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International Journal of Pharmaceutics 309 (2006) 94–100
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Development of pH-sensitive liposomes that efficiently retain encapsulated doxorubicin (DXR) in blood

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Received 9 July 2005; received in revised form 27 October 2005; accepted 11 November 2005
Available online 20 December 2005

Abstract

We have reported that targeted, pH-sensitive sterically stabilized liposomes are able to increase the cytotoxicity of DXR in vitro against B lymphoma cells, but the rate of release of DXR in plasma was too rapid to permit the results to be extended to in vivo applications. The purpose of the study reported here is two-fold. First, to understand the mechanism of the rapid release of DXR from pH-sensitive sterically stabilized liposomes (PSL) in human plasma. Second, to reformulate the above liposomes to improve their drug retention, while retaining their pH sensitivity. The stability of the PSL formulations in human plasma was evaluated by comparing the rate of release of encapsulated DXR with that of HPTS, a water-soluble fluorescent marker. Since DXR, but not HPTS, a water soluble-less membrane permeable fluorescence marker, was rapidly released from liposomes in the presence of plasma, the rapid release of DXR is likely caused by the diffusion of DXR molecules through the lipid bilayer, not by the disruption of the membrane. In order to develop more stable PSL formulations, various molar ratios of the membrane rigidifying lipid, hydrogenated soy HSPC and/or CHOL, were added to the lipid composition and the rate of release of encapsulated solutes and pH-sensitivity were evaluated. The compositions that showed the best drug retention and pH-sensitivity were a mixture of DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE at a molar ratio of 4:2:2:2:0.3 and DOPE/HSPC/CHEMS/CHOL at a molar ratio of 4:2:2:2. Our formulations, if targeted to internalizing antigens on cancer cells, may increase intracellular drug release rates within acidic compartment, resulting in a further increase in the therapeutic efficacy of targeted anticancer drug-containing liposomes.

Keywords: pH-sensitive liposomes; Encapsulation; Controlled drug release; Doxorubicin; Triggered drug release

1. Introduction

It has been reported that anti-CD19 targeted sterically stabilized (Stealth liposomes (SIL[anti-CD19])) selectively binds to CD19$^+$ human B cell lymphoma (Namalwa) cells, triggering endocytosis of the liposomes, resulting in a significantly improved therapeutic benefit in a murine model of Namalwa cells (Lopes de Menezes et al., 1998). Furthermore, they reported that encapsulated DXR accumulated in endosomes with very slow release of DXR from endosomes to the cytoplasm, indicating that most of the drugs are not bioavailable to the cells following receptor-mediated endocytosis (Lopes de Menezes et al., 1999). A more rapid rate of the drug release from endosomes/lysosomes would lead to more rapid delivery of the drug to its intracellular site of action, resulting in an improved therapeutic efficacy for the targeted liposomal drugs. One of possibilities for increasing the rate of drug release in endosomes/lysosomes would be to add pH-sensitivity to liposome formulation (Ellens et al., 1984; Straubinger et al., 1985; Chu and Szoka, 1991). However, current formulations of pH-sensitive liposomes are unstable in blood and are rapidly cleared from the circulation (Liu and Huang, 1989). One strategy for increasing

Abbreviations: CHEMS, cholesteryl hemisuccinate; CHOL, cholesterol; DOPE, dioleoylphosphatidylethanolamine; DPX, p-Xylene-bis-pyridinium bromide; ^{3}H-CHE, ^{3}H-cholesterylhexadecyl ether; DXR, doxorubicin; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; HPTS, trisodium 8-hydroxypyrenetrisulfonate; HSPC, hydrogenated soy phosphatidylcholine; MES, 2-[*N*-morpholino]ethanesulfonic acid; mPEG2000-DSPE, 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; mPEG2000-S-S-DSPE, N-[2-ω-methoxy-(polyethylene glycol)2000-αaminocarbonyl-ethyl-dithio-propionyl]-distearoyl phosphatidylethanolamine; MPS, mononuclear phagocyte system; PEG, polyethylene glycol; PSL, pH-sensitive sterically stabilized liposomes; SIL, sterically stabilized immunoliposomes; SL, sterically stabilized liposomes

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doi:10.1016/j.ijpharm.2005.11.010

their circulation time would be to include lipid derivatives of a hydrophilic polymer such as $mPEG_{2000}$-DSPE in the liposome formulations (Litzinger and Huang, 1992; Kono et al., 1997; Slepushkin et al., 1997; Guo and Szoka, 2001), but this leads to a decrease in the pH-dependent release of dye from liposomes (Holland et al., 1996; Slepushkin et al., 1997). To circumvent this problem, Kirpotin et al. (1996) synthesized a PEG-lipid derivative with a disulfide linkage (cleavable PEGylated lipid, mPEG-S-S-DSPE) to incorporate pH-sensitivity into the formulation. Thiolytic cleavage of the grafted PEG from the surface of pH-sensitive formulations restored pH-sensitivity, leading to release of the liposomal contents (Kirpotin et al., 1996; Zalipsky et al., 1999).

Based on these concepts, we developed DXR-loaded, pH-sensitive liposomes stabilized with a cleavable PEGylated lipid and attempted to further increase the efficacy of anti-CD19-targeted liposomal formulations by increasing the rate of intracellular drug release through a strategy that employed pH-sensitive targeted liposomes (Ishida et al., 2001). Logically, the targeted pH-sensitive liposomes, including the cleavable PEGylated lipid, allow the liposomes to circulate long enough to bind to the target cells. Enzymatic cleavage of the linker within the targeted cells subsequently leads to a restoration of pH sensitivity, thus permitting a rapid content release and an increased cytotoxicity, following internalization by the target cells. In spite of the significant higher in vitro cytotoxicity compared to non-pH-sensitive liposomes (SIL[anti-CD19]), the in vivo therapeutic efficacy of liposomes was similar or moderately higher than that of SIL[anti-CD19]. This lowered in vivo efficacy is thought to be due to the rapid release of the encapsulated DXR in blood and the rapid clearance of the liposomes from the circulation even though the formulation was stabilized by the inclusion of $mPEG_{2000}$-S-S-DSPE. These results suggest that, if the retention of an encapsulated drug and the stability of liposomes in circulating blood were improved, the new formulations might be able to substantially increase the therapeutic index of an encapsulated drug in vivo.

The focus of this study was on drug retention and attempts to design more stable formulations in the blood circulation. A variety of liposomes were formulated, containing DXR or a fluorescent dye, and the effect of lipid compositions on their plasma stabiliity and pH-sensitivity was examined in vitro. In addition, we investigated the pharmacokinetic profile of the newly formulated liposomes to determine whether lipid composition that could improve the in vitro retention of an encapsulated drug, improve the circulation time of liposomes in vivo.

2. Materials and methods

2.1. Materials and animal

Dioleoylphosphatidylethanolamine (DOPE), hydrogenated soy phosphatidylcholine (HSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were generous donated by the Nippon Oil and Fat (Tokyo, Japan). Cholesterol (CHOL) was of analytical grade (Wako Pure Chemical, Osaka, Japan). Doxorubicin (DXR), cholesteryl hemisuccinate (CHEMS), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) and 2-[*N*-morpholino]ethanesulfonic acid (MES) were purchased from Sigma Chemical (MO, USA). *p*-Xylene-bis-pyridinium bromide (DPX) and trisodium 8-hydroxypyrenetrisulfonate (HPTS) were purchased from Molecular Probes (OR, USA). ^{3}H-cholesterylhexadecyl ether (^{3}H-CHE) was purchased from NEN Research Products (MA, USA). Sepharose CL-4B and Sephadex G-50 were purchased from Amersham-Pharmacia Biotech (Upsala, Sweden). All lipids were used without further purification. All other reagents were of analytical grade.

Male ddY mice weighing 20–23 g were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Faculty of Pharmaceutical Sciences, The University of Tokushima.

2.2. Preparation of liposomes

The liposomes were composed of DOPE:HSPC:CHEMS:CHOL at various molar ratios either with or without $mPEG_{2000}$-DSPE at 5 mol% to phospholipids. The molar ratios of lipid in the liposomes are indicated in the text or figure legends. Small unilamellar vesicles were prepared as previously described (Ishida et al., 2003). Briefly, chloroform solutions of lipids were mixed and the solvent was evaporated using a rotary evaporator; residual solvent was removed under high vacuum. The dried lipid films were hydrated with an appropriate buffer and sequentially extruded through a series of polycarbonate membrane filters (Nucleopore, CA, USA) with pore sizes of 400–100 nm, using a Lipex Extruder (Lipex Biomembranes, BC, Canada). The mean diameter of the resulting liposomes was determined by dynamic light scattering using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The diameters of the extruded liposomes were in the range of 120 ± 10 nm. The concentration of phospholipid was determined by colorimetric assay (Bartlett, 1959).

For liposomes loaded with the HPTS-DPX complex as an aqueous phase maker, the lipid films were hydrated with an HPTS-DPX solution (30 mM HPTS, 30 mM DPX, pH 9.0, adjusted to 290 mOsmol with NaCl). Following extrusion, the unentrapped dye was removed by chromatography on a Sephadex G-50 column, by elution with HEPES buffered saline (25 mM HEPES, 140 mM NaCl, pH 7.4).

DXR was encapsulated into the liposomes by remote loading using an ammonium sulfate gradient (Bolotin et al., 1994) with minor modifications (Ishida et al., 2001). Briefly, the lipid film was hydrated in a 250 mM ammonium sulfate solution at pH 8.5. Following extrusion, the external buffer was exchanged by eluting through a Sephadex G-50 column equilibrated with 10% sucrose, with 25 mM Trizma base (Sigma Chemical, MO, USA), at pH 9.0. The higher pH was necessary for the DOPE formulations to self-assemble into bilayers (Ellens et al., 1984; Ellens et al., 1985). DXR was added to

the liposomes at a DXR/phospholipids ratio of 0.2:1 w/w. Liposomes including DOPE and liposomes not including DOPE were then incubated for 1.5 h at 37 °C and 1 h at 65 °C, respectively. The liposome-encapsulated DXR was separated from free DXR by chromatography on a Sephadex G-50 column eluted with HEPES buffered saline (pH 7.4). The concentration of liposome-entrapped DXR was determined by spectrophotometry ($\lambda = 480$ nm) following methanol extraction. The concentration of phospholipid was determined as described above. The encapsulation efficiency of DXR was consistently above 90–95%.

To determine the pharmacokinetic profiles, the liposomes were labeled with a trace amount of ^{3}H-CHE as a nonexchangeable lipid phase marker (40 μCi/μmol phospholipids).

2.3. In vitro leakage experiments

The in vitro leakage assays were carried out according to the method described previously (Ishida et al., 2001) with minor modification. Briefly, liposomes containing either entrapped HPTS-DPX or DXR were passed over a Sephadex G-50 column immediately prior to use to remove any residual free dye or drug. The release of entrapped HPTS was determined by means of a fluorescence-depending assay. Liposomes containing entrapped dye were incubated at a final phospholipid concentration of 0.6 mM at 37 °C in human plasma (final 80% (v/v)) and buffers at various pH. At various time points, the leakage of HPTS was determined in an aliquot of the incubation mixture in HEPES buffered saline (pH 7.4) by the increase in sample fluorescence at an emission wavelength of 512 nm and an excitation wavelength of 406 nm (Daleke et al., 1990) relative to that of the encapsulated HPTS by destabilization with 10% Triton-X 100 (100% release) using a F-4500 Fluorescence spectrophotometer (Hitachi, Tokyo, Japan). To determine DXR leakage, liposomes containing entrapped DXR were incubated at a final phospholipids concentration of 0.6 mM at 37 °C in HEPES buffered saline (pH 7.4) or human plasma (final 80% (v/v)). Since the fluorescence of DXR released from the liposomes is easily quenched due to their interactions with plasma proteins, the samples collected at various time points were passed through a Sepharose CL-4B column to separate the liposomal DXR from the leaked drug. The drug retention following incubation was calculated by dividing the drug/phospholipid ratio at indicated time point following incubation by the initial drug/phospholipid ratio.

2.4. Biodistribution of the liposomes

To determine the biodistribution of the liposomes, mice were injected intravenously with radio-labeled (^{3}H-CHE) liposomes (0.5 μmol phospholipids/mouse, approximately 25.0 μmol phospholipids/kg body weight) via tail vein. Mice were sacrificed at selected time points, and blood was sampled by heart puncture. The amount of radio activity in blood was determined as described previously (Harashima et al., 1993).

3. Results and discussion

3.1. Leakage of the encapsulated solute in human plasma

Fig. 1 indicates the retention of the loaded DXR or HPTS-DPX in the conventional pH-sensitive formulation (DOPE/CHEMS = 6:4 mol/mol) stabilized with mPEG$_{2000}$-DSPE at 5 mol% to phospholipids (PSL), followed by incubation in either human plasma (final 80% (v/v)) or HEPES buffered saline (pH 7.4) at 37 °C. The formulation was stabilized with mPEG$_{2000}$-DSPE instead of mPEG$_{2000}$-S-S-DSPE to exclude an automatically cleavage of PEG in human plasma. A rapid and large extent of DXR leakage was observed in the presence of human plasma, while no DXR leakage was detected in HEPES buffered saline. A rapid DXR release was also observed even in 20% (v/v) human plasma (data not shown). In contrast, conventional sterically stabilized liposomes (SL) (HSPC/CHOL/mPEG$_{2000}$-DSPE = 2:1:0.1 mol/mol) did not release DXR, when incubated in human plasma. It appears that the presence of human plasma, presumably plasma proteins, induces the leakage of the encapsulated DXR in the PSL, not the DXR encapsulated in SL.

In order to determine if the human plasma-dependent DXR leakage resulted from collapse of the liposomal membrane due to the exchange/transfer of phospholipids from the membrane or due to other plasma properties such as osmolarity and pH, the leakage of the hydrophilic, membrane-impermeable dye, HPTS from the PSL was examined. More than 90% of encapsulated HPTS remained inside the vesicles at over 12 h of incubation in

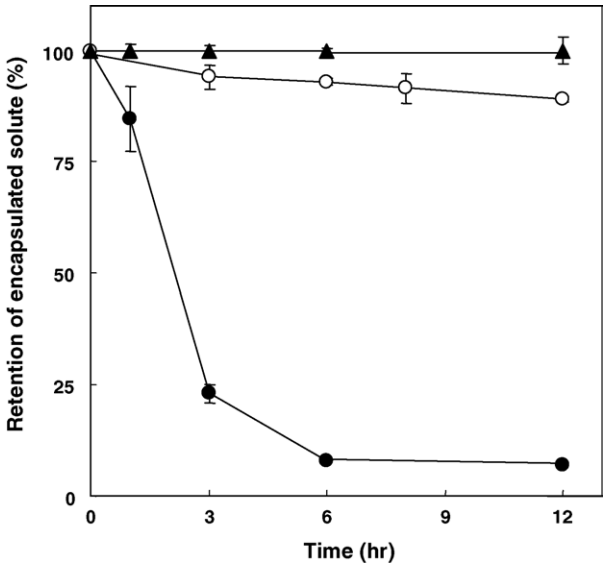

Fig. 1. Retention of encapsulated solutes in liposomes following incubation in either human plasma (80%) or HEPES buffered saline (pH 7.4) at 37 °C: (●) and (○) denotes incubation of DXR-containing conventional pH-sensitive liposomes stabilized with mPEG$_{2000}$-DSPE (PSL) (DOPE/CHEMS/mPEG$_{2000}$-DSPE = 6:4:0.3 mol/mol) in human plasma (80%) and HEPES buffered saline (pH 7.4), respectively; (▲) denotes the incubation of DXR-containing sterically stabilized liposomes (SL) (HSPC/CHOL/mPEG$_{2000}$-DSPE = 2:1:0.1 mol/mol) in human plasma (80%). The amount of leaked DXR following the incubation was determined according to the method described in Section 2. Each value represents the mean ± S.D. of three separate experiments.

80% human plasma. This finding indicates that the rapid leakage of DXR was caused by the diffusion of DXR molecules through the lipid bilayer, and not by disruption of the vesicles.

There are several possible explanations for the rapid DXR release observed in the PSL, and not in the SL, in human plasma (Fig. 1). DXR is a weak base: the neutral form of the drug (which is membrane-permeable) is favored at basic pH, and the charged form of the drug (which is membrane-impermeable) is favored at acidic pH. In the loading method, DOPE does not hydrate at acidic pH to form bilayer liposomes, and, because of this, the liposomes were prepared in a pH 8.5–9.0 ammonium sulfate solution. After the loading of DXR, the neutral form of DXR at interior pH (8.5–9.0) may not be able to form a stable precipitate with ammonium sulfate, and hence the drug molecules readily diffuse from the formulations. Horowitz et al. (1992) reported that exposure to nigericin, which causes the proton gradient of liposomes to collapse, resulted in >90% leakage of DXR from liposomes within 30 min. This finding clearly suggests that the lack of a proton gradient in our loading method may lead to the rapid release of DXR. In addition, the presence of negatively charged lipids at a basic pH as well as a low transition temperature of the lipid membrane might drive the release of DXR from the PSL. DOPE is negatively charged at a pH of around 9.0 (Stollery and Vail, 1977) and CHEMS, an anionic CHOL ester, when added to the pH-sensitive formulation to stabilize DOPE vesicles at neutral pH (Ellens et al., 1984; Lai et al., 1985), is also negatively charged at pH above 7.5 (Ellens et al., 1984). Several studies have shown that DXR binds strongly to anionic lipids in model membranes at a binding level of 1–2 mol DXR/mol anionic lipids (Henry et al., 1985; Goormaghtigh et al., 1987; de Wolf et al., 1992, 1993). In our liposome preparation, the lipid film was hydrated at a basic pH (8.5–9.0) and the exterior buffer was then exchanged with a 10% sucrose solution at pH 9.0. Under these conditions, the DOPE and CHEMS in the bilayer would be charged negatively, and then positively charged DXR, slightly exists at the high pH (8.5–9.0), would bind reversibly to these molecules. Banuelos et al. (1993) reported that the presence of negatively charged phospholipids permits the deeper penetration of the drug into the bilayer. The lower transition of the bilayer containing DOPE (transition temperature (T_c) = −16 °C) (Demel et al., 1977; Cullis, 1978) also enhances the penetration of the drug in the bilayer. The enhanced penetration by the charge and high membrane fluidity may result in the flip-floping the DXR molecules from the inner to the outer layer. The drugs flip-floped to the outer layer may be easily released by protein binding in an opposite direction to the initial remote loading mechanism. It is noteworthy, in this respect, that DXR binds strongly to plasma proteins (the percentage of protein binding is 83–83.9%).

3.2. Effect of lipid composition on the leakage of encapsulated DXR in human plasma

In order for PSL to be used as a drug delivery system in vivo, they must be designed in such a way that they are stable in the blood for an extended period of time, allowing the liposomes to reach a target cell, prior to releasing their contents.

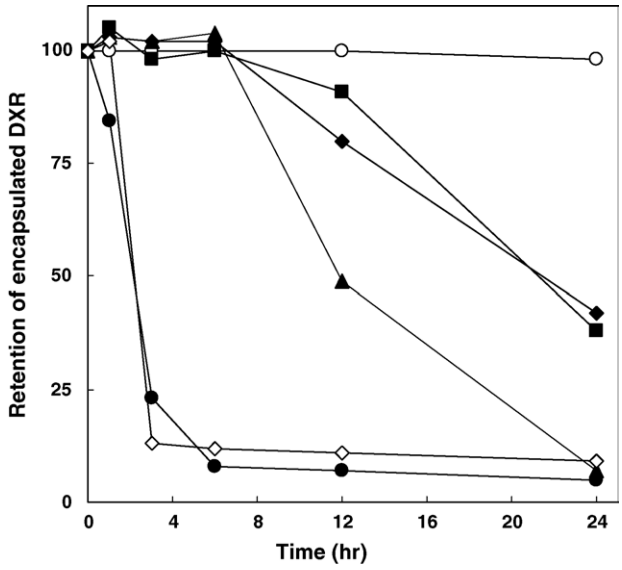

Fig. 2. Retention of encapsulated DXR in liposomes following incubation in human plasma (80%) at 37 °C. DXR-containing liposomes were incubated in human plasma (80%). The amount of leaked DXR following the incubation was determined according to the method described in Section 2: (◯) SL (HSPC/CHOL/mPEG$_{2000}$-DSPE = 2:1:0.1 mol/mol); (●) PSL (DOPE/CHEMS/mPEG$_{2000}$-DSPE = 6:4:0.3 mol/mol); (▲) (DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE = 6:0:2:2:0.3 mol/mol); (■) DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE = 4:2:2:2:0.3 (mol/mol); (♦) DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE = 3:3:2:2:0.3 (mol/mol); (◇) DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE = 3:3:4:0:0.3 (mol/mol).

Hence, on the basis of the rapid drug release mechanism that is apparent in Fig. 1, we attempted to develop a more stable formulation with respect to drug retention in human plasma. For this purpose, we focused on the lipid composition to inhibit the penetration and permeation of the drug molecules through the lipid bilayer. Earlier studies have already established a connection between liposome permeability, lipid phase transition and CHOL content (Papahadjopoulos et al., 1973; Luna and McConnell, 1977; Ben-Yashar and Barenholz, 1989). Therefore, the stability of the basic formulation (DOPE/CHEMS with 5 mol% mPEG$_{2000}$-DSPE) mixed with varying amounts of HSPC, to increase the phase transition temperature and CHOL, to increase the rigidity of the bilayer, was examined by the incubation in 80% human plasma. The time course for the release of DXR during incubation is shown in Fig. 2 for liposomes composed of DOPE/CHEMS/HSPC/CHOL/mPEG$_{2000}$-DSPE at six different molar ratios. The substitution of HSPC for 50 mol% of DOPE (DOPE/HSPC/CHEMS/mPEG$_{2000}$-DSPE = 3:3:4:0:0.3 mol/mol) failed to improve drug retention, but the substitution of CHOL for 50 mol% of CHEMS (DOPE/CHEMS/CHOL/mPEG$_{2000}$-DSPE = 6:2:2:0.3 mol/mol) resulted in a significant improvement. The addition of CHOL led to 100% retention of loaded DXR for 6 h. Interestingly, the addition of HSPC mixed with CHOL to the basic formulation at various molar ratios (DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE = 4:2:2:2:0.3 or 3:3:2:2:0.3 mol/mol) further improved the retention of DXR, permitting a good drug retention for approximately 12 h. It appears that the combination of HSPC and CHOL has a synergetic effect on drug retention. These results

indicate that the inclusion of 20–30 mol% HSPC and 20 mol% CHOL to the total lipid are required in order to achieve a minimal release of encapsulated DXR under the conditions used here.

As shown in Fig. 2, the inclusion of CHOL in the formulation significantly improved the drug retention of the formulation in human plasma. This result is not surprising, since it is well know that CHOL, when added to fluid phase lipids, stabilizes the lipid membrane and decreases its permeability (Mayhew et al., 1979) and the CHOL inhibits the incorporation of anthracycline into polar regions of the bilayer in a concentration-dependent manner (Banuelos et al., 1993). Contrary to the remarkable CHOL effect, the addition of HSPC to PSL led to no improvement in the retention (Fig. 2). This result is somewhat unexpected, since the rate of drug release is also dependent on liposome composition: liposomes formed from high phase-transition lipids release less rapidly than liposomes formed from low-phase transition. It has been known for some time that the maximal release of low molecular weight liposome contents occurs at a temperature higher than the gel-to-liquid crystalline transition (Papahadjopoulos et al., 1973). It is likely therefore that the inclusion of 20–30 mol% HSPC is not sufficient to significantly change physical state of the lipid membranes. It should also be noted that the inclusion of HSPC mixed with CHOL to the PSL showed a synergetic effect on the drug retention (Fig. 2). The included HSPC might increase to some extent on the phase-transition of the lipid membrane and CHOL increased the bilayer order by limiting the probability of trans-gauche isomerization of phospholipid acyl chains (Ben-Yashar and Barenholz, 1989).

3.3. Effect of lipid composition on pH-sensitivity

Since the drug retention of the liposomes in human plasma was improved by the inclusion of either CHOL or HSPC plus CHOL in the PSL, we investigated the impact of CHOL and HSPC plus CHOL on their pH-sensitivity. All formulations for a pH sensitive assay were prepared in the absence of $mPEG_{2000}$-DSPE, since the pH-sensitivity of the liposomes decreased as the mol percentage of $mPEG_{2000}$-DSPE increased an in vitro system (Slepushkin et al., 1997). The pH-sensitivity of the liposome formulations was assayed by incubation for 1 h in buffers at various pH values (Fig. 3). To assay the disruption of the liposomes as function of pH, a water soluble, membrane-impermeable dye, HPTS, was passively loaded into the liposomes.

In the series of assays, it appeared that pH-sensitivity increased with increasing mol% DOPE in the formulations. As expected, a DOPE/CHEMS (6:4 mol/mol) formulation released the encapsulated dye in a pH-dependent manner. As a negative control, pH-stable liposomes composed HSPC/CHOL (2:1 mol/mol) were used. HSPC/CHOL did not show any pH-sensitive release pattern of HPTS over the pH range examined. One of the most stable formulations in human plasma, DOPE/HSPC/CHMS/CHOL (3:3:2:2 mol/mol) (Fig. 2) showed no pH-sensitivity following a 1 h incubation. Some pH-sensitivity was detected in DOPE/HSPC/CHEMS/CHOL, 4:2:2:2. Extensive pH sensitivity was detected in 6:0:2:2 (mol/mol) formulations. The pH-dependent membrane disruption would increase rate and extension of release of encapsulated

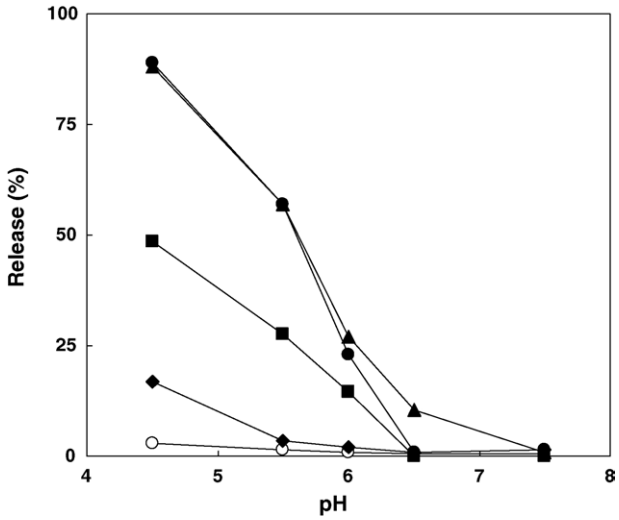

Fig. 3. Effect of lipid composition on the pH-sensitivity of DOPE-containing liposomes. HPTS-containing liposomes were incubated in buffers with various pH for 1 h at 37 °C. The amount of leaked HPTS was determined according to the method described in Section 2: (◯) conventional liposomes (HSPC/CHOL = 2:1 mol/mol); (●) conventional pH-sensitive liposomes (DOPE/CHEMS = 6:4 mol/mol); (▲) DOPE/HSPC/CHEMS/CHOL = 6:0:2:2 (mol/mol); (■) DOPE/HSPC/CHEMS/CHOL = 4:2:2:2 (mol/mol); (♦) DOPE/HSPC/CHEMS/CHOL = 3:3:2:2 (mol/mol).

DXR. In addition, these formulations showed reasonable levels of drug retention time in human plasma (Fig. 2). Therefore, these formulations are good for in vivo use, if the formulations would be proposed with a cleavable PEGylated lipid such as $mPEG_{2000}$-S-S-DSPE. Experiments exploring this possibility are currently in progress in our laboratory with newly synthesized cleavable PEGylated lipid.

3.4. Elimination of the formulation from blood

The formulations composed of DOPE/HSPC/CHEMS/CHOL, 6:0:2:2 or 4:2:2:2 (mol/mol), if stabilize with a PEG-lipid derivative, may be applicable for in vivo use. In order to confirm partly this assumption, we examined the blood concentration of these liposome preparations including $mPEG_{2000}$-DSPE instead of cleavable PEGylated lipid following intravenous administration. PSL and SL were employed as negative and positive controls, respectively. As expected, the injection of the PSL resulted in the rapid clearance of the liposomes, while SL cleared slowly (Fig. 4). The addition of HSPC and CHOL to the PSL composition significantly increased the circulation time of the preparations, compared to the original PSL. Approximately 9–13% of the injected liposomes still remained in the blood at 8 h, although their circulation longevities were shorter than that of SL.

We recently reported on a murine model of B cell lymphoma study, in which the therapeutic efficacy of a DXR-containing, anti-CD19-targeted, sterically stabilized, pH-sensitive formulation (DXR-PSIL[anti-CD19], DOPE/CHEMS/$mPEG_{2000}$-S-S-DSPE = 6:4:0.3 mol/mol) was superior to that of a stable long-circulating formulation of targeted liposomes (DXR-SIL[anti-CD19], HSPC/CHOL/$mPEG_{2000}$-

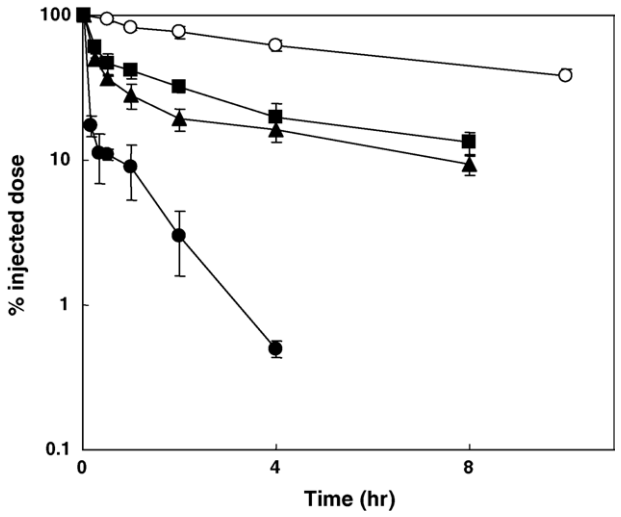

Fig. 4. Blood clearance of ^{3}H-CHE labeled liposomes in mice. Radio-labeled liposomes were intravenously injected into mice at a dose of 0.5 μmol phospholipid/mouse. At selected time points, mice were sacrificed and blood was sampled by heart puncture. Radioactivities in blood were assayed as described in Section 2: (◯) SL (HSPC/CHOL/mPEG$_{2000}$-DSPE = 2:1:0.1 mol/mol); (●) PSL (DOPE/CHEMS/mPEG$_{2000}$-DSPE = 6:4:0.3 mol/mol); (▲) DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE = 6:0:2:2:0.3 (mol/mol); (■) DOPE/HSPC/CHEMS/CHOL/mPEG$_{2000}$-DSPE = 4:2:2:2:0.3 (mol/mol). Each value represents the mean ± S.D.

DSPE = 2:1:0.1 mol/mol) despite the enhanced drug release and clearance of the PSIL[anti-CD19] (Ishida et al., 2001). With respect to drug retention and longevity in the circulation, our new formulations developed in this study are likely to be superior to the PSIL formulations. This superiority may leads to positive therapeutic results for the formulation.

In addition, the formulation developed herein may indicate an increased therapeutic index of encapsulated anti-cancer agents in the treatment of solid tumors, even though they are not targeted. It has recently been reported that long circulating liposomes enhance the antitumor effect of DXR by increasing the delivery of DXR to the tumor site (Woodle and Lasic, 1992; Allen et al., 1995), since liposomes accumulate extensively in tumor tissues due to extravasation via the immature capillary endotherium within the rapidly growing tumor (Wu et al., 1993). After carriers have accumulated in the tumor interstitial space, the encapsulated drug must be released. Our laboratory examined the optimum drug release rate from non-targeted, long circulating liposomes to maximize the therapeutic index of liposomal DXR using a pharmacokinetic/pharmacodynamic (PK/PD)-simulation system (Harashima et al., 1999; Tsuchihashi et al., 1999). The optimum rate of drug release was at a release rate constant of around 0.06 h^{-1} and this rate is much faster than that for conventional SL. The relationship between liposomal carrier stability or leakiness and the activity of liposomal anticancer agent in vivo has been examined and it has been suggested that the liposomal carrier should display properties that favor an enhanced blood residence time and good drug retention in the circulation, but should then transform into a leaky vesicle to release their contents following extravasation (Lim et al., 1997; Adlakha-Hutcheon et al., 1999). Our formulations, which have a much faster drug release rate than conventional SL, may enhance the therapeutic index of liposomal anti-cancer agents.

Acknowledgments

We would like to thank Dr. Theresa M. Allen (University of Alberta, Canada) for her helpful discussion. We also thank Dr. Milton S. Feather for his helpful advice in writing the English manuscript. This study was supported, in part, by research grants from Osaka Cancer Research Foundation and Grant-in-Aid for Young Scientist (B) (14771310), The Ministry of Education, Culture, Sports, Science and Technology.

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