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Revisiting the use of $sPLA_2$-sensitive liposomes in cancer therapy

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ABSTRACT

The first developed secretory phospholipase A_2 ($sPLA_2$) sensitive liposomal cisplatin formulation (LiPlaCis®) is currently undergoing clinical evaluation. In the present study we revisit and evaluate critical preclinical parameters important for the therapeutic potential and safety of platinum drugs, here oxaliplatin (L-OHP), formulated in $sPLA_2$ sensitive liposomes. We show the mole percentage of negatively charged phospholipid needed to obtain enzyme-sensitivity for saturated systems is ≥ 25% for 16-carbon chain lipid membranes, and > 40% for 18-chain lipid membranes, which was surprising as 25% is used clinically in LiPlaCis®. Efficient $sPLA_2$-dependent growth inhibition of colorectal cancer cells was demonstrated *in vitro*, where cell membrane degradation and cytolysis depends on the sensitivity of the formulation towards the enzyme and is governed by the amount of lysolipids generated and the presence of serum proteins. We found that serum proteins did not affect the lipase activity of the enzyme towards the membranes but instead sequester the lysolipid byproducts consequently inhibiting their detergent-like cytotoxic properties. *In vivo* therapeutic potential and safety of the liposomes was investigated in nude mice bearing $sPLA_2$-deficient FaDu squamous carcinoma and $sPLA_2$-expressing Colo205 colorectal adenocarcinoma. After intravenous injections, the tumor growth was suppressed for liposomal L-OHP relative to free drug, but only a weak response was observed for both slow- and fast-releasing $sPLA_2$-sensitive formulations compared to non-sensitive liposomes. Also, the mice did not show longer survival. In turn, for the highly $sPLA_2$-sensitive liposomes, multiple high doses caused petechial cutaneous hemorrhages, along with multifocal hepatonecrotic lesions, suggestive of premature activation in skin and liver irrespective of $sPLA_2$-status of the tumor engraft. These results indicate that although liposomal carriers can improve the antitumor efficacy of platinum drugs, $sPLA_2$-triggered release suffers from a narrow therapeutic index and has safety concerns.

1. Introduction

In the design of nanotherapeutics, controlled and site-specific drug release in diseased tissue remains one of the main challenges for the field and ideally should increase both therapeutic efficacy and minimize therapy-associated side effects. Long-circulating pegylated liposomes are designed to retain encapsulated drugs, altering drug deposition and improve efficacy and drug toxicity profiles [1,2]. Oxaliplatin (L-OHP) is a first-line chemotherapy in the combination FolFox regimen indicated for the treatment of advanced colorectal cancer [3]. In attempting to minimize the peripheral neuropathy, myelotoxicity and gastrointestinal toxicities commonly associated with L-OHP, pegylated-liposomes have been rigorously investigated for safer delivery both preclinically [4–7] and in patients [8]. Despite being well-tolerated and lowering toxicities, in many cases insufficient release rates of hydrophilic drugs at the target site has limited their therapeutic potential and more sophisticated liposomal delivery systems are therefore required [9]. Several approaches exist to trigger drug release by employing environmentally-

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sensitive liposomes that respond to either external stimuli, *e.g.* induced hyperthermia [10–12] and light in photodynamic therapy [13], or intrinsically by taking advantage of pathological changes arising in the diseased state. The elevated expression of endogenous enzymes in cancerous tissue represents a promising strategy to control and obtain a site-specific drug release intrinsically [14–16]. Apart from LiPlacis®, thermo-sensitive liposomes are among the most progressed liposome systems with triggered-release properties that have been investigated clinically, however, without sufficient success [17]. From a therapeutic point of view secretory phospholipase A_2 type IIa ($sPLA_2$) is an attractive target, as it is overexpressed in its active form in various types of cancer including colon, breast, pancreatic, and prostate [18–22], and thereby can function as a release trigger intrinsically build-in in the tumor. Additionally, $sPLA_2$ exhibits preferential substrate specificity for organized lipid structures (such as bilayers) over monomeric lipids in solution, making it particularly suitable for liposomal drug delivery [23]. The validity of this principle have been established *in vitro* [24–27], and to some extent *in vivo* using mouse models [16,28].

The $sPLA_2$ enzyme has a high specificity for anionic lipid membranes, *e.g.* composed of phosphatidylglycerol (PG) [29]. Thus, for controlled delivery purposes the sensitivity of the drug carrier to $sPLA_2$ degradation, and thereby the level of drug release, can be modulated by producing liposomes with varied amounts of negatively charged lipids and increasing or decreasing the chain length of the fatty acids [29].

$sPLA_2$ hydrolyzes the ester linkage of *sn*-2-acyls of phospholipids, which yields free fatty acids and 1-acyl-lysophospholipids [9,29]. In this sense liposome membrane destabilization by $sPLA_2$ is thought not only to liberate the encapsulated drug, but also to yield high concentrations of lysolipids and free fatty acids locally at the site of activation. In turn, these can then serve as permeability enhancers across biological membranes, or at high amounts, directly induce cellular toxicity by forming aggregated structures with detergent-like properties [16,28,30]. Thus, the carrier by itself is considered to be a prodrug. As premature activation therefore bears a risk of releasing bioactive molecules in unwanted sites, the sensitivity of the particles needs to be finely tuned in order to avoid harmful side effects.

The first liposomal formulation with a $sPLA_2$-triggered release mechanism encapsulating cisplatin, LiPlaCis®, has completed clinical phase I testing in patients with advanced or refractive solid tumors. Despite early cessation of an initial trial in 2009 owed to formulation-related safety concerns requiring reformulation, LiPlaCis® has reentered clinical testing to establish dose recommendations and is scheduled to be completed by mid-2017. In the original phase I study the formulation (DSPC:DSPG:DSPE-PEG2000 70:25:5 molar ratio) was reported to be too unstable, with high levels of platinum excreted *via* urine along with a high incidence of dose-related renal toxicity characteristic of free cisplatin [31]. The reason for this stability issue was not clear, but did not appear to be related to a premature activation of the liposomes during circulation by $sPLA_2$, as no correlation could be found between serum $sPLA_2$ levels and the plasma half-life of the particles. On the other hand, an unusually high incidence of non-dose related grade 1–2 infusion reactions (39%) was observed despite pre-medication with clemastine and dexamethasone and reducing infusion rates by 50% [31]. In comparison, other clinically relevant liposome formulations also induce similar symptoms, but at a much lower rate of up to 9% [31]. It was believed that this could be associated to activation of the complement system by LiPlaCis® from the observed concurrent immediate upsurge in SC5b plasma levels. Along these lines, PG-containing liposomes have been shown to have the capacity to activate the complement system [32].

The aim of the present work is to revisit the potential of $sPLA_2$-sensitive liposome formulations of platinum drugs motivated by the current clinical development stage of these systems; to get deeper insight into the $sPLA_2$ dependency of previous developed formulations and understand the therapeutic window that is obtainable with such formulations. We report here L-OHP loaded liposomes with low- or high sensitivity towards $sPLA_2$-triggered degradation as drug delivery nanocarriers. Hereby we compare slow- and fast-releasing formulations to non-sensitive nanocarriers, and assess their *in vitro* cytotoxicity against cultured cancer cells, as well as their efficacy and tolerability *in vivo* in mice bearing human tumors.

2. Materials and methods

2.1. Materials

The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DPPG), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DSPG), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2k), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1′-rac-glycerol) (LysoPPG) were purchased from Avanti Polar Lipids, Inc. (Alabama, USA). Lyophilized mixture of hydrogenated L-α-phosphatidylcholine (HSPC), cholesterol (chol), and DSPE-PEG2k (57:38:5 mol%) was acquired from Lipoid GmbH (Ludwigshafen, Germany). Oxaliplatin was purchased from Shanghai Yingxuan Chempharm (Shanghai, China). Chloroform, methanol, hydrochloric acid, HEPES, glucose, calcium carbonate, Triton X-100, Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptavidin (pen/strep) were purchased from Sigma-Aldrich (Schnelldorf, Germany), as well as the gallium and iridium standards for ICP-MS.

2.2. Preparation of liposome vesicles

Liposomes were prepared following a previously reported method [33]. Briefly, accurate amounts of lipids were dissolved in chloroform:methanol (9:1 v/v), followed by solvent evaporation at room temperature (RT) under a gentle stream of nitrogen. To ensure complete solvent removal, the lipid films formed were placed under vacuum overnight. Multilamellar vesicles (MLV) were prepared by hydrating the lipids with a buffered solution (10 mM HEPES, 5% glucose, pH 7.4), containing (if desired) the encapsulate molecule, at a temperature 15 °C above the main phase transition temperature with vortexing every 5–10 min. For the preparation of calcein loaded liposomes, calcein was firstly dissolved in water with NaOH and then the pH was adjusted to pH 7.4 prior to addition to the hydrating solution to a final calcein concentration of 20 mM. The preparation of oxaliplatin-loaded liposomes was carried out by previously dissolving 15 mg/mL oxaliplatin in the buffered solution at 65 °C for 1 h under stirring conditions. The MLV suspensions were extruded 21 times through two-stacked 100 nm pore size polycarbonate filters at 55 °C forming homogeneous large unilamellar vesicles (LUV) (< 130 nm) with a narrow size distribution (PDI < 0.1). For *in vivo* applications, liposomes were instead extruded using a high-pressure extrusion device (Northern Lipids Inc., Burnaby, Canada) and were sequentially downsized through 400/200/100 nm polycarbonate filters. Calcein containing liposomes were purified by gel filtration through Sephadex G-50 size exclusion column using HEPES buffer as eluent and liposomes loaded with oxaliplatin were purified by dialysis for 3 days using cassettes of 100 kDa molecular cutoff and HEPES buffer containing 1 mM $CaCO_3$ ($> 99\%$ encapsulation). All liposomes were stored at 4 °C.

2.3. Physicochemical characterization of liposomal drug carriers

2.3.1. Size and surface charge

Liposome hydrodynamic diameter and size distribution was analyzed using dynamic light scattering (DLS) and the vesicles surface charge was determined by zeta-potential using a ZetaPALS system (Brookhaven Instruments Corporation, New York, USA). Liposome suspensions were diluted 100-fold in filtered (0.2 μm) buffer, placed in plastic cuvettes and degassed for 5 min to expel any air in the samples