

Effective Prodrug Liposome and Conversion to Active Metabolite

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Abstract: Some antitumor agents encapsulated in liposomes have been used clinically. However, the usefulness of liposomes is limited to the liposomalization of active compounds. Irinotecan hydrochloride (CPT-11) is a prodrug of closed lactone ring form of SN-38, which is an active metabolite with antitumor activity and side toxicity. The plasma concentrations of closed CPT-11 and SN-38 increased with the liposomalization, and their blood circulation was prolonged by the polyethyleneglycol (PEG) modification. The antitumor activity of CPT-11 increased due to the elevated tumor distribution of closed CPT-11 and SN-38 levels by the PEG-modified liposomes. In the tumor, CPT-11 was converted to SN-38. Thus, it is considered that passive targeting to the tumor by liposomalization elevated the SN-38 level in the tumor especially and increased the antitumor activity of CPT-11. The closed/total ratio of SN-38 in the tumors of the liposomes group was greater than that of the CPT-11 solution group. Namely, SN-38 was thought to be generated in intact liposomes containing CPT-11. The generation of SN-38 in the liposomal membrane was shown after the incubation of liposome containing CPT-11 with carboxylesterase. It is therefore considered that part of CPT-11 is converted to SN-38 in intact liposomes. Furthermore, intestinal disorder, a side toxicity of CPT-11, decreased to depend on the closed SN-38 concentrations in the bile by liposomalization. Although the liposomes induce the improved tissue distribution of the prodrug, the tissue distribution of active metabolites do not always improve. However, CPT-11 entrapped liposome was useful.

1. INTRODUCTION

Liposomes are the principal means of effectively using medicines and are broadly recognized as a drug delivery system (DDS). Most detailed studies on liposomes have been performed on the antitumor agent. Some antitumor agents encapsulated in liposomes have been used clinically and have been shown to be effective against Kaposi's sarcomas in patients with AIDS in USA and Europe [1].

The liposomalization of antitumor agent prolongs their circulation in the blood and increases their accumulation in the tumor [2-5]. However, one of the biggest obstacles to the *in vivo* used of liposomes as drug carriers is their trapping by the cell of the reticuloendothelial system (RES) such as the liver and spleen. Many attempts have been made to overcome such as drawbacks. For example, the prolongation of liposome circulation in the blood has been achieved by the modification of the liposome surface with monosialogangliosides, GM₁s [6-8]. In addition, synthetic lipid derivatives of

polyethyleneglycol (PEG) have often been used as a lipid component to prolong the circulation time of liposomes because of their ease of synthesis with low cost and easy quality control of the molecular weight and distribution of PEG [9-12]. We have shown that doxorubicin (DOX)-encapsulated liposomes containing 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG) elevated DOX concentration in the serum and the tumor, reducing its distribution to the liver and spleen [13]. Furthermore, we have reported the increase of antitumor activity with the decrease of side toxicity by the liposomalization and the efficacy of intraperitoneal administration of DOX encapsulated liposome [14].

The pharmacokinetic profiles of liposomes containing antitumor agents are influenced by the physical property of liposomes, the pharmacokinetic property of the antitumor agent and the factors of the body [15]. Thus, for effective usefulness, it is necessary to examine the best combination of these factors. From these points, some effective liposomes were researched. However, the usefulness of liposomes is limited to the liposomalization of active compounds. In addition, there is a little information available on the efficacy of liposomal prodrugs.

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Irinotecan hydrochloride (CPT-11, (Fig. 1)) was semisynthesized as a water-soluble derivative of camptothecin, which is an alkaloid isolated from *Camptotheca acuminata* [16] and has been known to have unique antitumor activity, preventing DNA synthesis by inhibiting topoisomerase I [17]. Although CPT-11 has a potent antitumor activity [18, 19], it has lethal side effects such as myelosuppression and gastrointestinal disorders, with dehydration and electrolyte disorder to maintain serious diarrhea [20-22]. It has been on the market in Japan since 1994, whereas it is used with severe limitations today. In the liver, CPT-11 is enzymatically hydrolyzed by carboxylesterase to the active metabolite, SN-38 [23, 24]. Namely, CPT-11, shows antitumor activity in the body after conversion to SN-38, is a prodrug. Therefore, the targeting of CPT-11 liposomes, as prodrug, into the tumor is not always useful, because CPT-11 is not always converted to SN-38, active metabolite, in the tumor. So, we have examined the effect of the composing lipid of liposome and the efficacy of PEG modification of liposome on the antitumor activity and tissue distribution of CPT-11. Furthermore, it appears that CPT-11 induced intestinal disorder is mainly caused by the excretion of SN-38 into the bile [25]. We have investigated the effects of liposomalization on the SN-38 level in the bile and water content in the feces as indices of CPT-11 induced delayed diarrheal symptoms. We have concluded that the liposomalization of the CPT-11 as a prodrug is effective as same as that of active compounds liposomes [26-28].

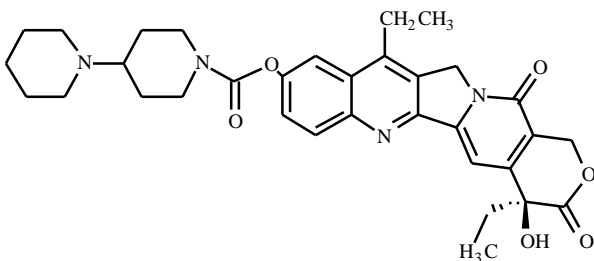


Fig. (1). Structure of CPT-11

2. CPT-11

2-1 Conversion to SN-38 from CPT-11 in vitro

CPT-11 was mainly converted to SN-38, an active metabolite, by the carboxylesterase in the liver, and SN-38 has strong antitumor activity [18, 19]. On the other hand, the liposome uptake by the RES in the liver and spleen prevented the

usefulness of liposome delivery. Therefore, the PEG modification on the surface of the liposomes was attempted to avoid RES [10, 13]. However, when a prodrug such as CPT-11 was delivered in liposomes, PEG-modification of the liposome was not always an improvement due to the superior to be requirement for prodrug processing, in the case for processing of CPT-11 to SN-38 in the tumor. Namely, the interaction of CPT-11 in liposome was found to enhance antitumor effect due to liver metabolism, but to decrease its effectiveness due to reduce the liposome uptake by PEG modification. Therefore, to be usefulness on the targeting of CPT-11 liposome into the tumor, it is necessary to be converted to SN-38 from CPT-11 by the carboxylesterase in the tumor.

The liver and tumor in Ehrlich ascites carcinoma (solid)-bearing mice were removed and a 5.0% homogenate was prepared in an isotonic saline. CPT-11 solution (Sol, 10 $\mu\text{g}/\text{ml}$) added in each homogenate and incubated at 37 $^{\circ}\text{C}$ for 4 h *in vitro*. By the incubation in the liver homogenate, CPT-11 was transiently converted to SN-38 (Fig. 2)). The SN-38 concentration was 3.19 $\mu\text{g}/\text{g}$ liver and 4.92 $\mu\text{g}/\text{g}$ liver at the 2nd and 4th h after incubation, respectively. On the other hand, in the tumor homogenate, 37% and 26% SN-38 was generated at the 2nd and 4th h, compared with that in the liver, respectively. Namely, the conversion of CPT-11 to SN-38 in the liver homogenate was confirmed. Similarly, conversion to SN-38 in the tumor was shown, and the conversion ratio was a quarter of that in the liver; thus, it is expected that the targeting of CPT-11 to the tumor by liposomalization may elevate the SN-38 level in the tumor and increase the antitumor activity of CPT-11. Furthermore, because SN-38 is not only associated with the antitumor activity of CPT-11 but also with the CPT-11 induced side toxicity, the SN-38 accumulation in the tumor may reduce its toxicity.

2-2 Physical Properties of Liposomal CPT-11

From above results, we have considered that it is worthy of note to the preparation of CPT-11 entrapped liposomes.

Liposome preparation was performed according to the method of Bangham [29]. Namely, dimyristoylphosphatidylcholine (DMPC) / cholesterol / dimyristoylphosphatidylglycerol (DMPG) (100:100:60 μmol) and CPT-11 10 mg (15 μmol) were dissolved in a chloroform / methanol mixture (2:1, v/v). The chloroform and

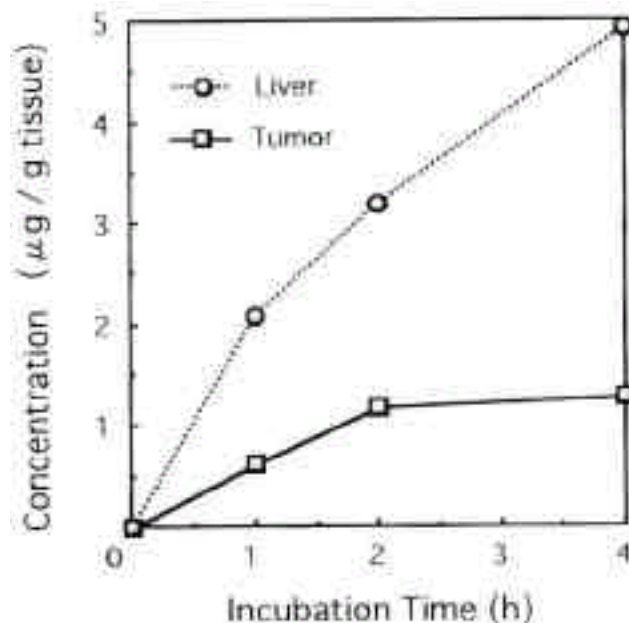


Fig. (2). Conversion to SN-38 from CPT-11 in the tumor and liver of mice (*in vitro*). Each point represents the mean of the duplicate of three samples.

methanol were evaporated to dryness under a stream of nitrogen gas. The thin lipid film was evacuated in a desiccator and the lipid film was then hydrated with 8.0 ml of 9.0% sucrose in 10 mM lactate buffer (pH 4.0) in a water bath at 50-60 °C for 10 min. The suspension was sonicated for 20 min above the phase transition temperature (T_c) after nitrogen gas bubbling. The liposome suspension was extruded through two stacked polycarbonate membrane filters with 0.2 μ m pores followed by five times with 0.1 μ m pores above T_c , and homogeneously sized liposome suspension was thus obtained. PEG modified liposome (M-PEG) and plain liposome (M-Lip) were prepared by adding 2.0 ml of 9.0% sucrose in 10 mM lactate buffer (pH 4.0) with or without 5.0 mol% PEG-DMG, respectively to 8.0 ml of the liposome suspension and sonicating the mixture. Liposome suspension dialyzed in 9.0% sucrose in 10 mM lactate buffer (pH 4.0) for 16 h. The trap ratio of CPT-11 in all liposome indicated above 90 %.

Examples of particle size distribution of CPT-11 entrapped liposomes are shown in Fig. (3). The mean particle size diameters of M-Lip and M-PEG were 165.7 ± 27 nm and 160.2 ± 25 nm, and the zeta potentials were -22.2 mV and -13.4 mV, respectively. The remaining percentages of CPT-11 in liposomes after 1 and 8 h incubation with 50 % fetal bovine serum / Hepes buffer (pH 7.0) at

37°C were 96.4 % and 77.2 %, respectively. Namely, these results have suggested to be able to prepare small sized, negative charged and stabilized liposomes encapsulating CPT-11 by the above method.

2-3 Tissue Distribution of Liposomal CPT-11

2-3-1 Effect of Composed Lipid

The effects of the composition of the phospholipid of the liposome containing CPT-11 on the CPT-11 distribution in mice were examined. S-Lip and S-PEG were prepared by the same method, except DMPC was changed to DSPC as the composing lipid of DMPC liposome. Ehrlich ascites carcinoma (5×10^5 cells/animal) was transplanted onto the backs of the mice. On the 14th day after transplantation, mice were injected via the tail vein with Sol, M-Lip, M-PEG, S-Lip and S-PEG at a dose of 10 mg/kg as CPT-11.

In the plasma (Fig. 4), CPT-11 concentration at the 1st h after injection in the M-Lip and S-Lip groups were increased 2.7 fold ($P < 0.01$) and 3.6 fold ($P < 0.001$), respectively, compared to that in the Sol group. Furthermore, each liposome prolonged the circulation in the plasma by PEG modification of liposomes, and the CPT-11

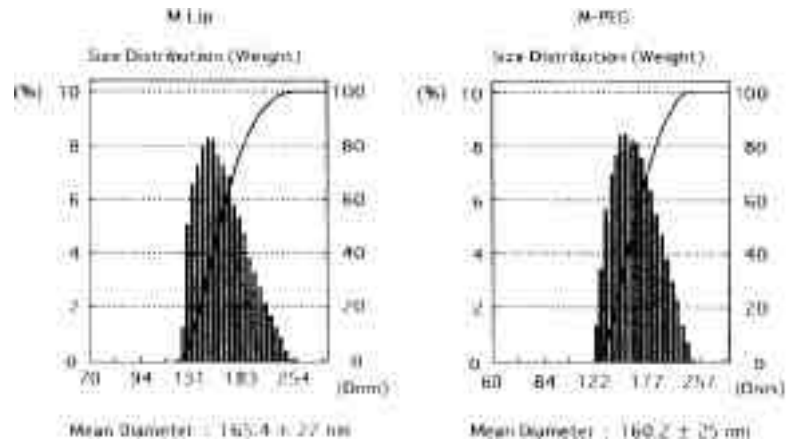


Fig. (3). Size distribution of M-Lip and M-PEG

concentration at the 8 h post injection in the M-PEG and S-PEG groups were shown to be 100 fold ($P < 0.001$) and 136 fold ($P < 0.001$), respectively, compared to that in the Sol group (Fig. 4). Namely, the liposomalization and PEG modification of the liposomes increased the plasma circulation of CPT-11. In particular, the CPT-11 concentration in the S-PEG group markedly increased. SN-38 concentration in the

plasma increased by the liposomalization, suggesting the same tendency to CPT-11 concentration. In particular, the SN-38 concentrations in the M-PEG and S-PEG groups at 8 h post injection were 11.4 fold ($P < 0.001$) and 9.2 fold ($P < 0.001$), respectively, that of the Sol group (Fig. 4).

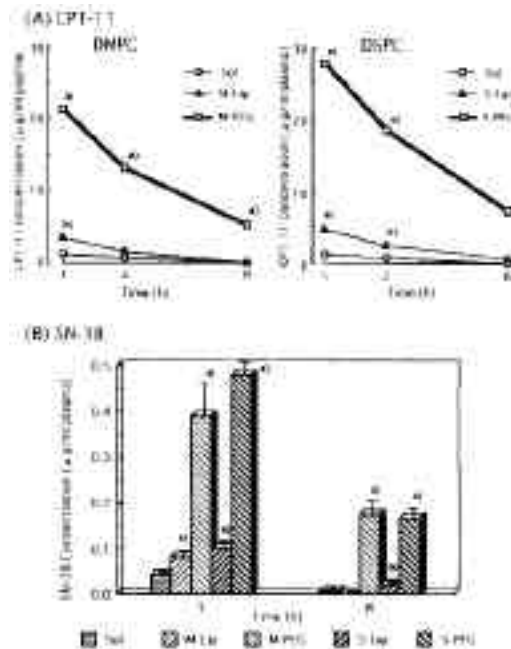


Fig. (4). CPT-11 and SN-38 concentrations in the plasma.

Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. (A) Each point represents the mean of three mice, each with no more than 10 % variation between them. (B) Each column represents the mean \pm S.D. of three mice. Significant differences from the level of Sol group are indicated by a) $P < 0.001$ and b) $P < 0.01$.

The order of the CPT-11 concentration in the tumor (Fig. 5) was M-PEG (S-PEG) > M-Lip (S-Lip) > Sol. This phenomenon is explained as follows: PEG modification forms the fixed aqueous layer around the surface of the liposomes [30]. This fixed aqueous layer prevents attack on liposomes by opsonins in the plasma and, therefore, avoids RES. The tumor accumulation of CPT-11 then increases by passive targeting, not active targeting. The change in SN-38 concentration in the tumor was similar to that of CPT-11 concentration, and these levels 8 h post injection in the S-Lip and S-PEG group increased to 2.5 fold ($P < 0.001$) and 3.7 fold ($P < 0.001$), of that in the Sol group, respectively. The increment of CPT-11 and SN-38 concentration in the tumor by the liposomalization and PEG modification suggests the possibility of an increase in CPT-11 induced antitumor activity.

The order of liver accumulation by the liposomalization and the RES avoidance by the PEG modification was as follows: DSPC > DMPC (Fig. 6). In the liver, the SN-38 concentration 8 h after the PEG-modified liposome injection decreased compared to that of the Sol group (data not shown). The decrease in SN-38 concentration in the liver, where CPT-11 was mainly converted to SN-38, is expected to be connected with the reduction of side toxicity. This speculation was supported from CPT-11 and SN-38 concentrations in the bile.

CPT-11 has severe side toxicity such as myelosuppression and intestinal disorder in the clinical use, and there are reports of CPT-11 induced deaths [31]. The intestinal toxicity is connected with SN-38 [32], which is conjugated with glucuronic acid in the liver and excreted in the bile. After deconjugation in the intestine, SN-38

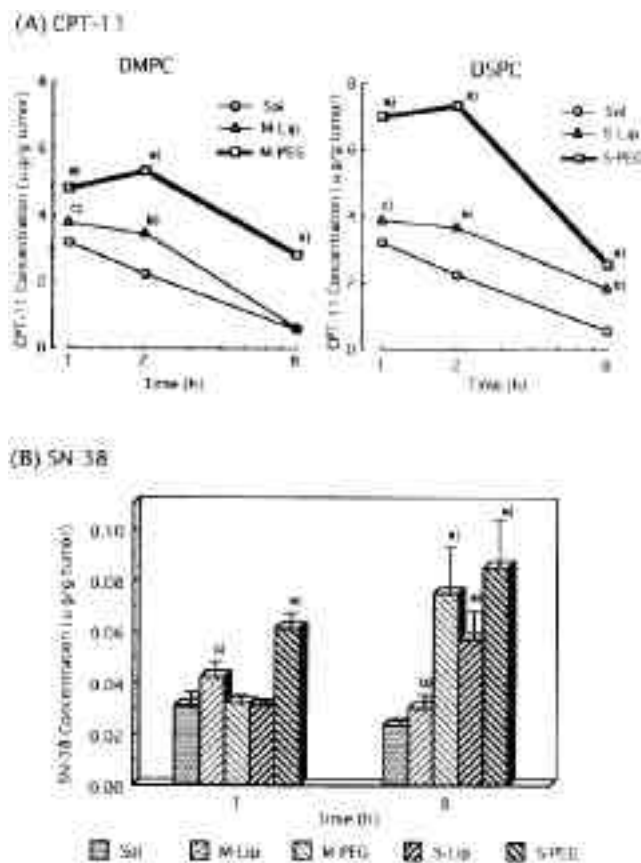


Fig. (5). CPT-11 and SN-38 concentrations in the tumor

Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. (A) Each point represents the mean of three mice, each with no more than 10 % variation between them. (B) Each column represents the mean \pm S.D. of three mice. Significant differences from the level of Sol group are indicated by a) $P < 0.001$, b) $P < 0.01$ and c) $P < 0.05$.

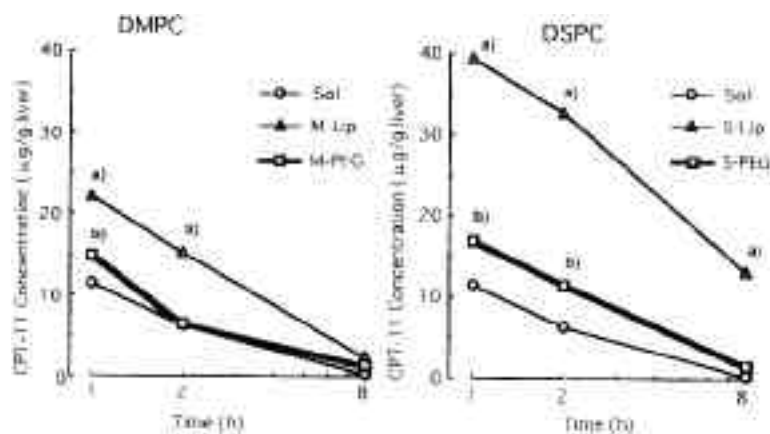


Fig. (6). CPT-11 concentration in the liver

Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. Each point represents the mean of three mice, each with no more than 10 % variation between them. Significant differences from the level of Sol group are indicated by a) $P < 0.001$ and b) $P < 0.01$.

was again regenerated. This SN-38 in the intestine is likely to cause intestinal disorder [25, 33, 34]. CPT-11 concentration in the bile (Fig. 7) was reduced by the liposomalization, in particular, that in the S-PEG group showed only 30% ($P < 0.001$) of that in the Sol group. The change in SN-38 concentration indicated the same tendency to that of the CPT-11 concentration and SN-38 concentration in the S-Lip and S-PEG groups was lower than that in the M-Lip and M-PEG groups, respectively. Therefore, it may be possible to reduce the CPT-11 induced intestinal disorder by the liposomalization of CPT-11.

The CPT-11 and SN-38 concentrations in the bone marrow after the S-Lip treatment were 109 % and 86 %, compared to those level in the Sol group, respectively (Figure not shown). Thus, there was no increase in CPT-11 and SN-38 concentrations in the bone marrow by liposomalization. Furthermore, CPT-11 induced reduction of the numbers of the bone marrow cells was not enhanced by liposomalization (109 % of that in the Sol group). Therefore, it is expected that CPT-11 induced myelosuppression was not amplified by liposomalization.

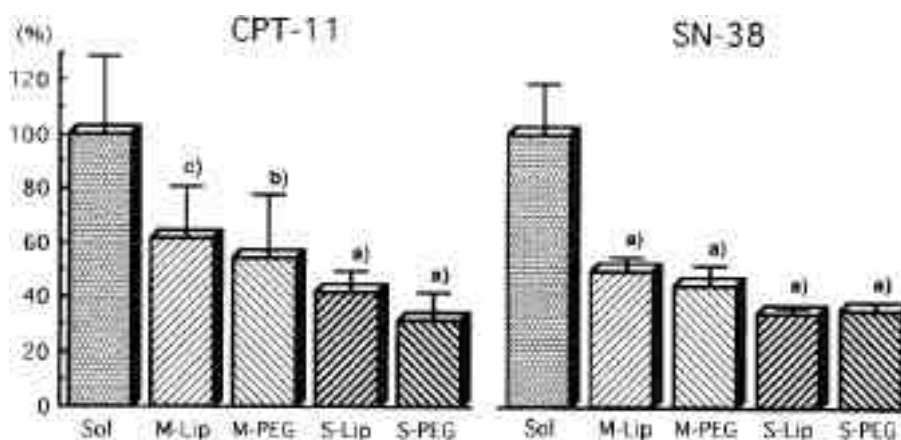


Fig. (7). CPT-11 and SN-38 concentrations in the bile

Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. Each column represents the mean \pm S.D. of three mice. Significant differences from the level of Sol group are indicated by a) $P < 0.001$, b) $P < 0.01$ and c) $P < 0.05$.

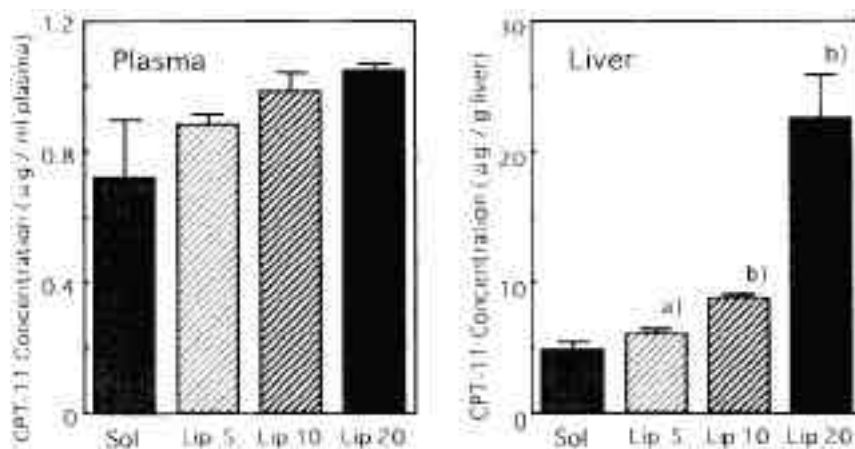


Fig. (8). Effect of CPT-11 entrapped levels in liposomes on the distribution in the plasma and the liver at 2 h after each liposome administration

Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, Lip5, Lip10 and Lip20. Each column represents the the mean ± S.D. of three mice. Significant difference from the level of Sol group are indicated by a) P < 0.05 and b) P < 0.001.

From these results, S-PEG is considered to be superior in these liposomes.

2-3-2 Effect of Entrapped Amount of CPT-11

The usefulness of CPT-11 entrapped liposomes on the tissue distribution has been demonstrated. Next, to search in more effective liposomes, the effects of the entrapped amount in liposomes on the tissue distribution *in vivo* were examined. We prepared liposomes, changing the entrapped amount of CPT-11 per amount of the liposome-comprising lipid. Lip10 (final concentration, 1.0 mg/26 µ mol lipid/ml) was prepared by the above method. Similarly, Lip5 (final concentration, 0.5

mg/26 µ mol lipid/ml) and Lip20 (final concentration, 2.0 mg/26 µ mol lipid/ml) were prepared by adding the respective amounts of CPT-11 when the lipid films were prepared.

After the administration of these liposomes with the same dose of CPT-11 (therefore, with different doses of lipid), the tissue distribution of CPT-11 was difference. At 2 hours after CPT-11 entrapped liposome administration (CPT-11 dose, 10 mg/kg, i.v.), an increase in the amount of CPT-11 entrapped in the liposomes induced an increase in the CPT-11 concentrations in the plasma and a tumor (Fig. 8, 9). It is suggested that the increase in the CPT-11 level per amount of the lipid

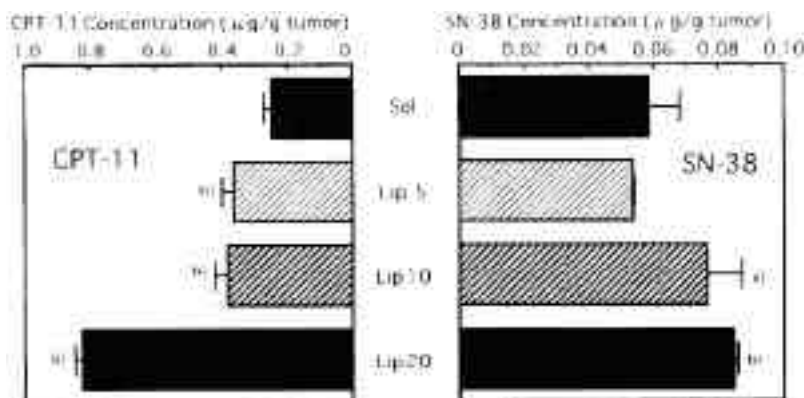


Fig. (9). Effect of CPT-11 entrapped level in liposomes on the distribution in the tumor at 2 h after each liposome administration

Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, Lip5, Lip10 and Lip20. Each column represents the the mean ± S.D. of three mice. Significant difference from the level of Sol group are indicated by a) P < 0.05 and b) P < 0.001.

comprising the liposomes increased the usefulness of liposomalization. However, the CPT-11 concentration in the liver (Fig. 9) increased with an increase in the amount of CPT-11 entrapped in the liposomes, too. In particular, CPT-11 concentration in the Lip20 group was greater than that in other group. In the tumor, SN-38 concentrations in the Lip10 and Lip20 groups were same level and greater than that in other group. Namely, it appeared that the tissue distributions of CPT-11 entrapped liposome *in vivo* change by the change of the entrapped amount of CPT-11 in liposomes. Furthermore, it is expected that Lip20 have effective antitumor activity with a severe toxicity, whereas Lip10 may show useful activity with moderate toxicity.

2-3-3 Efficacy on Lactone Ring of SN-38

From these results, the use of liposomal CPT-11 improved the tissue distributions of CPT-11 and SN-38 in Ehrlich ascites carcinoma-bearing mice. However, the activities of CPT-11 and SN-38 are known to depend on the closed lactone ring form of SN-38 [35]. The effect of the liposomalization on the closed-open reaction of the lactone ring of CPT-11 or SN-38 is not clear. In this section, we examined the tissue distributions of the closed- and open-forms CPT-11 and SN-38 in Lewis lung carcinoma-bearing mice after the administration of liposomal CPT-11.

Lewis lung carcinoma cells (5×10^5 cells/animal) were subcutaneously transplanted onto the backs of the mice. On the 21st day after transplantation, tumor-bearing mice were injected via a tail vein with Sol, S-Lip or S-PEG at a dose of 10 mg/kg as CPT-11. The CPT-11 and SN-38 concentrations in the plasma and each tissue were determined as follows. The tissues were homogenized in saline to obtain a 5.0% homogenate. One ml of tissue homogenate or 10% plasma was added to 0.5 ml of 0.1 M lactate buffer (pH 4.0) followed by mixing. This mixture was added to 3.0 ml of 1-butanol as an extracting solvent, and then the mixture was shaken in a vortex mixer and centrifuged at 1,200 g for 15 min. After centrifugation, CPT-11 and SN-38 in the upper layer were quantitated by fluorescence spectrophotometry (CPT-11, Ex: 374 nm, Em: 435 nm and SN-38, Ex: 380 nm, Em: 556 nm) as the total levels. The closed levels of CPT-11 and SN-38 were determined by the same procedure except for the addition of 0.1 M Hepes buffer (pH 7.5) in place of lactate buffer [36]. Total level is open level plus closed level.

The lactone ring of CPT-11 is nonenzymatically hydrolyzed under neutral and basic conditions, and an equilibrium is reached between the open ring (carboxyl acid form) and closed ring (lactone form) forms (Fig. 10). For CPT-11 and SN-38, the closed form of the lactone

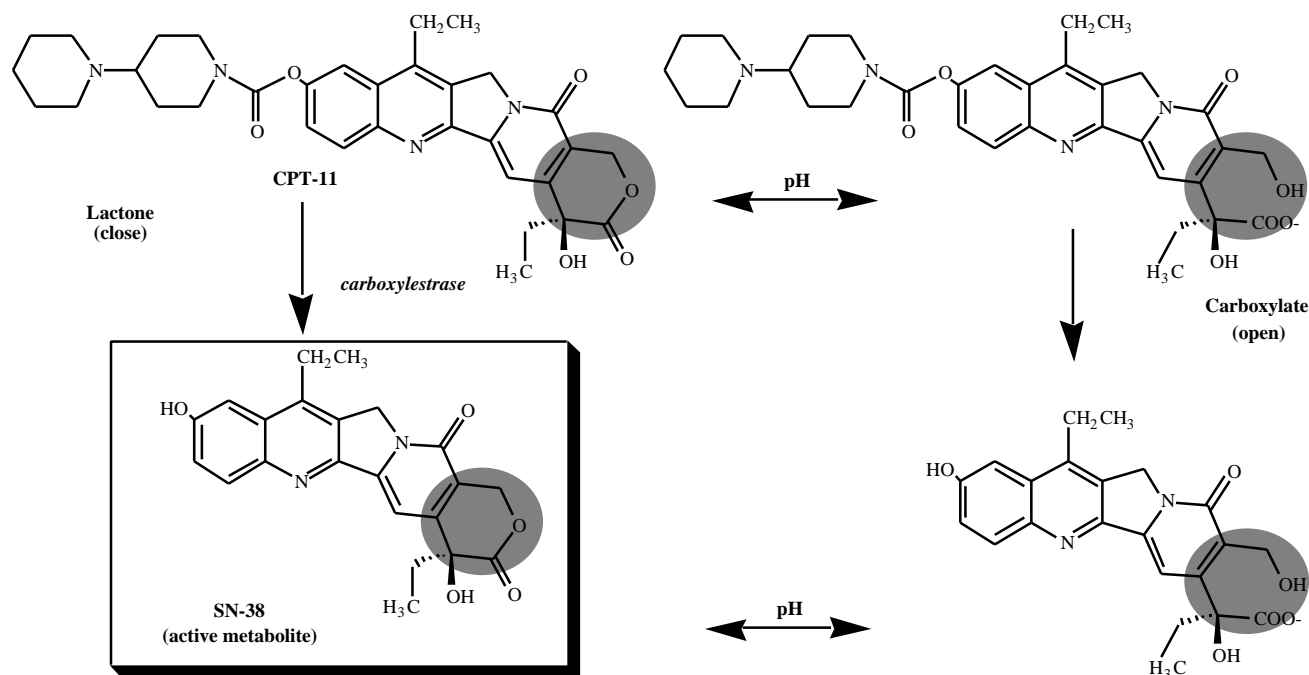


Fig. (10). Chemical structures of the lactone and carboxylate forms of CPT-11 and its metabolite SN-38

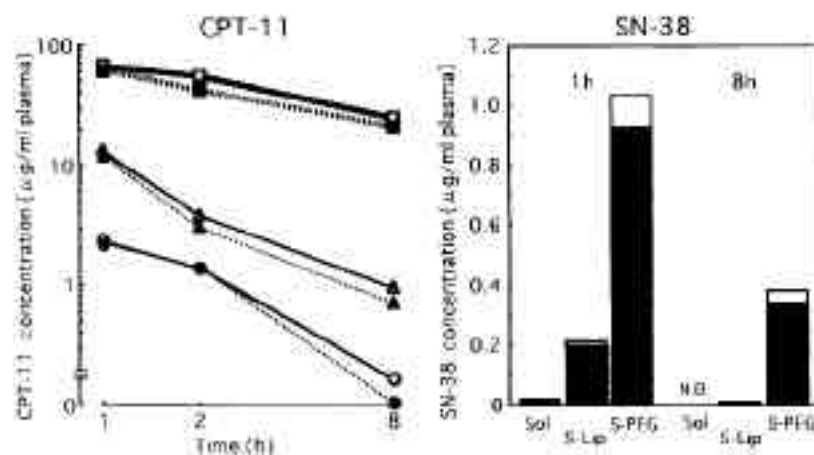


Fig. (11). CPT-11 and SN-38 concentrations in the plasma

Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, S-Lip or S-PEG. The data represent the means for three mice, each with duplicate data with no more than 10 % variation between them. Open- and closed-columns express open- and closed SN-38, respectively. Total level is open level plus closed level. Each point and each column for the S-Lip and S-PEG groups showed significant differences ($P < 0.001$) compared to the corresponding data for the Sol group.

Total; ○Sol, □S-Lip, □S-PEG; closed: ●Sol, ▲ S-Lip, ■S-PEG.

ring is essential for antitumor activity. In addition, the rate and equilibrium conditions of the open-closed reaction are each changed on the binding of CPT-11 and SN-38 with serum albumin [37, 38]. The closed lactone ring form of administered CPT-11 was cleaved gradually, and this activity became weaker in the body. However, through encapsulation of CPT-11 in liposomes containing acidic water (pH 4.0), it was expected that the more stable lactone form of CPT-11 can be targeted to tumors. In the plasma of the Lewis lung carcinoma-bearing mice (Fig. 11), the closed CPT-11 and SN-38 concentrations were increased by the liposomalization, and prolongation of their circulation in the blood was produced by the PEG modification. In addition, the closed CPT-11 and SN-38 concentrations in the tumors (Fig. 12) were elevated by both the liposomes. Burke attempted the liposomalization of camptothecin in order to stabilize its lactone ring, and reported that the lactone ring of camptothecin exists stably in the liposomal membrane [39]. If the lactone ring of CPT-11 enters the liposomal membrane, the closed lactone ring could be expected to be maintained stably. If CPT-11, which is released from disrupted liposomes, is converted to SN-38, the closed/total ratio of SN-38 in the S-PEG group should be the same as that in the Sol group. However, increase in the closed SN-38 concentration and closed/total ratio of SN-38 in the tumors occurred on the liposomalization of CPT-11. Thus, it is suspected that SN-38 was generated in the intact liposomes containing CPT-11. With the effective targeting of liposomal CPT-

11, the conversion of closed SN-38 in tumors is thought to cause the possible reduction of CPT-11 induced side toxicity, because closed SN-38 exhibits side toxicity. In fact, the closed SN-38 concentration in the bile is associated with CPT-11 induced side toxicity such as in an intestine disorder, and the closed SN-38 concentration in the bile was decreased by the liposomalization (figure not shown).

2-4 Antitumor Activity of CPT-11 Encapsulated Liposome

2-4-1 Tumor Weight of Ehrlich Ascites Carcinoma

The increment of CPT-11 and SN-38 concentrations in the tumor by the liposomalization and PEG modification suggests the possibility of an increase in CPT-11 induced antitumor activity. We examined the antitumor activity of these liposomes.

In Ehrlich ascites carcinoma (solid) bearing mice, Sol or each liposome (CPT-11 : 10 mg/kg/day x 3 days) was administered intravenously 14, 17 and 20 days after tumor inoculation. As shown in (Fig. 13), on the 23rd day after inoculation, the tumor weight of the control level was 2.51 ± 0.79 g. That level in the Sol group was 1.87 ± 0.85 g and decreased to 74.5% of the control level. There was an increased

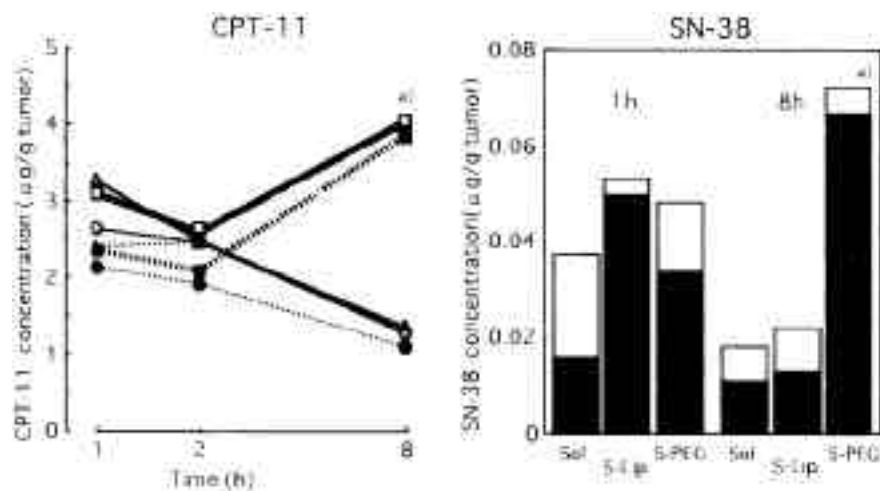


Fig. (12). CPT-11 and SN-38 concentrations in the tumor

Mice were injected with 10 mg/kg (i.v.) of CPT-11 in the form of Sol, S-Lip or S-PEG. The data represent the means for three mice, each with duplicate data with no more than 10 % variation between them. Open- and closed-columns express open- and closed SN-38, respectively. Total level is open level plus closed level. A significant difference from the Sol level is indicated by a) $P < 0.001$.

Total; ○Sol, S-Lip, □S-PEG; closed: ●Sol, ▲ S-Lip, ■S-PEG.

effect of the liposomalization and PEG modification on the reduction of the tumor weight. The order of this effect was tendency to; S-PEG > S-Lip = M-PEG > M-Lip > Sol. In particular, S-PEG enhanced by 2.6 fold (a significant difference from the level of Sol group : $p < 0.05$) the CPT-11 inhibitory effect of tumor growth. In this time, the CPT-11 and SN-38 concentrations in the tumor is elevated by the liposomalization ($P < 0.001$), compared to the Sol level. The order of the SN-38 concentration was as follows: S-PEG > S-Lip = M-PEG > M-Lip > Sol. In particular, the CPT-11 and SN-38 concentrations in the tumor of the S-PEG group increased to 29 fold ($P < 0.001$) and 4.2 fold ($P < 0.001$), of those levels in the Sol group, respectively. Therefore, the increase in CPT-11 induced antitumor activity by the liposomalization and PEG modification was connected with the CPT-11 and SN-38 concentrations in the tumor. Because the therapeutic dose of CPT-11 in the mice is about 50 mg/kg (i.v.), the administered dose, used in our experiments, enters into the area of therapeutic dose. There was no CPT-11 induced death or decrease in the body weight.

The liposomalization of CPT-11 as the prodrug has been indicated to also increase antitumor activity. In the liposomalization of the prodrugs, these results suggest that the targeting in the tumor

is useful in the case of the generation of active metabolite in the tumor.

2-4-2 CPT-11 Induced Prolongation of Survival in Mice with Lewis Lung Carcinoma

CPT-11 has shown strong activity against lung carcinomas clinically [40]. Therefore, the effect of liposomalization on the CPT-11 induced change in the lethality in Lewis lung carcinoma-bearing mice was examined. Lewis lung carcinoma cells (5×10^5 cells/animal) were subcutaneously transplanted onto the backs of the mice. On the 15th, 18th and 21st days after inoculation, tumor bearing mice were injected via a tail vein with Sol, S-Lip or S-PEG at a dose of 10 mg/kg/day x 3 days.

For Lewis lung carcinoma-induced death, the median number of survival days in the control group was 23.0 days (Fig. 14). The survival period of the Sol group with this experimental dose was not prolonged, whereas the numbers of survival days in the S-Lip and S-PEG groups were increased to 28.5 and 30.0 days ($P < 0.01$; compared to the levels in the control and Sol groups), respectively. Namely, it appeared that those of the S-Lip and S-PEG groups were prolonged with this dose and no significant difference between the S-Lip and S-PEG groups was observed. Normal mice did not die after CPT-11 administration of this dose as each formulation

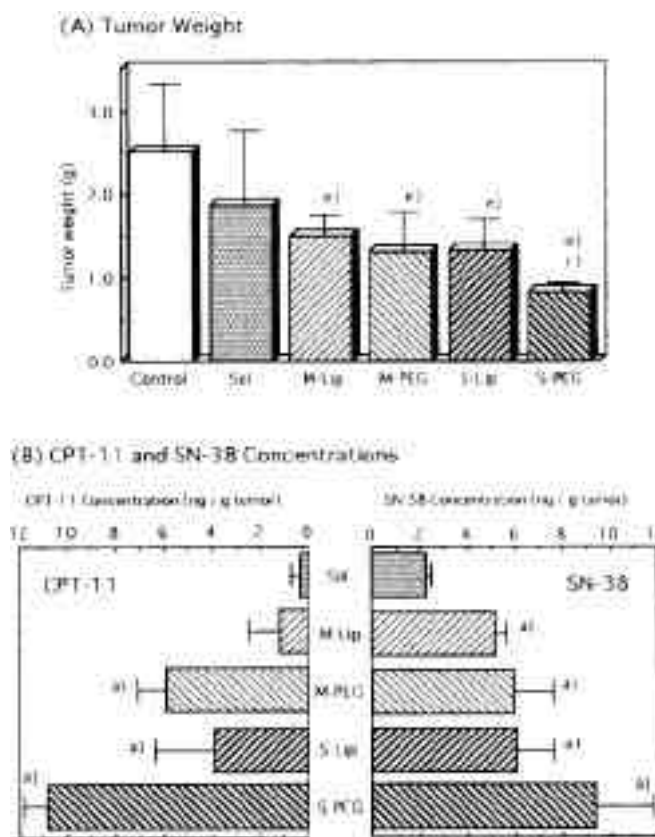


Fig. (13). Effects of liposomalization on (A) the changes in tumor weight induced by CPT-11 and (B) CPT-11 and SN-38 concentrations in the tumor

Mice were injected with 10 mg/kg/day (i.v.) x 3 days of CPT-11 in the form of Sol, M-Lip, M-PEG, S-Lip and S-PEG. Each column represents the mean \pm S.D. of eight mice. Significant differences from the level of the Sol group are indicated by a) $P < 0.001$ and c) $P < 0.05$. Significant differences from the level of the control group are indicated by d) $P < 0.01$ and e) $P < 0.05$.

and its body weight did not decrease. Therefore, the deaths of the mice were induced by the Lewis lung carcinomas, not by CPT-11 induced side toxicity.

2-5 CPT-11 Induced Intestinal Toxicity

CPT-11 occurs severe gastro-intestinal disorder, with dehydration and electrolyte disorder to maintain serious diarrhea [21, 22]. The effect of liposomalization on the water content in the feces as indices of CPT-11 induced delayed diarrheal symptoms was investigated. Sol, S-Lip and S-PEG (CPT-11 : 100 mg/kg/day x 4 days) were injected intraperitoneally into normal mice once a day for 4 days. The mice were killed by cervical dislocation on the 2nd day following the last injection. After observing the intestine by the naked eye, the wet and dry weights of the feces in the large intestine

were determined. The water content in the feces was calculated from these weights.

During the experimental periods, the body weight in the Sol group was observed to decrease by 15%, whereas the decreases in the S-Lip and S-PEG groups were smaller than those in the Sol group. Swelled intestine and intestinal disorder found in the intestine on the 2nd day after final administration in the Sol group. In contrast, these changes in the S-Lip and S-PEG groups were slight. The water content in the feces in the Sol group increased by 17% of the normal level, whereas the increase in the S-Lip and S-PEG groups was about one-half ($P < 0.01$) of that on the Sol group (Fig. 15). Thus, the liposomalization of CPT-11 appears to be able to suppress CPT-11 induced diarrhea as lethal toxicity. These results have been supported by the change of CPT-11 and SN-38 concentrations in the bile.

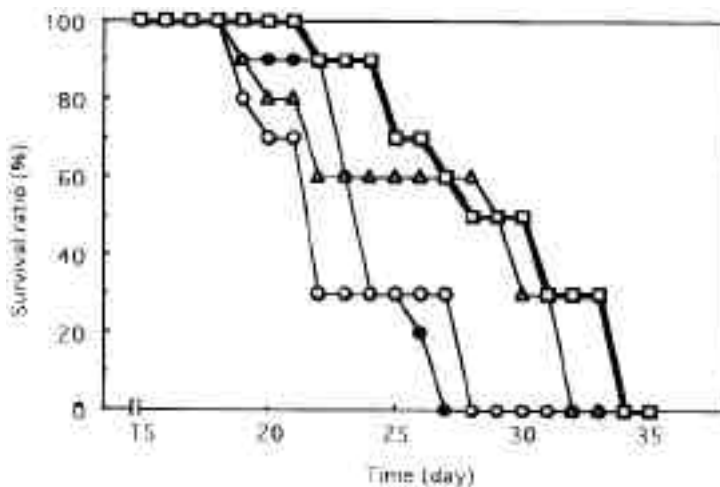


Fig. (14). Survival of tumor-bearing mice after administration of Sol, S-Lip or S-PEG.

Lewis lung carcinoma cells (5×10^5 cells/animal) were subcutaneously transplanted onto the backs of the mice. On the 15th, 18th and 21st days after inoculation, tumor bearing mice were injected via a tail vein with Sol, S-Lip or S-PEG at a dose of 10 mg/kg/day x 3 days. Each group consisted 10 mice.

●control, ○Sol, △S-Lip, □S-PEG.

2-6 Effect of Liposomalization on the uptake of CPT-11 by Tumor Cells

The usefulness of CPT-11 entrapped liposomes on the tissue distribution and antitumor activity has been demonstrated. To support these results *in vivo*, The effects of the liposomalization on the tumor cell uptake *in vitro* were examined.

Ehrlich ascites carcinoma cells suspensions (5.0×10^6 cells/ml) containing CPT-11 entrapped liposome were incubated at 37°C. The CPT-11 concentrations in the tumor cells for 30 min after the addition of S-Lip and S-PEG (CPT-11 concentration : 20 μ g/ml) were lower than that in the Sol group (Fig. 16). As shown in (Fig. 17), with the addition of CPT-11 at 40 μ g/ml, the CPT-11 concentration in the cells of the S-Lip group was half that of the Sol group, whereas in the case of CPT-11 at 5 μ g/ml, the concentration in the S-Lip group was not significantly different from that in the Sol group. The cell uptake of CPT-11 in the Sol group increased depending on the additional CPT-11 concentration, whereas this increase in the S-Lip group was suppressed. The amount of an administered lipid affects the tissue distribution of the liposomes and the liposome uptake by the liver shows saturation [41 - 43]. With the use of 30-40 μ g/ml of CPT-11 (this level is expected to be a lipid saturation level), the lipid concentration was 78-104 mM, whereas with 5 μ g/ml of CPT-11 (this level is below the saturation level, and there was no difference in the cell uptake between the Sol and S-Lip groups), the lipid concentration was 13 mM. The CPT-11 concentration in the tumors *in vivo* was below 5 μ g/g tumor, and the lipid concentration was below 13 mM. Namely, the lipid level did not reach the saturation level for cell uptake *in vitro*, and there was no difference in the cell uptake between the Sol and S-Lip groups. The CPT-11 concentration in the tumors of the S-Lip group was higher than

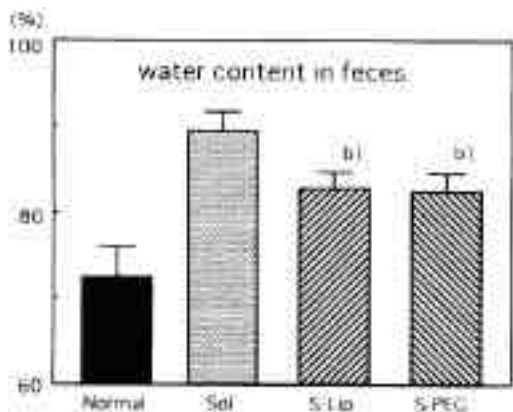


Fig. (15). Effect of liposomalization on CPT-11 induced delayed diarrheal symptoms in mice

Mice were injected with 100 mg/kg/day (i.p.) x 4 days of CPT-11 in the form of Sol, S-Lip and S-PEG. Each column represents the mean \pm S.D. of six mice. Significant difference from the level of Sol group is indicated by b) $P < 0.01$.

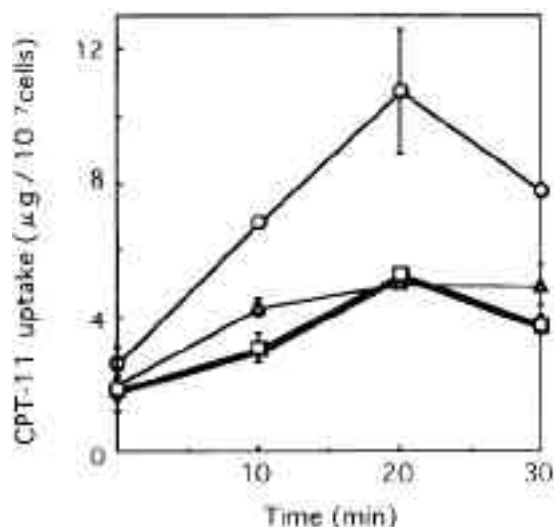


Fig. (16). Time course on uptake of CPT-11 by Ehrlich ascites carcinoma cells

The cell suspensions (5×10^6 cells/ml) in Eagle's MEM medium containing Sol, S-Lip or S-PEG (CPT-11 concentration : $20 \mu\text{g/ml}$) were incubated at 37°C . After incubation, each cells were collected and determined CPT-11 level. The data represent the means \pm S.D. for three experiments.

○Sol, □S-Lip, △S-PEG.

that of the Sol group *in vivo*. Therefore, liposomal CPT-11 is more effective than Sol. In contrast, the CPT-11 concentration in the liver *in vivo* was about $30 \mu\text{g/g}$ liver and the lipid concentration was 78 mM , i.e., the saturation level. Under these conditions, the liposomal uptake by the liver may be suppressed. In addition, the generation ratio of

SN-38/CPT-11 in the tumor cells of the S-Lip group was higher than that of the Sol group (Fig. 17). Thus, effective conversion of SN-38 in the tumor was produced by the liposomalization.

Because PEG-modified liposomes, which prevented the attack by opsonins and which was

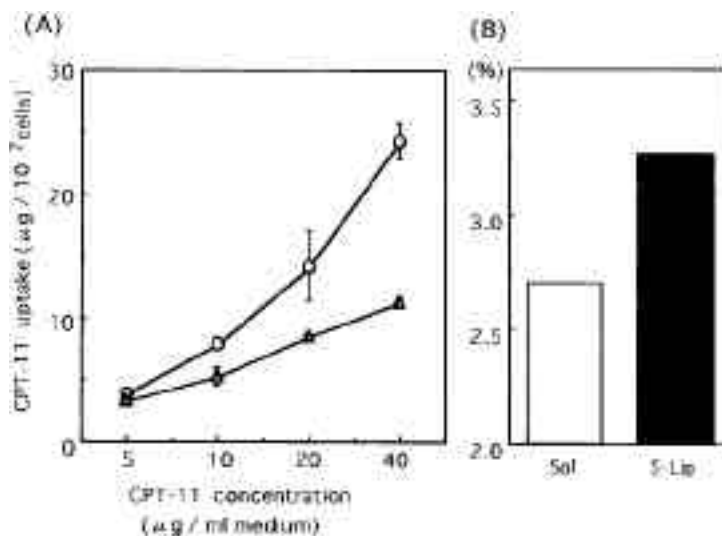


Fig. (17). Uptake of CPT-11 by Ehrlich ascites carcinoma cells

(A) The cell suspensions (5×10^6 cells/ml) in Eagle's MEM medium containing varied CPT-11 concentration of Sol or S-Lip for 20 min at 37°C . After incubation, each cells were collected and determined CPT-11 level. The data represent the means \pm S.D. for three experiments. ○Sol, □S-Lip.

(B) SN-38/CPT-11 ratio with $40 \mu\text{g/ml}$ of CPT-11.

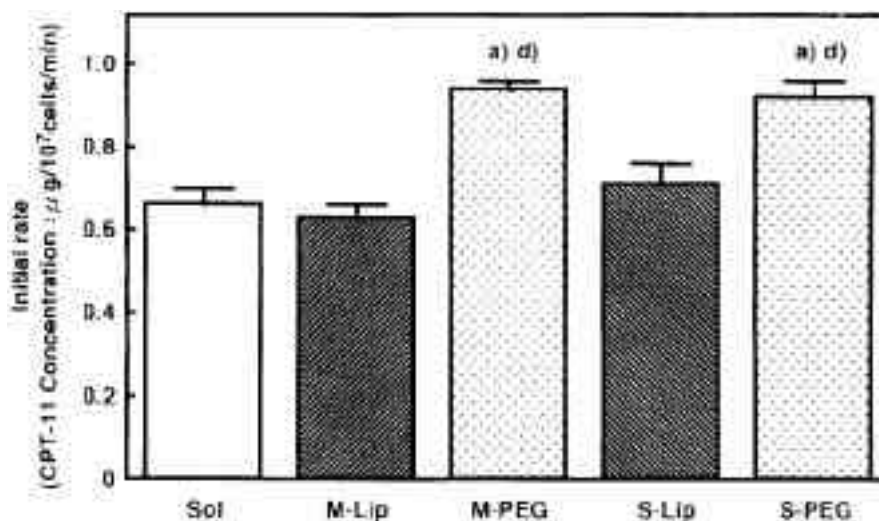


Fig. (18). Effect of PEG modification on the initial rate of tumor cell uptake *in vitro*

Each column represents the mean \pm SD for three experiments. Significant difference from the level of the Sol group is indicated by a) $P < 0.001$. Significant difference from the level of the M-Lip or S-Lip group is indicated by d) $P < 0.001$.

effective for avoidance of the RES, have a fixed aqueous layer around their surface [30], we feared suppression of the liposome uptake into the cells by the PEG modification. However, the PEG-modification did not have these effects, but rather facilitated the initial rate of liposome uptake by the tumor cells (Fig. 18). By the PEG-modification of the liposomes, this initial rates of CPT-11 uptake in the M-PEG and S-PEG groups showed 1.5- and 1.3-fold, that in the M-lip and S-Lip group, respectively. *In vivo*, PEG-modified liposomes avoided the RES through the inhibited attraction of the opsonins, and showed circulation in the blood and tumor accumulation through passive targeting. The possibility was then shown that PEG-modified liposomes are easier for tumor cells to take up than plain liposomes. This was caused by the lipophilic property of PEG. A fixed aqueous layer, containing a PEG layer, is formed on the surface of PEG-modified liposomes [27]. On the

other hand, PEG-DMG, of which the alkyl chain is buried, as an anchor, in the membrane of the liposomes, was shown to be drawn out partly from the membrane of the liposomes by the plasma and the buffer [44]. It is expected that the drawn PEG-DMG shortly exerted PEG-moiety in a fixed aqueous layer of the liposome through the shield efficacy of the fixed aqueous layer. During this time, the PEG-DMG sticks its alkyl moiety out of the fixed aqueous layer on the surface of the liposomes. Therefore, for the attack in the cell membrane by this alkyl moiety, we considered that PEG-modified liposomes are superior in cell uptake. These considerations were supported by the fact that in the 15th min on incubation in medium containing tumor cells and a PEG-DMG solution, PEG-DMG was incorporated by 33.8% (compared to the additional amount) into the cell fraction (Fig. 19).

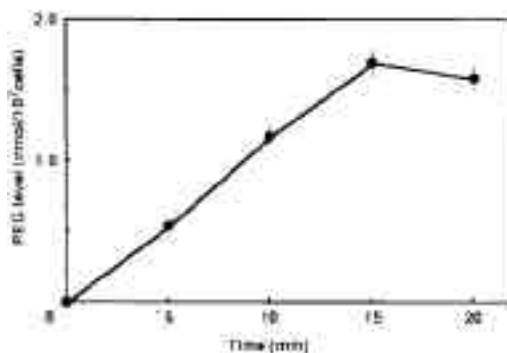


Fig. (19). Tumor cell uptake of PEG-DMG

In conclusion, PEG modification of the liposomes is expected to be effective on the cell uptake of liposomes *in vitro*, too.

2-7 Conversion of Liposomal CPT-11 to SN-38 *in vitro*

CPT-11 is constituted from a hydrophilic piperidinopiperidinocarboxyl moiety and lipophilic SN-38, and is converted to SN-38 by carboxylesterase in the body, particularly in the liver [45]. We have confirmed that the closed SN-38 concentration increases with the liposomalization of CPT-11. Namely, if CPT-11, which is released from disrupted liposomes, is converted to SN-38, the closed/total ratio of SN-38 in the liposome groups should be the same as that in the Sol group. However, increase in the closed SN-38 concentration and closed/total ratio of SN-38 in the tumors occurred on the liposomalization of CPT-11. Therefore, we expected the conversion of CPT-11 to SN-38 during the maintenance of intact liposomes.

We investigated the SN-38 generation from CPT-11 encapsulated in liposomes *in vitro*. After incubation of S-Lip with carboxylesterase, the generation of SN-38 occurred (Fig. 20). The closed/total ratios of CPT-11 or SN-38 was higher in the S-Lip than in the Sol group. In the S-Lip group, these ratios of CPT-11 and SN-38 were 100% and 89%, respectively (Fig. 20). Namely, *in vitro*, under the conditions of non-disruption of the

liposomes and no cells, SN-38 was generated after the incubation of S-Lip with carboxylesterase despite the absence of CPT-11 in the medium (extra-liposomal fraction). As adsorbed CPT-11 on the surface of the liposomes was removed by NaCl, we suspect that SN-38 was generated from CPT-11 in the liposomal membranes which were in contact with carboxylesterase in the medium. Furthermore, in the laser scanning confocal microscopy study, generation of SN-38 in the liposomal membranes was observed after the incubation of S-Lip with carboxylesterase [28]. The structural change of the surface of liposomes on the elimination of the piperidinopiperidinocarboxyl moiety is thought to result in a change in the zeta potentials of S-Lip on the incubation with carboxylesterase. Based on these results, as shown in (Fig. 21), the closed SN-38 moiety of liposomal CPT-11 is speculated to partly enter the double lipid membranes of the liposomes, and the piperidinopiperidinocarboxyl moiety projects out of the extra-liposomal fraction (medium). When these liposomes are distributed in some tissues, we suspect that CPT-11 in the membranes of the liposomes is converted by carboxylesterase and generates closed SN-38 during the maintenance of intact liposomes. Since the SN-38 generated under these conditions exists in the membranes of liposomes, it is not affected by the physiological pH in the body. This closed SN-38 behaves like the liposomes, i.e., avoids normal tissues and is distributed in the tumor. In mice and rat, carboxylesterase activity in the serum is high and SN-38/CPT-11 ratio is also high

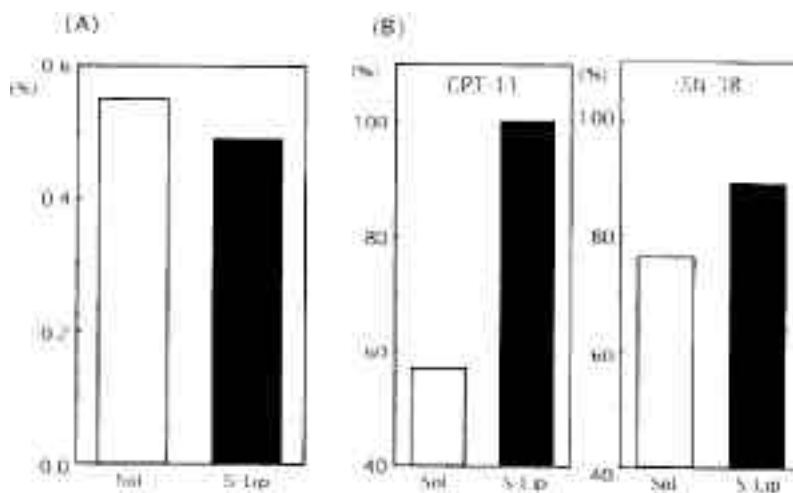


Fig. (20). The conversion of CPT-11 to SN-38 in liposomes

The reaction mixture contained Sol or S-Lip (CPT-11: 10 μ g/ml) and carboxylesterase (32.5 U/ml) in 0.1 M Tris-HCl buffer (pH 7.5). The generation of SN-38 was investigated in this reaction mixture at 37°C after 3h incubation. The CPT-11 and SN-38 closed-form and open-form concentrations were determined. (A) SN-38/CPT-11, (B) Closed/Total.

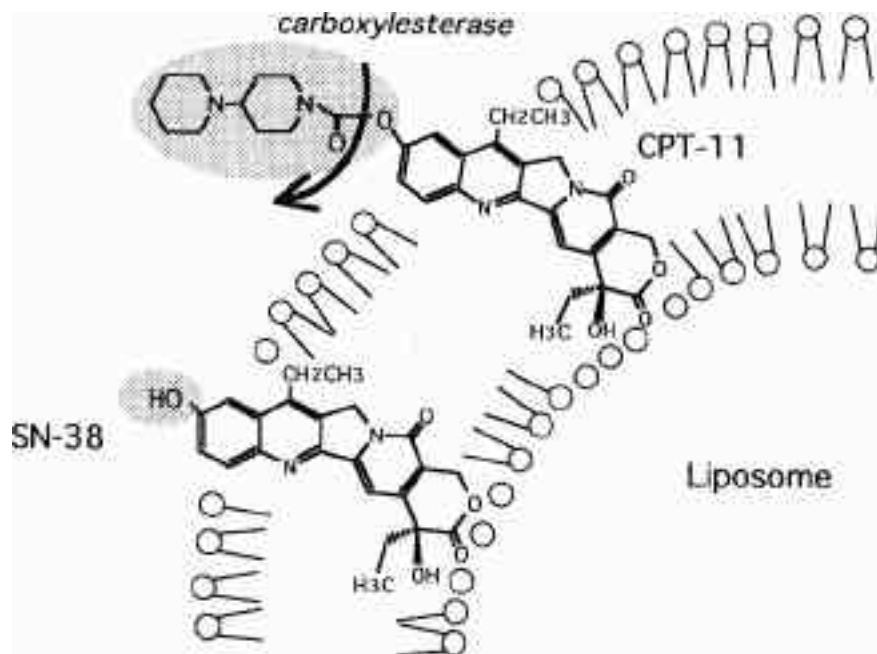


Fig. (21). A model of the conversion of CPT-11 to SN-38 in a liposomes

compared with human [45]. However, SN-38 generation by the carboxylesterase in the tumor of mice is similar with that of human [45]. Therefore, these results is considered to be important clinically.

In conclusion, the liposomalization of CPT-11 increased the closed/total ratios of CPT-11 and SN-38, is efficacious as to the conversion of closed SN-38. Regarding liposomal CPT-11, it is considered that part of CPT-11 as a prodrug is converted to SN-38 during the maintenance of intact liposomes. The usefulness of the liposomal prodrug and the generation of the active metabolite during the maintenance of intact liposomes in this study will be valuable for liposomal studies.

3. OTHER DRUGS

We have confirmed the usefulness of liposomal CPT-11 as a prodrug. We only have reported on the liposomal CPT-11 at aim of the drug targeting. There are some reports on the other prodrugs entrapped liposomes [46-57]. However, most papers have shown the studies on the prodrugs were derivatized to the liposomalization from the active compounds [46-52]. Namely, there is a little report on the targeting of liposomal prodrugs and the effective metabolism of liposomal prodrugs [53-57]. In these reports, the derivatives of FUdR [53, 54], araC [55], 5-FU [56] and cisplatin [57]

were liposomalized. On these prodrug liposomes, it has been suggested that the liposomal prodrug was converted to active metabolite in the targeting site [53, 54] and was degraded in the liposome, and the liposomalization decreased prodrug induced side toxicity.

In the prodrug liposomes, the metabolism of a prodrug to an active metabolite in the target site is considered to be very important. There is a little information available on the efficacy of liposomal prodrug, compared with that of the liposomalization of active compounds. However, it is hoped that these informations improve our chemotherapy.

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ABBREVIATIONS

CPT-11	=	Irinotecan hydrochloride
DDS	=	Drug delivery system
DMPC	=	Dimyristoylphosphatidylcholine

- DMPG = Dimyristoylphosphatidylglycerol
- DOX = Doxorubicin
- DSPC = Distearoylphosphatidylcholine
- Lip5 = Final concentration (CPT-11 0.5 mg/26 μ mol lipid/ml) of M-Lip
- Lip10 = Final concentration (CPT-11 1.0 mg/26 μ mol lipid/ml) of M-Lip
- Lip20 = Final concentration (CPT-11 2.0 mg/26 μ mol lipid/ml) of M-Lip
- M-Lip = Liposomes composed of DMPC:cholesterol:DMPG:CPT-11
- M-PEG = PEG-coated M-Lip
- PEG = Polyethyleneglycol
- PEG-DMG = 1-(Monomethoxypolyethylene-glycol)-2,3-dimyristoylglycerol
- RES = Reticuloendothelial system
- S-Lip = Liposomes composed of DSPC:cholesterol:DMPG:CPT-11
- Sol = CPT-11 solution
- S-PEG = PEG-coated S-Lip
- Tc = Phase transition temperature
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