Artificial Lipids Stabilized Camptothecin Incorporated in Liposomes

Yoshie MAITANI,*a Sayaka KATAYAMA,a Kumi KAWANO,a Akihiro HAYAMA,a and Kazunori TOMAa,b

a Institute of Medicinal Chemistry, Hoshi University; Shinagawa-ku, Tokyo 142–8501, Japan: and b The Noguchi Institute; Itabashi-ku, Tokyo 173–0003, Japan.

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Camptothecin (CPT) has anticancer activity. While only the lactone form of CPT is biologically active, this form exhibits poor aqueous solubility. Pharmaceutical formulation of CPT incorporated in liposomes is of significant importance to develop the therapeutic utilization of CPT. The aim of this study was to increase incorporation efficiency and stability of CPT in liposomes composed of hydrogenated soybean phosphatidylcholine, cholesterol, and oleic acid (7:3:1, molar ratio), by incorporating three kinds of artificial lipids (DBs) (DB-liposome); 4-n-(M12B), 3,5-bis(B12B) and 3,4,5-tris(dodecylxoy)benzoic acid (T12B). The interaction of CPT with DB in the state of liposomes, was examined. In DB-liposomes presenting mean diameters of 150 nm, incorporation efficiency of CPT up to 55% and final drug to lipid molar ratio up to 0.07 were obtained when the liposomes were prepared at a feeding ratio of 1/30 (w/w) CPT/total lipid. However, in the optimal formulations, incorporated DB mol% was different; T12B and D12B were incorporated about one third and half mol% of M12B, respectively. Moreover, we demonstrated that T12B stabilized CPT in liposomes significantly compared with other DBs as measured by CPT release, and by steady state fluorescence polarization degree of CPT using intrinsic fluorescence of CPT. These findings suggested that in addition of contribution of phenyl group of DB, dodecylxoy group may interact strongly with lactone ring of CPT. The capacity to contain CPT interacted with DBs may be limited in liposomes. T12B may be incorporated in the interior of the bilayers, resulting in increase of incorporation stability of CPT. This finding demonstrates a potential application of the novel liposome formulation of CPT in drug delivery.

Key words camptothecin; liposome; artificial lipid; release test; incorporation efficiency; incorporation stability

Camptothecin (CPT) is a naturally occurring cytotoxic alkaloid isolated from the Chinese plant Camptotheca acuminate.1) CPT and some of its analogs have shown a broad spectrum of antitumor activity against many solid tumors in xenografts including colorectal cancer.2,3) CPT inhibits the enzyme DNA topoisomerase I, initially by noncovalent binding and subsequently by stabilization of the complex through a nucleophilic attack by the enzyme at the acyl position of the CPT lactone ring.4) Of significant importance for pharmaceutical formulation is that, while only the lactone form of CPT is biologically active, this form exhibits poor aqueous solubility. The lactone of CPT is converted to carboxylate in a pH-dependent equilibrium.5) To overcome the aforementioned solubility problems and hydrolytic processes of CPT, several approaches have been investigated. Numerous attempts have been made to prepare water-soluble CPT analogs. The majority of these analogs were less potent in assays both in vitro and in vivo than the parent drug. In addition to the synthesis of new derivatives and pro-drug products,6–10) the development of adequate drug carriers is gaining increasing attention. There are many reports about effective formulation and utilization of CPT in cancer therapy by using drug delivery technologies such as liposomes,11,12) microemulsions,12) microspheres,13,14) and inclusion complexes with cyclodextrins.15) Previously we have reported that the stability of CPT loaded polymeric micelles in vivo was increased by benzyl esterification of hydrophobic segment of block copolymer.16–18) However, long circulation of CPT loaded polymeric micelles in vivo was not achieved yet. Other carriers such as liposomes were examined because release of drugs encapsulated in carriers depended on carriers. The designed amphipathic compounds are called artificial lipid, which has similar properties with phospholipid to form vesicles. Therefore, artificial lipid with a phenyl group, e.g., 3,5-bis(dodecylxoy)benzoic acid, was synthesized and added to the liposome formulation. The PE-gylated liposomes incorporating CPT were stable in vivo.19) However, there was not enough information about interaction between CPT and artificial lipid molecules in liposomes.

To develop the therapeutic utilization of CPT, it is necessary to prepare liposomes with high incorporation efficiency and stability of CPT. This study demonstrated that incorporation of 5 mol% of 3,4,5-tris(dodecylxoy)benzoic acid increased incorporation efficiency and stability of CPT in liposomes.

MATERIALS AND METHODS

Materials (S)-(+-)-Camptothecin (CPT), cholesterol (Ch), high performance liquid chromatography (HPLC) grade methanol and tetrahydrofuran (THF) were purchased from Wako Pure Chemicals (Tokyo, Japan). Hydrogenated soybean phosphatidylcholine (HSPC, >90% phosphatidylcholine), and oleic acid (OA) were purchased from NOF Corporation (Tokyo, Japan). 4-n-Dodecylxoy benzoic acid (M12B) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 3,5-Bis(dodecylxoy)benzoic acid (B12B) and 3,4,5-tris(dodecylxoy)benzoic acid (T12B) were synthesized as reported previously20) (Fig. 1). Other chemicals were of reagent grade.

Preparation of Liposomes Liposomes incorporating CPT were prepared as described elsewhere.10) Briefly, HSPC, Ch, OA, DB and CPT (molar ratio, HSPC:Ch:OA:DB:CPT = 7:3:1:0—3:1; weight ratio, total lipid:CPT = 30:1) were dissolved in methanol/chloroform mixture (1/4 (v/v)). The solvent was evaporated in a rotary evaporator at 55°C under stream of N2 gas. The lipid film containing the drug was hydrated with 2.5 ml of sodium phosphate-buffered solu-
ODS-AA-302 column (150 set at 254 nm). Separation was performed with an YMC-Pack Co., Ltd., Japan) apparatus equipped with a Shimadzu RF-

Fig. 1. Chemical Structure of 4-n-Dodecyloxy Benzoic Acid (A), 3,5-Bis(dodecyloxy)benzoic Acid (B), and 3,4,5-Tris(dodecyloxy)benzoic Acid (C)

**Determination of CPT and DB Content in Liposomes**

Drug incorporation efficiency was determined using the ultra-centrifugation method. Liposomes incorporating CPT were centrifuged at 52000 g for 1 h at 4 °C to separate free CPT. Then, the incorporation efficiency was obtained using two methods: determination of the CPT concentration of the supernatant containing free CPT, and determination of the amount of CPT entrapped in the precipitate, which was disrupted using chloroform. The incorporation efficiencies estimated using both methods were similar. The former method was used in the following experiment. The total drug concentrations in liposomes before centrifugation (liposome A) and in the supernatant after centrifugation (supernatant B) were determined using a one-centimeter cuvette in a spectrophotometer (HITACHI, Electronics). One-centimeter rectangular quartz fluorometer cell was used, and the excitation and emission wavelengths were set at 369 and 437 nm, respectively. The fluorescence polarization of liposomes in sodium phosphate-buffered solution (pH 6.04) was measured at room temperature (24 °C). I|| (I⊥) is the intensity of photons with electric vectors parallel (perpendicular) to the beam direction.

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polarization\ degree = \frac{I_{||} - I_{⊥}}{I_{||} + I_{⊥}}
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**Statistical Analysis**

The statistical significance of the data was evaluated with Student’s t test. A p value of 0.05 or less was considered significant.

**RESULTS AND DISCUSSION**

**Preparation and Characterization of CPT Incorporated in Liposomes**

The incorporation stability of CPT in polymeric micelles in vitro and in vivo was increased by benzyl esterification of hydrophobic segment of block copolymer, supposed to be π–π interaction of phenyl group with CPT.16—19 Therefore, the artificial lipids with a phenyl group, DBs were synthesized and added to liposome formulation to obtain stable liposomes incorporating CPT (DB-liposome). Basic formulation, HSPC : Ch : OA : DB : CPT (7:3:1:1, molar ratio) aqueous suspension with CPT concentration varied from 5.7×10^-1 μg/ml to 0.1 mg/ml (corresponding on x molar ratio). Steady-state fluorescence polarization measurements were performed on F-4500 fluorescence spectrophotometer (HITACHI, Electronics). One-centimeter rectangular quartz fluorometer cell was used, and the excitation and emission wavelengths were set at 369 and 437 nm, respectively. The fluorescence polarization of liposomes in sodium phosphate-buffered solution (pH 6.04) was measured at room temperature (24 °C). I|| (I⊥) is the intensity of photons with electric vectors parallel (perpendicular) to the beam direction.

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**Effect of DB/lipid Ratio on Incorporation to Liposomes**

CPT and DB contents in liposomes were determined at liposome formulation as HSPC : Ch : OA : DB : CPT = 7:3:1:1:1 (mol), as shown in Figs. 2 and 3. When the ratio of each DB to starting total lipid of M12B-, B12B- and T12B-liposome was increased to 15.3 mol%, the incorporation efficiency of M12B, B12B and T12B was increased to 84, 61.

Briefly, 1 ml of CPT liposomes was placed in a dialysis tube (Spectra/Por CE (MWCO 12000—14000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, U.S.A.)) and then suspended in a temperature-controlled, jacketed flask containing 100 ml of PBS. After various time intervals, aliquots were withdrawn and assayed for CPT content by fluorophotometry. Drug release profiles (percent release versus time) were plotted.

**Fluorescence Polarization Measurements**

To evaluate distribution of CPT in the liposomes, we examined mobility of CPT using intrinsic fluorescence of CPT by fluorescence polarization measurements. We prepared about 10 μl of lipid concentration of M12B-, B12B- and T12B-liposome (HSPC : Ch : OA : M12B : B12B : T12B : CPT = 7:3:1:1:1, HSPC : Ch : OA : B12B : CPT = 7:3:1:1:x, and HSPC : Ch : OA : T12B : CPT = 7:3:1:1:1:x, molar ratio) aqueous suspension with CPT concentration varied from 5.7×10^-1 μg/ml to 0.1 mg/ml (corresponding on x molar ratio). Steady-state fluorescence polarization measurements were performed on F-4500 fluorescence spectrophotometer (HITACHI, Electronics). One-centimeter rectangular quartz fluorometer cell was used, and the excitation and emission wavelengths were set at 369 and 437 nm, respectively. The fluorescence polarization of liposomes in sodium phosphate-buffered solution (pH 6.04) was measured at room temperature (24 °C). I|| (I⊥) is the intensity of photons with electric vectors parallel (perpendicular) to the beam direction.

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group of DBs and lactone ring of CPT over increase of acidity by carboxyl group of DBs.

**In Vitro Drug Release** The incorporation stability of CPT in M12B-L, B12B-L and T12B-L was examined from drug release test by incubation in PBS at 37°C, as shown in Fig. 4. The CPT released from Control-L was 100% for 3 h while that from M12B-L, B12B-L and T12B-L were 36.3, 22.3 and 20.5%, respectively. The CPT released from M12B-L was significantly higher than that from B12B-L and T12B-L for 5 h. CPT incorporated in M12B-L may be distributed at the surface of liposomes more than that in B12B-L and T12B-L, therefore CPT was released highly. During 24-h-period, M12B-L, B12B-L and T12B-L released 68.8, 69.0 and 54.5%, respectively. Release of CPT among DB-liposomes was higher T12B-L, B12B-L and M12B-L showed significantly lower release than B12B and M12B at 24 h ($p<0.05$). This result indicated that DBs increased incorporation stability of CPT, and incorporation stability of CPT was increased with increase of the numbers of dodecyloxy-group of DBs. Dodecyloxy-group of DBs might induce drug’s lactone ring to penetrate into lipid bilayers.

**Fluorescence Measurements** Anisotropy measurements were worthwhile as a strong indication of incorporation stability. Polarization degree of intrinsic fluorescence of CPT incorporated in the liposomes was evaluated. Figure 5 revealed that the polarization degree was CPT concentration-dependant. The polarization degree values were directly related to the kind of environment where the CPT was distributed. Free rotations in DMSO solution were related to the smallest values of polarization degree, compared to the state in liposomes, indicating that CPT molecule can move freely. M12B-, B12B- and T12B-liposomes at 0.11—0.13 $\mu$g/ml.

and 54%, respectively (Fig. 2). Also, incorporation efficiency of CPT up to 55% in all liposomes was obtained (Fig. 3). However, in the optimal formulation, DB (mol%) was different. Without DB, 26.3% of CPT was incorporated in liposomes (Control-L). The maximum CPT incorporation efficiency was obtained at the formulations of DB-liposomes: HSPC:Ch:OA:M12B:CPT=7:3:1:2:1 (molar ratio, M12B-L), HSPC:Ch:OA:B12B:CPT=7:3:1:1:1 (B12B-L), and HSPC:Ch:OA:T12B:CPT=7:3:1:0.67:1 (T12B-L), as reflected about 2-fold increase (55% for M12B-L and B12B-L, and 54% for T12B-L, final drug to lipid molar ration up to 0.07) compared with Control-L. Among DB-liposomes, T12B seemed most effective to incorporate CPT in liposomes since it worked at the smallest addition amount. The excess amount of T12B decreased incorporation of CPT in liposomes, suggesting that the capacity to contain the complex of T12B with CPT might be limited in liposomes. When increase of initial CPT amount in T12B-liposomes, the incorporation efficiency of CPT was decreased (data not shown). The incorporation of DBs in liposomes seemed to decide incorporation of CPT in liposomes. Because CPT could not complex with B12B (data not shown), DBs may distribute in liposomes, and then CPT may interact with DBs and could be incorporated into the interior of the bilayers. CPT molecules may be accumulated in hydrophobic region of membranes. The presence of DB contributed increased 30% of incorporation efficiency. These findings suggested that increased CPT incorporation efficiency by DBs might be due to interaction between phenyl and dodecyloxy...
CPT in T12B-L showed the lowest release among DB-liposomes. These findings suggested that CPT in T12B-L would be incorporated into the interior of the bilayers by interaction of dodecyloxy-group of T12B with drug’s lactone ring, and release slowly, while CPT in Control-L would be in contact with the water/lipid interface, be changed into the ionized form, and be released quickly.

Liposomal CPT delivery systems may be promising to cancer therapy. We have reported presently to apply this PE-Gylated formulation in vivo to evaluate anticancer effect.19)

CONCLUSIONS

By incorporating various amounts of artificial lipid, DB, incorporation efficiency of CPT in liposomes increased. Additionally, we demonstrated, 4,5-tris(dodecyloxy)benzoic acid (T12B) stabilized significantly CPT in liposomes at about one third of M12B amount compared with other DBs as measured by CPT release. These findings suggested that incorporation stability of CPT in liposomes was increased, likely due to the interaction between lactone ring of CPT and dodecyloxy group more than phenyl group of DB, resulting in CPT incorporated into the interior of the bilayers.

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REFERENCES