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Phospholipase A_2-susceptible liposomes of anticancer double lipid-prodrugs

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ABSTRACT

A novel approach to anticancer drug delivery is presented based on lipid-like liposome-forming anticancer prodrugs that are susceptible to secretory phospholipase A_2 ($sPLA_2$) that is overexpressed in several cancer types. The approach provides a selective unloading of anticancer drugs at the target tissues, as well as circumvents the necessity for "conventional" drug loading. In our attempts to improve the performance of the liposomes *in vivo*, several PEGylated and non-PEGylated liposomal formulations composed of a retinoid prodrug premixed with the $sPLA_2$-hydrolyzable DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) were prepared. Besides favorably modifying the physicochemical properties of the liposomes, the incorporation of DPPC and PEG-lipids in the liposomes should substantially enhance the enzymatic activity, as concluded from literature. In addition, one can reap benefits from the presumed permeability enhancing effect of the liberated fatty acids and lysolipids. The size distribution of the prepared liposomes as well as their phase behavior, enzymatic hydrolysis, and cytotoxicity, in the presence and absence of $sPLA_2$, were determined. The liposomes were around 100 nm in diameter and in the gel/fluid coexistence region at 37 °C. The enzymatic hydrolysis of the prodrug was pronouncedly accelerated upon the premixing with DPPC, and the hydrolysis was further enhanced by PEGylation. Interestingly, the faster hydrolysis of the prodrug and the released fatty acids and lysolipids from DPPC did not improve the cytotoxicity of the mixture; the effect of combining the prodrug with DPPC was additive and not synergistic. The data presented here question the significance of the permeability enhancing effects claimed for fatty acids and lysolipids at the target cell membrane, and whether these effects can be achieved using physiologically achievable concentrations of fatty acids and lysolipids.

1. Introduction

The use of sub-micro-scale systems, like liposomes, to deliver anticancer drugs is a very appealing approach to increase the efficiency and minimize the severe systematic cytotoxicity of chemotherapy. Although the leaky vasculature and impaired lymphatic drainage in cancer tissues, the so-called enhanced permeability and retention phenomenon (EPR) (Matsumura and Maeda, 1986), will cause the drug vehicles to accumulate in leaky cancer tissues, the selective unloading of the anticancer drugs at the target areas as well as promoting their uptake by the target cancer cells remain the bottleneck for this treatment approach. Several active and passive strategies have been applied to enhance the performance and targeting of liposomes *in vivo*, including the design of liposomes that are sensitive to the tumor conditions, like pH-, thermo-, hypoxia-, and enzyme-sensitive liposomes (Andresen et al., 2005a, 2010; Chen and Hu, 2009; Das et al., 2009; Kaasgaard and Andresen, 2010; Malam et al., 2009; Minko et al., 2004; You et al., 2010). Our approach is based on benefiting from secretory phospholipase A_2 ($sPLA_2$) to trigger the unloading of anticancer drugs from liposomes (Davidsen et al., 2003, 2001; Jorgensen et al., 2002), since $sPLA_2$ subtype IIA is overexpressed in several cancer types (Graff et al., 2001; Jiang et al., 2002; Kashiwagi et al., 1999; Kennedy et al., 1998; Kiyohara et al., 1993; Murata et al., 1993; Tribler et al., 2007).

1.1. Secretory phospholipases A_2 ($sPLA_2$)

Secretory phospholipases A_2 ($sPLA_2$) specifically cut off the acyl chain at the *sn*2 position in phospholipids, releasing fatty acids and lysolipids as hydrolysis products (Kudo, 2004; Lambeau and Gelb, 2008; Six and Dennis, 2000). The enzyme is solely activated at the water/membrane interface of assembled lipid structures, that is, it does not hydrolyze single lipid molecules in solution (Mouritsen et al., 2006). The mechanism of action of the enzyme is rather complex; the enzymatic activity does not follow Michaelis–Menten kinetics, it requires Ca^{2+} as a co-factor, and is governed by the composition, morphology, and physicochemical

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properties of the lipid membrane (Halperin and Mouritsen, 2005; Honger et al., 1996; Hoyrup et al., 2004; Mouritsen et al., 2006). Once bound to lipid membranes, the enzyme can show two modes of hydrolysis; a slow one and a fast one. The transition from the slow mode to the fast mode occurs after a characteristic lag-time, which can be determined by monitoring the change in the hydrolysis rate of the lipid substrate, the change in the release rate of the hydrolysis products, and the increase in the intrinsic tryptophan fluorescence of the $sPLA_2$ enzyme that was found to increase when the enzyme undergoes the transition (Apitz-Castro et al., 1982; Burack and Biltonen, 1994; Honger et al., 1996; Mouritsen et al., 2006). During the lag-time, there is an increase in both the amount of $sPLA_2$ bound to the lipid membrane as well as the depth of insertion of the enzyme (Wacklin et al., 2007). The transition from the slow to the fast mode can be sudden or broad, and the lag-time can range from seconds to more than hours (Honger et al., 1996; Hoyrup et al., 2001a; Mouritsen et al., 2006). The fast hydrolysis mode can be up to two or three orders of magnitude higher than the slow mode, and is usually triggered by the accumulation of the hydrolysis products and the formation of membrane defects caused by the action of the enzyme in the slow mode (Mouritsen et al., 2006). Therefore, pre-existing membrane defects, domain boundaries and heterogeneities that arise, for instance, in lipid mixtures or during the membrane phase transition will boost the activity of $sPLA_2$ (Honger et al., 1996). It is not uncommon that lipid substrates are fully consumed by $sPLA_2$ in the slow mode before the enzyme enters the fast mode. In addition, due to the continuously changing composition and properties of the target lipid membrane during the hydrolysis, it can happen that $sPLA_2$ enzymes in the fast mode become deactivated back to the slow mode before the full consumption of the lipid molecules (Hoyrup et al., 2001b).

1.2. $sPLA_2$-susceptible liposomes and prodrugs

The feasibility of utilizing the overexpression of $sPLA_2$ in the interstitial fluid of some cancer types to release drugs from liposomes was demonstrated before in several studies using cisplatin- and doxorubicin-loaded liposomes (Andresen et al., 2005a,b; Mouritsen et al., 2006) and $sPLA_2$-degradable anticancer ether lipids (Andresen et al., 2004, 2005c; Jensen et al., 2004). In addition to the role of phospholipids in forming the drug carrier, i.e., liposomes, the hydrolysis products of phospholipids can serve other functions that will augment the efficiency of the encapsulated drugs: (1) lysolipids are substantially cytotoxic in micromolar concentrations (Andresen et al., 2004, 2005c; Traikia et al., 2005) which will add to the toxicity of the formulation, (2) fatty acids by themselves can inhibit cancer cell growth and sensitize cancer cells to the effects of anticancer drugs (Lu et al., 2010; Pardini, 2006; Sauer et al., 2000), and (3) both fatty acids and lysolipids can reduce the permeability barrier of lipid membranes (Grit and Crommelin, 1992; Jespersen et al., 2011; McKersie et al., 1989).

In the past few years, several lipid-like prodrugs were designed based on replacing the acyl chain at position *sn*2 with anticancer drugs, like chlorambucil (Pedersen et al., 2009), prostaglandin (Pedersen et al., 2010a), and retinoids (Christensen et al., 2010; Pedersen et al., 2010b). The prodrugs can form liposomes and once subjected to $sPLA_2$ the anticancer drug will be cleaved and released from the liposomes.

One of the prodrugs with the capabilities mentioned above is 1-*O*-stearyl-2-RAR-C6-*sn*-glycero-3-phosphoglycerol, and it will be called the "C6-RAR prodrug" throughout the article. Once hydrolyzed by $sPLA_2$, C6-RAR and lyso-*O*-SPG (1-*O*-stearoyl-2-hydroxy-*sn*-glycero-3-phosphoglycerol) will be released. The structures of the prodrug and of the hydrolysis products are illustrated in Fig. 1. The RAR compound (4-(4-octylphenyl)-benzoic acid) is a selective retinoic acid receptor β2 (RARβ2) agonist (Lund et al., 2009, 2005). The RAR compound directly esterified to the *sn*2 position of lyso-*O*-SPG was not cleavable by $sPLA_2$, therefore, a C6 spacer had to be introduced between the RAR compound and the glycerol backbone of lyso-*O*-SPG (Pedersen et al., 2010b); the C6 spacer linked to the RAR compound will be called the "C6-RAR compound". Despite the limited aqueous solubility of the C6-RAR compound (Lund et al., 2005), the C6-RAR prodrug is fairly water dispersible (Arouri and Mouritsen, 2011; Pedersen et al., 2010b).

As reported earlier, the C6-RAR prodrug behaves comparably to synthetic lipids, that is, the prodrug can form bilayer liposomes with a diameter around 100 nm as well as be cleaved by $sPLA_2$ (Pedersen et al., 2010b). In addition, the prodrug shows significant anticancer cytotoxicity only when hydrolyzed by $sPLA_2$ with IC_{50} of a few micromolar (Pedersen et al., 2010b). In a recent study (Arouri and Mouritsen, 2011), we characterized lipid mixtures of the C6-RAR prodrug with the ester-linked $sPLA_2$-hydrolyzable DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and its ether-linked non-hydrolyzable analogue Di-*O*-PPC (1,2-di-*O*-palmityl-*sn*-glycero-3-phosphocholine). The main finding of the study is that the physicochemical properties of the prodrug liposomal formulations, like the phase transition temperature, stability, and permeability, can be favorably modified via the premixing of the prodrug with other lipid components.

1.3. The current study

The main aim of the present study was to investigate the hydrolysis profile and cytotoxicity of prodrug-containing liposomal formulations. Two formulations were tested: (1) DPPC/prodrug binary mixtures (10–50 mol% prodrug), and (2) DPPC/prodrug/DPPE-PEG ternary mixtures (30 mol% prodrug, 5–10 mol% DPPE-PEG with average molecular weights of 350, 750, 2000, and 5000). The structures of DPPC and of the hydrolysis products, lyso-PPC (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) and palmitic acid (PA), are depicted in Fig. 1. We chose DPPC, which undergoes the gel-to-fluid main phase transition at ~42 °C, for two reasons; first, the enzymatic hydrolysis of DPPC by $sPLA_2$ is well characterized (Honger et al., 1996; Hoyrup et al., 2001a; Mouritsen et al., 2006), and secondly, it facilitates the formation of liposomes that have a transition region near human body temperature, which can be an advantage due to the formation of membrane defects that will enhance the enzymatic activity of $sPLA_2$ on the liposomes. Moreover, the prodrug and DPPE-PEG are expected to be highly miscible with DPPC, as it was found before that phospholipids with an acyl chain length difference of up to two methylene units are completely miscible and form homogeneous bilayers in both gel and liquid-crystalline phases (Garidel and Blume, 2000; Garidel et al., 1997).

Besides enhancing the stability and improving the pharmacokinetics of our liposomes *in vivo* (Torchilin et al., 1994), the incorporation of lipopolymers, like PEG-lipids, was found to shorten the lag-time of $sPLA_2$, proportionally to the chain length, mole percentage (mol%), and surface coverage of the lipopolymer (Davidsen et al., 2001; Jorgensen et al., 1999; Mouritsen et al., 2006). This enhancement in the activity of $sPLA_2$ was attributed to a stronger binding of the cationic $sPLA_2$ enzyme to membranes with anionic PEG-lipids as well as to the higher protrusions at the membrane surface induced by the presence of these lipopolymers. Assuming fluid-phase membranes, the transition from polymer mushroom to brush regimes is anticipated to begin at PEG-lipid mol% of 11, 4.5, 1.4, and 0.5 for PEG lipids with polymer average molecular weights of 350, 750, 2000, and 5000, respectively (Marsh et al., 2003).

In the present study, we used DSC and DLS to measure respectively the lipid phase transition and size distribution of the various