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Comparative studies of irinotecan-loaded polyethylene glycol-modified liposomes prepared using different PEG-modification methods

Keisuke Yoshino [a,*], Koji Nakamura [a], Yoko Terajima [a], Akinobu Kurita [b], Takeshi Matsuzaki [b], Keiko Yamashita [a], Masashi Isozaki [a], Hiroaki Kasukawa [a]

[a] Terumo Corporation, R&D Center, 1500 Inokuchi, Nakai-machi, Ashigarakami-gun, Kanagawa 259-0151, Japan
[b] Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi-shi, Tokyo 186-8650, Japan

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ABSTRACT

Recently, a polyethylene glycol (PEG)-modification method for liposomes prepared using pH-gradient method has been proposed. The differences in the pharmacokinetics and the impact on the antitumor effect were examined; however the impact of PEG-lipid molar weight has not been investigated yet. The main purpose of this study is to evaluate the impact of PEG-lipid molar weight against the differences in the pharmacokinetics, the drug-release profile, and the antitumor effect between the proposed PEG-modification method, called the post-modification method, and the conventional PEG-modification method, called the pre-modification method. Various comparative studies were performed using irinotecan as a general model drug. The results showed that PEG-lipid degradation could be markedly inhibited in the post-modification method. Furthermore, prolonged circulation time was observed in the post-modification method. The sustained drug-release was observed in the post-modification method by the results of the drug-releasing test in plasma. Moreover, a higher antitumor effect was observed in the post-modification method. It was also confirmed that the same behaviors were observed in all comparative studies even though the PEG molecular weight was lower.

In conclusion, the post-modification method has the potential to be a valuable PEG-modification method that can achieve higher preservation stability of PEG-lipid, prolonged circulation time, and higher antitumor effect with only half the amount of PEG-lipid as compared to the pre-modification method. Furthermore, it was demonstrated that PEG_{5000}-lipid would be more desirable than PEG_{2000}-lipid since it requires much smaller amount of PEG-lipid to demonstrate the same performances.

1. Introduction

Currently, drug delivery system studies focus on achieving selective delivery and distribution of drugs to the target disease sites to enhance safety and effectiveness. Therefore, the feasibility of various types of particulate systems such as liposomes, emulsions, and polymeric nanoparticles has been evaluated as effective drug delivery systems [1–3].

To selectively deliver an encapsulated drug to the target site, the control of its pharmacokinetics is essential. The clearance of liposomes has been considered to occur through their capture by the mononuclear phagocyte system (MPS), which takes up liposomes circulating in the blood stream and removes them. It is especially important to avoid capture by the phagocytic cells in the liver and spleen to prolong circulation time in the blood. Various liposome characteristics affect circulation time in the blood, such as lipid composition, size, and Zeta-potential [4–6]. Among these, liposome membrane surface modification using monosialoganglioside GM1 or polyethylene glycol (PEG)-conjugated lipid (PEG-lipid) has been shown to greatly improve the pharmacokinetics [7,8]. PEG is a highly hydrophilic polymer with very low toxicity; hence, PEG and its derivatives have been widely used to improve the stability and pharmacokinetics of drug carriers and parent drug [9]. In liposomal drug delivery, PEG-lipid has been widely used for liposome surface modification, and this technique, called PEGylation, has been already employed in the preparation of liposomal drug delivery systems, which are known as PEGylated liposomes [10–23]. Specifically, doxorubicin-loaded PEGylated liposomes (Doxil®) have a strong pharmacological effect and low toxicity. Therefore, these have been widely used in clinical applications and approved in more than 80 countries for the treatment of cancer [24,25]. To manufacture this product, a unique technology known as the pH-gradient method has been utilized to achieve high drug-loading efficiency [26–29].

To achieve the prolonged circulation time using PEGylated liposomes, it is important to maintain the physiological and physicochemical stabilities of the PEG-lipid. To our knowledge, however, excess PEG-lipid degradation has been observed; especially in the PEGylated liposomes prepared using the pH-gradient method. Owing to the pH-gradient method, the pH of the internal aqueous phase

* Corresponding author at: 1500 Inokuchi, Nakai-machi, Ashigarakami-gun, Kanagawa 259-0151, Japan. Tel.: +81 465 81 4187; fax: +81 465 81 4114.
E-mail address: Keisuke_Yoshino@terumo.co.jp (K. Yoshino).

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decreases, thus leading to the rapid hydrolysis of the phospholipids. In general, phospholipids are a component of PEG-lipid, and this group is easily hydrolyzed in a bell-shaped manner, with the greatest stability at around neutral pH [30]. Our recent research demonstrated that the post-modification method could markedly inhibit PEG-lipid degradation and prolong the circulation time [31]. Recently, the differences in the pharmacokinetics and the impact on the antitumor effect were examined using vinorelbine, which is vinca alkaloid group, as a model drug [32]. However, the impact of PEG-lipid molar weight has not been investigated yet.

In this study, we selected irinotecan (CPT-11) as a general model drug instead of vinorelbine since it shows extremely fast drug-release profiles due to its high hydrophobicity [33]. In addition, the liposomal CPT-11 showed a great feasibility as the drug delivery system with less toxicity and high pharmacological effect [34]. We demonstrated various comparative studies with CPT-11 liposomes prepared using the proposed PEG-modification method, called the post-modification method [35], and the conventional PEG-modification method, called the pre-modification method. The main purpose of this study is to evaluate the impact of PEG-lipid molar weight against the differences in the pharmacokinetics, the drug-release profile, and the antitumor effect between the pre- and post-modification methods using CPT-11 liposomes.

2. Material and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) was purchased from Lipoid GmbH (Ludwigshafen, Germany), cholesterol (Chol) was obtained from Dishman (Veenendaal, Netherlands). Methoxypolyethyleneglycol (Mw: 2000)-distearylphosphatidylethanolamine (PEG_{2000}-lipid). Methoxypolyethyleneglycol (Mw: 5000)-distearylphosphatidylethaxnolamine (PEG_{5000}-lipid) was purchased from NOF (Tokyo, Japan). Irinotencan hydrochloride (CPT-11) was obtained from Yakult Honsha. (Tokyo, Japan). All other chemicals used were of analytical grade.

Rats (Crl:CD (SD), male, 7 weeks old) for pharmacokinetics experiments and BALB/c nude mice (CAnNCg-Foxnl^{nu}/CrlCrlj, male, 5 weeks old) for antitumor effect experiments were purchased as test animals from Charles River Japan, Inc. (Yokohama, Japan). Human colon cancer cells (HT-29) were purchased from American Type Culture Collenction (ATCC).

2.2. Preparation of CPT-11 liposomes by the pre- and post-modification methods

Our recent study demonstrated that PEG_{5000}-lipid could be distributed only on the outer surface of the liposomes in the post-modification method, whereas it was equally distributed both on the inner and outer surfaces in the pre-modification method [31,36]. CPT-11 liposomes were prepared using the above 2 different PEG-modification techniques.

2.2.1. Preparation of CPT-11 liposomes by the pre-modification method (Pre)

PEGylated liposomes composed of HSPC, Chol (molar ratio, 54:46) and 1.5 mol% of PEG_{5000}-lipid were prepared as follows. HSPC (7.06 g), Chol (2.94 g) and PEG_{5000}-lipid (1.51 g) were dissolved in dehydrated ethanol (10 ml) at 72 °C and hydrated in 250 mM aqueous solution of ammonium sulfate (90 ml) for 10 min at 72 °C to afford crude liposomes. The obtained crude liposomes were firstly filtered through 200 nm of double filters three times using Extruder T100. After this, the particle size was further regulated through 100 nm of filters until the particle size came to approximately 100 nm [PEGylated liposomes (Pre)].

The outer aqueous solution of PEGylated liposomes was exchanged with 10% sucrose and 10 mM histidine (pH 6.5) using cross flow to generate pH-gradient between outer and inner aqueous solution (pH-gradient liposomes). An aqueous CPT-11 solution was added to pH-gradient liposomes at CPT-11/total lipid weight ratio of 0.18 and incubated at 50 °C for 20 min. Unloaded CPT-11 was removed using cross flow with 10% sucrose and 10 mM histidine solution (pH 6.5) as an eluent. Finally, sterile filtration using a 0.2-μm membrane filter (Minisart Plus, Sartorius, Goettingen, Germany) was carried out for PEGylated liposomes encapsulated with CPT-11 [CPT-11 liposomes (Pre)]. CPT-11 liposomes (Pre) containing 4.0 mol% of PEG_{2000}-lipid was prepared using the same method.

2.2.2. Preparation of CPT-11 liposomes by the post-modification method (Post)

Bare liposomes composed of HSPC and Chol (molar ratio, 54:46) were prepared as follows. HSPC (21.17 g) and Chol (8.83 g) were dissolved in dehydrated ethanol (30 ml) at 72 °C and hydrated in 250 mM aqueous solution of ammonium sulfate (270 ml) for 10 min at 72 °C to afford crude liposomes. The obtained crude liposomes were firstly filtered through 200 nm of double filters three times using Extruder T100. After this, the particle size was further regulated through 100 nm of filters until the particle size came to approximately 100 nm. Then, a 60 ml of PEG_{5000}-lipid aqueous solution (37.67 mg/ml) was added to the liposome suspension and heated at 65 °C for 30 min to afford PEGylated liposomes with 0.75 mol% of PEG_{5000}-lipid [PEGylated liposomes (Post)].

The same process described in Section 2.2.1 was then conducted. Finally, sterile filtration using a 0.2-μm membrane filter (Minisart Plus, Sartorius, Goettingen, Germany) was carried out for PEGylated liposomes encapsulated with CPT-11 [CPT-11 liposomes (Post)]. CPT-11 liposomes (Post) containing 2.0 mol% of PEG_{2000}-lipid was prepared using the same method.

2.3. Characterization of CPT-11 liposomes

2.3.1. Determination of the lipid component

HSPC, Chol, and PEG-lipid were analyzed by HPLC [31]. Briefly, Each CPT-11 liposomes (Pre and Post) (2 ml) was dissolved in a mixture of water, chloroform, 2-propanol, and internal standard solution. Standard solutions of HSPC, Chol, and PEG-lipid were prepared separately. The lipid component of the prepared solutions (30 μl) was determined using the following HPLC conditions: column, Inertsil Ph column (4.6×250 mm, 5 μm; GL Science, Japan); mobile phase, acetate buffer/methanol/ethanol; detector; refractive index detector (Shimadzu, Japan); and flow rate, approximately 1 ml/min.

The PEG-modification ratio was calculated according to the following formula:

$$\text{PEG-modification ratio (mol\%)} = \text{measured PEG-lipid (mol)} / [\text{measured HSPC (mol)} + \text{measuredChol (mol)}] \times 100$$

2.3.2. Determination of CPT-11 concentration

Each CPT-11 liposomes (Pre and Post) (0.4 ml) were dissolved in a mixture (9.6 ml) of internal standard solution and methanol. 2 ml of above sample was taken and further dilution by mixture (18 ml) of phosphate solution and methanol. A small aliquot (10 μl) of the test solution was applied to an HPLC system (Shimadzu, Kyoto, Japan) equipped with an Inertsil ODS-2 column (4.6×250 mm, 5 μm, GL Science, Tokyo, Japan) and UV detector at 254 nm. The mobile phase was a mixed solution of acetonitrile, formic acid, methanol, and water.

2.3.3. Other methods

Particle size and Zeta-potential were determined using Zetasizer 3000HS (Malvern Instruments, UK). To measure particle size (nm), 20 μl of liposomes was diluted with 3 ml of physiological saline

solution, and 20 μl of liposomes and PBS were diluted with 2.5 ml of purified water to measure Zeta-potential.

2.4. Various comparative studies of CPT-11 liposomes

2.4.1. Accelerated preservation stability experiments

Each CPT-11 liposomes (Pre and Post) was stored at 25 °C and 60% RH to measure PEG-lipid (PEG_{5000}-lipid or PEG_{2000}-lipid) and HSPC residual ratio after predetermined periods (4, 8 and 12 weeks). PEG-lipid (PEG_{5000}-lipid or PEG_{2000}-lipid) and HSPC amounts were measured using HPLC system and each residual ratio were calculated.

2.4.2. Pharmacokinetics experiments

Each CPT-11 liposomes (Pre and Post) was intravenously administered via tail vein of a rat [Crl: CD (SD), male, 7 weeks old] as a dose of 30 mg/kg equivalent of CPT-11 (n = 6 or 5). At 1, 2, 4, 8, 24, 48 and 72 h after administration, 0.3 ml of blood plasma was obtained by centrifuge (15,000 rpm, 3 min, 0 °C). 0.1 ml of obtained supernatant was diluted with 0.4 ml of 0.15 H_3PO_4 solutions. After diluting 0.1 ml of the diluted blood plasma with 0.4 ml of methanol, the resultant solution was treated in a centrifuge (15,000 rpm, 3 min, 0 °C). 0.1 ml of obtained supernatant was diluted with 0.4 ml of an internal standard solution (camptothecin 1 μg/ml in 0.15 M H_3PO_4), to thereby obtain a sample for total CPT-11 concentration measurement. Analysis of pharmacokinetics parameters was made using WinNonlin (Pharsight Inc.).

2.4.3. In vitro release experiments in blood plasma of rats

A laparotomy was performed on a rat (Crl: CD (SD), male, 7 weeks old) under nembutal anesthesia for collection of whole blood from an abdominal aorta of the rat. After adding 0.2 ml of heparin to the collected blood, the resultant solution was treated in a centrifuge (3000 rpm, 15 min, 4 °C), to thereby obtain supernatant (plasma). The obtained supernatant was stored in frozen state under −20 °C. After thawing the frozen blood plasma of the rat on the ice and preheating 0.5 ml of the thawed blood plasma for 5 min at 37 °C, 5 μl of both CPT-11 liposomes (Pre and Post) was added to begin the release experiments. At 0, 1, 2, and 4 h after the incubation, 0.1 ml of the sample was taken and diluted with 0.4 ml of 0.15 H_3PO_4 solutions. After diluting 0.1 ml of the diluted blood plasma with 0.4 ml of methanol, the resultant solution was treated in a centrifuge (15,000 rpm, 3 min, 0 °C). 0.1 ml of obtained supernatant was diluted with 0.4 ml of an internal standard solution (camptothecin 1 μg/ml in 0.15 M H_3PO_4), to obtain a sample for total irinotecan concentration measurement. Meanwhile, subjecting 0.2 ml of the diluted blood plasma to centrifugal separation (100,000 *g*, for 30 min, 10 °C), and the obtained 0.05 ml in the upper layer was diluted with 0.2 ml of methanol, the resultant solution was treated in a centrifuge (15,000 rpm, 3 min, 0 °C). 0.1 ml of obtained supernatant was diluted with 0.4 ml of the internal standard solution, to obtain a sample for released irinotecan concentration measurement. Then, each concentration of total irinotecan and released irinotecan were measured using HPLC system and fluorescence detector. The encapsulated irinotecan concentration was calculated by subtracting the released irinotecan concentration from the total irinotecan concentration. The number of n used in these release experiments was 3.

2.4.4. Antitumor effects in vivo

Human colon cancer cells (HT-29) were subcultured under the condition of 5% CO_2 and 37 °C by using Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. 2×10^6 cells/0.1 ml/mouse of HT-29 cells were implanted subcutaneously in left inguinal region of a nude mouse by using a glass syringe and injection needle. The group was separated so that a day in which a presumptive tumor volume calculated by $1/2ab^2$ (a: represents a longitudinal diameter of a tumor and b: represents a short axis diameter) achieved about 100 mm^3 was set as day 0. At day 1, each CPT-11 liposomes (Pre and Post) (12.5 mg/kg/dose per) was intravenously administered. A physiological saline solution which is a solvent was administered for a control group. After extracting tumors and measuring a weight at day 21, an inhibition rate of tumor growth (I.R.) (%) was calculated by the following formula to determine a main effect. A presumptive tumor volume was secondarily measured for the total comparison. Each group was consisted of 10 mice.

$$\text{I.R.}(\%) = (1 - \text{average tumor weight in a treatment group} / \text{average tumor weight in a control group}) \times 100$$

2.4.5. Ethics in animal experiments

All the in vivo experimental protocols were approved by the animal care committee of the Yakult Central Institute for Microbiological Research.

2.4.6. Statistical analysis

For group comparisons, one-way analysis of variance (ANOVA) with duplication was applied. Significant differences in the mean values were evaluated by Student's unpaired *t*-test. A p value of less than 0.05 was considered significant.

3. Results and discussion

3.1. Chromatographic analysis of CPT-11 liposomes

Each CPT-11 liposomes prepared using the pre-and post-modification methods was analyzed by various equipments and the characteristics of resultant liposomes, PEG-lipid content (PEG_{5000}-lipid or PEG_{2000}-lipid), diameter, Zeta-potential, and CPT-11 content are listed in Table 1. As seen in Table 1, the particle size with the post-modification method was slightly larger than that with the pre-modification method. This behavior is reasonable because in the pre-modification method, the sizing process is initiated after PEGylation; therefore, the final particle size is adjusted to the intended size, irrespective of the PEG-lipid content. On the other hand, in the post-modification method, the sizing process is initiated before PEGylation; therefore, the final particle size means sum total of bare liposomes and PEG layer thickness.

With respect to the Zeta-potential of the PEGylated liposomes, no clear differences were observed between the pre- and post-modification methods in not only the PEG_{5000}-lipid modified liposomes (−12 mV) but also the PEG_{2000}-lipid modified liposomes (−27 mV). This negative charge density comes from the PEG-lipid itself, and is thought to be balanced by the charge-shielding effect of PEG-chains. In principle, PEG-chains of lower molecule weight show smaller charge-shielding effect. This means that the apparent Zeta-potential would be lower in the case of lower molecule weight PEG-lipid. Hence, the difference in Zeta-potential between PEG_{5000}-lipid and PEG_{2000}-lipid was reasonable. The liposomes with 0.75 mol% of PEG_{5000}-lipid prepared using the post-modification method was almost equivalent to the liposomes with 1.5 mol% of PEG_{5000}-lipid prepared using the pre-modification method in terms of the charge-shielding effect [31,36]. Considering that the distribution of PEG-lipid on the liposome contributes directly

Table 1
Characterization of prepared CPT-11 liposomes.

PEG-modification method	Mw	HSPC: Chol:PEG (molar ratio)	PEG-lipid content (mol%)	Diameter (nm)	Zeta-potential (mV)	CPT-11 content (mg/ml)
Pre	5000	54:46:1.5	1.48	102	−12.5	5.03
Post	5000	54:46:0.75	0.75	114	−12.7	5.08
Pre	2000	54:46:4.0	4.0	97	−27.8	5.00
Post	2000	54:46:2.0	2.0	107	−26.5	4.92

Pre: pre-modification method, Post: post-modification method.

to the charge-shielding effect, the acquired Zeta-potential results were well supports those work since no clear differences were observed between the pre- and the post-modification methods.

3.2. Preservation stability of HSPC and PEG-lipid in liposomes

In order to examine the differences in physicochemical stability arising from the use of different PEG-modification methods, the preservation stability study was performed at 25 °C and 60% RH for 12 weeks with each CPT-11 liposomes (Pre and Post) modified with PEG_{5000}-lipid (Table 1). Fig. 1 (A) shows the results of the HSPC residual ratio and (B) the PEG_{5000}-lipid residual ratio. As seen in Fig. 1 (A), the degradation of HSPC gradually proceeded with time, and the HSPC residual ratio finally reached to around 88% in both liposomes after 12 weeks. Yet, no difference was observed between both. As seen in Fig. 1 (B), about 25% of PEG_{5000}-lipid degradation was observed after 12 weeks in the pre-modification method, whereas less degradation was observed in the post-modification method.

The degradation (hydrolysis) rate of PEG_{5000}-lipid on the inside of liposomal membrane is expected to be higher than that of PEG_{5000}-lipid on that outside of liposomes, because the pH of internal aqueous phase is kept considerably low when conventional pH-gradient method is applied. HSPC is considered to distribute equally on the inside and outside of the liposomal bilayer in both methods. Therefore, it is reasonable that no differences were observed in the HSPC degradation behavior. On the other hand, clear differences in the preservation stability of PEG_{5000}-lipid were observed between both pre- and post-modification methods, which strongly suggest that the distribution of PEG_{5000}-lipid is completely different between both methods. That is, as mentioned above, in the case of the pre-modification method, PEG_{5000}-lipid would be distributed equally on the inner and outer surfaces of the liposomal membrane; therefore, the internal PEG_{5000}-lipid is subjected to hydrolysis due to the low pH of the internal aqueous phase. However, in the post-modification method, all PEG_{5000}-lipid would exclusively be located on the outer surface of the liposomes, thus reducing hydrolysis. The same behavior was observed in our recent research with other drugs as well [30]. Since this finding was quite important, further study was conducted using CPT-11 liposomes (Pre and Post) modified with PEG_{2000}-lipid (Table 1). Fig. 1 (C) shows the results for the HSPC residual ratio and (D) the PEG_{2000}-lipid residual ratio. As a result, it was confirmed that both HSPC and PEG_{2000}-lipid degradations occurred in the same manner, even though the PEG molecular weight was lower than Mw: 5000. These findings suggest that the PEGylated liposomes prepared using the post-modification method are superior to those obtained using the pre-modification method from the viewpoint of preservation stability.

3.3. Pharmacokinetics experiments of CPT-11 liposomes (Pre and Post)

The pharmacokinetics studies were performed with each CPT-11 liposomes (Pre and Post) modified with PEG_{5000}-lipid [Fig. 2 (A)] and PEG_{2000}-lipid [Fig. 2 (B)], respectively. The total CPT-11 concentration profiles in plasma after intravenous administration (30 mg/kg) are shown. As seen in both figures, CPT-11 liposomes (Post) provided higher total CPT-11 concentrations in plasma than CPT-11 liposomes (Pre), not only (A) for PEG_{5000}-lipid but also (B) for PEG_{2000}-lipid. On the other hand, no clear difference was observed between PEG_{5000}-lipid and PEG_{2000}-lipid. This means that both would have the same potential pharmacokinetics when the same modification method is used.

The pharmacokinetics parameters in a treatment group with PEG_{5000}-lipid of dosage shown in the above Fig. 2 (A) were analyzed. The results are shown in Table 2. There were significant differences in

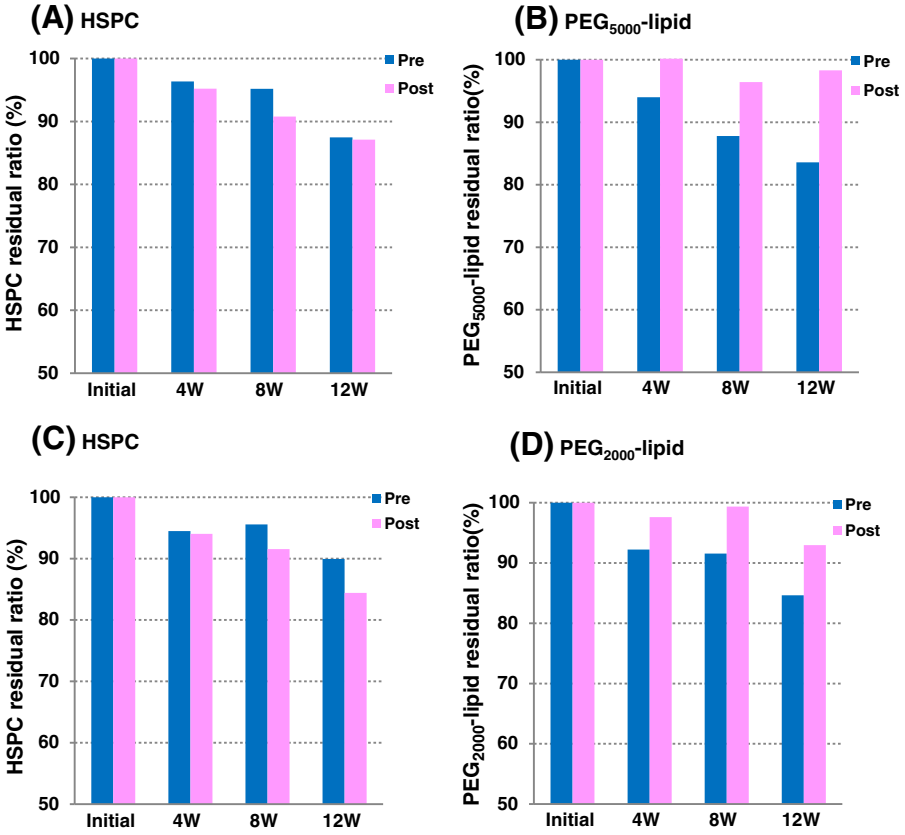

Fig. 1. Percent of HSPC and PEG-lipid residual ratio in PEGylated liposomes prepared using the pre- or post-modification method after storage at 25 °C for 0, 4, 8, and 12 weeks. PEG_{5000}-lipid modified liposomes (A): remaining HSPC and (B): remaining PEG_{5000}-lipid. PEG_{2000}-lipid modified liposomes (C): remaining HSPC and (D): remaining PEG_{2000}-lipid.

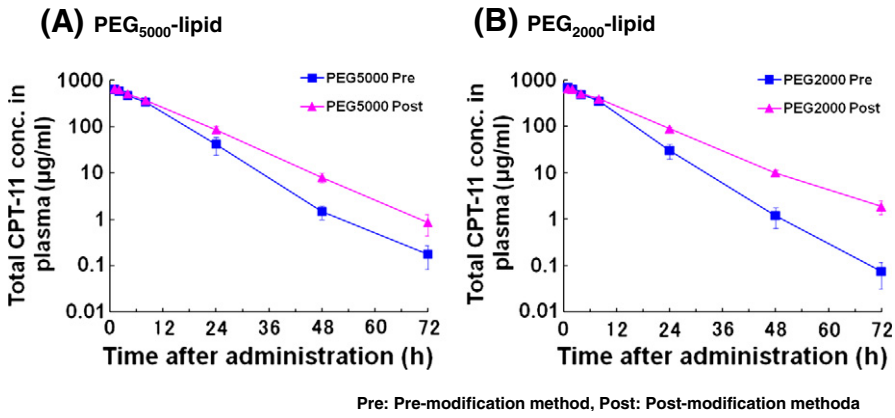

Fig. 2. The drug-concentration profiles in plasma after intravenous administration of CPT-11 liposomes to male rats (30 mg/kg). CPT-11 liposomes modified with (A): PEG_{5000}-lipid and (B): PEG_{2000}-lipid. CPT-11 liposomes prepared using the pre-modification method (□) and PEGylated liposomes prepared using the post-modification method (△). Data are presented as mean ± standard deviation (n = 3).

the AUC and CLtot between liposomes prepared using the pre- and post-modification methods ($p<0.01$), and more significant differences were observed in the MRT and $T_{1/2}$ ($p<0.001$). As a result, it was confirmed that CPT-11 liposomes (Post) significantly allowed CPT-11 to disappear more slowly (high values of MRT and $T_{1/2}$) and to be maintained at a high concentration for a long time. In our recent research, the same behavior was observed with other drugs [30]. Hence, it is considered that the present result is not related to the drug itself but to the PEG-modification method. These findings suggest that the PEGylated liposomes prepared using the post-modification method are superior to those modified using the pre-modification method from the viewpoint of pharmacokinetics.

3.4. In vitro CPT-11 release experiments in blood plasma of rats

To investigate further details, each CPT-11 liposomes (Pre and Post) was incubated with rat plasma at 37 °C to evaluate the difference in the CPT-11 release profiles. The results are shown for each CPT-11 liposomes (Pre and Post) modified with PEG_{5000}-lipid [Fig. 3 (A)] and PEG_{2000}-lipid [Fig. 3 (B)], respectively. As seen in both the figures, there were significant differences in the released CPT-11 ratio between liposomes prepared using the pre- and post-modification methods at 1 hour ($p<0.01$) and 2 h ($p<0.001$), respectively. This result indicates that CPT-11 liposomes (Post) release the drug in rat plasma more slowly as compared to CPT-11 liposomes (Pre). That is, it was ascertained that CPT-11 liposomes (Post) could hold encapsulated CPT-11 for a longer time, compared to CPT-11 liposomes (Pre). It was recently reported that the drug-release rate from vesicles increased with increasing PEG contents [32,37]. In the pre-modification method, twice the amount of PEG-lipid is required to achieve the same charge-shielding effect; therefore, membrane permeability would be higher in the pre-modification method than the post-modification method. From this, it is considered that the membrane permeability of CPT-11 liposomes (Pre) would be higher than that of CPT-11 liposomes (Post) and that it would be a reasonable. From the result of Fig. 1, the faster PEG-lipid degradation rate might cause faster drug release in vitro and in vivo since hydrolysis of PEG-lipid like lyso PEG-lipid and stearic acid might disturb the lipid membrane. Although various unknown factors may still be involved in this phenomenon, the present in vitro CPT-11 releasing study (Fig. 4) is well consistent with the results of the pharmacokinetics study (Fig. 3), in which CPT-11 liposomes (Post) showed prolonged circulation time as compared to CPT-11 liposomes (Pre). Therefore, it is considered that the hypothesis shown above would be reasonable.

Table 2
Pharmacokinetic parameters following intravenous administration of 30 mg/kg of both CPT-11 liposomes to male rats. Data are presented as mean ± standard deviation (n = 6 for the pre-modification method and n = 5 for the post-modification method).

Parameter	Pre (n = 6)	Post (n = 5)
AUC_{0-72} (mg·h/ml)	7607 ± 588	9102 ± 813**
$AUC_{0\cdot inf}$ (mg·h/ml)	7609 ± 586	9111 ± 817**
$MRT_{0\cdot 72}$ (h)	7.53 ± 0.83	9.82 ± 0.54***
$MRT_{0\cdot inf}$ (h)	7.54 ± 0.83	9.90 ± 0.56***
$T_{1/2}$ (h)	5.74 ± 0.32	7.19 ± 0.53***
CL_{tot} (L/h/kg)	0.00396 ± 0.00033	0.00331 ± 0.00033**

Pre: pre-modification method, Post: post-modification method.
**: $p<0.01$, ***: $p<0.001$.

3.5. In vivo antitumor effects with each CPT-11 liposomes (Pre and Post)

Through the various studies, it was confirmed that the post-modification method showed prolonged circulation time and slower drug-release profile. However, there is no information regarding the antitumor effects. Therefore, the in vivo antitumor effects with each CPT-11 liposomes (Pre and Post) were compared using nude mice subcutaneously implanted with human colon cancer cells (HT-29). The results are shown for each CPT-11 liposomes (Pre and Post) modified with PEG_{5000}-lipid [Fig. 4 (A)] and PEG_{2000}-lipid [Fig. 4 (B)], respectively. As seen in both figures, the tumor volume in the control group gradually increased with time after tumor inoculation. On the contrary, in all the administered groups, the increase in tumor volume was suppressed as compared to that in the control group. To compare the differences between the pre- and the post-modification methods, a suppressive effect on tumor growth was finally determined at 21 days, and the results are shown in Table 3. The tumor volume in the control groups was an average of 1258 mm^3 and 1286 mm^3, respectively. As shown in Table 3, on comparing both CPT-11 liposomes (Pre and Post) with the control groups, a significant low tumor weight was found ($p<0.001$). Comparison of CPT-11 liposomes (Pre) and CPT-11 liposomes (Post) modified with PEG_{5000}-lipid showed that although the inhibition rate was higher for CPT-11 liposomes (Post), the difference was not statistical significant. On the other hand, comparison of CPT-11 liposomes (Pre) and CPT-11 liposomes (Post) modified with PEG_{2000}-lipid showed that the inhibition rate was significantly higher in CPT-11 liposomes (Post) ($p<0.01$).

In general, the abnormal and leaky vasculature of tumor causes enhanced permeability of liposomal drugs in tumors. Moreover, tumor tissues usually lack effective lymphatic drainage. Therefore, PEGylated

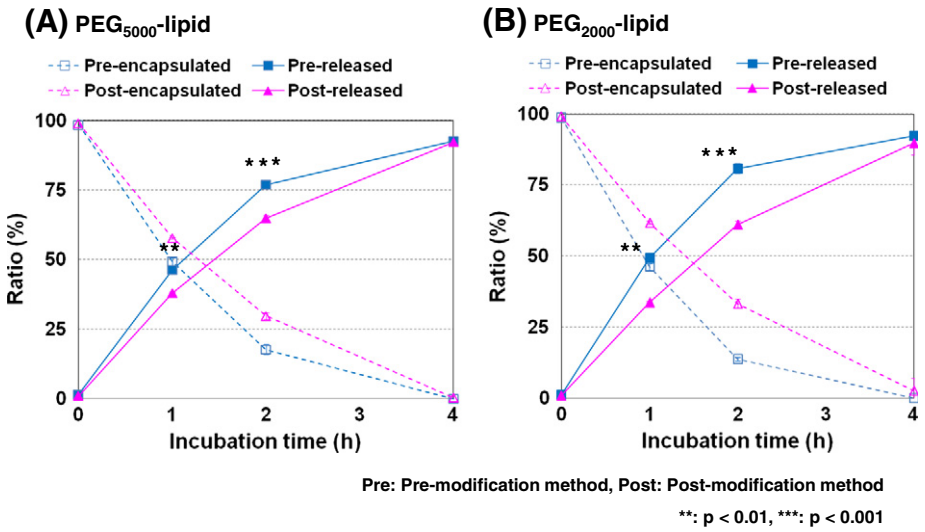

Fig. 3. CPT-11 release profiles in blood plasma from CPT-11 liposomes modified with (A): PEG_{5000}-lipid and (B): PEG_{2000}-lipid. CPT-11 liposomes prepared using the pre-modification method (□) and PEGylated liposomes prepared using the post-modification method (△) after incubated at 37 °C for 0, 1, 2, and 4 h. Data are presented as mean ± standard deviation (n = 3).

liposomes can be drained through the leaky blood vessels and be retained, resulting in an increased accumulation of liposomal anticancer agents in tumors. This phenomenon is called the enhanced permeation and retention (EPR) effect [38,39]. As described earlier, CPT-11 liposomes (Post) showed prolonged circulation time (Fig. 2) and slower CPT-11 release profile (Fig. 3); therefore, it is considered that a large amount of CPT-11 could be delivered to the tumor cells through the leaky vasculature owing to the EPR effects. These findings suggest that the PEGylated liposomes prepared using the post-modification method are superior to those obtained using the pre-modification method from the viewpoint of the antitumor effect.

4. Conclusions

Through a series of comparative studies, it was shown that the post-modification method could markedly inhibit PEG-lipid degradation. Furthermore, a prolonged circulation time was observed with the post-modification method. In addition, the CPT-11 release profile in blood plasma was restrained in the post-modification method as compared to the pre-modification method. Moreover, the post-modification method showed a higher inhibition rate for tumor growth as compared to the pre-modification method. Using the post-modification method, only half the amount of PEG-lipid is needed to have the same charge-shielding effect as the pre-modification method. Besides, the CPT-11 release from liposomes was slower since the membrane permeability was decreased. As a result, it achieved the prolonged circulation time and the higher antitumor effect. In addition, the same behaviors were observed in all studies even though the PEG molecular weight was lower.

In conclusion, CPT-11 liposomes prepared using the post-modification method can achieve higher preservation stability of PEG-lipid, prolonged circulation time, sustained drug-release and higher antitumor effect with only half the amount of PEG-lipid as compared to that needed in the pre-modification method. Furthermore, it was also demonstrated that

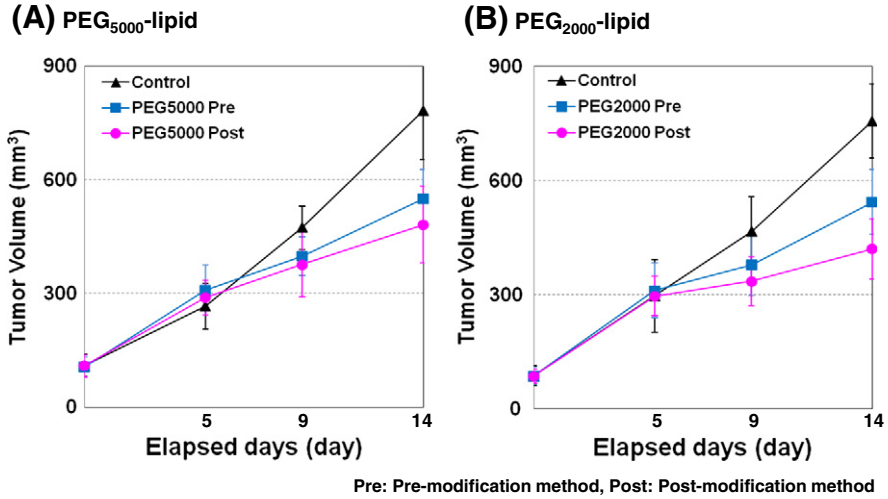

Fig. 4. Suppressive effect on tumor growth after administration of CPT-11 liposomes (12.5 mg/kg/dose) prepared using the pre- and post-modification methods on days 0, 5, 9, and 14. Data are presented as mean ± standard deviation (n = 10).

Table 3
Inhibition rate for tumor growth at day 21. A 12.5 mg/kg/dose of CPT-11 liposome prepared using pre- or post-modification method was intravenously administered. Data are presented as mean ± standard deviation (n = 10).

Group	Tumor weight (g) (Mean ± S.D.)	Vs Cont.	Vs Pre.	Inhibition rate (%)
Control	0.78 ± 0.10	–	–	–
PEG5000 Pre	0.56 ± 0.12	***	–	27.8
PEG5000 Post	0.52 ± 0.09	***	N.S.	33.2
Control	0.72 ± 0.12	–	–	–
PEG2000 Pre	0.51 ± 0.05	***	–	29.0
PEG2000 Post	0.43 ± 0.06	***	**	40.7

Pre: pre-modification method, Post: post-modification method.
N.S.: not significant, **: $p<0.01$, ***: $p<0.001$.

PEG_{5000}-lipid would be more desirable than PEG_{2000}-lipid since it requires much smaller amount of PEG-lipid to demonstrate the same performances.

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