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

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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

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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