In view of these observations, we report here the successful incorporation of CPT into a liposome-based drug delivery system (LCPT) composed of DPPC : Sph : CHOL : PI (2.4 : 6.6 : 1.0 : 0.05 M ratio) that can be used as a suitable formulation for clinical testing of the drug. Higher incorporation efficiency was observed when the total phospholipids : drug ratio = 40 and the cholesterol content = 1%. Image analysis of the CPT-containing liposomes with freeze-fracture electron microscopy has indicated that CPT significantly increased the interlamellar space of the vesicles as a result of its intercalation between lipid bilayers. This has occurred with no major disruptive effects on the bilayer structure. The *in vitro* drug release study in human serum was characterized by an initial rapid loss of 50% of contents during 4 h, followed by a slow leakage of the remaining 50% of the total drug over a 20 h period. When tested for its antitumor activity on nude mice xenografted with human malignant melanoma and breast carcinoma, LCPT displayed effective antitumor activity with minimal host toxicity. For example, single i.m. injection of LCPT at 10 mg/kg has produced complete tumor regression to nude mice xenografted with CLO beast carcinoma. Likewise, similar results were obtained with the nude mice xenografted with human malignant BRO cells melanoma. These results appear to suggest that i.m. administration of liposome-incorporated CPT has considerable potential for the treatment of human neoplastic diseases, especially lymph node metastases.

Key words: Breast carcinoma, camptothecin, freeze-fracture, liposomes, melanoma.

This work is supported in part by funds from WSU (SSD) and The Stehlin Foundation for Cancer Research (BCG). MIF is the recipient of an Al-Azhar University Postdoctoral Fellowship.

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Introduction

The chemotherapy of cancer is perhaps the most difficult of all therapeutic endeavors. Effective antineoplastic drugs need to kill selectively or impair the growth of malignant cells which differ subtly from normal host cells. The design of genuinely selective chemotherapeutic agents has thus far eluded investigators in the cancer field. Many agents are available which are highly toxic to tumor cells; however, these agents also display very predictable toxicities, usually on rapidly dividing normal cell populations. The low therapeutic ratio and poor selectivity of most anticancer drugs has prompted a search for new targets of drug action.[1] Targets, such as cell adhesion molecules that are essential for metastasis formation, receptors for autocrine and paracrine, microtubules and topoisomerases, largely unknown a decade ago can provide opportunities to develop new drugs with discrete mechanisms of action. Good examples for this approach would be the use of the topoisomerase I (topo I) inhibitors, camptothecin (CPT) and its derivatives. CPT is a plant-derived alkaloid that first demonstrated high antiproliferative and toxic activity against murine leukemia L1210 cells.[2] Subsequently, it was shown that CPT is also cytotoxic against large numbers of human malignant tumors xenografted in immunodeficient (nude) mice.[3–7] The clinical trials of the water-soluble salt of CPT (Na-CPT) have been limited by the severe toxicities and the loss of its antitumor activity.[8–10] This is presumably due to the opening of the CPT's lactone ring which generates a less effective molecule (Na-CPT) at normal physiological pH.[11] It has to be mentioned that even CPT has been shown to exhibit high antitumor activity *only* when administered orally or by the i.m. route in suspension form.[3,5–7]

To improve the therapeutic utilization of CPT, we have prepared and evaluated the antitumor activity of the drug using liposome technology as a delivery system. Liposomes as drug carriers can modify the

therapeutic profiles of selected antitumor drugs in a vary favorable manner and can also create opportunities for therapeutic applications of chemical moieties not previously considered for drug use (reviewed in 12,13). In this study, we report the successful incorporation of CPT into multilamellar liposomes of appropriate composition with the maintenance of its antitumor activity in nude mice xenografted with human malignant melanoma and breast carcinoma cells. Part of this study has been presented previously as an abstract.[14]

Materials and methods

Materials

CPT and 9-aminocamptothecin (9-AC) were prepared and purified at the Stehlin Foundation according to published procedures.[15] Dipalmitoylphosphatidylcholine (DPPC), phosphatidylinositol (PI) and sphingomyelin were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesterol (CHOL) and monosialoganglioside (GM_1) were purchased from Sigma (St Louis, MO). Human plasma was from Biological Specialty (Landsdale, PA). [^{3}H]20(*S*)-camptothecin was obtained from the Stehlin Foundation, 9-amino-[10,12,-^{3}H(*N*)]20(*S*)-camptothecin was provided by Dr R Haugwitz of NCI and [^{14}C]cholesteryloleate was purchased from DuPont/NEN (Boston, MA). All other chemicals were reagent grade. All glassware used in this study was sterile and pyrogen-free.

Preparation of CPT liposomes

Multilamellar vesicles (MLVs) contained CPT or 9-AC were prepared as described elsewhere.[16,17] Briefly, DPPC, sphingomyelin, CHOL and PI or GM_1 were mixed with CPT or 9-AC (DPPC : sphingomyelin : CHOL : PI or GM_1 : CPT or 9-AC molar ratio 2.4 : 6.6 : 1.0 : 0.05 : 1) and dissolved in chloroform. A trace amount of [^{3}H]CPT or [^{3}H]9-AC was added such that the specific activity of the final liposome solution was 37 kBq/ml. The solvent was evaporated in a rotavapor at 40°C and the lipid film containing the drug was dissolved in borate buffer (pH 6.5). Complete dispersion of the lipid film into the aqueous phase was obtained within a few minutes by mild hand-shaking of the hydration mixture. Liposomes were concentrated by centrifugation at 100 000 *g* for 1 h to remove the unincorporated materials and the free CPT or 9-AC was removed by washing the liposomes three times in borate buffer. Following the final washing step, the liposome pellet was made up into the desired concentration by the addition of the buffer. The final osmolality of these solutions was equal to 320 mOsmol/kg, the osmolality of mouse plasma.

The incorporation efficiency was determined by measurement of the radioactivity in the aqueous milieu followed by assessment of radioactivity in the liposome pellet obtained after centrifugation of the liposome suspension at 100 000 *g* for 1 h. The percentage of incorporation efficiency (%IE) was calculated by the following equation: % IE = c.p.m. in liposome − c.p.m. in supernatant ÷ c.p.m. in liposome × 100.

Release of CPT from liposomes

The stability of liposome-incorporated CPT in human plasma was determined by mixing the particular phospholipids with 5 μCi [^{3}H]CPT (1 mCi/mg), 1 μCi [^{14}C]cholesteryloleate (156 μCi/mg) and 1 mg CPT. [^{14}C]cholesteryloleate was used as a marker for the lipid phase. The solvent was evaporated to dryness and the liposomes were obtained as mentioned above. The double-labeled liposomes (0.5 mg/ml) were incubated, while shaking, with 50% human plasma at 37°C for periods ranging from 1 to 24 h. At specific times, aliquots of 0.5 ml were then diluted with borate buffer, centrifuged at 100 000 *g* for 1 h. Aliquots of 50 μl samples of the supernatant as well as pellet suspension were counted for the presence of released [^{3}H]CPT using a Tri-Carb 1500 liquid scintillation counter in preset ^{3}H/^{14}C channels. The rate of leakage was determined by the following equation: % release = $\text{c.p.m.}_x - \text{c.p.m.}_0 \div \text{c.p.m.}_0 \times 100$, where c.p.m._0 is the radioactivity of the pellet at time zero and c.p.m._x is the radioactivity of the pellet at time *x* at 37°C.

Electron microscopy study

The physical stability of the obtained MLVs was determined by (1) differential interference microscopy to observe aggregation of liposomes or precipitation of CPT and (2) freeze-fracture of lipid dispersions using transmission electron microscopy (Electron Microscopy Center, WSU). Aliquots of 'empty' liposomes or liposome-incorporated CPT were mixed with glycerol (25% v/v) and al-

lowed to incubate for approximately 30 min. A droplet (1–2 μl) of the lipid suspension was placed on a flat-top, gold support disc which was then plunged into liquid nitrogen-cooled liquid freon. After 3–4 s, the sample was transferred into a specimen table immersed in liquid nitrogen prior to insertion into the freeze-fracture apparatus (Balzers, BAF 400). Fracturing was carried out at −110°C and 10^{-6} to 10^{-7} torr. Platinum/carbon replicas were then produced and examined on a Hitachi H-300 at 100 kV. Image analysis of the fractured samples was performed by the NIH Image 1.52. Statistical analysis to obtain differences between 'empty' liposomes and LCPT with respect to the distance or the actual space between lamellae was determined by the Kruskal–Wallis test using Stat View Set Graphics 1.03.

Cells and nude mice

In this study, we used human melanoma and breast carcinoma cell lines maintained by serial passages in nude mice.[3] BRO melanoma cells were derived from a biopsy of a human primary melanoma of the skin and then transplanted in nude mice, where they induce highly invasive tumors without a characteristic pattern of metastatis.[18] CLO breast carcinoma was established as a xenograft from a biopsy taken from a patient with breast carcinoma.[19] For xenotransplant, we used Swiss immunodeficient (nude) mice of the NIH-1 high fertility strain, routinely bred and maintained under strict pathogen-free conditions in the Stehlin Foundation Laboratory as previously described.[20,21] Nude mice were 75–85 day old males. No sex-related differences in growth rates have been observed for any of the tumors used.

Xenografts and liposomal CPT (LCPTII) treatment

Approximately 2×10^7 cells were inoculated subcutaneously in each nude mouse as previously described.[22,23] Tumor-implanted animals were randomly assigned to LCPTII-treated or control groups that were treated with 'empty' liposomes. LCPTII was administered to the animals i.m. or i.v. at 5 mg/kg twice a week for 1 week or at 10 mg/kg once a week for 1 week. The precise number of mice used in each study is indicated in the appropriate text section or figure legend. In general, treatment was initiated when the tumor size reached about 500 mm^3. The tumors were measured in three dimensions using a caliper and their volumes were calculated. Tumor growth and drug toxicity in animals were monitored by measuring the tumor size and the body weight, respectively. Drug toxicity was initially determined on normal animals as a 15% or more reduction of total body weight following the period after the first drug administration according to generally accepted guidelines.[24] The time of appearance of toxicity may vary depending on the dose, regimen and site of drug administration. A tumor-bearing mouse was euthanized when the tumor neared its maximum size. All procedures involving the use and handling of animals were strictly followed according to guidelines defined by the Laboratory Animal Committee.

Results

Characteristics of multilamellar liposomes incorporated with CPT and 9-AC

Initially, we studied the role of total phospholipids and the amount of cholesterol content of the prepared liposomes on the incorporation efficiency of CPT and 9-AC. Figure 1 shows that the incorporation efficiency of the MLVs composed of DPPC : Sph (molar ratio 2.4 : 6.6) and 26 mol% cholesterol increases when the total amount of the phospholipids also increased. At pH 7.0, an incorporation efficiency of 9% was achieved when the molar ratio of the phospholipids : CPT was 40 : 1. Similar results with 9-AC were also obtained (Figure 1B), although the incorporation efficiency in this case was lower than that of CPT. However, when the cholesterol content was reduced from 26 mol% to a lower amount, a higher incorporation efficiency with CPT was achieved. At pH 7.0 and cholesterol content 1 mol%, the incorporation efficiencies of CPT and 9-AC were 30 and 10%, respectively (Figure 2A and B). At pH 6.5, however, the incorporation efficiencies of both drugs were 45 and 30%, respectively. This data indicates that lowering the cholesterol content of the MLVs to 1 mol% at pH 6.5 can markedly increase the incorporation efficiency of CPT from 9 to 45%. In general, lowering the cholesterol content in the lipid vesicle to 1 mol% can usually result in reducing the stability of the prepared liposomes which in turn can lead to an increase in the release of its content.[25] Therefore, 1% of GM_1 or PI

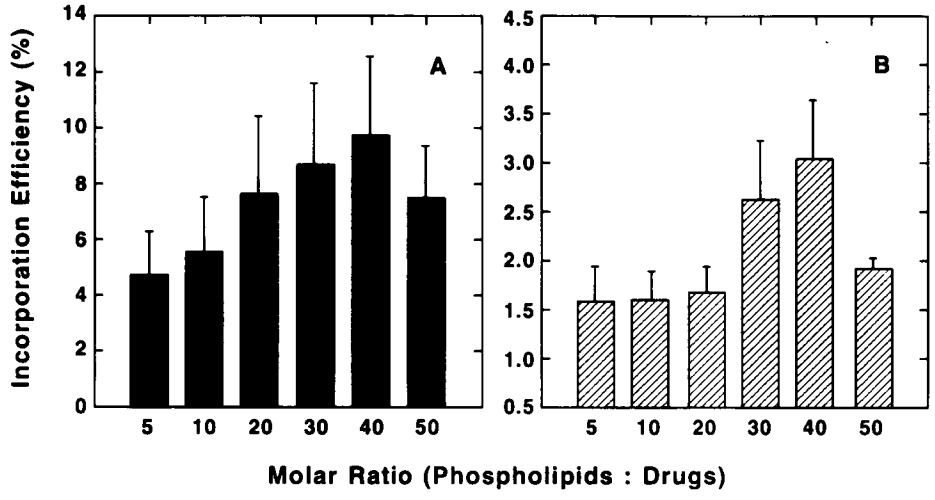


Figure 1. The role of total phospholipids on the incorporation efficiency of CPT (A) or 9-AC (B) into multilamellar liposomes composed of DPPC : Sph : CHOL (2.4 : 6.6 : 2.6 M ratio). Data represent mean values ± SD.

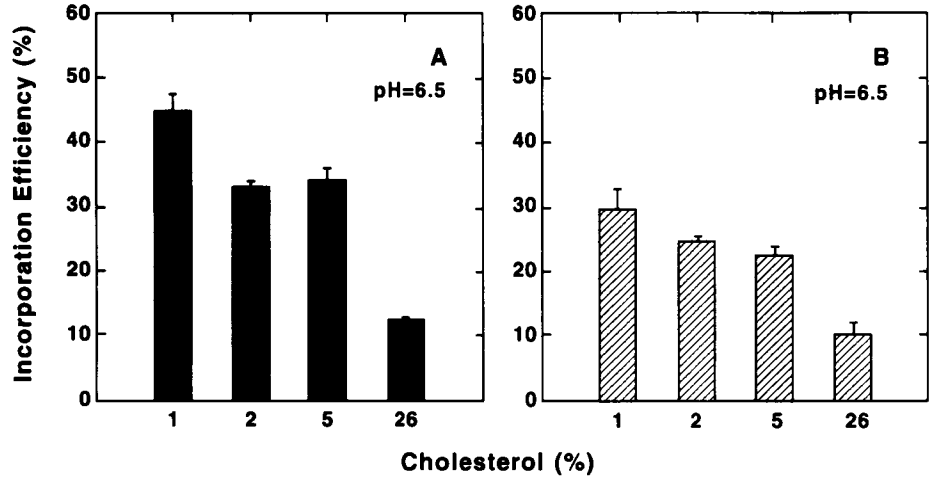
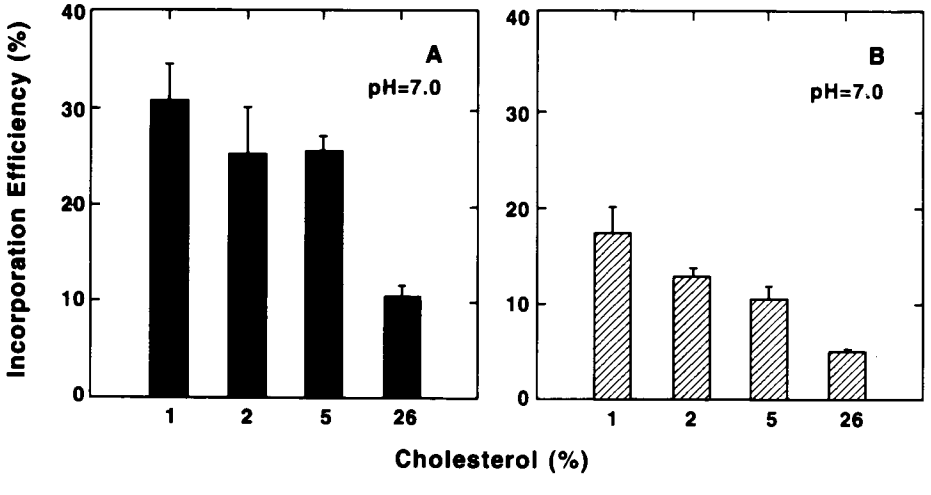


Figure 2. The role of cholesterol in the incorporation of CPT (A) or 9-AC (B) into multilamellar liposomes at different pH. Data represent mean value ± SD.



was included in the LCPT preparation to enhance its stability and to protect against plasma-induced solute release. The physical stability of the obtained liposomes composed of a 2.4 : 6.6 : 1.0 : 0.05 molar ratio of DPPC : Sph : CHOL : GM_1 or PI was then examined by electron microscopy. Freeze-fracture electron microscopy employs a simple preparation technique in which a droplet of sample suspension is rapidly frozen to liquid nitrogen temperatures and fractured prior to platinum shadowing. The fracture plane exposes the membrane hydrophobic interior. In our study, a short period of etching also preceded the shadowing step in order to expose a thin rim of the liposome outer surface. The technique resolves features down to 2.5 nm and provides 100 times higher resolution than light microscopy. Figure 3 shows detailed views of LCPTI and LCPTII that contain GM_1 and PI. Bilayers of rigid DPPC (Figure 3A and C) are well known to have a banded fracture plane upon freezing from below 41°C.[26] This banded pattern has been shown to be present in most of liposomes with a 2.4 : 6.6 : 1.0 : 0.05 molar ratio of DPPC : SpH : CHOL : GM_1 or Pi (Figure 3A and C). The presence of GM_1 and PI in these liposomes did not affect the banded fracture plane. However, the incorporation of 1 mol% CPT in these liposomes seems to increase the interlamellar distance between the branded pattern with no major structural changes (Figure 3B and D). This indicates that CPT molecules—which are highly lipophilic—are incorporated into the lipid bilayers rather than simply coexisting with them as free drug. Extensive scrutiny of numerous preparations showed no evidence of non-bilayer or amorphous structures. All liposomes have the classic fracture face and etch face appearance unique to the lipid bilayer.

Several studies have indicated that the inclusion of GM_1 or PI in liposome formulations can improve the stability of the lipid vesicles in serum[27,28] and thus prolong their circulation half-times. To examine the effect of GM_1 and PI on the plasma stability of LCPT, we measured the release of [^{3}H]CPT and [^{14}C]cholesteryloleate (lipid marker) during incubation with 50% human plasma at 37°C. The results, presented in Figure 4, indicate that incubation of LCPT in plasma for 4 h at 37°C typically induced the release of approximately 50% of the incorporated drug. However, PI-containing liposomes (Figure 4B) were more resistant to plasma-induced disruption of lipid vesicles than GM_1-containing liposomes (Figure 4A). After 24 h of LCPT incubation in plasma, approximately 90% of CPT was equally released from both formulations.

Inhibition of tumor growth followed by tumor regression with LCPT

In pilot studies, we initially determined the effectiveness of a single injection of i.p. and i.v. doses (5 and 10 mg/kg) of LCPT and 'free' CPT against P388 leukemia in female mice (data not shown). The results of this study have shown that 'free' CPT had no inhibitory effects on the growth of the P388 leukemia model as compared with LCPT. Therefore we studied the inhibitory effects of only LCPT in nude mice xenografted with CLO breast carcinoma and BRO malignant melanoma.

The nude mice were inoculated with CLO or BRO cells, and then divided randomly into six groups of five or six mice per group. Treatment was initiated when all mice developed visible tumors of about 0.5 cm^3. LCPT was administered either i.v. or i.m. at doses of 10 mg/kg once or 5 mg/kg twice for 1 week. Control groups received 'empty' liposomes. Tumor size and body weight were estimated once a week. Figure 5 depicts the results of a typical experiment with CLO breast carcinoma. It can be seen that the size of CLO tumors increased in an exponential manner in the control groups receiving 'empty' liposomes, whereas a dramatic decrease in tumor size was clear in i.m. treated animals with LCPT twice (5 mg/kg) or once at 10 mg/kg (Figure 5A). Mice receiving i.m. treatment became virtually tumor free by growth examination; no tumor re-growth was observed after the termination of LCPT treatment. The experiment was terminated when the control mice developed tumors of about 7 cm^3 in size. Tumor-free animals had no loss of body weight except with mice that were treated with 10 mg/kg, which showed a transient loss of body weight that was completely regained by the third week of LCPT administration (Figure 5B). No improvement in animal survival with i.v. treatment of LCPT was observed. A similar pattern of tumor regression was also observed with BRO malignant melanoma (Figure 6). Thus a single i.m. injection of LCPT at 10 mg/kg or multiple injections of 5 mg/kg induced compete remission in animals xenografted with BRO cells (Figure 6A). However, i.v. administration of the same doses of LCPT resulted in partial inhibition of tumor growth. Similar patterns of transient weight loss of animals receiving a single injection of 10 mg/kg i.m. was also observed (Figure 6B). This toxic effect had no major impact on animal survival since the weight loss due to i.m. administration of LCPT was reversed after 35 days of drug treatment.

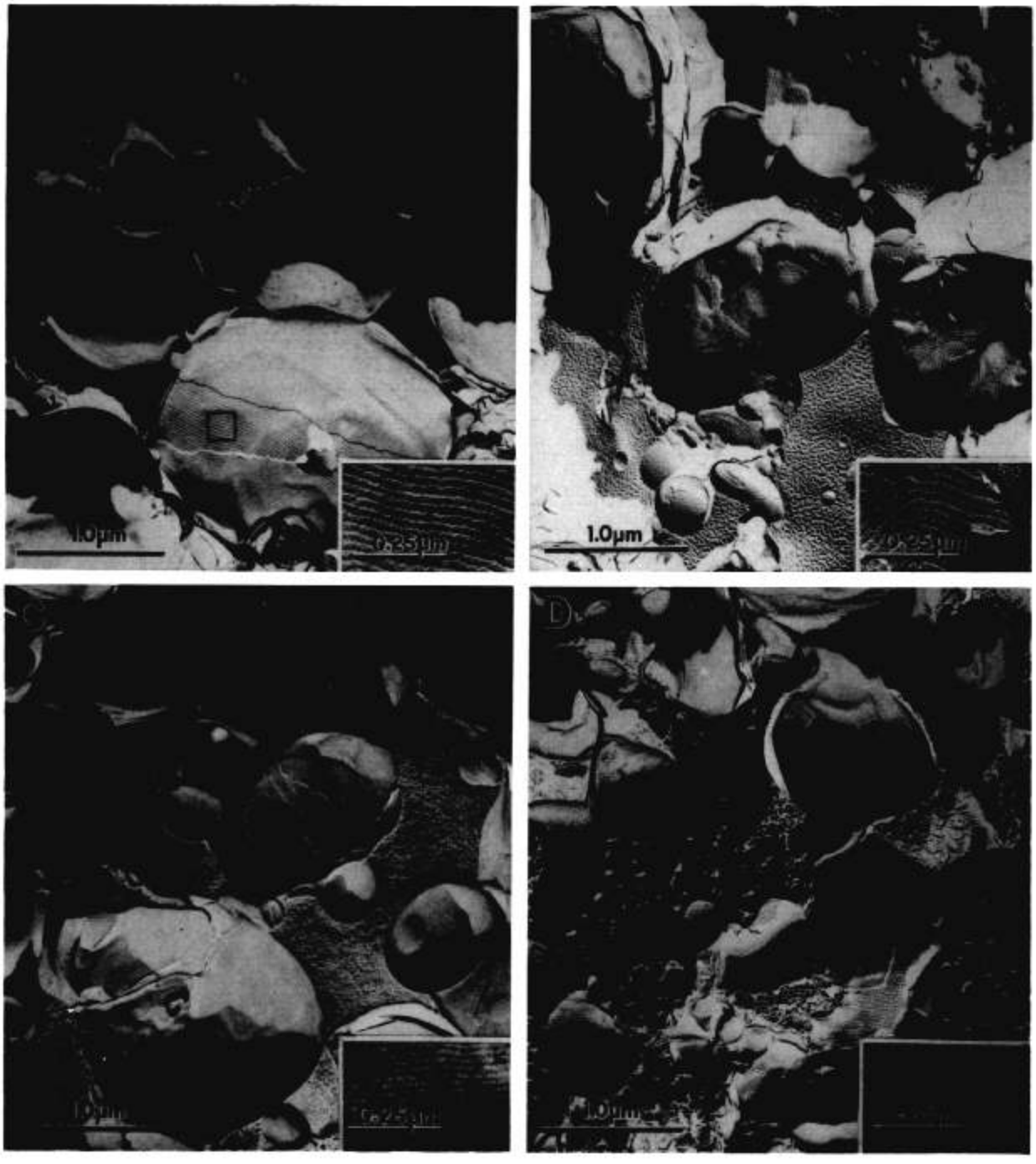


Figure 3. Freeze-fracture electron micrographs of 'empty' liposomes composed of DPPC : Sph : CHOL : GM_1 (2.4 : 6.6 : 1.0 : 0.05 M ratio) (A); LCPTI (B); 'empty' liposomes composed of DPPC : Sph : CHOL : PI (2.4 : 6.6 : 1.0 : 0.05 M ratio) (C); LCPTII (D) prepared in borate buffer at pH 6.5. Multilamellar liposomes are of various size range (0.5–3 μm) and show banded fracture planes. Magnification × 18 600. Insert, the portion of the multilamellar bilayers at higher magnification, × 60 450. Ten different measurements were used for the calculation of interlamellar space as indicated in Materials and methods.

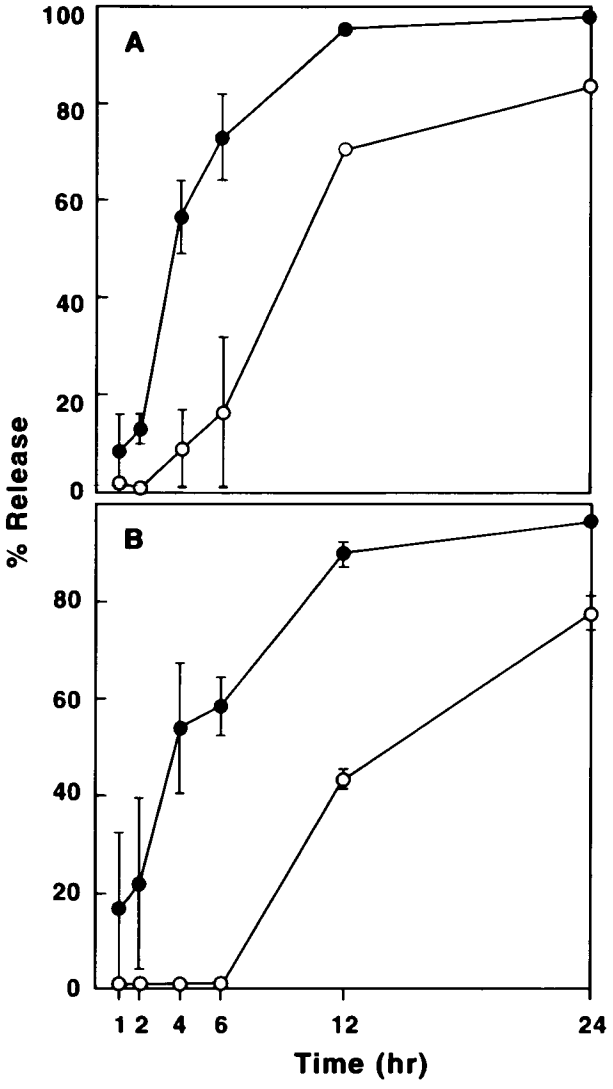


Figure 4. Time-dependent release of double-labeled GM_1-containing liposomes (A) and PI-containing liposomes (B) when incubated in 50% human plasma at 37°C. Data represent mean values ± SD. ●, [^{3}H]CPT; ○, [^{14}C]cholesteryl oleate.

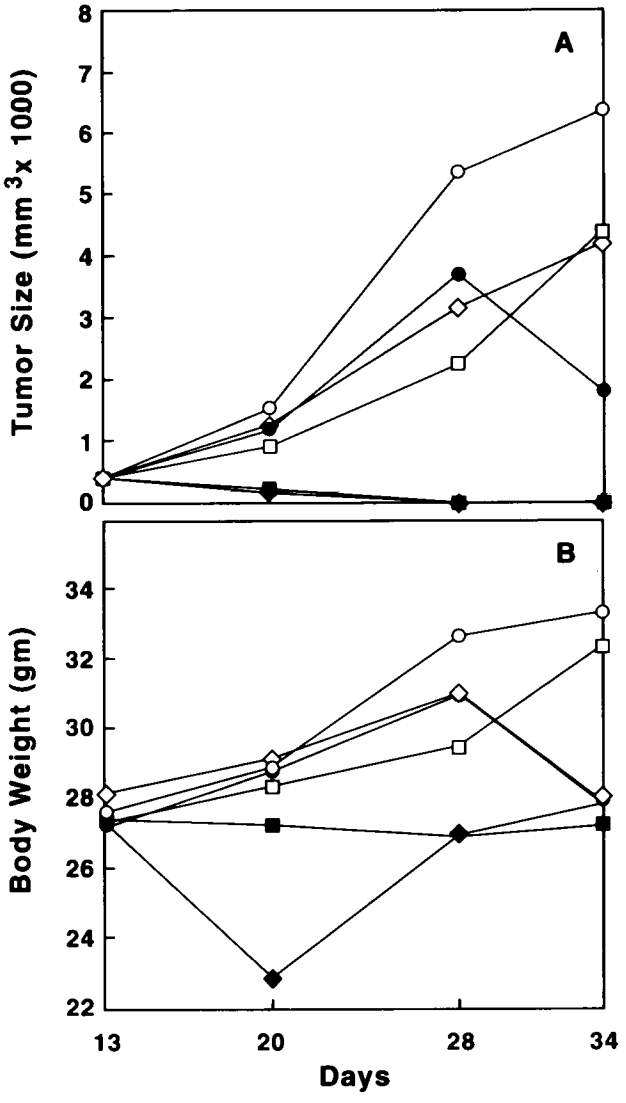


Figure 5. Treatment of CLO breast carcinoma with various doses of LCPTII. Thirty-six nude mice were xenografted with human breast carcinoma CLO cells, and then randomly and equally divided into six groups. The animals received 5 mg/kg twice a week for 1 week either i.v. (□), i.m. (■), or single injection of 10 mg/kg i.v. (◇) or i.m. (◆). Control groups received 'empty' liposomes either i.v. (○) or i.m. (●). Drug treatment was initiated when the tumors reached an average size of 500 mm^3. Measurements of the change in tumor size (A) and body weight (B) were then recorded.

Discussion

In this report we demonstrate that a topo I inhibitor can be efficiently incorporated into multilamellar liposomes, and that the liposomal form of CPT displays activity against a human malignant melanoma and breast carcinoma when administered i.m. in nude mice. In contrast to other studies of liposome-encapsulated antitumor drugs, we are not trying to improve upon the efficacy of a currently accepted antitumor agent; rather we are attempting to develop an approach which will permit the utilization of a distinct class of agents (CPTs) which are not now used in clinical practice due to the lack of suitable drug carrier system. Thus the use of drug delivery technologies such as liposomes should permit effective formulation and utilization of these potent agents in cancer therapy.

Based on our previous studies with lipophilic drugs,[16,29] it seems likely that CPT is intercalated into the bilayers of the multilamellar liposomes used in this study rather than being entrapped in the internal aqueous compartments.[12,30] Most drugs of

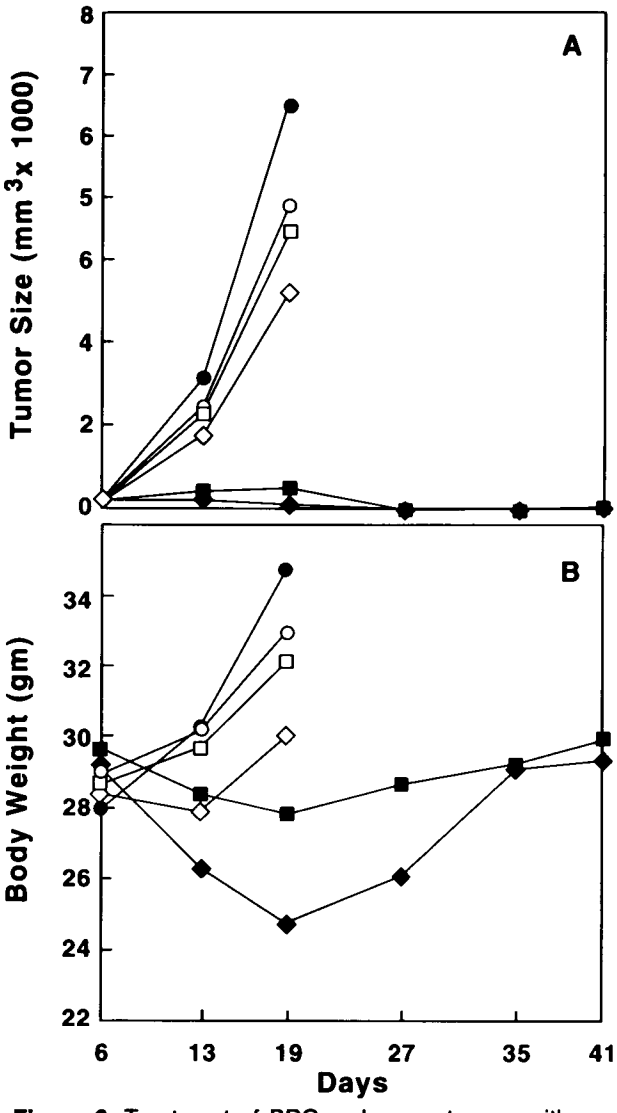


Figure 6. Treatment of BRO melanoma tumors with various doses of LCPTII. Thirty-six nude mice were xenografted with human BRO malignant tumor cells, and then randomly and equally divided into six groups. The animals received the same doses of LCPTII as in the case of CLO breast carcinoma with the same routes of administration. Similarly, control groups received 'empty' liposomes either i.v. (○) or i.m. (●). Measurements of the change in tumor size (A) and body weight (B) were then recorded. ■, 5 mg/kg i.m.; □ 5 mg/kg i.v.; ◆, 10 mg/kg i.m.; ◇, 10 mg/kg i.v.

this type are highly hydrophobic entities and, as such, can be readily accommodated by intercalation into the phospholipid bilayer membranes of liposomes. Furthermore, the freeze-fracture studies (Figure 3) indicate that incorporation of CPT into liposomes had extended the interlamellar distance between the lipid bilayers. This usually occurs with the incorporation of a lipophilic drug into the phospholipid bilayers. Image analysis measurements of the mean values (n = 10) of the actual bilayer space between 'empty' liposomes and liposome-incorporated CPT were 0.023 and 0.034 μm, respectively. Mean ranks of the measurements using the Kruskal–Wallis test (Table 1) indicate that the interlamellar distance of LCPT was significantly different from that of the 'empty' liposomes (p = 0.001). Therefore, we conclude that the incorporation of CPT into multilamellar liposomes resulted in extending the space between the lamellae in a manner differently from that of the 'empty liposomes. This is an indication of a drug intercalation between lipid bilayers.

The composition and physical form of the liposomes used seem to have important effects on the incorporation efficiency of CPT and on the stability of LCPT. Thus both the total ammount of the phospholipid present in the dispersion and the cholesterol content seem to be important in achieving a high loading capacity of CPT and 9-AC into liposomes (Figures 1 and 2). A higher incorporation efficency was achieved when the molar ratio of the total phopholipid : drug was 40 : 1 and the cholesterol content was 1%. Furthermore, the liposomal formulation of CPT at pH 6.5 produced a higher incorporation efficiency as compared with formulations at pH 7.0. This could be due to the fact that the stability of the CPT molecule in its keto-form (closed lipophilic form) favors lower pH conditions than the open form, which is more hydrophilic at pH 7.0.[11] Thus, liposome formulations of CPT were performed at pH 6.5.

Our recent work[14] has demonstrated that 90% of CPT is rapidly released from the multilamellar liposomes that contain a low amount (1%) of cholesterol in approximately 2 h. Inclusion of GM_1 or PI in the lipid bilayer of the obtained lipsomes in the present study, however, has resulted in the modulation of CPT release when incubated in human serum at 37°C. The CPT release from GM_1- or PI-containing liposomes was characterized by an initial rapid loss of contents (50% in 4 h), followed by a slow leakage of the remaining 50% over a 20 h period (Figure 4). Furthermore, PI-containing liposomes seem to be

Table 1. Ranks of outcomes for interlamellar measurements of lipid bilayers following the Kruskal–Wallis test[a]

Experimental subject	No. of measurements	∑ rank	Mean rank
'Empty' MLV-I	10	102.0	10.20
LCPTI	10	177.5	17.75
'Empty' MLV-II	10	187.5	18.75
LCPTII	10	353.0	35.30

[a] p = 0.0001.

more stable than GM_1-containing liposomes (Figure 4B). Thus we choose to use the DPCC/Sph/CHOL/PI formulation in our *in vivo* antitumor study. The DPCC/Sph/CHOL/PI preparation was also the most promising in terms of reduced toxicity (body weight loss) of the limited number of preparations screened in our initial study. However, it is not clear at this time if further reductions in drug toxicity could be attained using other formulations. Toxicity by the i.v. route but not i.m. toxicity seems to be strongly affected by the average size of the liposomal population; presumably this may relate to differences in the blood clearance rate and organ distributions of differently sized liposome preparations.[12,31]

There have been a large number of investigators using liposomes as carriers for currently utilized antitumor drugs, usually with water soluble compounds such as methotrexate[32] or 1-β-D-arabinofuranosylcytosine,[27] whereas other studies have used anthracyclines,[28,33–35] platinum-based drugs[36] and taxol[37] in liposomes. The delivery of these liposome-incorporated antitumor agents, in this case, is mainly by the i.v. route where the liposomes are shown to affect the pharamcodynamic behavior of incorporated drugs in a variety of ways. However, in the present studies, we have shown that liposome-incorporated CPT produced effective antitumor activity and indeed complete tumor regression when administered by the i.m. route once (10 mg/kg) or twice (5 mg/kg) in nude mice xenografted with a human malignant melanoma or breast carcinoma (Figure 5 and 6). The suspension form of CPT has also been shown to inhibit the growth of a wide variety of human malignant xenografts in nude mice when administered by the i.m. route, but in multiple doses for 3 weeks.[3,6–7] This indicates that the liposomes, in our case, acted as a sustained-release system for CPT since a single injection of LCPT produced a complete tumor regression as compared with multiple injections of 'free' CPT. The ability of liposomes to release the encapsulated drug slowly after i.m. injection has been reported repeatedly in the literature. Most studies demonstrate the ability of liposomes to release the laden drug slowly over a prolonged period of time after i.m. injection.[38] Depending on the drug used, the aim of the therapy might be either decreased drug toxicity or sustained systemic therapy in the case of chronic treatment. In our study, it seems likely that sustained systemic therapy has been attained with the use of LCPT.

Absorption of liposome-incorporated drug from i.m. injection sites usually occurs by several ways. Both liposome-incorporated drug and 'free' drug can reach the blood compartment via lymphatic absorption and by absorption through the capillary wall of blood vessels, respectively. The dynamic flow is able to drain the particles away from the site of administration and drainage of the lymph into the bloodstream will carry the particulates into the blood circulation. Whether the liposomes are drained through the lymph or stay at the injection site depends on their size and resistance against destabilization. Thus the exact mechanism of continuous CPT liberation from the MLVs at the injection site is currently under active investigation by our laboratory. Several variables are also known to influence the rate and extent of release of drugs from liposomes following i.m. injection. Through variations in size, number of bilayers, lipid composition, bilayer rigidity, charge and surface characteristics, injection volumes and amount of liposome administered, the efficacy of the incorporated agents can be manipulated. These variables are also being actively investigated.

Clearly, there are many questions which remain to be answered in this system. For example, we have no information on the basis for the cellular toxicity to BRO melanoma and CLO breast carcinoma of LCPT and whether it is the same as for the free drug. Further we have no clear insights into the mechanism of the host toxicity of LCPT and whether it is the same as for 'free' CPT. Finally, there is some indication that the i.v. LCPT may be quite schedule-dependent compared with i.m. administration, but we have not yet determined the optimum scheduling. Despite these limitations, the present study seems quite promising in that it offers an approach for improving the use of a pharmaceutical formulation of CPT by i.m. administration which in turn creates opportunities for drug delivery to the blood capillaries and the draining lymph nodes. Thus this approach can enhance the possible utilization of CPT as a chemotherapeutic agent for treatment of lymph node metastases.

Acknowledgments

The authors would like to thank Drs Vincent Franceschi and Christine Davitt of the Electron Microscopy Center at WSU for their expert assistance with the electron microscopy study.

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(Received 10 August 1994; accepted 18 August 1994)