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Research Paper

Fabrication of liposomal doxorubicin exhibiting ultrasensitivity against phospholipase A_2 for efficient pulmonary drug delivery to lung cancers

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ABSTRACT

Phospholipase A_2 (PLA_2) is expressed in inflammation-related tissue, including cancer tumors. We report that a hybrid liposome composed of phospholipid (DPPC) and PEGylated block-copolymer (Poloxamer 188) can rapidly release an encapsulated hydrophilic drug in the presence of PLA_2. DPPC/P188 liposomes released approximately 80% of the encapsulated calcein (a fluorescence marker) within 10 min in the presence of 120 mU of PLA_2 at 37 °C *in vitro*, whereas several other liposomal compositions used for inhalation therapy did not. DPPC/P188 liposomes were stable in the absence of PLA_2 at 37 °C after 60 min incubation and drug release by PLA_2 was dependent on the amount of P188 incorporated into the DPPC liposomes. Drug release from doxorubicin (DOX, anticancer drug)-loaded DPPC/P188 liposomes was facilitated at higher PLA_2 concentrations and was dependent on the temperature and the presence of calcium ion, thus partially explaining PLA_2-responsive drug release. DOX release from liposomes triggered by PLA_2 exhibited the same cytotoxic effects on the A549 lung cancer cell line as did DOX in free solution. These findings suggest that DPPC/P188 liposomes are a promising drug carrier for delivering drug efficiently at PLA_2-expressing sites such as inflammatory lung cancer.

1. Introduction

Liposomal drugs have been used to treat various challenging diseases by taking advantage of their drug delivery characteristics (Dawidczyk et al., 2014; Samad et al., 2007; Yingchoncharoen et al., 2016), and specifically the biocompatibility of liposomes. Liposomes are composed of phospholipid, the major component of cell membranes. Encapsulation in liposomes can prevent the side effects associated with hydrophilic and lipophilic drugs, which typically have a narrow therapeutic window, by preventing non-specific drug distribution to non-target organs. For example, Doxil® is a polyethylene glycol (PEG)-conjugated (PEGylated) liposome formulation that remarkably reduces the cardiotoxicity of the encapsulated anticancer drug (Barenholz, 2012; Xing et al., 2015).

The pulmonary route holds promise for the delivery of liposomal drugs both to local lung tissue and systemically (Chattopadhyay, 2013; Jaafar-Maalej et al., 2012; Mansour et al., 2009). Phospholipids are similar to the components of lung surfactant and thus do not elicit immunogenicity; in addition, liposome-mediated delivery prolongs the retention time of the drug at high concentration (de Jesus Valle et al., 2013; Omri et al., 1994; Wong et al., 2003). The retention time of an unencapsulated drug may only be several hours, whereas a liposomal drug can reside longer in pulmonary tissue. Therefore, direct drug delivery into lung tissue by inhalation enhances drug delivery efficiency. No inhalation liposomal drug is currently on the market but inhalable liposomal formulations of amikacin (ClinicalTrials.gov-NCT02628600) and ciprofloxacin are presently undergoing clinical trials (ClinicalTrials.gov-NCT01515007; ClinicalTrials.gov-NCT02104245; Serisier et al., 2013).

The triggered release of a drug from nanocarriers such as liposomes has been studied both as a strategy for controlling drug delivery (Mura et al., 2013) and various applied triggers (*e.g.*, heat (Ta and Porter, 2013), ultrasound (Schroeder et al., 2009), light (Jin and Zheng, 2011)) and *in vivo* environmental triggers (*e.g.*, pH (Ferreira Ddos et al., 2013), hypoxia (Thambi et al., 2016), enzyme (Andresen et al., 2010; Hu et al., 2012)) have been investigated. For example, ThermoDOX® is a thermosensitive liposomal formulation currently undergoing clinical trials that allows selective drug delivery into cancer tissue by the application of mild hyperthermia *via* localized heating (ClinicalTrials.gov-NCT02112656). In the present study, phospholipase A_2 (PLA_2) was used to efficiently trigger drug release from liposomes. PLA_2 is an enzyme that cleaves

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the *sn*-2 position of a phospholipid to generate the lysolipid lysophosphatidylcholine and the fatty acid arachidonic acid. There are ten secretary-type active isoforms of PLA_2 and PLA_2 is present in various tissues (Murakami et al., 2015). PLA_2 is over-expressed in inflammatory tissues and its over-expression is associated with cancers (Brglez et al., 2014). Liposomal carriers are composed of phospholipid, the substrate for PLA_2, and thus liposomes become leaky in the presence of PLA_2 due to the degradation of phospholipid. Increasing the amount of single-chain lysolipid destabilizes the liposome. Consequently, the use of PLA_2 as a trigger in conjunction with liposome-encapsulated drugs should allow effective drug delivery. Andresen's group has reported PLA_2-triggered drug release from liposomes for cancer treatment following systemic administration, (Andresen et al., 2005, 2010; Davidsen et al., 2003) but overall there is little information regarding the effectiveness of PLA_2-triggered drug release.

The aim of the present study was to develop a liposome with high sensitivity towards PLA_2. We previously reported a thermosensitive liposome formulation composed of dipalmitoyl phosphatidylcholine (DPPC) as the phospholipid and Poloxamer 188 (P188) to facilitate drug release (Tagami et al., 2015). P188 is an amphiphilic block-copolymer with two PEG chains. DPPC/P188 hybrid liposomes may exhibit enhanced lipid membrane fluidity compared with DPPC liposomes and therefore enhanced sensitivity to temperature. P188 is a widely used excipient in drug formulations (Patel et al., 2009; Schmolka, 1991). DPPC liposomes incorporating P188 and administered *via* the pulmonary route exhibited minimum cytotoxicity *in vitro* and immune response *in vivo* (Tagami et al., 2015), suggesting that DPPC/P188 hybrid liposomes hold promise as an inhalation formulation and that degradation by PLA_2 might trigger drug release. In the current study, we characterized DPPC/P188 liposomes to determine their suitability for triggered drug release.

2. Materials and methods

2.1. Reagents

DPPC, hydrogenated soybean phosphatidylcholine (HSPC), distearoyl phosphatidylcholine (DSPC), and cholesterol (CHOL) were purchased from Wako Pure Chemical (Osaka, Japan). P188 has the molecular formula $HO(C_2H_4O)_{80}(C_3H_6O)_{27}(C_2H_4O)_{80}H$ and was donated by BASF Japan (Tokyo, Japan). 1,2-Distearoyl-*sn*-*glycero*-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG_{2000}-DSPE) was kindly donated by NOF Corporation (Tokyo, Japan). Calcein and doxorubicin (DOX) were purchased from Wako Pure Chemical. PLA_2 from porcine pancreas was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Preparation of calcein-loaded liposomes

Calcein-loaded liposomes were prepared as described previously (Tagami et al., 2015). Briefly, different combinations of lipids were dissolved in organic solvent and the lipid solutions were transferred to glass test tubes. Thin lipid films were obtained by evaporating the organic solvent under vacuum overnight. Isotonic calcein aqueous solution (60 mM calcein) was added to each glass tube and the tubes were alternately gently vortexed and immersed in hot water to obtain liposome suspensions. Each liposome suspension was passed through a mini-extruder (Avanti Polar Lipid, Alabaster, AL, USA) with polycarbonate membranes (200 nm, 100 nm, 80 nm) to adjust the diameters of the liposomes. The liposome solutions were then loaded separately onto a Sepharose CL-4B (Sigma Aldrich) gel filtration column equilibrated with isotonic HEPES buffered saline (HBS, pH 7.5) to replace the exterior phase.

2.3. Preparation of DOX-loaded DPPC/P188 liposomes (remote-loading method)

DOX-loaded DPPC/P188 liposomes were prepared using the remote-loading method as previously described (Tagami et al., 2015). Briefly, a thin-lipid film of DPPC/P188 (molar ratio 3/0.4) was prepared as described in Section 2.2. The lipid film was hydrated with 300 mM citric acid (pH 2), the liposome suspension was extruded, then the liposome solution was passed through a Sepharose CL-4B column as described above. The phospholipid concentration in the liposomal suspension was determined using a Phospholipid Test-Wako kit (Wako Pure Chemical). The DOX solution (20 mg/mL) was mixed with the liposome suspension at a drug to phospholipid ratio of 1/20 (w/w) in a 1.5 mL microtube and gently mixed. The sample was inserted into a floater and immediately placed in a water bath (Isotemp 2340 model; Thermo Fisher Scientific, Waltham, MA USA) maintained at 37.0 °C. The sample was removed after 1 h and then the microtube was chilled on ice for more than 5 min. The liposomal suspension was run through a gel column (Sepharose CL-4B) to separate DOX-loaded liposomes from unencapsulated DOX. The phospholipid and DOX concentrations in the liposomal suspension were determined; the DOX concentration was measured by fluorescence using a plate reader (Wallac ARVO multi-label counter; PerkinElmer, Waltham, MA, USA; wavelength: ex. 485 nm, em. 590 nm). The encapsulation efficiency was determined by: Encapsulation efficiency (%) = (DOX/phospholipid ratio after loading)/(theoretical DOX/phospholipid ratio, *i.e.,* 1/20) × 100%. The encapsulation efficiency of DOX into DPPC/P188 liposomes was 96.3 ± 12.6% (n = 3). The mean dimeter and zeta potential of the DOX-loaded DPPC/P188 liposomes were measured using a ZetaSizer (Malvern Instrument Ltd, Malvern, UK) and were 141.7 ± 7.6 nm (n = 3) and −0.95 ± 1.0 mV (n = 3), respectively.

2.4. Drug release

Drug release was measured as described previously (Tagami et al., 2015). In a typical experiment, liposome samples prepared in Sections 2.2 and 2.3 were diluted with HBS containing 10 mM Ca^{2+} to a final phospholipid concentration of approximately 0.04 μmol. Diluted liposome suspension (200 μL) was mixed with the same volume of PLA_2 solution (dissolved in HBS containing 10 mM Ca^{2+}, final concentration is 120 mU) in a microtube. The optimal amount of PLA_2 was determined by testing various concentrations of PLA_2 with the liposomes and the effect of calcium ion was investigated by conducting experiments in HBS with and without added calcium ion. Temperature dependency was investigated by floating microtubes in a water bath as described above but adjusted to 20 °C, 30 °C, 37 °C, or 42 °C, or by placing the microtubes on ice (0 °C). After 10 min, the microtubes were transferred onto ice for at least 5 min. Incubation time experiments were conducted by incubating samples for the indicated time. Then the samples were transferred into a 96-well black plate and the fluorescence was measured using a plate reader (ex. 355 nm, em. 535 nm for calcein; ex. 485 nm, em. 590 nm for DOX). Drug release was calculated by: Drug release (%) = $(F_{samples} - F_{0\%})/(F_{100\%} - F_{0\%})$. $F_{100\%}$ is the fluorescence intensity of a sample treated with Triton X-100: 10 μL of 1% Triton X-100 was added to 400 μL of sample in a microtube and the mixture was incubated at 70 °C for 5 min to release the drug completely. The presence of Triton X-100 decreased the fluorescence intensity and thus the fluorescence intensity was normalized between calcein aqueous solution and calcein-Triton X-100 solution. $I_{0\%}$ is the fluorescence intensity of an untreated sample kept on ice and I_{sample} is the fluorescence intensity of a sample incubated at the condition described for each specific experiment.