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Prolongation of time interval between doses could eliminate accelerated blood clearance phenomenon induced by pegylated liposomal topotecan

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ABSTRACT

Repeated injection of pegylated liposomes could elicit the disappearance of long-circulating characteristic, referred to as "accelerated blood clearance phenomenon." ABC phenomenon typically occurs when entrapped drugs are not cytotoxic, but recently it was reported that multiple doses of pegylated liposomal topotecan, a cytotoxic drug, could also induce the phenomenon in rats. To reveal whether the phenomenon could be induced in dogs and the effect of time interval between doses on the magnitude of ABC, pLT was repeatedly injected into beagle dogs with a time interval of 7, 21 and 28 days. The anti-PEG Ig M levels were detected using ELISA. It was found that ABC phenomenon could be induced in dogs by pLT. Inter-individual difference in anti-PEG antibody production could be observed, and antibody levels were directly correlated with the magnitude of ABC. Furthermore, time interval between doses had marked effect on the magnitude of ABC phenomenon. When the time interval was prolonged from 1 week to 4 weeks, ABC phenomenon could be eliminated. By comparing the pharmacokinetic profiles of lipid vesicles and entrapped topotecan, it was found that "empty pegylated vesicles" be formed in circulation, which might be responsible for the occurrence of ABC phenomenon.

1. Introduction

Liposomes could alter the pharmacokinetics and biodistribution of entrapped drugs, thus resulting in improved safety and efficacy (Fenske and Cullis, 2008). For years liposomes have been extensively used as the carrier of antineoplastic drugs since rationally designed liposomes could effectively accumulate into malignant zones due to "enhanced permeability and retention" effects (Allen et al., 2006). To increase delivery efficiency of drugs into tumors, liposomes must possess the following properties: stable drug encapsulation in circulation, long circulation time and small vesicle size of ~100 nm (Fenske and Cullis, 2008).

Stable drug encapsulation could prevent premature drug release and undesirable drug distribution in healthy tissues. Small vesicle size could guarantee the effective accumulation of liposomes into tumors via the leaky blood vessels of rapid-growth tumors. To realize long circulation, liposomes must be stealthy to the reticulo-endothelial systems so that the rapid clearance could not occur (Allen and Cullis, 2004).

Typically, the surface of liposomes was coated with hydrophilic polymer such as polyethylene glycol (PEG) to achieve long circulating properties and the resulting liposomes were named pegylated liposomes (Allen, 1994). To prepare pegylated liposomes, methoxy polyethylene glycol (mPEG, molecular weight: ~2000)-distearoyl phosphatidylethanolamine (mPEG$_{2000}$-DSPE) was used commonly, which could be incorporated into lipid membrane via the lipid anchor, DSPE portion (Papahadjopoulos et al., 1991). It is hypothesized that the presence of PEG on the liposomes could provide a steric barrier against opsonins and prevent the recognition by cells of RES, thus resulting in the decreased elimination of liposomes from blood and long circulation time (Papahadjopoulos et al., 1991).

Recent studies revealed that repeated injection of pegylated liposomes (empty vesicles or non-cytotoxic drug-containing vesicles) could lead to the loss of long-circulating characteristic, referred to as the accelerated blood clearance (ABC) phenomenon (Yamamoto et al., 2008). The production of anti-PEG Ig M, induced by the first dose of pegylated liposomes, might be responsible for

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the occurrence of the phenomenon (Wang et al., 2007). Moreover, it was demonstrated that the magnitude of the ABC phenomenon depended on the properties of injected liposomes as a first dose, time interval between injections, lipid dose and drug encapsulation (Laverman et al., 2001; Ishida et al., 2004, 2006; Yamamoto et al., 2008).

Based on previous experimental data (Ishida et al., 2006; Cui et al., 2008), the intravenous administration of cytotoxic drug-containing pegylated liposomes could not induce ABC phenomenon since the kind of liposomes might be toxic to B cells in spleen that are responsible for the production of Ig M. In addition, the ABC phenomenon was only observed in several animal species including mouse, rat and monkey (Dams et al., 2000; Laverman et al., 2001; Yamamoto et al., 2008).

Recently, it was reported by Ma et al. (2012) that pegylated liposomal topotecan with a standard PEG grafting density (HSPC/cholesterol/DSPE-PEG2000: 3/1/1; PEG grafting density of ~9% relative to HSPC; drug/lipid of 1/10, wt/wt) could induce accelerated blood clearance phenomenon (ABC) in rats upon the second dose. Moreover, it was proved that increasing dose level of the first injection could reduce the magnitude of ABC phenomenon of the second dose. The results were inconsistent with previous reports since topotecan is a cytotoxic drug, which could be used for the treatment of solid tumors in the clinical practice (Cheong et al., 2006) by means of selective inhibition of topoisomerase I.

Recently, a pegylated liposomal topotecan (pLT) has been evaluated in our lab (Li et al., 2012), which was slightly different from the formulation used by Ma's group. The formulation had a low PEG grafting density (~3%) and a low drug/lipid ratio (1/28, wt/wt). Despite that it was a low PEG formulation, in rats the clearance of the formulation followed linear kinetics. In the antitumor studies, multiple doses were injected into tumor-bearing mice and smaller split doses were more therapeutically active than larger doses when the overall dose intensity was equivalent. Furthermore, it was found that the formulation could not induce hand–foot syndrome. The latter two phenomena might be associated with its rapid drug release rate.

Subsequently, the pharmacokinetics of the formulation was investigated in beagle dogs. Unlike in small animals such as mouse and rats, the liposomes were administrated in dogs via slow intravenous infusion, a drug administration manner extensively used in the clinical practice. Preliminary studies revealed that the repeated injection of pLT could induce ABC phenomenon in beagle dogs. Thus, a series of questions were raised. Is anti-PEG antibody also responsible for the occurrence of ABC phenomenon in dogs? Do anti-PEG antibody levels positively correlate with the magnitude of ABC phenomenon? Whether the prolongation of time interval between doses could eliminate ABC considering the time-dependency of anti-PEG antibody production in small animals?

To resolve the questions, pLT was repeatedly injected into beagle dogs at different time points to investigate whether the prolongation of time interval between injections could eliminate ABC phenomenon. The plasma Ig M levels were determined using an ELISA method following the first dose of pLT to observe the correlation of Ig M levels with the magnitude of ABC phenomenon. In addition, the clearance kinetics of lipid vesicles was explored using doxorubicin as a membrane-impermeable marker. By comparing the pharmacokinetics of both lipid vesicles and total topotecan (entrapped and leaked), whether "empty lipid vesicles" could be formed in circulation following the injection pLT or not could be determined, which might be associated with ABC phenomenon.

2. Materials and methods

2.1. Materials

Topotecan hydrochloride was provided by Chengdu Tianyuan Natural Product Co., Ltd. (Chengdu, China). Hydrogenated soybean phosphatidylcholine (HSPC) was a kind gift from Degussa (Freising, Germany). *N*-(carbonyl-methoxypolyethyleneglycol$_{2000}$)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt (MPEG$_{2000}$-DSPE) was purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol (Chol) and Sephadex G-75 (medium) were obtained from the Sigma Chemical Company (St. Louis, MO). All other chemicals used in this study were of analytical or high-performance liquid chromatography (HPLC) grade. Beagle dogs were obtained from Hebei Medical University.

2.2. Preparation of liposomes and remote loading

Pegylated liposomes were prepared according to the following procedure. Briefly, the mixtures of HSPC, cholesterol and MPEG$_{2000}$-DSPE (mass ratio: 9.58:3.19:1.06; 3% PEG grafting density) were solubilized in chloroform and dried to a thin lipid film under a stream of N_2 gas, followed by incubation overnight under vacuum to remove residual solvent. The dried lipid films were subsequently hydrated in 0.25 L of 250 mmol/L ammonium sulfate to a final lipid concentration of 13.83 mg/mL. The hydration process was performed at 60 °C for 1 h. The dispersion was extruded eight times through polycarbonate filters of 0.10 μm employing a LiposoFast-100 jacketed extruder obtained from Avestin (Ottawa, Canada) at 60 °C. This procedure formed unilamellar vesicles of ~100 nm.

Before drug loading, a transmembrane ammonium sulfate gradient must be created firstly. Thus, the extraliposomal ammonium sulfate solution was replaced by sucrose/histidine (250/10 mM, pH 6.5) buffer using a Millipore Labscale TFF System (with 50,000 nominal molecular weight limit polysulfone filters). During tangential flow filtration process, constant feed volume was maintained and at least seven-fold volumes of sucrose/histidine buffer were used, resulting in liposomes suspended in an exterior aqueous phase composed of 250 mM sucrose and 10 mM histidine.

After diafiltration, 0.25 L of empty liposomes were mixed with 12.5 mL of topotecan solution (10 mg/mL). Accordingly, the drug to total lipid mass ratio was 1:27.65. The mixture was rapidly heated to 60 °C and incubated at the same temperature for 40 min, after which the mixture was rapidly cooled in an ice–water bath. After drug loading, a sample of the liposomes was taken to determine the percent encapsulation and to measure the mean particle diameter.

The final liposome preparation was firstly concentrated to a drug concentration of 0.5 mg/mL for pLT, and then sterilely filtered using a 0.22 μm cellulose acetate syringe filter, and stored, refrigerated and protected from light until use.

Pegylated liposomal doxorubicin (pLD) was prepared and loaded with the same procedure, but for pLD the drug to total lipid mass ratio was 1:6.91. The other formulation composition was the same for both pegylated liposomal formulations. Here liposome-entrapped doxorubicin was used as a membrane-impermeable marker to reveal the clearance of pegylated lipid vesicles (HSPC:cholesterol:MPEG-DSPE = 9.58:3.19:1.06, mass ratio) since doxorubicin could be stably encapsulated into pegylated HSPC/cholesterol vesicles using ammonium sulfate method.

The measurements of size and zeta potential were performed at 25 °C using Zetasizer Nano ZS (Malvern Instruments, UK). Prior to the measurement of mean size, the samples were diluted in 5% glucose solution with a volume ratio of 1/100. The zeta potential of vesicles was also determined using Nano ZS, but the measurement was carried out after 50-fold dilution. DTS software (version 4.0) was employed to collect the data that were analyzed using