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Biochimica et Biophysica Acta 1515 (2001) 144–158

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Targeted delivery and triggered release of liposomal doxorubicin enhances cytotoxicity against human B lymphoma cells

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Received 7 March 2001; received in revised form 28 August 2001; accepted 30 August 2001

Abstract

Dioleoylphosphatidylethanolamine (DOPE)-containing liposomes that demonstrated pH-dependent release of their contents were stabilized in the bilayer form through the addition of a cleavable lipid derivative of polyethylene glycol (PEG) in which the PEG was attached to a lipid anchor via a disulfide linkage (mPEG-S-S-DSPE). Liposomes stabilized with either a non-cleavable PEG (mPEG-DSPE) or mPEG-S-S-DSPE retained an encapsulated dye at pH 5.5, but treatment at pH 5.5 of liposomes stabilized with mPEG-S-S-DSPE with either dithiothreitol or cell-free extracts caused contents release due to cleavage of the PEG chains and concomitant destabilization of the DOPE liposomes. While formulations loaded with doxorubicin (DXR) were stable in culture media, DXR was rapidly released in human plasma. pH-Sensitive liposomes, targeted to the CD19 epitope on B-lymphoma cells, showed enhanced DXR delivery into the nuclei of the target cells and increased cytotoxicity compared to non-pH-sensitive liposomes. Pharmacokinetic studies suggested that mPEG-S-S-DSPE was rapidly cleaved in circulation. In a murine model of B-cell lymphoma, the therapeutic efficacy of an anti-CD19-targeted pH-sensitive formulation was superior to that of a stable long-circulating formulation of targeted liposomes despite the more rapid drug release and clearance of the pH-sensitive formulation. These results suggest that targeted pH-sensitive formulations of drugs may be able to increase the therapeutic efficacy of entrapped drugs.

Keywords: pH-sensitive liposome; Stealth liposome; Targeted drug delivery; Polyethylene glycol; Triggered release; Chemotherapy

Abbreviations: CFE, cell-free extract; CHEMS, cholesteryl hemisuccinate; CHOL, cholesterol; [^{3}H]CHE, cholesteryl-(1,2-[^{3}H](N)]-hexadecyl ether; DOPE, dioleoylphosphatidylethanolamine; DPX, *p*-xylene-bis-pyridinium bromide; DSPE, distearoylphosphatidylethanolamine; DTT, dithiothreitol; DXR, doxorubicin; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HPTS, trisodium 8-hydroxypyrenetrisulfonate; HSPC, hydrogenated soy phosphatidylcholine; IC_{50}, drug concentration at which cell growth is inhibited by 50%; mAb, monoclonal antibody; mPEG-DSPE, methoxypoly(ethylene glycol) (M_r 2000) covalently coupled to DSPE; Mal-PEG-DSPE, maleimide-terminated polyethylene glycol (M_r 2000) covalently coupled to DSPE; mPEG-S-S-DSPE, *N*-[2-ω-methoxypoly(ethylene glycol)-α-aminocarbonylethyl-dithiopropionyl]-DSPE; MES, *N*-morpholinoethanesulfonic acid; MPS, mononuclear phagocyte system; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PL, phospholipid; SL, sterically stabilized (Stealth) liposomes composed of HSPC/CHOL/mPEG-DSPE; DXR-SL, SL loaded with doxorubicin; SIL[anti-CD19], sterically-stabilized (Stealth) immunoliposomes composed of HSPC/CHOL/mPEG-DSPE/Mal-PEG-DSPE covalently coupled to mAb anti-CD19; DXR-SIL, SIL loaded with doxorubicin; TI, tyraminylinulin

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0005-2736/01/$ – see front matter
PII: S0005-2736(01)00409-6

1. Introduction

Long-circulating liposomal doxorubicin (DXR), Caelyx/Doxil, is currently approved for clinical use in the treatment of refractory Kaposi's sarcoma and ovarian cancer [1–3]. However, the passive targeting approach that has made liposomal drugs successful in the treatment of solid tumors will be less successful when applied to haematological malignancies. To increase the uptake of liposomal drugs by malignant B cells, a ligand-mediated targeting approach has been employed [4]. Many investigators have evaluated long-circulating targeted liposomes (sterically-stabilized immunoliposomes, SIL), which have antibodies, their fragments or other ligands attached to the PEG termini, for their ability to increase the selective delivery of liposomal contents to target tissues and cells in vivo and in vitro [4–14]. We have reported that anti-CD19-targeted sterically-stabilized liposomes (SIL[anti-CD19]) bound selectively to $CD19^+$ human B-lymphoma cells, triggering endocytosis of the liposomes [15]. The encapsulated DXR accumulated in the endosomes of the target cells and was released from the endosomes into the cytoplasm only very slowly. This has led us to hypothesize that a more rapid rate of release of the drug from the endosomes would lead to more rapid delivery of the drug to its intracellular site of action, resulting in improved therapeutic efficacy for the targeted liposomal drugs.

The lipid dioleoylphosphatidylethanolamine (DOPE) adopts a nonbilayer (hexagonal, H_{II}) structure in an aqueous medium at neutral pH [16–18] but, when combined with stabilizing components such as cholesteryl hemisuccinate (CHEMS) [16], the lipids can assemble into a bilayer. These liposomes are postulated to destabilize in the acidic environment of the endosomes, rapidly releasing their contents [16,19,20]. Current formulations of pH-sensitive liposomes are unstable in blood and are rapidly cleared from the circulation by the mononuclear phagocyte system (MPS) [21,22]. One way to increase their circulation half-life is to include lipid derivatives of PEG in the liposome formulations, but this leads to a decrease in the pH-dependant release of dye from these liposomes [23,24]. A PEG-lipid derivative with a disulfide linkage (mPEG-S-S-DSPE) has been synthesized by Zalipsky [25]. Thiolytic cleavage of the grafted PEG from the surface of pH-sensitive formulations restored pH sensitivity, leading to release of the liposomal contents [25,26]. This linker is hypothesized to resist cleavage in the circulation. Targeting ligands bound to these liposomes may allow the liposomes to circulate long enough to bind to, and be internalized by, the target cells. Subsequently, cleavage of the disulfide linkage by lysosomal enzymes within the target cells should lead to a restoration of the pH sensitivity and allow rapid content release and increased cytotoxicity.

Here we report the development of anti-CD19-targeted, DXR-loaded, pH-sensitive liposomes stabilized with PEG-lipid derivatives, which can be expected to selectively deliver their contents to target cells with rapid intracellular drug release. Comparisons are made of liposomes containing either cleavable PEG (mPEG-S-S-DSPE) or non-cleavable PEG (mPEG-DSPE) as stabilizing components. The liposomes are characterized in vitro as to drug release properties and cytotoxicity against a $CD19^+$ human B-lymphoma cell line (Namalwa). In addition, we test the therapeutic efficacy of the formulations in vivo in SCID mice implanted with the Namalwa cell line.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC), $mPEG_{2000}$-distearoylphosphatidyl-ethanolamine ($mPEG_{2000}$-DSPE, also abbreviated mPEG-DSPE) and doxorubicin (DXR) were generous gifts from Alza Corp. (Mountain View, CA). Maleimide-derivatized PEG_{2000}-DSPE (Mal-PEG-DSPE) [27] was custom synthesized by Shearwater Polymers (Huntsville, AL). Cholesterol (CHOL) and dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). *N*-[2-ω-methoxy-poly(ethylene glycol)-α aminocarbonyl-ethyl-dithiopropionyl]-DSPE (mPEG-S-S-DSPE, disulfide-linker) was synthesized as described elsewhere [25]. Sephadex G-50 and Sepharose CL-4B were purchased from Pharmacia Biotech (Uppsala, Sweden). $Na^{125}I$ and cholesteryl-[1,2-[^{3}H](N)]-hexadecyl ether ([^{3}H]CHE) were purchased from Mandel Scientific

(Guelph, ON). Cholesteryl hemisuccinate (CHEMS), 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT) and iminothiolane were purchased from Sigma Chemicals (Oakville, ON). *p*-Xylene-bis-pyridinium bromide (DPX) and trisodium 8-hydroxypyrenetrisulfonate (HPTS) were purchased from Molecular Probes (Eugene, OR). Iodination of IgG was performed according to the method described elsewhere [4]. Tyraminylinulin (TI) synthesis and preparation of [^{125}I]TI have been described previously [28]. The murine monoclonal antibody (mAb) anti-CD19 was prepared from the FMC-63 murine anti-CD19 hybridoma cell line obtained from Dr. H. Zola (Children's Health Research Institute, Australia). The human B-lymphoma cell line Namalwa (ATCC CRL 1432) was obtained from American Type Culture Collection (MD, USA). Human plasma was obtained from healthy volunteers at the University of Alberta, Department of Pharmacology. Nuclepore polycarbonate membranes (0.08, 0.1 and 0.2 μm pore size) were purchased from Northern Lipids (Vancouver, BC). All other chemicals were of analytical grade.

2.2. Preparation of liposomes

Sterically stabilized pH-sensitive liposomes were prepared from a mixture of DOPE or DOPE/CHEMS (6:4 molar ratio) and either mPEG-DSPE or mPEG-S-S-DSPE. The lipid molar ratios of the different formulations are indicated in the text or in Table or Figure legends. SIL (not pH-sensitive) for coupling to anti-CD19 were composed of HSPC/CHOL/mPEG-DSPE/Mal-PEG-DSPE, 2:1:0.08:0.02. 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 filters with pore sizes ranging from 0.2 to 0.08 μm, using a Lipex Extruder (Lipex Biomembranes, Vancouver, BC). The mean diameter of liposomes was determined by dynamic light scattering using a Brookhaven BI-90 Particle Sizer (Brookhaven Instruments, Holtsville, NY). The diameters of extruded liposomes were in the range of 120 ± 10 nm. For liposomes loaded with either HPTS-DPX or [^{125}I]TI, the lipid films were hydrated with HPTS-DPX solution (30 mM HPTS, 30 mM DPX, pH 9.0, adjusted to 290 mosmol with NaCl) or [^{125}I]TI solution (pH 9.0). Following extrusion, the untrapped dye or [^{125}I]TI was removed by chromatography on Sephadex G-50 or Sepharose CL-4B columns, respectively, eluted with HEPES buffer (25 mM HEPES, 140 mM NaCl), pH 7.4.

DXR-SIL[anti-CD19] were prepared and DXR loaded by the ammonium sulfate loading method at 65°C as previously described [4]. The lipid molar ratios of the additional targeted formulations of liposomal DXR, composed of DXR-DOPE/mPEG-DSPE[anti-CD19], DXR-DOPE/CHEMS/mPEG-DSPE[anti-CD19], DXR-DOPE/mPEG-S-S-DSPE-[anti-CD19], or DXR-DOPE/CHEMS/mPEG-S-S-DSPE[anti-CD19] are indicated in the text or in Table or Figure legends; the drug was encapsulated by remote loading using an ammonium sulfate gradient [29] with minor modifications. Briefly, the lipid films were hydrated in 250 mM ammonium sulfate at pH 8.5 for formulations containing DOPE/CHEMS/PEG-DSPE or at pH 9.0 for formulations containing DOPE/PEG-DSPE, with addition of minute amounts of NaOH until complete hydration was obtained. Following extrusion, the external buffer was exchanged by eluting through a Sephadex G-50 column equilibrated with 10% sucrose, 25 mM Trizma base at pH 8.5 or pH 9.0, as appropriate. The higher pH was necessary to get DOPE formulations to self-assemble into bilayers [16,30]. DXR was added to the liposomes at a DXR/PL ratio of 0.2:1 (w/w), and the liposomes were incubated for 15 min at 22°C. The liposome-encapsulated DXR was separated from free DXR by chromatography on a Sephadex G-50 column eluted with degassed HEPES buffer. Coupling of anti-CD19 mAb to Mal-PEG-DSPE on the liposomes was carried out according to a previously described method [15], using ^{125}I-labeled anti-CD19 mAb as a tracer. The concentration of liposome-entrapped DXR was determined by spectrophotometry ($\lambda = 490$ nm) following methanol extraction. Phospholipid concentrations were determined using the Fiske–Subbarow colorimetric assay [31]. All mAb densities were routinely in the range of 30–60 μg anti-CD19/μmol PL for in vivo experiments and 65–80 μg anti-CD19/μmol PL for in vitro experiments.

2.3. In vitro leakage experiments

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 solute was studied using a fluorescence-dequenching assay. Fifty μl of liposomes containing entrapped dye (HPTS-DPX) or DXR were incubated at 0.5 mM final PL concentration at 37°C in 450 μl of various pH buffers, cell-free extract (CFE), human plasma, or cell-culture medium containing 10% fetal bovine serum (FBS). At various time points, the percentage of released HPTS was determined in an aliquot of the incubation mixture by the increase in sample fluorescence at an emission wavelength of 512 nm and an excitation wavelength of 413 nm [32] relative to that of the pre-incubation sample (zero release) using an SLM-Aminco Model 8100 fluorimeter (Spectronic Instruments, Rochester, NY); values were normalized to the increase in fluorescence obtained after lysis of a pre-incubation sample with 10% Triton X-100 [25]. The fluorescence of DXR contained in liposomes was quenched due to its self-association when loaded by the ammonium sulfate method; DXR leakage was determined by fluorescence dequenching at excitation and emission wavelengths of 485 and 590 nm, respectively. Liposomes were incubated at 37°C with various buffers, CFE, cell-culture medium containing 10% FBS, or human plasma. At designated intervals, aliquots were taken, and the amount of released DXR was determined as described above. Total DXR (100% dequenched) was measured by lysing the liposomes at a final Triton X-100 concentration of 0.5% (v/v).

2.4. Preparation of cell-free extracts

Namalwa cells were maintained in logarithmic growth conditions in RPMI 1640 supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Cells (1.0×10^8) were collected by centrifugation (1000 rpm for 10 min) and washed with 20 ml of TEA buffer (10 mM triethanolamine, 0.25 M sucrose, 10 mM acetic acid, and 1 mM EDTA, pH 7.4). The washed cells were resuspended in 4 ml TEA buffer and a protease inhibitor cocktail formulated for mammalian cell extracts (4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucylamino(4-guanidino)butane, bestatin, leupeptin, and aprotinin; Sigma, MO, USA) was added at 100 μl per gram of cells. The cells were ruptured at 4°C using 40 firm strokes with a tight-fitting Dounce homogenizer. Unbroken cells were pelleted by centrifugation at 1000 rpm for 10 min at 4°C. The CFE was carefully removed from the cell pellet and then diluted to 6 ml with the addition of TEA buffer.

2.5. Nuclear accumulation assay

Nuclear accumulation of DXR was determined according to the method of Kirchmeier et al. [33]. Briefly, 4.5×10^8/500 ml Namalwa cells, maintained under logarithmic growth conditions in RPMI 1640 supplemented with 10% FBS, were treated with various DXR formulations at a DXR concentration of 8 μM. At various time points (0, 2, 4, 8, 12 h), 100 ml of cells were pelleted by centrifugation at 1000 rpm for 10 min and washed with 20 ml of TEA buffer. Washed cells were resuspended in 3 ml TEA buffer and ruptured at 4°C using 40 firm strokes with a tight-fitting Dounce homogenizer. Unbroken cells were pelleted and the CFE, which contained the nuclei, was carefully removed from the cell pellet. To obtain more complete homogenization, the pellet of unbroken cells was suspended in TEA buffer (3 ml) and ruptured a second time, followed again by removal of the unbroken cells. The combined supernatants were centrifuged at 1000 rpm for 10 min to remove any remaining unbroken cells. The supernatant from this centrifugation step was spun at 2000 rpm for 2.5 min at 4°C to pellet nuclei. After removal of the supernatant, the pellet was diluted to 1 ml with TEA buffer, then vortexed and sonicated until the nuclei were evenly dispersed, as determined by visual inspection.

For each time point, three aliquots of the nuclear fractions (0.2 ml each) were placed in 1.3 ml TEA. DNA was enzymatically digested by the addition of 10 μl digitonin solution (25 mg/ml in sterile PBS, Sigma, St. Louis, MO), 10 μl $MgCl_2$ solution (57 mg/ml in sterile PBS) and 50 μl DNase 1 solution (3 mg/ml in sterile PBS, Sigma). Following digestion at 22°C for 2 h, the DXR fluorescence was recorded (excitation at 480 nm and emission at 595 nm). The

purity of the nuclear fraction was checked by determining the levels of enzyme markers for various cellular organelles [15].

2.6. In vitro cytotoxicity experiments

A comparison of the in vitro cytotoxicity of free DXR and various liposomal formulations was performed on Namalwa cells with an in vitro proliferation assay using a tetrazolium dye (MTT) [34], as described in [4].

2.7. Blood elimination of liposomes in BALB/c (inbred) mice

Female BALB/c Cr Alt B/M mice, in the weight range of 17–22 g, were obtained from University of Alberta Health Sciences Laboratory Animal Services, and were injected via the tail vein with a single bolus dose of 0.2 ml of liposomes of various formulations containing encapsulated [^{125}I]TI (0.5 μmol PL/mouse). At selected times post-injection, mice were anesthetized with halothane and sacrificed by cervical dislocation. A blood sample (100 μl) was collected by cardiac puncture. Blood samples, various organs and the carcass were counted for ^{125}I label in a Beckman 8000 gamma counter. Data were analyzed using PKAnalyst (MicroMath Scientific Software).

2.8. In vivo therapeutic experiments

Namalwa cells were passaged i.p. in CB.-17/ICR Tac SCID mice (Charles River Laboratories, Quebec, Canada) to develop a more virulent strain with reproducible tumor take [4]. Namalwa cells were harvested in sterile PBS and implanted into SCID mice. Cell viability was assessed by dye exclusion using Trypan Blue dye before and after the implantation process. For therapeutic experiments, CB-17/ICR Tac SCID mice (Charles River Laboratories), 6–8 weeks of age, were injected with cells i.v. (5×10^6) and treated i.v. at 24 h after implantation with a single dose of 3 mg DXR/kg as either free DXR, DXR-SIL[anti-CD19] or targeted DXR-loaded DOPE/CHEMS formulations stabilized with either mPEG-DSPE or mPEG-S-S-DSPE. Mice were monitored routinely for weight loss, and euthanized as they became moribund; survival times were recorded. All animal experiments were approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta (Edmonton, Alberta, Canada).

2.9. Statistics

Statistical analyses were performed using GraphPad Instat software (v.3.01, GraphPad Software, San Diego, CA) using analysis of variance for multiple comparisons. Data were considered significant at $P<0.05$.

3. Results

3.1. pH sensitivity of liposomal HPTS in buffer

Leakage of HPTS from DOPE (Fig. 1) and DOPE/CHEMS (Fig. 2) liposomes, stabilized with mPEG-DSPE (Fig. 1A and 2A) or mPEG-S-S-DSPE (Fig. 1B and 2B), was examined as a function of pH and reducing conditions. At physiological pH and ionic strength, DOPE does not form bilayers, but rather exists in an inverted hexagonal (H_{II}) phase [17]. However, DOPE will form liposomes readily at higher pH, upon deprotonation of the amine group. Dilution of DOPE liposomes, made at pH 8.5–9.0, into pH 7.4 or pH 5.5 buffers led to rapid release of contents (Fig. 1A). Incorporation of 5 mol% mPEG-DSPE (Fig. 1A) or mPEG-S-S-DSPE (Fig. 1B) into DOPE liposomes resulted in the formation of liposomes which did not leak at physiological or acid pH over 24 h. Treatment of liposomes with dithiothreitol (DTT) was used to induce thiolytic cleavage of mPEG-S-S-DSPE from the lipid membrane anchor (DSPE) in liposomes containing mPEG-S-S-DSPE (Fig. 1B). DOPE liposomes containing 5 mol% mPEG-S-S-DSPE had little contents release over 24 h in the presence of DTT at pH 7.4 (Fig. 1B); however at pH 5.5, treatment with DTT led to a gradual release of HPTS (50% release in about 10 h). At pH 5.5, a DOPE formulation that contained 3 mol% mPEG-S-S-DSPE had a much more rapid release of contents (100% release in 2 h, data not shown).

DOPE liposomes can also be stabilized in the bilayer state by an amphiphile with bulky and/or charge-repulsing hydrophilic moieties [35]. CHEMS

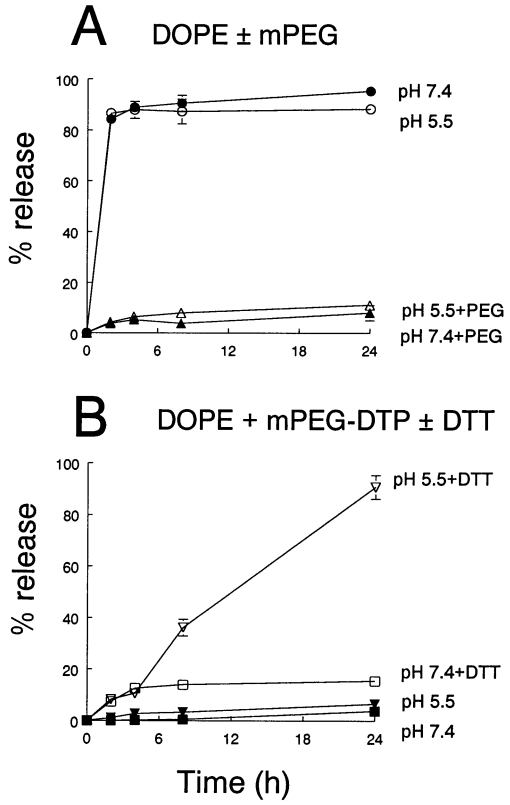

Fig. 1. Leakage of encapsulated HPTS from DOPE formulations ± mPEG-DSPE or mPEG-S-S-DSPE in buffers of varying pH at 37°C (± DTT). HPTS encapsulated in DOPE stabilized with various PEG-DSPE conjugates (cleavable or non-cleavable) at pH 9.0 was diluted into buffer at either pH 7.4 or pH 5.5, at 37°C. Formulations containing mPEG-S-S-DSPE were also incubated with DTT (100 mM) at pH 7.4 or pH 5.5. At specified time points, aliquots were added to HEPES buffer (pH 7.4) and the fluorescence intensity of the sample was determined using a spectrofluorimeter. Released HPTS was determined as the percentage increase in the sample fluorescence over that of the preincubation sample treated with Triton X-100 (100% release). The formulations consisted of DOPE, or DOPE/mPEG-DSPE, or DOPE/mPEG-S-S-DSPE, 1:0.05 molar ratio. Results are from a representative experiment, and are means of triplicate analyses ± S.D. (error bars are smaller than markers in many cases). (A) ●, DOPE, no PEG-DSPE, pH 7.4; ○, DOPE, no PEG-DSPE, pH 5.5; ▲, DOPE/mPEG-DSPE, pH 7.4; △, DOPE/mPEG-DSPE, pH 5.5. (B) ■, DOPE/mPEG-S-S-DSPE, pH 7.4; ▼, DOPE/mPEG-S-S-DSPE, pH 5.5; □, DOPE/mPEG-S-S-DSPE+DTT, pH 7.4; ▽, DOPE/mPEG-S-S-DSPE+DTT, pH 5.5.

(cholesteryl hemisuccinate), which is net negatively charged at pH 7.4, has been widely used to stabilize DOPE in the bilayer state at neutral pH. Protonation of CHEMS at low pH (<6.0) accelerates the destabilization of DOPE vesicles by promoting the formation of the hexagonal (H_{II}) phase [16,36]. DOPE/CHEMS formulations without mPEG-DSPE had little leakage at pH 7.4, but rapidly released encapsulated dye at pH 5.5 (Fig. 2A). At pH 5.5 or 7.4 less than 10% leakage occurred over 24 h when these liposomes were stabilized with 5 mol% mPEG-DSPE (Fig. 2A). In the presence of DTT, DOPE/

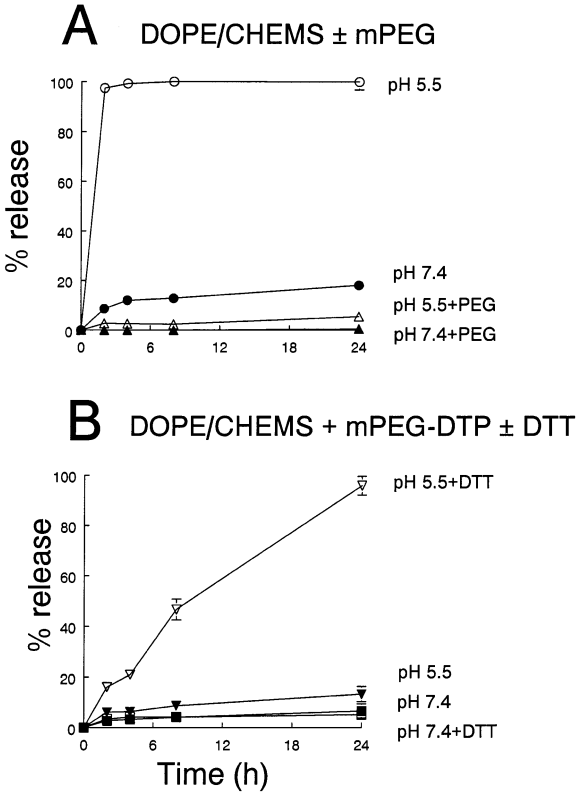

Fig. 2. Leakage of encapsulated HPTS from DOPE/CHEMS formulations ± mPEG-DSPE or mPEG-S-S-DSPE in buffers of varying pH at 37°C (± DTT). HPTS encapsulated in DOPE/CHEMS formulations stabilized with various PEG-DSPE conjugates (cleavable or non-cleavable) at pH 9.0 were diluted into buffer at either pH 7.4 or pH 5.5, at 37°C. Incubation with DTT, determination of released fluorescence and determination of released HPTS were as described in the legend for Fig. 1. The formulations consisted of DOPE/CHEMS alone (6:4 molar ratio), or DOPE/CHEMS/mPEG-DSPE or DOPE/CHEMS/mPEG-S-S-DSPE, 6:4:0.3 molar ratio. Results are from a representative experiment, and are means of triplicate analyses ± S.D. (A) ●, DOPE/CHEMS, no mPEG-DSPE, pH 7.4; ○, DOPE/CHEMS, no mPEG-DSPE, pH 5.5; ▲, DOPE/CHEMS/mPEG-DSPE, pH 7.4; △, DOPE/CHEMS/mPEG-DSPE, pH 5.5. (B) ■, DOPE/CHEMS/mPEG-S-S-DSPE, pH 7.4; ▼, DOPE/CHEMS/mPEG-S-S-DSPE, pH 5.5; □, DOPE/CHEMS/mPEG-S-S-DSPE+DTT, pH 7.4; ▽, DOPE/CHEMS/mPEG-S-S-DSPE+DTT, pH 5.5.

CHEMS liposomes containing 5 mol% mPEG-S-S-DSPE had a release half-life of approximately 8 h at pH 5.5, while little release occurred at pH 7.4 (Fig. 2B); DTT treatment of DOPE/CHEMS liposomes stabilized with 3 mol% mPEG-S-S-DSPE resulted in intermediate leakage rates (data not shown).

3.2. Release of liposomal HPTS by cell-free extracts (CFE)

Since CFE is expected to contain cytoplasmic and lysosomal enzymes, the leakage of the hydrophilic, membrane-impermeable dye HPTS from formulations exposed to CFE may mimic the process of destabilization, in an intracellular environment, of liposomes stabilized with either mPEG-DSPE or mPEG-S-S-DSPE. CFE was adjusted to pH 5.5, approxi-

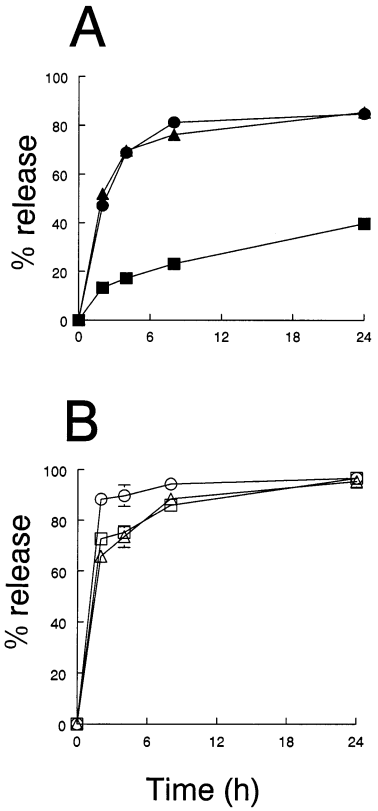

Fig. 3. Leakage of encapsulated HPTS from formulations ± mPEG-S-S-DSPE in cell-free extract. DOPE liposomes (A) or DOPE/CHEMS liposomes (B) containing encapsulated HPTS, and different proportions of mPEG-S-S-DSPE in the lipid mix, were incubated in cell-free extract adjusted to pH 5.5 at 37°C. The fluorescence intensity of the sample and the percentage of released HPTS were determined as described in the legend of Fig. 1. Results are from a representative experiment, and are means of triplicate analyses ± S.D. (A) ●, DOPE alone; ▲, DOPE/mPEG-S-S-DSPE (1:0.03); ■, DOPE/mPEG-S-S-DSPE (1:0.05). (B) ○, DOPE/CHEMS (6:4); △, DOPE/CHEMS/mPEG-S-S-DSPE (6:4:0.18); □, DOPE/CHEMS/mPEG-S-S-DSPE (6:4:0.3).

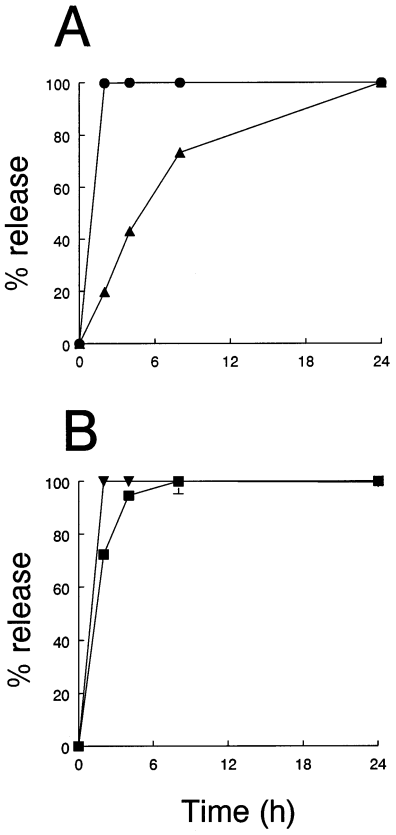

Fig. 4. Leakage in human plasma of encapsulated DXR from DOPE or DOPE/CHEMS formulations containing mPEG-DSPE or mPEG-S-S-DSPE. DOPE or DOPE/CHEMS liposomes, containing mPEG-DSPE or mPEG-S-S-DSPE, with anti-CD19 as a targeting moiety, were incubated in human plasma (pH 7.4) at 37°C. At specified time points, aliquots were collected from the incubation mixture, and diluted in HEPES buffer (pH 7.4), the fluorescence intensity of the sample was determined using a spectrofluorimeter, and released DXR was determined as the percentage increase in the sample fluorescence over that of samples preincubated with Triton X-100. Results are from a representative experiment, and are means of triplicate analyses ± S.D. (A) ●, anti-CD19-targeted, DXR-loaded DOPE/mPEG-DSPE/Mal-PEG-DSPE (1:0.04:0.01); ▲, anti-CD19-targeted, DXR-loaded DOPE/CHEMS/mPEG-DSPE/Mal-PEG-DSPE (6:4:0.2:0.1). (B) ■, anti-CD19-targeted, DXR-loaded DOPE/mPEG-S-S-DSPE/Mal-PEG-DSPE (1:0.04:0.01); ▼, anti-CD19-targeted, DXR-loaded DOPE/CHEMS/mPEG-S-S-DSPE/Mal-PEG-DSPE (6:4:0.2:0.1).

mating the lysosomal pH of between 5 and 6.5 [37,38]. DOPE formulations, or DOPE formulations with 3 mol% mPEG-S-S-DSPE, released their contents in CFE with half-lives of 1.7 h (Fig. 3A). The release rate in CFE of the DOPE formulation with 5 mol% mPEG-S-S-DSPE was considerably slower; only 40% of encapsulated dye was released over 24 h (Fig. 3A). All three DOPE/CHEMS formulations, with or without mPEG-S-S-DSPE, released HPTS rapidly in CFE at pH 5.5; this release was almost complete by 8 h (Fig. 3B). DOPE/CHEMS and DOPE/CHEMS/mPEG-S-S-DSPE formulations incubated in CFE adjusted to pH 7.4 released HPTS at much slower rates (data not shown).

3.3. Leakage of HPTS or DXR from pH-sensitive liposomes incubated in human plasma

Having evaluated the leakage rates of the hydrophilic compound HPTS from various liposome formulations in buffer or CFE, we proceeded to evaluate leakage in 90% human plasma at 37°C. The chemical structure of the loaded drug will have an effect on the rate of its leakage from a liposome formulation. HPTS is passively loaded into liposomes as the water-soluble (but fluorescence-quenched) complex, HPTS-DPX. When HPTS-DPX leaks from liposomes, it dissociates into free HPTS and DPX, increasing the HPTS fluorescence when excited at 413 nm. Less than 20% of encapsulated HPTS leaked from DOPE/mPEG-DSPE, DOPE/CHEMS/mPEG-DSPE, DOPE/mPEG-S-S-DSPE or DOPE/CHEMS/mPEG-S-S-DSPE formulations over 24 h in human plasma (not shown).

Doxorubicin is normally actively loaded into liposomes at a pH of about 5.5, using an ammonium sulfate gradient, resulting in the precipitation of a $(DXR\text{-}NH_3)_2SO_4$ gel inside the liposomes [29]. However, at the higher pH required to assemble DOPE/CHEMS/PEG-DSPE into liposomes, it is not known whether DXR forms a precipitate inside liposomes during active loading, and this may affect the rate at which DXR leaks from these pH-sensitive liposomes. The leakage of DXR in human plasma was examined for anti-CD19-targeted liposomes of DOPE/mPEG-DSPE, DOPE/CHEMS/mPEG-DSPE (Fig. 4A), DOPE/mPEG-S-S-DSPE or DOPE/CHEMS/mPEG-S-S-DSPE (Fig. 4B) liposomes. There was rapid leakage of DXR from all formulations with the exception of DOPE/CHEMS/mPEG-DSPE, which had a somewhat slower (but still rapid) leakage rate (Fig. 4A). There was no significant difference in the rate of DXR release between the anti-CD19-targeted and similar non-targeted formulations (containing 5 mol % mPEG) in the presence of human plasma (data not shown).

Before using these liposomes in cell-culture experiments, we also examined whether anti-CD19-targeted DXR-loaded formulations of the same lipid compositions described in the previous paragraph were sta-

Table 1
Cytotoxicity of free doxorubicin (DXR) and liposomal formulations of DXR (with or without anti-CD19) to $CD19^+$ Namalwa cells

Formulation of DXR-loaded liposomes	IC_{50} (μM DXR)
DOPE/mPEG-DSPE (1:0.05)	7.0 ± 2.2
DOPE/mPEG-DSPE/Mal-PEG-DSPE[anti-CD19] (1:0.04:0.01)	0.2 ± 0.1
DOPE/mPEG-S-S-DSPE (1:0.03)	8.9 ± 4.7
DOPE/mPEG-S-S-DSPE/Mal-PEG-DSPE[anti-CD19] (1:0.02:0.01)	1.5 ± 0.7
DOPE/CHEMS/mPEG-DSPE (6:4:0.3)	4.2 ± 1.1
DOPE/CHEMS/mPEG-DSPE/Mal-PEG-DSPE[anti-CD19] (6:4:0.24:0.06)	0.4 ± 0.1
DOPE/CHEMS/mPEG-S-S-DSPE (6:4:0.18)	6.0 ± 0.8
DOPE/CHEMS/mPEG-S-S-DSPE/Mal-PEG-DSPE[anti-CD19] (6:4:0.12:0.06)	3.3 ± 1.0
HSPC/CHOL/mPEG-DSPE	> 200
HSPC/CHOL/mPEG-DSPE/Mal-PEG-DSPE[anti-CD19] (2:1:0.08:0.02)	35.4 ± 12.7
Free DXR	0.8 ± 0.7

Namalwa cells (5×10^5) were incubated with free DXR or various formulations of liposome-encapsulated DXR with or without anti-CD19 mAb. mAb coupling via the PEG-Maleimide method was 65–80 μg mAb/μmol PL; DXR loading was 140–160 μg DXR/μmol PL (0.24–0.28 μmol DXR/μmol PL). After 1 h, cells were washed free of drug and then incubated for a further 48 h. Cytotoxicities were determined by the MTT assay and are expressed as mean IC_{50} ± S.D. (n = 3–6).

ble in cell culture media containing 10% FBS at 37°C. Over 24 h there was less than 10% DXR leakage (data not shown).

3.4. In vitro cytotoxicity of targeted, PEG-DSPE-stabilized, pH-sensitive liposomes

We compared the cytotoxicity of anti-CD19-targeted DOPE or DOPE/CHEMS formulations of liposomal DXR, stabilized with either mPEG-DSPE or mPEG-S-S-DSPE, to the cytotoxicity for non-targeted formulations stabilized with mPEG-DSPE or mPEG-S-S-DSPE, and to DXR-SL, DXR-SIL[anti-CD19] or free DXR (Table 1). All the DOPE or DOPE/CHEMS formulations, either targeted or non-targeted, had significantly lower IC_{50}s than the DXR-SL or DXR-SIL[anti-CD19] formulations ($P < 0.001$). Possibly this is because the overall release rates of DXR from the DOPE or DOPE/CHEMS formulations (Figs. 1–4) are higher than the release rates of DXR from DXR-SL or DXR-SIL[anti-CD19], which have a negligible release of drug on the time scale of our experiments [4]. Targeted DOPE and DOPE/CHEMS formulations had IC_{50}s that were comparable in most instances to those for free DXR (Table 1). The IC_{50}s of DXR-loaded targeted formulations of either DOPE or DOPE/CHEMS (stabilized with mPEG-DSPE or mPEG-S-S-DSPE) were significantly lower than the IC_{50}s of the non-targeted formulations ($P < 0.05$ to $P < 0.001$). Notably, there was a trend for formulations that contained mPEG-DSPE to be slightly more cytotoxic than those that contained mPEG-S-S-DSPE, whether targeted or non-targeted. Targeted formulations without encapsulated DXR were not toxic at concentrations below 0.06 μM DOPE, which would correspond to a DXR concentration of 34.5 μM, if the formulations contained DXR (not shown).

3.5. Nuclear accumulation of doxorubicin within Namalwa cells

In theory, the rate of release of encapsulated DXR from the lysosomal apparatus, following internalization of targeted liposomes, is a determining factor in the cytotoxicity of liposome-encapsulated DXR. To demonstrate the kinetics of release of DXR from the

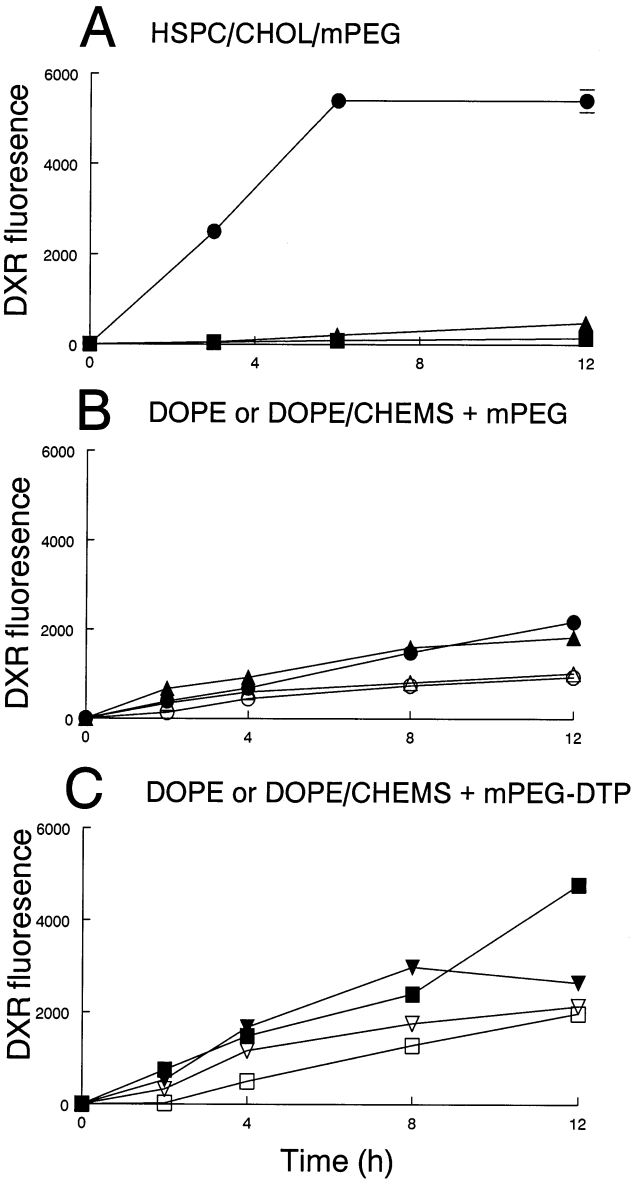

Fig. 5. In vitro nuclear accumulation of doxorubicin in Namalwa cells. The DXR nuclear accumulation assay is as described in Section 2. Results are from a representative experiment, and are means of triplicate analyses ± S.D. (A) ●, free DXR; ■, DXR-HSPC/CHOL/mPEG-DSPE, 2:1:0.1; ▲, anti-CD19 targeted DXR-HSPC/CHOL/mPEG-DSPE/Mal-PEG-DSPE, 2:1:0.08:0.02. (B) ●, anti-CD19-targeted DXR-DOPE/mPEG-DSPE/Mal-PEG-DSPE, 1:0.04:0.01; ▲, anti-CD19 targeted DXR-DOPE/CHEMS/mPEG-DSPE/Mal-PEG-DSPE, 6:4:0.24:0.06; ○, DXR-DOPE/mPEG-DSPE, 1:0.05; △, DXR-DOPE/CHEMS/mPEG-DSPE, 6:4:0.3. (C) ▼, anti-CD19 targeted DXR-DOPE/CHEMS/mPEG-S-S-DSPE/Mal-PEG-DSPE, 6:4:0.12:0.06; ■, anti-CD19-targeted DXR-DOPE/mPEG-S-S-DSPE/Mal-PEG-DSPE, 1:0.02:0.01; □, non-targeted DXR-DOPE/mPEG-S-S-DSPE, 1:0.03; ▽, non-targeted DXR-DOPE/CHEMS/mPEG-S-S-DSPE, 6:4:0.18.

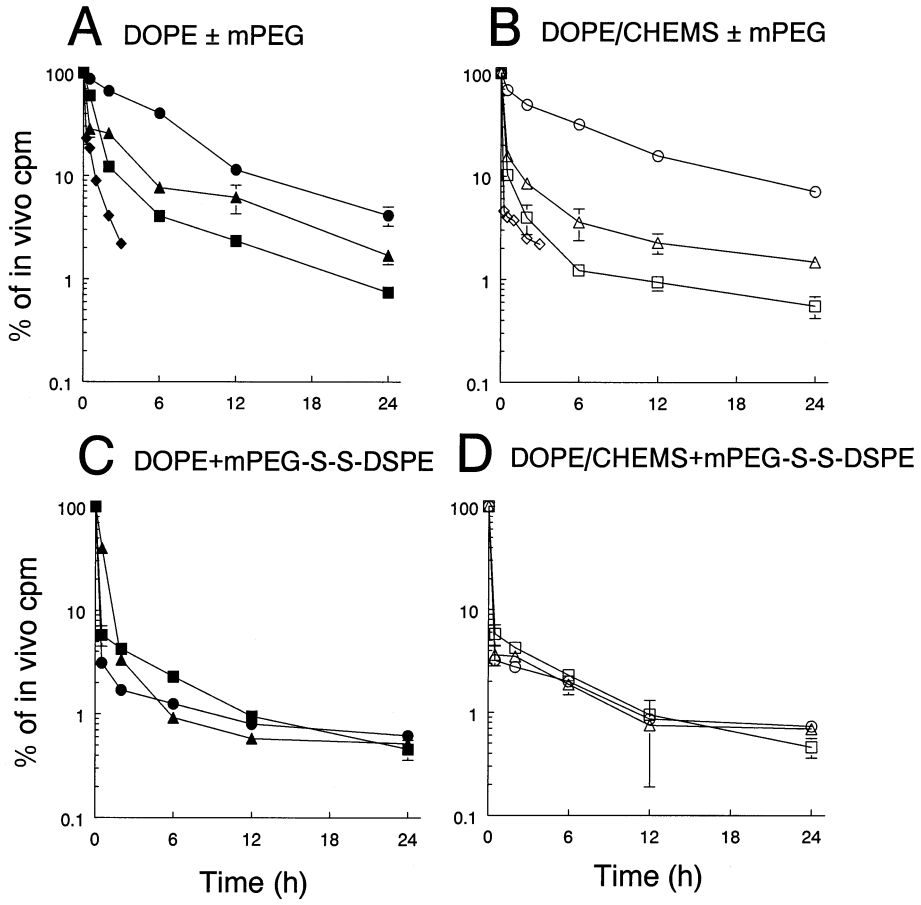

Fig. 6. Pharmacokinetics of DOPE or DOPE/CHEMS liposomes in BALB/c mice. Female BALB/c mice were injected i.v. via the tail vein with a single dose of 0.2 ml of [^{125}I]TI encapsulated in various liposomal formulations (0.5 μmol PL/mouse). At selected times after injection blood samples were collected and counted for ^{125}I. Results are from a representative experiment, and are means of triplicate analyses ± S.D. (A) ♦, DOPE; ■, DOPE/mPEG-DSPE, 1.0:0.03; ▲, DOPE/mPEG-DSPE, 1.0:0.05; ●, DOPE/mPEG-DSPE, 1.0:0.1. (B) ◇, DOPE/CHEMS; □, DOPE/CHEMS/mPEG-DSPE, 6:4:0.18; △, DOPE/CHEMS/mPEG-DSPE, 6:4:0.3; ○, DOPE/CHEMS/mPEG-DSPE, 6:4:0.6. (C) ■, DOPE/mPEG-S-S-DSPE/mPEG-DSPE, 1.0:0.02:0.01; ▲, DOPE/mPEG-S-S-DSPE/mPEG-DSPE, 1.0:0.04:0.01; ●, DOPE/mPEG-S-S-DSPE/mPEG-DSPE, 1.0:0.09:0.01. (D) □, DOPE/CHEMS/mPEG-S-S-DSPE/mPEG-DSPE, 6:4:0.012:0.06; △, DOPE/CHEMS/mPEG-S-S-DSPE/mPEG-DSPE, 6:4:0.024:0.06; ○, DOPE/CHEMS/mPEG-S-S-DSPE/mPEG-DSPE, 6:4:0.054:0.06.

endosomes, the rate of accumulation of DXR in cell nuclei was determined by using a DXR nuclear accumulation assay (Fig. 5). For each formulation, DXR was detectable in the nuclear fraction of cells after 2 or 3 h incubation. As expected in this in vitro assay, free DXR accumulated within the nuclei at the fastest rate (Fig. 5A). Rates of DXR release for targeted mPEG-DSPE-stabilized (Fig. 5B) or mPEG-S-S-DSPE-stabilized (Fig. 5C) DOPE or DOPE/CHEMS formulations were much faster than that of targeted DXR-SIL[anti-CD19] (Fig. 5A). The order of nuclear accumulation of DXR from liposomal formulations was: free drug > targeted mPEG-S-S-DSPE > targeted mPEG-DSPE > non-targeted mPEG-S-S-DSPE > non-targeted mPEG-DSPE > targeted DXR-SIL > SL. Interestingly, although they were not more cytotoxic than mPEG-containing formulations (Table 1), formulations containing mPEG-S-S-DSPE appeared to result in more rapid nuclear accumulations of DXR over the time course of these experiments (Fig. 5).

3.6. Blood elimination of PEG-DSPE-stabilized pH-sensitive liposomes

In order to determine the pharmacokinetics of the

Table 2
In vivo survival studies with triggered release formulations in SCID mice bearing $CD19^+$ human B-lymphoma (Namalwa) cells

Treatment	MST ± S.D.(days)	% ILS
Saline	21.0 ± 1.6	–
Free DXR	23.0 ± 1.4	9.5
DOPE/CHEMS/mPEG-DSPE	27.8 ± 1.6	32.4
DOPE/CHEMS/mPEG-S-S-DSPE	25.8 ± 4.0	22.9
HSPC/CHOL/mPEG-DSPE	24.2 ± 1.3	15.2
DOPE/CHEMS/mPEG-DSPE[anti-CD19]	42.2 ± 4.1	101
DOPE/CHEMS/mPEG-S-S-DSPE[anti-CD19]	53.8 ± 6.5	156.2
HSPC/CHOL/mPEG-DSPE[anti-CD19]	43.2 ± 5.7	105.7

SCID mice were implanted i.v. with 5×10^6 cells, then injected i.v. 24 h later with either saline, free DXR, or DXR-loaded in either anti-CD19 targeted or non-targeted, mPEG-DSPE-stabilized or mPEG-S-S-DSPE-stabilized formulations composed of HSPC/CHOL/mPEG-DSPE/Mal-PEG-DSPE (2:1:0.08:0.02) or DOPE/CHEMS/mPEG-DSPE/Mal-PEG-DSPE (6:4:0.24:0.06) or DOPE/CHEMS/mPEG-S-S-DSPE/Mal-PEG-DSPE (6:4:0.24:0.06). Liposomes composed of DOPE/CHEMS/mPEG-DSPE/Mal-PEG-DSPE, DOPE/CHEMS/mPEG-S-S-DSPE/Mal-PEG-DSPE, or HSPC/CHOL/mPEG-DSPE/Mal-PEG-DSPE contained 33.3, 31.2 or 58.4 μg anti-CD19/μmol PL, respectively. All treatment groups received DXR 3 mg/kg. MST, mean survival time; ILS, increased life span over MST of control.

liposome preparations, liposomes containing radiolabeled [^{125}I]TI were prepared and injected into the tail vein of mice at a PL dose of 0.5 μmol/mouse. Circulation times increased with increasing concentration of mPEG-DSPE in either DOPE or DOPE/CHEMS liposome formulations (Fig. 6A,B). Approximately 5–10% of the injected liposomes still remained in the blood 24 h after injection of liposomes containing 10 mol% mPEG-DSPE. Injection of DOPE or DOPE/CHEMS liposomes that were not stabilized with mPEG-DSPE resulted in rapid clearance of the liposomes. Liposomes accumulated primarily in the liver and spleen (not shown). Inclusion of from 2 to 9 mol% of mPEG-S-S-DSPE did not increase the circulation times for either the DOPE (Fig. 6C) or DOPE/CHEMS (Fig. 6D) formulations. All mPEG-S-S-DSPE formulations contained 1 mol% mPEG-DSPE to mimic the effect of adding 1 mol% coupling lipid to the formulations in targeting experiments. Increasing amounts of mPEG-S-S-DSPE in the formulations did not increase circulation half-lives of the formulations to any significant extent (Fig. 6A,B), likely because the mPEG-S-S-DSPE was rapidly cleaved in plasma.

3.7. In vivo therapeutic experiments

For therapeutic experiments, SCID mice (5 mice/group), implanted i.v. with 5×10^6 Namalwa cells, were treated i.v. at 24 h after implantation with single doses of 3 mg/kg free DXR or liposome-encapsulated DXR (Table 2). No evidence of drug toxicity was observed in any experimental group. None of the non-targeted DXR-loaded formulations showed improved therapeutic efficacy over the control group ($P > 0.05$). All groups treated with targeted formulations had significantly higher increased life spans (%ILS) than did the control group or groups treated with non-targeted formulations ($P < 0.001$). The group treated with DXR-DOPE/CHEMS/mPEG-S-S-DSPE/Mal-PEG-DSPE[anti-CD19] had a significantly increased %ILS compared to the other targeted treatment groups (vs. DXR-DOPE/CHEMS/mPEG-DSPE/Mal-PEG-DSPE[anti-CD19], $P < 0.001$; vs. DXR-SIL[anti-CD19], $P < 0.01$).

4. Discussion

Ligand-mediated targeting of drug carrier systems like SIL can improve site-specific drug delivery [4–14]. We have previously demonstrated that the targeting of DXR-SIL[anti-CD19] to CD19, an internalizing epitope expressed on cells of B-cell lineage, increases its therapeutic index against B-cell-derived haematological malignancies [4,39]. In this study we have attempted to further increase the efficacy of anti-CD19-targeted liposomal formulations of DXR by increasing the rate of intracellular drug release through the strategy of employing pH-sensitive tar-

geted liposomes. Liposomes exhibiting pH sensitivity have been described previously, but their potential for use in vivo has been hampered by their rapid removal from circulation into the MPS. Although inclusion of mPEG-DSPE into pH-sensitive, DOPE-containing liposome formulations increased both the stability and the circulation half-lives of the liposomes, these liposomes did not destabilize at low pH, as determined by an in vitro dye release assay [23–25]. Theoretically, this loss of pH sensitivity could be avoided through the incorporation of cleavable PEG-lipid derivatives into the formulations. As previously reported, addition of 3 mol% of a thiol cleavable PEG-derivative, mPEG-S-S-DSPE, significantly increased the stability of DOPE or DOPE/CHEMS formulations in vitro; the pH-dependent leakage of HPTS was restored by treatment with a thiolytic agent, DTT, which cleaves the PEG chains from mPEG-S-S-DSPE [25].

At physiological pH, DOPE is in the H_{II} state and is incapable of entrapping contents (since there is no aqueous interior space). Bilayer liposomes of DOPE can be made at pHs above the pK_a for the amine group, i.e., above a pH of approximately 8.5, and these structures will retain contents within their aqueous interior [17]. DOPE can also be stabilized in the bilayer state in the presence of CHEMS at pHs where the acid is negatively charged, i.e., above a pH of approximately 6.0 [36]. These considerations explain the results we presented in Figs. 1 and 2 for release of HPTS from DOPE or DOPE/CHEMS liposomes in the absence of PEG-lipid. It can also be seen from Figs. 1 and 2 that inclusion of a small mol% of a molecule having a bulky headgroup, e.g., a PEG-lipid derivative, can stabilize DOPE or DOPE/CHEMS in the bilayer state in the pH range of 5.5–7.4. When a disulfide-containing lipopolymer, mPEG-S-S-DSPE, is included in the lipid bilayer, thiolytic cleavage of the PEG headgroup with DTT leaves a bilayer that is inherently unstable, being composed of only DOPE or DOPE/CHEMS and a small amount of DSPE. As can be seen in Fig. 1B and 2B, liposomes containing mPEG-S-S-DSPE have an increased rate of contents release at pH 5.5, although contents release is less rapid than that seen for similar liposomes that never contained PEG (Fig. 1A and 2A). A slow rate of cleavage of mPEG-S-S-DSPE, or incomplete cleavage of mPEG-S-S-DSPE likely contributes to the slower release rate. Cellular enzymes extracted from cells are capable of mimicking the action of DTT in cleaving mPEG-S-S-DSPE (Fig. 3).

DXR, but not HPTS, was rapidly released from liposomes in 90% human plasma (Fig. 4). This might be due to the mechanism used for DXR loading. DXR is a weak base: the charged form of the drug is favored at acid pH, and the neutral form of the drug (which is membrane permeable) is favored at basic pH. In the loading method we used for DXR, the interior of the liposomes contains ammonium sulfate. The drug in the neutral form crosses the liposomal membrane from exterior to interior where it is protonated, leading to the formation of an insoluble DXR-sulfate precipitate [29]. These preparations are very stable, with extremely slow rates of drug release. However, DOPE will not hydrate to form bilayer liposomes at acid pH, so we prepared DOPE and DOPE/CHEMS liposomes in a pH 8.5–9.0 ammonium sulfate solution. Although the loading efficiency at this pH is greater than 95%, the neutral form of DXR is favored at pH 8.5, and hence it may not be able to form a stable precipitate with ammonium sulfate. We have tried forming and hydrating the pH-sensitive liposomes at basic pH ($>$pH 9.0) in the presence of mPEG-S-S-DSPE, then lowering the pH prior to drug loading. Although the DOPE will stay in the bilayer state when the pH is lowered, we have not yet been successful in achieving pH-sensitive formulations of DXR that do not have fairly high rates of drug release in human plasma. Future experiments may need to be done with other antineoplastic drugs that are more amenable to stable loading into these DXR-containing pH-sensitive formulations.

To demonstrate the kinetics of release of DXR from the lysosomal apparatus, the rate of accumulation of DXR in cellular nuclei was measured as a function of time by using a DXR nuclear accumulation assay (Fig. 5). As expected, little nuclear uptake of DXR was seen for DXR-SL, since it has a very slow rate of drug release [40] and the only mechanism for the drug to reach the nucleus is through passive diffusion into the cell of drug released from the liposomes into the culture medium. When these liposomes are targeted with anti-CD19 (DXR-SIL[anti-CD19]), nuclear levels of DXR in Namalwa

cells increased approximately 3-fold, most likely because the liposome drug packages were taken up into the cell by receptor-mediated internalization. However, the intracellular rate of drug release from endosomes is slow [15], hence the comparatively low levels of nuclear DXR. The more rapid rates of drug release for the non-targeted pH-sensitive formulations (at all pHs) compared with the DXR-SIL[anti-CD19] and DXR-SL formulations led to more drug release into the media and hence more drug delivery to the nucleus via a passive diffusion mechanism. It is possible, but not confirmed, that the DOPE and DOPE/CHEMS formulations may also deliver some drug into the cell cytoplasm by a fusion mechanism, and from there it would be available to traffic to the cell nucleus. As might be predicted from their in vitro drug release rates and their ability to trigger receptor-mediated endocytosis, the targeted mPEG-S-S-DSPE formulations have the highest rates and levels of nuclear accumulation among the liposomal formulations.

In Table 1, the cytotoxicity (IC_{50}) values for these formulations parallel the nuclear accumulation data, supporting the hypothesis that not only the total amount of uptake of liposomal drug, but also the rate of release of the encapsulated drug governs the cytotoxicities of liposomal drugs. Total cellular uptake of liposomal drug can be increased via the mechanism of receptor-mediated internalization, and intracellular drug release can be increased through mechanisms such as the pH-sensitive triggered-release mechanism, described here. One anomaly is the observation that liposomes containing mPEG-S-S-DSPE have higher nuclear accumulations (Fig. 5), and faster rates of drug release in CFE or buffer, but do not have higher cytotoxicity than mPEG-containing liposomes (Table 1). These results might be explained in light of the recent observations of Goren et al. in experiments where the cytotoxicity of folate-targeted liposomal DXR was examined in MDR cells that overexpress the folate receptor [14]. In spite of up to 6-fold higher accumulation of DXR in the cell nucleus, when it was delivered in targeted liposomes relative to free drug, they were unable to reverse multidrug resistance in their cells. The authors interpret these results to mean that a long period of time is necessary to allow for the disaggregation of DXR (dissolution of the insoluble DXR-SO_4 and solubilization of DXR dimers) following the internalization of liposomes and their degradation within the cell interior. The 48 h incubation time that we used in our cytotoxicity experiments after washing away the drugs may not have been long enough to allow for sufficient disaggregation of the DXR to see a difference between the liposomes containing mPEG-S-S-DSPE versus mPEG-DSPE.

Previous studies have shown that inclusion of mPEG-DSPE increases the circulation half-life of DOPE and DOPE/CHEMS formulations [24]. However, the inclusion of mPEG-S-S-DSPE did not increase the circulation time of the DOPE and DOPE/CHEMS formulations (Fig. 6). Since 10 mol% of mPEG-DSPE in either DOPE or DOPE/CHEMS liposomes gave significantly longer circulation half-lives than 9 mol% mPEG-S-S-DSPE plus 1 mol% mPEG-DSPE (Fig. 6), we believe that the short half-life of liposomes containing mPEG-S-S-DSPE is probably due to rapid cleavage of the disulfide linkage by blood components, e.g. cysteine, in vivo. The loss of steric hindrance due to the loss of PEG from the liposomes would decrease their stability in blood and increase the uptake of liposomes by the MPS.

We have previously shown that DXR-SIL[anti-CD19] binds to, and is internalized by, human B-lymphoma (Namalwa) cells within a few minutes [4,15]. If this process occurs rapidly enough in vivo, then we may still be able to achieve good therapeutic results with targeted, mPEG-S-S-DSPE-stabilized, pH-sensitive formulations even though these formulations release the encapsulated DXR rapidly and have short circulation half-lives in vivo. To test this hypothesis, we conducted therapeutic studies with these formulations in SCID mice inoculated with $CD19^+$ Namalwa cells. In spite of their rapid leakage and clearance, it appears that the therapeutic efficacy of anti-CD19-targeted, mPEG-DSPE-stabilized DOPE/CHEMS liposomes was similar to that of DXR-SIL[anti-CD19] liposomes, and the efficacy of mPEG-S-S-DSPE-stabilized DOPE/CHEMS formulations was moderately higher (Table 2). The longer time of exposure of the liposomal drugs to the cells in vivo may have allowed more time for drug disaggregation, supporting the studies of Goren et al. [14]. These results strongly suggest that if retention of encapsulated drug and stability in blood circulation

could be further improved, then triggered release formulations targeted against internalizing epitopes will be able to substantially increase the therapeutic index of encapsulated drug in vivo. Development of a formulation that has both good drug retention properties in the presence of human plasma and prolonged circulation time is in progress in our laboratories.

In this study, we developed targeted, pH-sensitive liposomes sterically stabilized by PEG, which was surface-grafted via a chemically-cleavable bond (mPEG-S-S-DSPE). These targeted, pH-sensitive formulations stabilized with mPEG-S-S-DSPE (triggered-release formulations) delivered encapsulated DXR efficiently into the cytoplasm of target cells and improved the cytotoxicity of encapsulated DXR in vitro, relative to a targeted formulation that lacked triggered release properties, i.e., DXR-SIL[anti-CD19]. The targeted triggered release formulations resulted in a modest increase in therapeutic efficacy in vivo. The usefulness of this approach has been demonstrated in this study. Further optimization of these formulations may result in additional increases in their therapeutic efficacy in vivo. In addition, the unique properties of these targeted, triggered-release formulations may be used for the intracytoplasmic delivery of plasmids, antisense oligonucleotides, and ribozymes in vivo for the treatment of cancer and viral infections.

Acknowledgements

This work was supported by the Medical Research Council of Canada (MT-9127) and Alza Corp., Menlo Park, CA. We thank Susan Cubitt for her assistance in the preparation of the monoclonal antibody.

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