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Secretory phospholipase A$_2$ responsive liposomes exhibit a potent anti-neoplastic effect *in vitro,* but induce unforeseen severe toxicity *in vivo*

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Abstract

The clinical use of liposomal drug delivery vehicles is often hindered by insufficient drug release. Here we present the rational design of liposomes optimized for secretory phospholipase A_2 ($sPLA_2$) triggered drug release, and test their utility *in vitro* and *in vivo*. We hypothesized that by adjusting the level of cholesterol in anionic, unsaturated liposomes we could tune the enzyme specificity based on membrane fluidity, thus obtaining liposomes with an improved therapeutic outcome and reduced side effects. Cholesterol is generally important as a component in the membranes of liposome drug delivery systems due to its stabilizing effects *in vivo*. The incorporation of cholesterol in $sPLA_2$ sensitive liposomes has not previously been possible due to reduced $sPLA_2$ activity. However, in the present work we solved this challenge by optimizing membrane fluidity. *In vitro* release studies revealed enzyme specific drug release. Treatment of two different cancer cell lines with liposomal oxaliplatin revealed efficient growth inhibition compared to that of clinically used stealth liposomes. The *in vivo* therapeutic effect was evaluated in nude NMRI mice using the $sPLA_2$ secreting mammary carcinoma cell line MT-3. Three days after first treatment all mice having received the novel $sPLA_2$ sensitive liposome formulation were euthanized due to severe systemic toxicity. Thus the present study demonstrates that great caution should be implemented when utilizing $sPLA_2$ sensitive liposomes and that the real utility can only be disclosed *in vivo*. The present studies have clinical implications, as $sPLA_2$ sensitive formulations are currently undergoing clinical trials (LiPlaCis®).

1. Introduction

The clinical use of most conventional chemotherapeutics is often hampered by inadequate tumor accumulation and severe toxic effects on healthy organs. Although stable entrapment in long-circulating liposomes can improve tumor accumulation and tolerability through the enhanced permeability and retention (EPR) effect [1,2], drug bioavailability after tumor accumulation remains a challenge. Prolonged and uncoordinated release kinetics from the carrier results in drug concentrations that are insufficient to achieve significant therapeutic effects [3].

Currently clinically approved liposomal formulations such as Doxil® have shown moderately improved antitumor activity and improved safety [4,5], likely due to disruption of the ammonium-sulfate gradient and slow diffusion of the drug through the liposomal membrane [6]. However, for hydrophilic drugs such as cisplatin and oxaliplatin, which are unable to diffuse over an intact liposomal membrane [3], an active trigger mechanism is a requisite for drug release.

This underlines the paradoxical problem of how to maintain the stability of liposomes in circulation and at the same time obtain a high local drug bioavailability in the cancerous tissue. One solution is utilizing an endogenous triggered release mechanism that relies on a difference in the microenvironment of tumor and healthy tissue, such as the elevated expression of endogenous enzymes in cancerous tissues. An attractive candidate is secretory phospholipase A_2 type IIA (sPLA$_2$-IIA). Both *in vivo* and *in vitro* studies have reported elevated levels of sPLA$_2$ in several human tumor types, including colon and breast cancer [7–9], as well as elevated levels during tumor vascularization [10] and metastases [9].

sPLA$_2$ catalyzes the hydrolysis of glycerophospholipids at the sn-2 position, yielding equimolar amounts of lyso-lipids and free fatty acids [11]. For liposomes this may result in rupture of the lipid bilayer and release of the encapsulated drug (Fig. 1). In addition, high local concentrations of lyso-lipids and fatty acids may serve as permeability enhancers, thus further contributing to transport of the released drug across the cellular membrane [12,13]. However, at too high concentrations they can cause cytotoxicity in the form of apoptosis or cellular lysis [14].
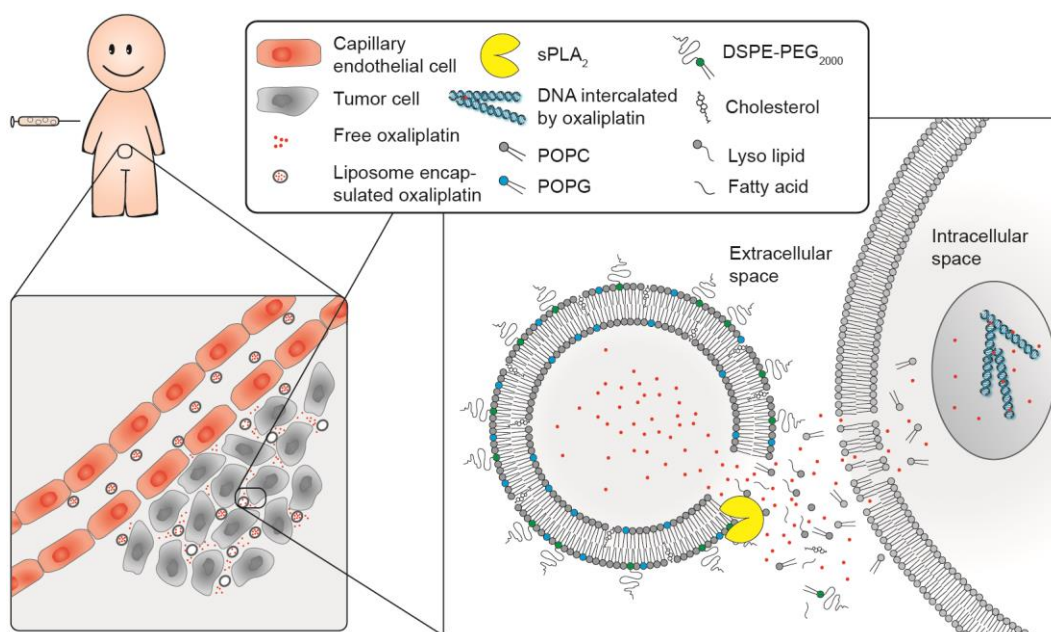
Fig. 1. Illustration of the secretory phospholipase A_2 (sPLA$_2$) concept showing the intended action of the sPLA$_2$ activated drug delivery systems. Liposome encapsulated oxaliplatin will circulate until it encounters the fenestrated capillaries in the tumor tissue, where it extravasates. Here it encounters an elevated level of secretory phospholipase A_2 (sPLA$_2$), which hydrolyze the phosphoglycerolipids, causing release of the drug. In addition, the hydrolysis products, lyso-lipids and free fatty acids, may act as permeability enhancers, thus further contributing to drug transport across the cellular membrane.

With reference to its substrate specificity, sPLA$_2$ is an interfacially active lipase which functions mainly on organized substrates such as lipid bilayers, making it optimal for liposomal drug delivery [15]. The enzyme activity is highly dependent on the physical properties and the small-scale lateral organization of the lipid membrane [16]. Specifically sPLA$_2$ seems to be particularly active at sites of structural defects such as domain boundaries [17,18], and no activity is observed in highly rigid cholesterol-doped membranes in the liquid-ordered phase [18,19]. In contrast, incorporation of poly(ethylene-glycol) (PEG) in the membrane seems to enhance the sPLA$_2$ catalytic activity [16], likely due to an electrostatic interaction between the positively charged sPLA$_2$ and the negative charges on the PEG group. In accordance with this, sPLA$_2$ also has high specificity towards anionic membranes, particularly at phosphatidylglycerol (PG) levels above ~ 38 mol% [17].

The concept of sPLA$_2$ sensitive liposomes has been widely studied, especially *in vitro* [13,17,20], and in a few cases *in vivo* [18,21], for delivery of conventional chemotherapeutics and in combination with pro-drug activation [22,23]. Despite this, only one formulation has made it to clinical trials. In 2008 LiPlaCis®, a liposomal formulation of cisplatin, entered a clinical phase I trial. However, severe renal toxicity and acute infusion reactions following complement activation led to a termination of the trials [24]. Despite these adverse effect, LiPlaCis re-entered clinical trials in 2013, and are currently recruiting patients (NCT01861496 at clinicaltrials.gov).

Recently, we have developed a highly sensitive sPLA$_2$ responsive liposome formulation loaded with oxaliplatin, which is not limited by nephrotoxicity [25]. However, an *in vivo* efficacy study resulted in severe toxicity, which was suspected to be caused by an excess concentration of permeability enhancing components released into the bloodstream, locally in the skin and potentially in other tissues (unpublished results).

In the present study we set out to design liposomes optimized for controlled sPLA$_2$ catalyzed drug release, attempting to avoid hemolytic adverse effects, and show the potential of the liposomes both *in vitro* and *in vivo*. Based on phase diagrams [26,27] of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/cholesterol liposomes, we reasoned that by adjusting the cholesterol level we could tune membrane disorder, and thereby closely tune the enzyme sensitivity, thus obtaining liposomes with a significant therapeutic effect but without substantial side effects.

2. Materials and Methods

2.1 Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG), 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphocholine (TopFluor PC) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabama, USA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2k) and lyophilized mixture of hydrogenated soy phosphocholine (HSPC), cholesterol and DSPE-PEG2k (3:1:1 weight %) were acquired from Lipoid GmbH (Ludwigshafen, Germany). Oxaliplatin (OxPt) was purchased from Shanghai Yingxuan Chempharm (Shanghai, China). All other chemicals were from Sigma Aldrich (Brøndby, Denmark) and of analytical grade.

2.2 Preparation of liposomes

Liposomes for *in vitro* experiments were prepared by thin-film hydration. Briefly, lipids were dissolved in chloroform/methanol (9:1), mixed and placed under a gentle stream of nitrogen to evaporate the solvent, before being placed under vacuum over night. For drug loading, 15 mg/mL OxPt was dissolved in hydration buffer (10 mM HEPES, 5% Glucose, pH 7.4) at 66 °C for 1 h, followed by hydration of lipid films at 66 °C for 1 h with vortexing every 15 min. Liposomes were downsized by extrusion 21 times through a 100 nm polycarbonate filter at 72 °C. Non-encapsulated drug was removed by three days of dialysis (10 mM HEPES, 5% Glucose, 1 mM Calcium Gluconate, pH 7.4) using cassettes of 100 kDa molecular cut-off.

For large-scale production for *in vivo* experiments liposomes were prepared by lyophilization and pressure extrusion. Briefly, lipids were dissolved in tert-butanol, mixed, frozen in liquid nitrogen, and dried over night. Following hydration, liposomes were downsized by extrusion five times through three stacked polycarbonate filters of 200 nm, 100 nm and 100 nm, respectively, using a high-pressure extrusion device (Northern Lipids Inc., Burnaby, Canada). Following dialysis, liposomes were filtered using 0.45 μm sterile filters. All liposomes were stored at 4 °C.

2.3 Size and zeta potential

Particle size, polydispersity and zeta potential were analyzed by light scattering using a ZetaPals system (Brookhaven Instruments Corporation, NY, USA). Samples were diluted 200-fold (10 mM HEPES, 5 % Glucose, pH 7.4), and particle size distribution was determined by five sub runs of 30 sec each, and zeta potential was determined by 10 sub runs with a target residual of 0.04.

2.4 Drug and lipid concentrations

The lipid and drug concentrations were determined by measuring the total phosphor and platinum content of the samples using inductively coupled plasma mass spectrometry (ICP-MS) using an ICAPQ (Thermo Scientific[TM], Dreieich, Germany). For phosphor measurements samples were diluted 5000-fold in 2% HCl containing 10 ppb gallium as internal standard, and for platinum measurements samples were diluted 500,000-fold in 2% HCl containing 0.5 ppb iridium as internal standard. Non-encapsulated drug was separated by spin-filtration at 2000 g for 10 min using Amicon spin filters with 100,000 kDa molecular cut-off, and diluted 100,000-fold for quantification.

2.5 Release of oxaliplatin

Oxaliplatin loaded liposomes were diluted in colo205 cell conditioned media or fresh RPMI media, both supplemented with 10% FBS, to a final concentration of 500 μM lipid. Samples were mixed and incubated at 37 °C for 24 h with gentle magnetic stirring. At time points 0, 6 and 24 h, aliquots were removed. Total and released oxaliplatin concentrations were determined by ICP-MS, as described above.

2.6 Phospholipid hydrolysis

Empty liposomes were diluted in buffer (10 mM HEPES, 30 μM CaCl$_2$, 10 μM EDTA, 150 mM KCl, pH 7.4), colo205 cell conditioned media or human tear fluid (diluted 30-fold in buffer), to a final concentration of 500 μM lipid. Samples were mixed and incubated at 37 °C for 6 h with gentle magnetic stirring. Following incubation, 100 μL of the reaction mixture was quickly mixed with 1 mL chloroform/methanol/acetic acid (2:4:1) to stop the reaction, followed by 1 mL water for washing. The organic phase was collected and lipid hydrolysis was analyzed by MALDI-TOF MS. Briefly, samples were mixed with matrix (2:3), spotted in triplicates and analyzed by Bruker autoflex speed (Bruker Daltonics, Bremen, Germany). As matrix we utilized 2,5-dihydroxybenzoic acid (DHB) spiked with sodium trifluoroacetate (NaTFA), dissolved in methanol, with 85 μM DPPC as internal reference. Observed MW$_{POPC}$: 760.6 (M+H$^+$), 782.6 (M+Na$^+$). Expected MW$_{POPC}$: 760.6 (M+H$^+$), 782.6 (M+Na$^+$). Observed MW$_{POPG}$: 793.6 (M+2Na$^+$), 771.6 (M+H$^+$+Na$^+$). Expected MW$_{POPG}$: 793.5 (M+2Na$^+$), 771.5 (M+H$^+$+Na$^+$).

2.7 Cell culture

Colo205 and HT-29 human colon carcinoma cell lines were purchased from American Type Culture Collection (Virginia, USA), and the MT-3 human mammary carcinoma cell line was a kind gift from Dr. Iduna Fichtner. Colo205 and MT-3 cells were maintained in RPMI-1640 and HT-29 cells in DMEM, both supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin (pen/strep), in a humidified 5% CO$_2$ atmosphere at 37 °C. Colo205 and HT-29 cells were harvested every 3-4 days and used in passages 23-28 and 159-167, respectively. MT-3 cells were harvested every 6-7 days and used in passages 31-35. For production of Colo205 cell conditioned media (CM), cells were serum-deprived. Briefly, 15-20 million cells were seeded in a T75 flask and cultured for 24 h under normal conditions, followed by 24 h in RPMI-1640 supplemented with only pen/strep. The conditioned media was collected and stored at -20 °C for further use. Immediately before use the CM was supplemented with 10% FBS.

2.8 Cytotoxicity

The *in vitro* anti-proliferative effect was determined using the CellTiter 96 $^{\circledR}$ AQ$_{ueous}$ One Solution Cell Proliferation Assay (Promega Biotech AB, Stockholm, Sweden), according to the manufacturers protocol. Briefly, cells were plated in 96 well plates at a density of 10,000 cells/well for HT-29 cells and 15,000 cells/well for MT-3 cells. Following 24 h incubation, media was removed and replaced with fresh media or Colo205 CM containing free oxaliplatin, liposomal oxaliplatin or empty liposomes. Cells were incubated for 6 h, remaining drug was removed and replaced with fresh media, and cells were incubated for a further 66 h before determining the anti-proliferative effect.

2.9 Cellular uptake

HT-29 cells were seeded in 12 well plates at 10^5 cells per well and allowed to adhere for 24 h. Empty liposomes labeled with 0.05 mol% TopFluor PC were diluted in pre-warmed DMEM to a final concentration of ~ 400 μM lipid (normalized for fluorescence signal), and added to the cells in duplicates. At 6 h post treatment, cells were washed twice with PBS, trypsinized, diluted in DMEM, pelleted and resuspended in 500 μL PBS. Cellular association was analyzed using a BD Accuri^{TM} C6 flow cytometer (BD Biosciences, Erembodegem, Belgium). Samples were excited using a 488 nm argon laser and emission was determined using a 533/30 band pass filter. 10,000 events were counted per sample. Data were analyzed using the BD Accuri C6 analysis software.

2.10 Light microscopy

For time-course micrographs, HT-29 cells were seeded in 96 well plates at 10,000 cells per well. Following 24 h incubation, media was removed and replaced with CM containing 740 μM empty liposomes. Cells were incubated at 37 °C for 72 h. Using an Axiovert 25 inverted light microscope (Zeiss, Birkerød, Denmark), pictures were taken 10 min, 1, 2, 4, 6 and 72 h post treatment. Specific markings on the microscope were used to obtain pictures of the same area each time.

2.11 Animals

Five-week-old female NMRI-nu mice were purchased from Taconic (Lille Skensved, Denmark) and were allowed to acclimatize in the animal facility for a minimum of one week before initiation of experiments. All care and experimentation was performed according to the Danish Animal Experimentation Act (equivalent to the U.S. Public Health Service (PHS) Policy on humane Care and Use of Laboratory Animals), under the approval of the Danish Animal Welfare Council.

2.12 *In vivo* efficacy

MT-3 cells (10^7) suspended in 100 μL culture media were injected subcutaneously into the right and left flank of anaesthetized mice. When reaching a tumor size of approximately 10 x 10 mm, serial transplantations were performed. Donor mice were euthanized and tumors were excised. Recipient mice were anaesthetized and tumor sections (~3 x 3 mm) transplanted subcutaneously onto the right flank. When tumors reached ~ 200 mm^3 (15 days) animals were randomized into 5 groups of 7 mice and treatment was initiated. Animals received 10 mg/kg oxaliplatin, the liposomal equivalent or isotonic glucose solution, by tail vein injection once a week for a total of four injections. Tumor size and body weight were monitored 3 times per week. The tumor size was measured by an electronic caliper and the tumor volume was determined using the formula: volume = (length x width^2) / 2, where length is the longest measurement. Animals were euthanized when tumors reached 1000 mm^3, the body weight decreased by more than 15 % or the animals showed signs of discomfort or failure to thrive.

3. Results and Discussion

3.1 Formulation and characterization of liposomes

According to phase diagrams of POPC/cholesterol liposomes published elsewhere, none or minor amounts of cholesterol should render the liposomes in the liquid-disordered phase (L_d), whereas high amounts of cholesterol should render the liposomes in the liquid-ordered phase (L_o) [26,27]. As sPLA$_2$ has high sensitivity towards anionic membranes with structural defects, we hypothesized that by tuning the amount of cholesterol in anionic liposomes, we would tune the enzyme sensitivity, thus obtaining a controlled release. To test this, liposomes composed of the zwitterionic lipid POPC, the anionic lipid POPG, the polymer DSPE-PEG2000 and 0, 20 or 40 mol% cholesterol were synthesized. At these cholesterol concentrations, at 37 °C, the liposomes should be in the L_d phase, at the boundary of the L_d and L_d-L_o co-existence region and in the L_d-L_o co-existence region, respectively [27]. Although, it should be noted that due to large variations in proposed phase diagrams in literature, an exact estimate is not possible [26]. Non-hydrolysable HSPC/cholesterol/DSPE-PEG$_{2000}$ (Stealth) liposomes were included as a negative control. All liposomes were loaded with the hydrophilic, non-nephrotoxic drug oxaliplatin (OxPt) [25].

Size distribution, polydispersity index (PDI) and zeta-potential as well as lipid and drug concentrations were characterized for each formulation (Table 1). Homogenous size distributions of ~130 nm and zeta-potentials of ~ -20 mV were obtained for all formulations. The similar zeta-potential for the neutral and anionic liposomes was unexpected, but is most likely due to a shielding effect of the PEG layer. The encapsulation stability was closely followed for all formulations (Table

2). The unsaturated liposomes were slightly less stable than the Stealth liposomes, with an encapsulation of ~ 95% after three months versus ~ 99% for the Stealth formulation. As the main phase transition temperature of the two main lipids (POPC and POPG) is -2 °C, this slight instability is likely due to the liposomes being in a fluid phase.

Table 1: Physical/chemical characterization of liposomal formulations of oxaliplatin[a]

	Abbreviation	Size (nm)	PDI[b]	Zeta-potential (mV)	OxPt[c] (mg/mL)	Lipid (mg/mL)	ED[d] (%)	Drug-to-lipid weight ratio
HSPC/Chol/DSPE-PEG2k (57:38:5) + OxPt	Stealth	144 ± 2	0.02	- 19 ± 1	0.72	18.4	98.9	1:26
POPC/POPG/DSPE-PEG2k (55:40:5) + OxPt	0%chol	133 ± 1	0.03	- 23 ± 1	0.99	14.6	98.6	1:15
POPC/POPG/Chol/DSPE-PEG2k (35:40:20:5) + OxPt	20%chol	127 ± 2	0.07	- 19 ± 1	0.92	13.5	99.4	1:15
POPC/POPG/Chol/DSPE-PEG2k (15:40:40:5) + OxPt	40%chol	129 ± 1	0.03	- 19 ± 1	0.81	13.8	98.7	1:17

[a]Representative batches of liposomes used for *in vitro* experiments

[b]Polydispersity index (PDI)

[c]Oxaliplatin (OxPt)

[d]Encapsulated drug (ED) to free drug fraction in percentage

Table 2: Encapsulation stability

	Months[a]	Encapsulation (%)[b]	n
POPC/POPG/DSPE-PEG2k (55:40:5)	0	98 ± 1	2
	1	97.9 ± 0.2	2
	2	96 ± 2	2
	3	94 ± 3	2
	6	96	1
	9	95	1
POPC/POPG/Chol/DSPE-PEG2k (35:40:20:5)	0	98.7 ± 0.5	4
	1	97.9 ± 0.9	4
	2	97 ± 1	4
	3	96 ± 2	4
	6	95 ± 3	3
	9	93 ± 3	2
	12	86	1
POPC/POPG/Chol/DSPE-PEG2k (15:40:40:5)	0	99	1
	1	97	1
	2	95	1
	3	94	1

HSPC/Chol/DSPE-PEG2k	0	100	1
(57:38:5)	1	99	1
	2	99	1
	3	99	1

[a]Time after production

[b]Values are mean of n different batches ± standard deviation

3.2 Enzyme sensitivity and drug release

The reasoning for utilizing a trigger mechanism is to obtain a controlled, site-specific drug release. To investigate if the formulated liposomes posses this quality, the different formulations were incubated in the presence or absence of human sPLA$_2$ at 37 °C for 0, 6 and 24 h. Cell conditioned growth media (CM) from the human colorectal cancer cell line colo205 was used as the enzymatic source [23], and release of oxaliplatin was determined by ICP-MS (Fig. 2A). All three formulations exhibited a distinct release in the presence of enzyme, with minor alterations from 6 to 24 h. The formulations with 0 and 20% cholesterol exhibited a similar release of ~ 80%, whereas the formulation with 40% cholesterol showed a release of ~ 50%. It was unexpected that the latter showed any release at all as it should be in the L_d-L_o co-existence region and thus be inert to the enzyme. However, it is possible that the high negative charge reduces the requisite for disorder. Alternatively, the co-existence of L_d and L_o phase does not abolish the enzyme activity, only reduces it. Thus the study demonstrates that by applying anionic unsaturated liposomes, incorporation of cholesterol is possible without abolishing sPLA$_2$ specificity, and that by changing the amount of cholesterol one can alter the liposome release kinetics. This is important since incorporation of cholesterol has been reported to increase liposome stability *in vivo*.

In the absence of enzyme, all three formulations gave a release of 20-30%, which increased slightly from 6 to 24 h. This release is likely due to leakage upon heating, and seems to represent a small burst release during the heating process followed by a slow diffusion when kept at 37 °C. This would mean that the formulations would not be entirely stable in circulation. However, as the release does not seem to increase much when reaching 37 °C, the liposomal oxaliplatin may still represent an improvement compared to the free drug.

To verify that the enzyme-mediated release was actually due to phospholipid hydrolysis, the enzymatic process was analyzed directly by MALDI-TOF MS. Liposomes with 20% cholesterol were incubated in the absence or presence of sPLA$_2$ at 37 °C for 6 h. Enzyme from two different human sources was used; colo205 CM (~ 75 ± 20 ng/mL [23]) and human tear fluid (54.5 ± 33.9 µg/mL [28]). The hydrolysis was estimated based on the relative change in the MALDI-TOF peaks after addition of sPLA$_2$ (Fig. 2B and 2C). For both POPC and POPG the peak intensities decreased in the presence of enzyme. The decrease was greatest in the presence of tear fluid, which

corresponds well with this having the highest enzyme concentration, and for POPG, which corresponds well with the enzyme being sensitive towards anionic lipids. The disappearance of the di-acyls correlated well with the appearance of the hydrolysis products (data not shown). The hydrolysis was not complete for any of the lipids. This could be explained by the liposomes being disintegrated before complete hydrolysis, and the enzyme loosing its sensitivity towards the disorganized substrate. Control experiments carried out in the absence of enzyme at 0 and 24 h incubation proved the absence of non-enzymatic hydrolysis (data not shown).

In conclusion, the new liposomal formulations do seem to represent an enzyme specific trigger mechanism, where release kinetics is partially dependent on the level of cholesterol. It has been demonstrated that $sPLA_2$ from porcine pancreas (which is different from human $sPLA_2$-IIA [29,30]) is active towards membranes in the L_d-L_o co-existence region, and that hydrolysis results in an increase in the L_o phase up to a point where the enzyme looses its sensitivity towards the membrane [19]. It is possible that human $sPLA_2$ has a similar activity, and that the liposomes with 40 mol% cholesterol eventually shifted from an L_d-L_o co-existence phase to a pure L_o phase, thus resulting in reduced release from these. This underlines the possibility of controlling the release kinetics by altering the amount of cholesterol.
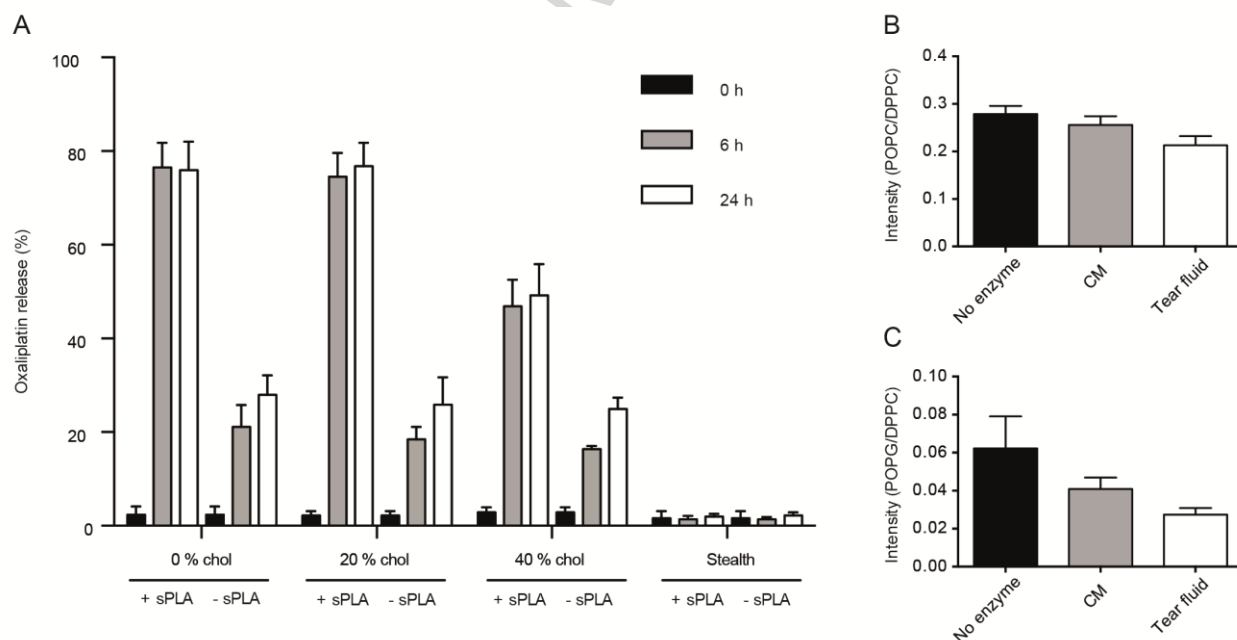
Fig. 2. Enzymatic hydrolysis and release of oxaliplatin. **A)** POPC/POPG/Chol/DSPE-PEG2k liposomes with 0, 20 and 40% cholesterol and HSPC/Chol/DSPE-PEG2k (Stealth) liposomes, loaded with oxaliplatin, were incubated in the presence or absence of secretory phospholipase A_2 (± $sPLA_2$) at 37 °C with gentle magnetic stirring for 0, 6 and 24 h. Release of oxaliplatin was

determined by ICP-MS. Values are mean of three individual experiments ± SD. POPC/POPG/Chol/DSPE-PEG2k (35:40:20:5) liposomes were incubated with colo205 cell conditioned media (CM), human tear fluid or no enzyme at 37 °C for 6 h. Hydrolysis of **B)** POPC and **C)** POPG was determined by MALDI-TOF MS. DPPC was included as internal reference. Values are mean of a minimum of three lipid extractions and three spots of each extraction.

3.3 Empty liposomes reveal sPLA$_2$ dependent *in vitro* cytotoxicity

The *in vitro* cytotoxicity of empty sPLA$_2$ sensitive liposomes was investigated, and compared to that of non-toxic stealth liposomes. The human colorectal cancer cell line HT-29, which does not secrete sPLA$_2$ [31], was used as model cell line. The anti-proliferative effect of increasing concentrations of empty liposomes in the absence of sPLA$_2$ was evaluated by MTS staining (Fig. 3A). All four formulations gave a slight decrease in cell survival, with ~ 80% survival at 740 μM lipid. It is not known what this decrease was caused by, but it was also the case for the stealth liposomes, and could be due to the presence of liposomes causing minor cellular stress responses. Accordingly, it was concluded that the sPLA$_2$ sensitive liposomes (SSLs) per se are non-toxic.

It has previously been reported that the sPLA$_2$ hydrolysis products may act as permeabilizing agents and at high concentrations be cytotoxic, possibly by inducing cellular lysis. It was therefore important to investigate the potential cytotoxicity of these. Therefore, the anti-proliferative effect of empty liposomes was examined in the presence of enzyme (Fig. 3B). Here the SSLs revealed a concentration dependent anti-proliferative effect with only ~ 50% cell survival at 740 μM liposomes, as compared to ~ 100% survival for the Stealth liposomes. A distinct difference between the three SSL formulations was not observed. At low lipid concentrations (below ~ 200 μM) none of the liposomes displayed toxicity. This is likely due to a neutralizing effect of serum albumin, which has previously been reported to bind up to five lyso-lipids [32]. Thus at high protein to lipid ratios, there may not be an effect of the hydrolysis products. This could be an advantage during circulation, as it would avoid disruption of both liposomes and blood-borne cells.

To investigate if the cytotoxicity at high lipid concentrations was caused by cellular lysis, the effect of empty liposomes was followed by time-course microscopy. Cells were treated with 740 μM empty SSLs for 72 h and imaged 10 min, 1, 2, 4, 6 and 72 h post treatment (Fig. S1). Although the cells showed clear signs of toxicity, even after 72 h, there was no apparent lysis (compared to complete lysis after two hours for fully saturated lipid formulations, previously studied in our lab). This would imply that these sPLA$_2$-sensitive liposomes do not cause lysis *in vitro*, and that the reduced proliferation is either due to increased permeability or induction of apoptosis. We have previously observed *in vivo* toxicity for fully saturated lipid formulations upon sPLA$_2$ activation,

and related this to the immense lysis seen *in vitro* for such formulations. We therefore reasoned that the new SSL formulations should be safer *in vivo* due to their substantially reduced lytic properties.
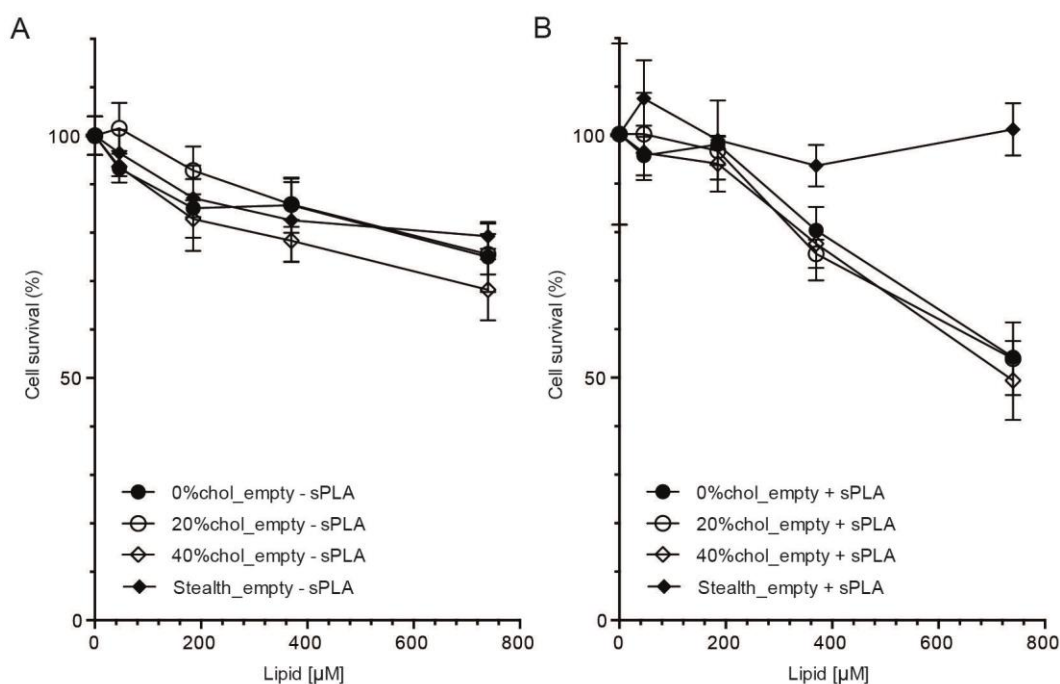
Fig. 3. *In vitro* cytotoxicity of empty sPLA$_2$-sensitive liposomes. HT-29 cells were treated with an increasing amount of empty POPC/POPG/Chol/DSPE-PEG2k liposomes with 0 (closed circles), 20 (open circles) or 40% cholesterol (open diamonds) or Stealth liposomes (closed diamonds), either **A)** in the absence or **B)** in the presence of colo205 cell conditioned media containig sPLA$_2$. Cell survival was evaluated by MTS staining. Values are mean of triplicates with blank (media with MTS reagent) subtracted, $\pm$ SD. All values are normalized to non-treated cells. The data is representative of minimum three separate experiments.
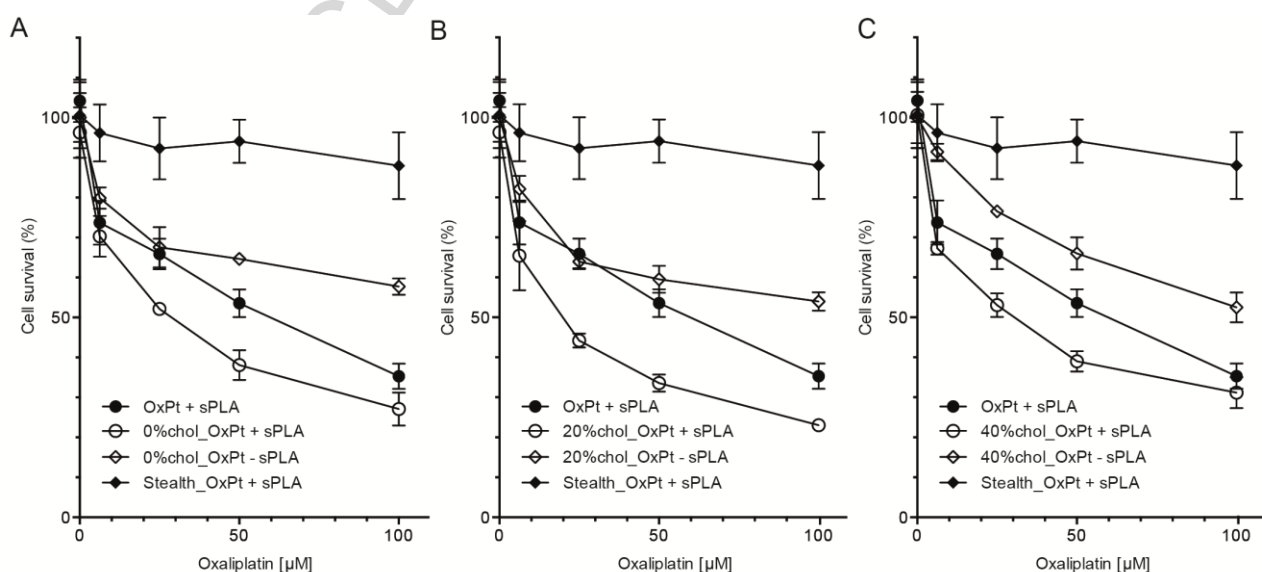
3.4 *In vitro* cytotoxicity of OxPt-loaded liposomes is enzyme dependent, but not cholesterol dependent.

The *in vitro* anti-neoplastic effect of oxaliplatin encapsulated in the three SSL formulations was examined and compared to that of free oxaliplatin and oxaliplatin encapsulated in stealth liposomes. HT-29 cells were treated with increasing concentrations of SSLs with 0% cholesterol (Fig. 4A), 20% cholesterol (Fig. 4B) or 40% cholesterol (Fig. 4C). Exogenous sPLA$_2$ was added using cell-conditioned media from colo205 cells. All three SSL formulations revealed a concentration dependent, enzyme-specific, anti-proliferative effect that was clearly higher than that of free oxaliplatin. This enhanced effect is likely due to the hydrolysis products, which may either be

cytotoxic or enhance cell permeability, thus enhancing the drug transport across the cellular membrane. It should be noted that even though the SSLs are toxic, there is a clear effect of loading them with oxaliplatin. At 25 μM oxaliplatin (corresponding to ~ 185 μM lipid) the loaded SSLs resulted in a cell survival of ~ 50% (Fig. 4) as compared to ~ 100% survival for the empty SSLs (Fig. 3B). Thus it seems that it is the combination of oxaliplatin encapsulated in SSLs that provide the superior anti-neoplastic effect.

There was no distinct difference between the three SSL formulations, although there might be a slight tendency towards a lesser effect of the liposomes with 40% cholesterol (Fig. 4). Thus it appears that the amount of cholesterol is not the determining factor in the anti-proliferative effect of these SSLs.

Surprisingly, all three oxaliplatin loaded SSL formulations revealed growth inhibition in the absence of enzyme (Fig. 4). Although this inhibition was markedly lower than that of free oxaliplatin, it was distinctly higher than that of loaded stealth liposomes. Thus it seems that the SSL-induced anti-proliferative effect is not entirely enzyme specific. Flow cytometry revealed a small but distinct uptake for all three SSL formulations (Fig. S2). However, the mean fluorescence intensity of the stealth liposomes was ~ 3.5-fold higher than that of the SSLs, suggesting that unspecific uptake is not a major determinant in sPLA$_2$-independent growth inhibition. As the unspecific drug leakage after 6 h at 37 °C was found to be ~ 20% for all three SSL formulations and ~0 % for the stealth formulation (Fig. 2A), it seems that the unspecific cytotoxicity is primarily due to extracellular leakage from unstable liposomes. Although this will effectively reduce the amount of liposomal oxaliplatin that can potentially reach the tumor site *in vivo*, 80% encapsulated drug represents a major improvement to free oxaliplatin.
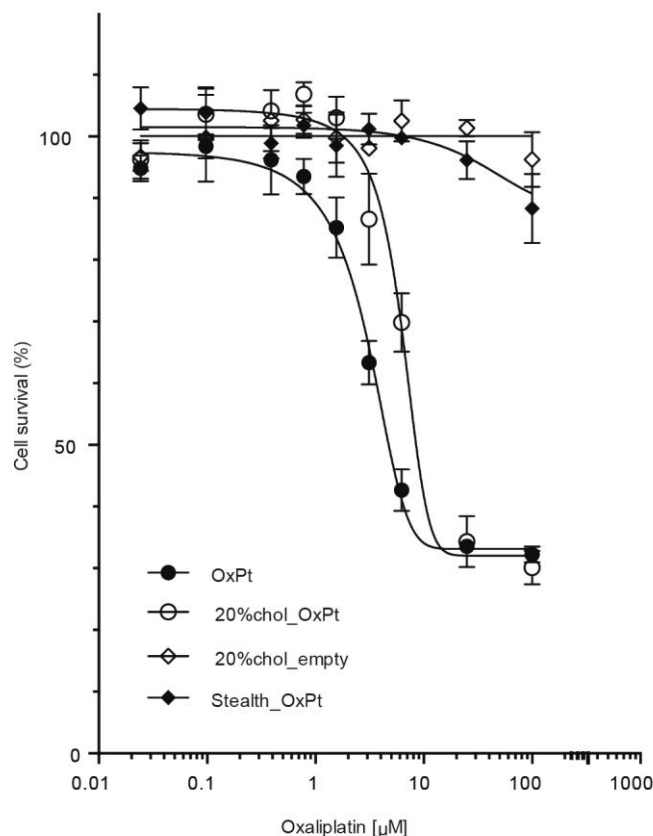
Fig. 4. *In vitro* cytotoxicity of oxaliplatin (OxPt) loaded sPLA$_2$-sensitive liposomes. HT-29 cells were treated with an increasing amount of OxPt loaded POPC/POPG/Chol/DSPE-PEG2k liposomes with **A)** 0, **B)** 20 or **C)** 40% cholesterol, either in the presence (open circles) or absence (open diamonds) of colo205 cell conditioned media containing sPLA$_2$. OxPt in free form (closed circles) or encapsulated in HSPC/Chol/DSPE-PEG2k (Stealth) liposomes (closed diamonds) were used as controls. Cell survival was evaluated by MTS staining. Values are mean of triplicates with blank (media with MTS reagent) subtracted ± SD. All values are normalized to non-treated cells. The data is representative of minimum three separate experiments.

3.5 sPLA$_2$ sensitive liposomes are toxic towards mammary carcinoma cell line

Since there did not seem to be a distinct difference in the anti-proliferative effect of the three SSL formulations, only one preparation was selected for further studies. As the formulation with 40% cholesterol gave a clearly lower *in vitro* release, this was excluded. Further, liposomes with no cholesterol have been shown to be less stable in circulation, with high protein adsorption and rapid clearance [33]. The 20% cholesterol formulation was therefore selected for the final *in vitro* and *in vivo* studies.

To investigate the versatility of this formulation, its anti-neoplastic effect was tested on MT-3 cells, a human, sPLA$_2$ secreting, mammary carcinoma cell line [34]. Cells were treated with increasing concentrations of empty SSLs, oxaliplatin loaded SSLs, free oxaliplatin and oxaliplatin loaded stealth liposomes (Fig. 5). The empty SSLs showed no toxicity, supporting the biocompatibility of these liposomes. Both the loaded SSLs and the free oxaliplatin gave a concentration-dependent anti-proliferative effect, with ~ 35% survival at 25 μM oxaliplatin. This is a slightly lower survival than what was seen for the HT-29 cells (Fig. 4), indicating that the MT-3 cells are somewhat more sensitive to treatment by oxaliplatin. At lower drug concentrations (below 25 μM), the loaded SSLs gave a slightly lower effect than the free drug towards the MT-3 cells.

Fig. 5. *In vitro* cytotoxicity of sPLA$_2$-sensitive liposomes towards sPLA$_2$ secreting MT-3 cells. Cells were treated with an increasing amount of free oxaliplatin (OxPt) (closed circles), empty (open diamonds) or OxPt loaded (open circles) POPC/POPG/Chol/DSPE-PEG2k (35:40:20:5) liposomes or OxPt loaded Stealth liposomes (closed diamonds). Cell survival was evaluated by MTS staining. Values are mean of triplicates with blank (media with MTS reagent) subtracted ± SD. All values are normalized to non-treated cells. The data is representative of minimum three separate experiments.

3.6 *In vivo* **efficacy**

Though the *in vitro* data were promising, *in vivo* evaluation is essential for signifying a clinical potential. For this purpose we utilized human MT-3 xenografts transplanted onto nude NMRI mice. The MT-3 model was chosen based on its high *in vitro* sensitivity towards oxaliplatin and *in vivo* sPLA$_2$ expression. Given its clear *in vitro* anti-neoplastic effect towards MT-3 cells, we evaluated the SSL formulation with 20% cholesterol. For comparison we included stealth liposomes. Mice received 10 mg/kg oxaliplatin, the liposomal equivalent (Table S1) or isotonic glucose solution, by tail vein injection once per week for a total of four injections.

Two days after first treatment one mouse having received the SSL formulation succumbed. Three days after first treatment the remaining six mice having received the SSL formulation were euthanized due to excessive weight loss, dehydration and subcutaneous bleedings (Fig. 6A and B). Mice having received control compounds showed no signs of discomfort. The symptoms observed for the SSL treated mice were similar to those previously observed for saturated liposomes that were highly sensitive to sPLA$_2$. However, for fully saturated liposomes the symptoms did not occur until after four administrations of 8 mg/kg every four days. Thus it seems that the new SSL formulation is even more toxic than the previous formulations we have investigated, even thought the liposomes developed in the present work were less lytic *in vitro*.

Macroscopically the mice had excessive amounts of sero-hemorrhagic fluid in the abdominal cavity. The livers were enlarged and heterogeneously darker colored and the gall bladder content was hemorrhagic in all mice investigated. No abnormalities were observed in the thoracic cavity and the urinary bladder content was not hemorrhagic. The liver was formalin fixed and paraffin wax embedded and investigated histologically (hematoxylin and eosin staining was performed on multiple 5 μm thick sections). Liver sections displayed acute multifocal necrosis of hepatocytes with collapse of hepatic sinusoids and peripheral vacuolar degeneration; some hepatocytes also had nuclear accumulations reminiscent of inclusion bodies. A distinct inflammatory reaction was observed in hepatic sinusoids and around necrotic regions with accumulation of granulocytes (Fig. 6C).

Tumor volume measurements were continued for the remaining therapy groups and controls. Stealth liposomes displayed improved tumor control compared to untreated controls, although all tumors displayed a continuous growth (Fig. S3). In contrast, treatment with free oxaliplatin had no effect compared to control. This was somewhat unexpected as the *in vitro* data suggested high sensitivity of MT-3 cells towards oxaliplatin, and support the need and potential of an effective drug delivery vehicle in cancer therapy.

The immense systemic toxicity caused by the new SSL formulation is alarming. Previous findings have shown that serum sPLA$_2$ levels are often elevated in cancer patients compared to healthy controls [7,35]. Further, it has been shown that many outbred mouse stocks have an intact murine sPLA$_2$ gene [36]. Together these findings suggest the possibility of an abnormally high sPLA$_2$ serum level, which could induce premature activation of the liposomes in circulation. Finally, nude mice are known to accumulate liposomes in the skin [37]. This could give a locally very high concentration of lytic agents, resulting in lysis of platelets and subsequent subcutaneous bleedings. However, the complete absence of lysis *in vitro* would still imply that the new SSL

formulation should be less prone to induce lysis *in vivo*, compared to the previously investigated saturated lipid formulation, suggesting that there may be another explanation.

An alternative theory is induction of complement activation. Previous studies have shown that liposomes with high negative charge are prone to activate complement [38]. As both the new SSL formulation and our previously investigated saturated lipid formulation contain 40 mol% negatively charged lipids, they are both within this category. Further, studies have indicated that liposomes made up of unsaturated lipids are more prone to activating the complement system compared to those prepared by saturated lipids [38]. This could explain why the new SSL formulation induced the greatest toxicity. Finally, complement activation has been correlated to platelet activation, endothelial damage, microvascular thrombosis and subsequent thrombocytopenia [39], which could explain the symptoms in the skin, seen for the SSL treated mice. However, complement activation is usually observed as an acute infusion reaction [40], and not as a slowly progressing state.

Instead of a direct effect of the liposomes, the difference in toxicity could be related to the different lysis products. Where hydrolysis of DPPC and DPPG results in the release of palmitic acid (PA), hydrolysis of POPC and POPG results in the release of oleic acid (OA). However, a direct comparison between the *in vitro* toxicity of PA and OA demonstrated that OA was significantly less damaging to hepatocytes in culture compared to PA [41]. Thus it seems unlikely that the *in vivo* toxicity was a direct cause of OA-induced hepatocyte damage. Instead, intravenous injection of oleic acid has been reported to elicit respiratory distress and acute lung injury demonstrated by inflammation and macroscopic hemorrhagic lesions on both pulmonary fields [42–45]. Contrary, no association between PA and lung injury seem to be reported. However, no reports seem to exist on oleic acid induced liver damage, and in our studies no macroscopic lung damage was observed, suggesting a somewhat different mechanism. In relation to this, there is inevitably a difference in the pharmacokinetics (PK) and biodistribution (BD) of free oleic acid and that potentially produced by hydrolysis of liposomes. Where intravenous administration of free oleic acid leads to accumulation in lungs, negatively charged liposomes do not normally demonstrate a high lung accumulation. Instead, clearance of liposomes, generally tend to result in a high accumulation in the liver and spleen. Although previous experiments in our laboratory have not indicated a relation between high liver accumulation of liposomal oxaliplatin and liver damage, other mechanism might be important in the case of SSLs. Even though the expression of $sPLA_2$-IIA is likely to be low in the liver, the presence of other subtypes might also be able to degrade the liposomes. Especially subgroup 1B ($sPLA_2$-IB) has been reported to be expressed in the liver [29]. $sPLA_2$-IB has been proposed to have a substrate specificity and activity similar to that of snake venom $sPLA_2$ [30], i.e. a high activity and broader specificity compared to human $sPLA_2$-IIA. Thus $sPLA_2$-IB might not

only be able to degrade the new SSLs, it might also induce a faster drug release compared to sPLA$_2$-IIA. The combination of potentially haemolytic agents and an acute burst release of oxaliplatin, causing a locally very high dose of oxaliplatin, could potentially result in acute liver failure. Both necrosis and inflammation seen in the liver histopathology implies a liver related toxicity. In accordance with this, oxaliplatin-based chemotherapy has been associated with portal hypertension, severe hepatic sinusoidal injury, thrombocytopenia and ascites (fluid in the peritoneal cavity) in patients [46], i.e. adverse effects similar to those observed here. Furthermore, sPLA$_2$ has been reported to leak out of necrotic hepatocytes and induce injury on healthy hepatocytes [47]. This could suggest a positive feedback loop, where a few necrotic hepatocytes secrete sPLA$_2$, which activates the liposomes, resulting in enhanced hepatocyte death, and subsequent enhanced sPLA$_2$ release etc. Combined with the generally higher accumulation of liposomal oxaliplatin compared to free oxaliplatin (which to a larger extent is cleared by the kidneys), this could explain the extensive toxicity observed.

Further evidence of a toxicity caused by the combination of SSLs and oxaliplatin could be obtained by investigating the effect of administering empty SSLs. However, the severe adverse effects imply that such an experiment would be highly unethical.

Finally, it should be noted that due to the severe toxicity the *in vivo* study has not been repeated. With this in mind, the present study demonstrates that great caution should be implemented when utilizing sPLA$_2$ sensitive liposomes. Even though many have shown potential *in vitro*, the real utility can only be disclosed *in vivo*. To the best of our knowledge no one has so far provided convincing proof of an sPLA$_2$ sensitive formulation with a significantly improved efficacy and reduced adverse effects. As such we remain skeptical to the actual potential of sPLA$_2$ sensitive liposomes in drug delivery, and considerable caution should be applied with the current formulations undergoing clinical evaluation.
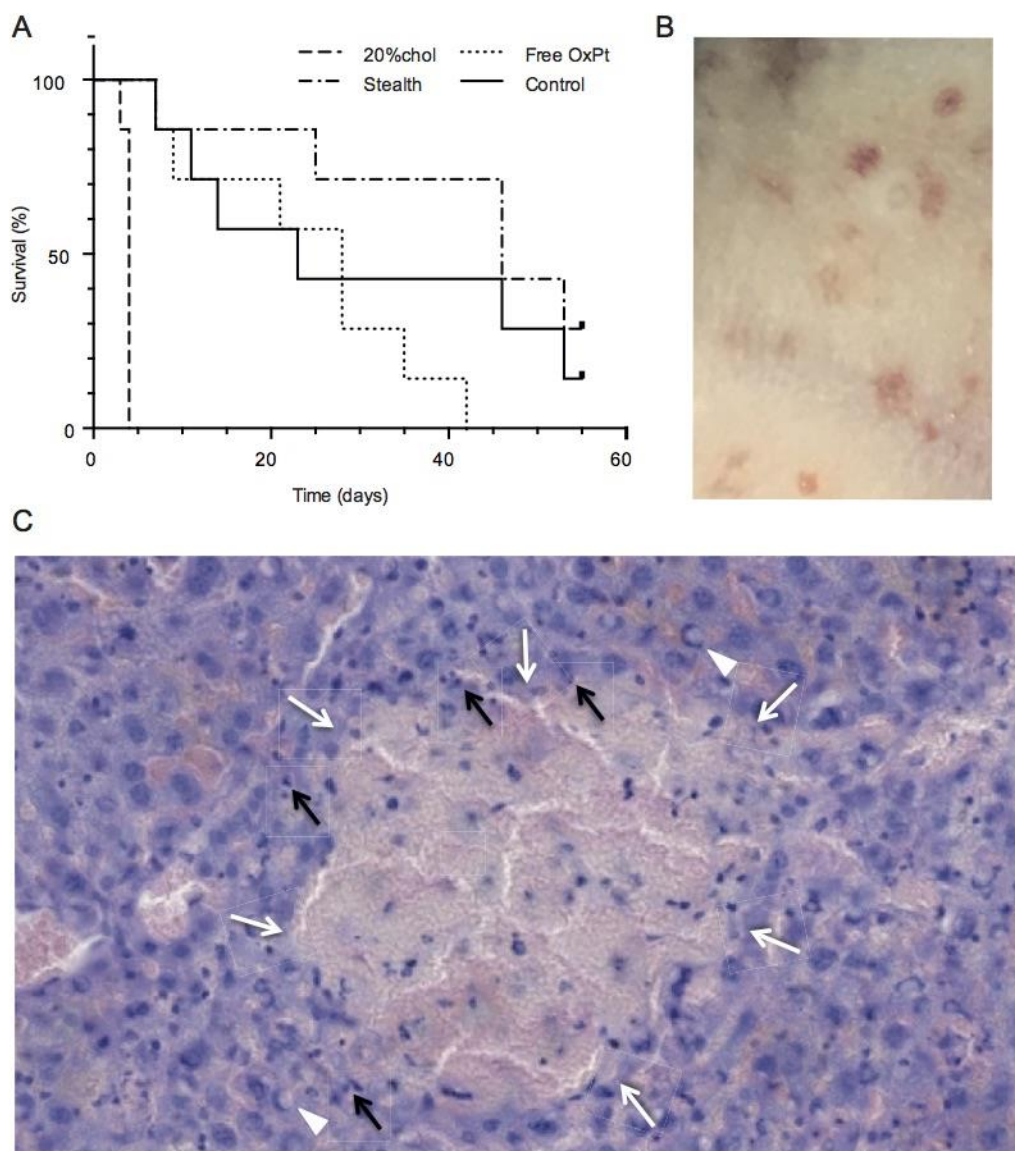
Fig. 6. *In vivo* effect of sPLA$_2$ sensitive liposomes. **A)** Kaplan-Meier analysis of survival. Nude NMRI mice bearing MT-3 xenograft tumors were treated with 10 mg/kg free oxaliplatin (OxPt) (dotted line), OxPt loaded POPC/POPG/Chol/DSPE-PEG2k (35:40:20:5) liposomes (dashed line) or OxPt loaded Stealth liposomes (dotted dashed line) once per week for a total of four treatments. Isotonic glucose solution (solid line) was included as control. **B)** The skin of mouse treated with OxPt loaded POPC/POPG/Chol/DSPE-PEG2k (35:40:20:5) liposomes, revealing subcutaneous bleedings. **C)** Liver section from a mouse treated with OxPt loaded POPC/POPG/Chol/DSPE-PEG2k (35:40:20:5) liposomes. The liver section displays multifocal necrosis of hepatocytes (white arrows) with collapse of hepatic sinusoids and vacuolar degeneration of primarily peripheral hepatocytes; some of the peripheral hepatocytes also present nuclear inclusion body-like accumulations (white arrowheads). Distinct inflammatory reaction is seen in the hepatic sinusoids

<mark>and around necrotic regions with accumulation of granulocytes (black arrows) (HE stained FFPE 5 um).</mark>

4. Conclusion

In conclusion, the present study demonstrates that by applying anionic unsaturated liposomes one can incorporate cholesterol <mark>into the liposome membranes</mark>, which is important for *in vivo* stability, without loosing sPLA$_2$ specificity. Although none of the liposomal formulations revealed cellular lysis *in vitro*, the tested SSLs induced severe toxicity *in vivo*, indicating that the related toxicity is not necessarily associated with lytic agents. Instead, histopathology studies demonstrated extensive necrosis and inflammation of the liver, indicating a direct toxic effect on the liver. <mark>We hypothesize that this is due to high local concentration of oxaliplatin, perhaps in combination with generation of lyosolipid and fatty acid hydrolysis products, as a result of extensive liposome activation by sPLA$_2$.</mark> As such our results demonstrate the necessity of evaluating SSLs *in vivo*, and underlines existing challenges with sPLA$_2$ sensitive liposomal approaches.

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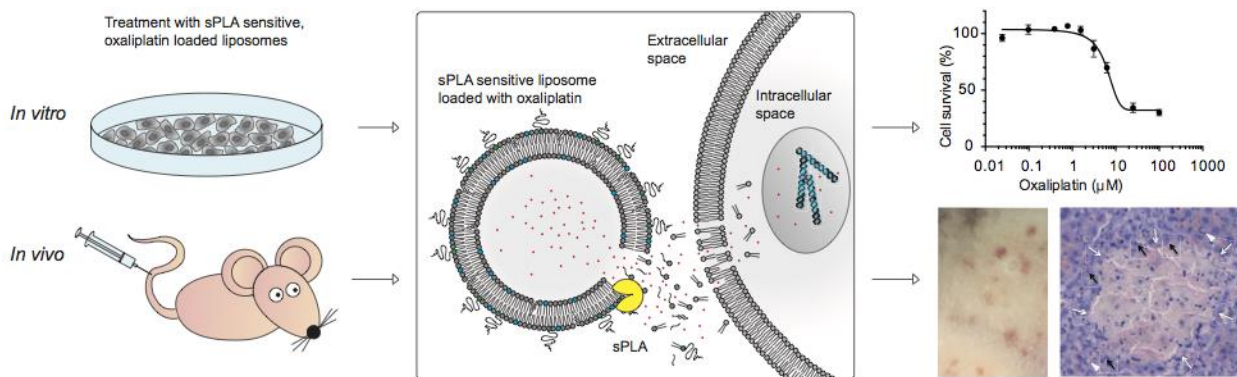[15]   O.G. Mouritsen, T.L. Andresen, A. Halperin, P.L. Hansen, A.F. Jakobsen, U.B. Jensen, M.O.

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Graphical abstract