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Porphyrin-phospholipid liposomes with tunable leakiness

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ABSTRACT

Drug bioavailability is a key consideration for drug delivery systems. When loaded with doxorubicin, liposomes containing 5 molar % porphyrin-phospholipid (HPPH liposomes) exhibited *in vitro* and *in vivo* serum stability that could be fine-tuned by varying the drug-to-lipid ratio. A higher drug loading ratio destabilized the liposomes, in contrast to standard liposomes which displayed an opposite and less pronounced trend. Following systemic administration of HPPH liposomes, near infrared laser irradiation induced vascular photodynamic damage, resulting in enhanced liposomal doxorubicin accumulation in tumors. In laser-irradiated tumors, the use of leaky HPPH liposomes resulted in improved doxorubicin bioavailability compared to stable standard liposomes. Using this approach, a single photo-treatment with 10 mg/kg doxorubicin rapidly eradicated tumors in athymic nude mice bearing KB or MIA Paca-2 xenografts.

1. Introduction

Liposomes are self-assembled, lipid-based nanocarriers that are used clinically for drug delivery [1–3]. Long-circulating liposomal doxorubicin (Dox) has been approved for treatment of various cancers [4]. Despite enhanced intratumoral deposition, the clinical efficacy of long-circulating liposomal Dox is not necessarily superior to that of free Dox [5,6] while the main benefit is reduced cardiotoxicity compared to the free drug [6]. Slow drug release from the carrier reduces bioavailability and efficacy [7]. PEGylated liposomes tend to produce greater tumoral drug deposition due to their longer circulation times [8–10]. However, to become bioavailable, the encapsulated drug needs to be released from the carrier. Drugs loaded in stable and long-circulating liposomes remain partially entrapped and inactive after extravasation into the tumor [11].

Numerous strategies have been proposed to improve drug bioavailability from liposomes and other nanocarriers [12]. These include pH-sensitive liposomes [13,14], heat sensitive liposomes [15,16] and enzymatic responsive liposomes [17], all of which release their content in response to local physiological or externally applied stimuli. An alternative and more generalized approach is to design liposomes with a faster drug release rate. This strategy has been demonstrated with liposomal mitoxantrone, where shorter-circulating formulations showed therapeutic advantages over more stable, longer-circulating ones [18–20]. Varying the drug to lipid ratio has been proposed as a simple way of potentially controlling the rates of drug release [21–23]. Here, a faster-releasing and shorter-circulating liposome system is explored which exhibits enhanced bioavailability and therapeutic efficacy following tumor vasculature permeabilization.

Vascular barriers and heterogeneous drug distribution are central challenges for delivery of nanoparticulate chemotherapeutics to solid tumors [24]. Despite the endothelial defects found in growing tumor blood vessels, the enhanced permeability and retention (EPR) effect alone is less than ideal to enable sufficient nanoparticle extravasation for tumor eradication and additional strategies can be beneficial [25–27].

Photodynamic therapy (PDT) can be used to permeabilize tumor vasculature, although it can also induce thrombus formation and blood flow stasis [28]. PDT generates singlet oxygen, which can damage vascular endothelial cells and induce the formation of endothelial intercellular gaps, resulting in leakier tumor microvasculature and an augmented EPR effect [29,30]. PDT enhances liposomal drug delivery in mouse models of cancer in different scenarios using photosensitizers that: 1) extravasate to the tumor after leaving circulation [31]; 2) remain in blood circulation for vascular PDT [29]; and 3) are specifically targeted to tumor neovasculature [32].

2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) is a second generation photosensitizer under clinical evaluation as a PDT agent [33]. Our group previously conjugated HPPH to a lysophosphatidylcholine to generate a porphyrin-phospholipid (PoP) and incorporated it into liposomes which could then be permeabilized with near infrared light [34]. PoP has been used for a variety of purposes including light-triggered release of Dox, a handle for radionuclides for positron emission tomography, optical imaging, and a scaffold for simple peptide functionalization

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of liposomes [35–38]. In this study, HPPH-lipid is used not for the effect of light-triggered drug release, but rather for two other purposes: 1) for serum-induced tunable Dox leakiness from liposomes and 2) for PDT-mediated tumor vasculature permeabilization. Relatively short-circulating, leaky formulations of Dox are shown to have superior anti-tumor efficacy following PDT-mediated tumor vasculature permeabilization.

2. Methods

2.1. Liposome preparation

Chemicals were obtained from Sigma unless noted otherwise. HPPH-lipid was synthesized as previously described [34]. HPPH liposome composition was 45 mol% 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti #850365P), 45 mol% cholesterol (CHOL, Avanti #700000P), 5 mol% HPPH-lipid and 5 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2K; Avanti #880120P). Stable standard (std.) liposomes were composed of 50 mol% DSPC, 45 mol% cholesterol and 5 mol% DSPE-PEG2K. Unless stated otherwise, leaky HPPH liposomes and stable std. liposomes used a 1:4 drug: lipid (D:L) loading molar ratio. Lipids in the indicated molar ratios were fully dissolved in 2 mL ethanol at 70 °C, then 8 mL 250 mM ammonium sulfate (pH 5.5) buffer was injected to the lipid solution. The lipid solution was passed 10 times at 70 °C through a high pressure lipid extruder (Northern Lipids) with sequentially stacked polycarbonate membranes of 0.2, 0.1 and 0.08 μm pore size. Free ammonium sulfate was removed by dialysis in a 10% sucrose solution with 10 mM HEPES (pH 7.4). To prepare HPPH liposomes or std. liposomes, doxorubicin (LC Labs #D-4000) was then loaded by adding the indicated ratio of drug into liposome solutions and incubating at 60 °C for 1 h. Liposome sizes were determined in phosphate buffered saline (PBS) at room temperature by dynamic light scattering to be 89–96 nm for both HPPH liposomes and std. liposomes. Loading efficiency was determined by running 500 μL of liposomes diluted 10 times over a Sephadex G-75 column. 24 × 1 mL fractions were collected and the loading efficiency was determined as the percentage of the drugs in the liposome-containing fractions (which elute in the first 3–8 mL). Dox was measured using fluorescence with an excitation of 480 nm and emission of 590 nm.

2.2. Cryo-electron microscopy

Approximately 3.4 μL of stable std. liposomes (D:L molar ratio 1:4, ~20 mg/mL lipids), leaky HPPH liposomes (D:L molar ratio 1:4) or empty HPPH liposomes in buffer containing 10% sucrose and 10 mM histidine were deposited in holey carbon grids (c-flat CF-2/2-2C-T) prepared with an additional layer of continuous carbon ~5–10 nm thick. Grids were treated with glow discharge at 5 mA for 15 s before the liposome samples were deposited on them. The grids were then blotted and plunged in liquid ethane at −180 °C using a Vitrobot (FEI) with the blotting chamber maintained at 25 °C and 100% relative humidity. Liposomes were imaged in a JEOL2010F transmission electron microscope at 200 kV using a Gatan 914 cryo-holder. Images were recorded in SO-163 films and collected using a total dose of ~15–20 electrons per $Å^2$, magnification ×50,000 and a defocus that ranged between −7 to −11 μm. Micrographs were digitized in a Nikon Super Coolscan 9000 scanner.

To quantify the morphological differences between leaky HPPH liposomes and stable std. liposomes as well as for the crystals they enclosed, we measured the two major dimensions of the liposomes (a and b) and the crystals (a′ and b′) (Fig. 2B, diagrams on the right). From these measurements the aspect ratio of both the liposomes themselves and those from the enclosed crystals were calculated. Aspect ratios were used to discriminate liposomes and crystals in both samples into brackets defined by the values indicated in the graphs. The number of liposomes or crystals in each bracket was expressed as a percentage of the total number of liposomes (n = 50). Percentages and aspect ratios were plotted as histograms that were fitted to a polynomial function producing the distributions in Fig. 2B (left panel).

2.3. In vitro stability

For serum stability measurements, HPPH liposomes or std. liposomes (~20 mg/mL lipids) were diluted 200 times in PBS containing 50% mature bovine serum (Pel-Freez #37218-5), or 50 mg/mL bovine serum albumin (BSA, AMRESCO #9048-46-8), or 25 mg/mL bovine gamma globulin (BGG, Pel-Freez #27005-1). Initial readings were taken and samples were incubated at 37 °C for 24 h. Triton X-100 was added to 0.25% to lyse the liposomes and final fluorescence values were read. Dox release was calculated according to the formula % Release = $(F_{Final} - F_{initial})/(F_{TX\text{-}100} - F_{initial}) \times 100\%$.

For gel permeation, 50 μL of leaky HPPH liposomes (D:L molar ratio 1:4, Dox 3.5 mg/ml), stable std. liposomes (D:L molar ratio 1:4, Dox 3.5 mg/ml) or an equal amount of free Dox were incubated in 500 μl of 50% bovine serum for 24 h at 37 °C. A 30% acrylamide solution was prepared with Acryl/Bis 19:1 premixed powder (Amresco # 0729); 0.05% ammonium persulfate and 0.05% TEMED were added and the solution was poured into 3.5 cm diameter petri dishes. Gel was polymerized overnight. 50 μL of sample was added to a hole in the center of the gel. Dox fluorescence was imaged with a LUMINA IVIS imager immediately and after 6 h of incubation at room temperature, using 465 nm excitation and a DS-red emission filter.

For ex vivo tumor permeation, MIA Paca-2 tumors from sacrificed mice were removed and incubated with 100 μL leaky HPPH liposomes (3.5 mg/mL Dox), stable std. liposomes (3.5 mg/mL Dox) or an equal amount of free Dox in 2 mL of 100% mature bovine serum for 24 h at 37 °C. The tumor slices were prepared and scanned as described below.

2.4. Pharmacokinetics study

All procedures in this work performed on mice were approved by the University at Buffalo Institutional Animal Care and Use Committee. Female mice (female CD-1, 18–20 g, Charles River) were injected via tail vein with HPPH liposomes or std. liposomes (5 mg/kg or 10 mg/kg Dox) at the indicated D:L loading ratios. Small blood volumes were sampled at sub-mandibular and retro-orbital locations at the indicated time points. Blood was centrifuged at 2000 g for 15 min. 10 μL serum was added to 990 μL extraction buffer (0.075 N HCl, 90% isopropanol) and stored for 20 min at −20 °C. The samples were removed and warmed up to room temperature and centrifuged for 10 min at 10,000 *g*. The supernatants were collected and analyzed by fluorescence. Dox and HPPH (excitation 400 nm and emission 660 nm) concentrations were determined from standard curves. Non-compartmental pharmacokinetics parameters were analyzed by PKsolver [39].

2.5. Tumor deposition of Dox

Five week old female nude mice (Jackson Labs, #007850) were inoculated with 2×10^6 KB cells on both flanks and randomly grouped into 1) leaky HPPH liposomes or 2) stable std. liposomes + empty HPPH liposomes groups when the sizes of the tumors reached 6–8 mm (n = 16 per group). Injection dose for both formulations was 10 mg/kg based on Dox (D:L molar ratio 1:4) and 3.9 mg/kg HPPH lipid. Liposomes were injected via tail-vein and 15 min later, tumors were irradiated for 12.5 min with a light dose of 200 mW/cm^2 from a 665 nm laser diode (RPMC laser, LDX-3115-665). Mice were sacrificed and tumors were collected immediately after irradiation, 0.5 h, 4 h and 24 h after irradiation, n = 4 for each time point. For tumor drug deposition determination and biodistribution study, tumors and indicated organs were collected and homogenated in nuclear lysis buffer [0.25 mol/L sucrose, 5 mmol/L Tris–HCl, 1 mmol/l $MgSO_4$, 1 mmol/L $CaCl_2$ (pH 7.6)] and