Sonosensitive dioleoylphosphatidylethanolamine-containing liposomes with prolonged blood circulation time of doxorubicin

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ABSTRACT

Ultrasound sensitive (sonosensitive liposomes) are drug delivery systems designed for releasing their drug load upon exposure to ultrasound (US). Inclusion of dioleoylphosphatidylethanolamine (DOPE) in liposome membranes was previously shown to induce sonosensitivity. For efficient US mediated drug delivery to solid tumours, a long blood circulation time of the liposomal drug providing high tumour uptake is required. In this study, blood pharmacokinetics of DOPE-based liposomal doxorubicin (DXR) were evaluated in mice. A markedly faster blood clearance of DXR was observed for DOPE-rich liposomes compared to Caelyx® (standard liposomal DXR). Subsequently, liposome membrane composition was altered to improve drug retention in the bloodstream, while maintaining sonosensitivity. Formulations with reduced blood clearance of DXR were obtained by reducing the content of DOPE from 62 to 32 or 25 mol%. These formulations showed long blood circulation time, as approximately 20% of the administered DXR dose was present in the bloodstream 24 h after intravenous injection. The reduction in liposomal DOPE content did not significantly reduce US mediated DXR release *in vitro*, indicating that DOPE is a potent modulator to sonosensitivity. The novel liposome formulations, containing moderate amounts of DOPE, displayed similar blood pharmacokinetic profiles as standard liposomal DXR, but a markedly improved sonosensitivity.

Keywords

Liposomes; Doxorubicin; Pharmacokinetics; Ultrasound; Sonosensitive; Drug delivery.

1. INTRODUCTION

Liposomes that exhibit controlled release properties have potential for delivery of chemotherapeutic drugs to solid tumours. Ideally, liposomes should accumulate intact at the tumour site, and subsequently release their drug load. The extent of liposome accumulation in the tumour is largely determined by the blood circulation time of the liposomal drug, where liposomes that retain their drug over several hours to days will provide greater tumour delivery and less non-specific toxicity. For reviews see Drummond *et al.*, 1999; Gabizon *et al.*, 2003. After liposome accumulation within the tumour tissue, the encapsulated drug must be released before it can be taken up by the neoplastic cells (Horowitz *et al.*, 1992). In the case of long circulating PEGylated doxorubicin (DXR)-liposomes, the drug has shown to leak slowly from the liposome (Horowitz *et al.*, 1992; Colbern *et al.*, 2000). Thus, triggered destabilization of the liposome carrier within the tumour area might lead to a substantial increase in drug availability and thus improved therapeutic efficacy.

Recently, there has been increasing interest in using ultrasound (US) as a means to physically and non-invasively trigger drug release from liposomes upon accumulation in tumour tissue (de Smet *et al.*, 2010; Schroeder *et al.*, 2009; Myhr and Moan, 2006; Pong *et al.*, 2006). Local application of US to tumours produces several effects, such as enhancing extravasation of liposomes to tumours, inducing drug leakage from the liposome carrier and enhancing cell membrane permeability with a resulting increased intracellular drug uptake (For recent reviews see Schroeder *et al*., 2009; Pitt *et al*., 2004; Frenkel, 2008). In order to maximize the therapeutic efficacy, liposomes should display properties that both impart high US sensitivity (sonosensitivity) and favour drug retention in the blood stream, to enable sufficient tumour accumulation. Several studies have shown that gas-containing particles are highly sonosensitive (Huang *et al.*, 2008; Unger *et al*., 1998; Huang and MacDonald., 2004). Unfortunately, gas-filled liposomes are micron-sized and thus too large to allow for effective extravasation into tumour tissue (Ferrara, 2008; Maeda *et al*., 2000). Another limitation of such particles is the rapid dissolution of the entrapped gas in blood circulation with consequent loss of sonosensitivity (Unger *et al.*, 1998; Ferrara, 2008).

An approach to formulate gas-free liposomes combining high sonosensitivity with long blood circulations times might be to modulate the liposome membrane composition. Previously, we demonstrated that PEGylated liposomes comprising phosphatidylethanolamine showed a several fold increase in drug release compared to Caelyx® (standard liposomal DXR) during *in vitro* exposure to US (Evjen *et al.*, 2010; Evjen *et al.*, 2011a; Evjen *et al*., 2011b). An US enhanced therapeutic activity of DSPE-based liposomal DXR was demonstrated in tumoured mice (Hagtvet *et al*., 2010). Recently, we

found that liposomes based on DOPE as the main phospholipid constituent displayed markedly improved sonosensitivity than DSPE-based liposomes (Evjen *et al*., 2011a). These DOPE-based liposomes, however, were not evaluated and optimized as drug carriers for *in vivo* applications.

In the present study, blood circulation time of various DXR-containing liposome formulations combining DOPE with distearoylphosphatidylcholine (DSPC), PEGylated distearoylphosphatidylethanolamine (DSPE-PEG 2000) and cholesterol, were investigated in non-tumoured mice. Novel formulations combining high sonosensitivity and long blood residence time of DXR were obtained.

2. MATERIALS AND METHODS

2.1 Materials

Cholesterol, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) ammonium sulfate, TritonX-100® solution and sucrose were obtained from Sigma Aldrich, Oslo, Norway. Serum of fetal bovine origin (Autonorm) was obtained from Sero (Billingstad, Norway). DSPE-PEG 2000) DSPC, DOPE, were purchased from Genzyme Pharmaceuticals, Liestal, Switzerland. Doxorubicin hydrochloride (DXR) was purchased from Nycomed, Asker, Norway. DXR-containing liposomes, Caelyx® (herein defined as standard liposomal DXR), comprising hydrogenated soy phosphatidylcholine (HSPC), DSPE-PEG 2000 and cholesterol (57:5:38 mol %) was supplied from the pharmacy at the Norwegian Radium Hospital, Oslo, Norway (European distributor Schering-Plough).

For anaesthesia of mice a mixture of 2.4 mg/ml tiletamine and 2.4 mg/ml zolazepam (Zoletil® vet, Virbac Laboratories, Carros, France), 3.8 mg/ml xylazine (Narcoxyl® vet, Roche, Basel, Switzerland) and 0.1 mg/ml butorphanol (Torbugesic®, Fort Dodge Laboratories, Fort Dodge, IA) was prepared and used.

2.2 *Liposome preparation*

Liposomes with different mol % of the lipids DOPE, DSPC, DSPE-PEG and cholesterol were prepared by the thin film hydration and sequential extrusion method similarly as previously described (Evjen *et al*., 2011a). (See Table 1, Section 3.2 for liposome membrane compositions). Briefly, dry lipid films were hydrated with 300 mM ammonium sulfate solution and exposed to three freeze/thaw cycles in a dry-ice/aceton/methanol mixture and water, respectively. The liposomes were reduced in size by stepwise extrusion (Lipex extruder, Biomembrane Inc., Vancouver B.C., Canada) through Nucleopore polycarbonate

filters with pore sizes of 800, 400, 200,100 and 80 nm (Nuclepore, West Chester, PA, USA). The mean target size of the liposomes was 85 ± 5 nm.

DXR was remote loaded into liposomes using an ammonium sulfate transmembrane gradient (Haran *et al.*, 1993), as previously described by Evjen *et al*., 2011a. The resulting nominal lipid and DXR concentrations were 16 mg/ml and 1 mg/ml, respectively.

2.3 Liposome characterization

The mean intensity-weighted hydrodynamic liposome diameter was determined by Photon Correlation Spectroscopy (Nanosizer, Malvern Instruments, Malvern, UK). The liposome dispersions were diluted prior to measurements in a ratio of 1:200 (v/v) with 0.22-μm- filtered sucrose/HEPES solution. The measurements were performed at 23 ºC and at a scattering angle of 90°. The polydispersity index (P.I) was given as an indicator for the width of the particle size distribution. Triplicate measurements of each liposome batch were performed.

Determination of liposomal entrapment efficiency of DXR was estimated by fluorescence measurements as previously described (Evjen *et al.*, 2011). All liposome formulations displayed entrapment efficiencies of DXR of more than 95% (drug:lipid ratio 1:16 wt/wt).

2.4 Ultrasound mediated drug release

US release measurements were conducted using a 40 kHz US transducer (VC 750, Sonic and Materials, Inc, Newtown, CT, USA) with a 19 mm diameter nonfocused probe mounted in a custom-built sample chamber as previously described (Evjen *et al.*, 2010). In brief, the liposome dispersions were diluted in a 1:500 (v/v) ratio with sucrose/HEPES solution containing 20% serum, just prior to the US experiments. The diluted liposome dispersions were exposed to 40 kHz US at a nominal intensity of 12 W/cm^2 in a continuous mode (100 % duty cycle) up to 6 min. Acoustic pressure measurements conducted with a Bruel and Kjaer hydrophone (Type 8103, Denmark) in the sample chamber gave 240 kPa (pk–pk). The temperature in the liposome samples never exceeded 30 °C during the US experiments, excluding the possibility of direct thermal effect of US on liposomal drug release.

DXR release could be monitored due to the relief of DXR-mediated fluorescence self-quenching in the external liposomal phase, and concomitant increase in fluorescence intensity. An optical fiber probe (Ocean optics, type QP600-2-VIS/BX, Florida, USA) was

used to measure *in line* the increase in fluorescence intensity as a result of DXR release, using the following equation:

Equation 1: $\%\ \text{Drug release} = (F_t - F_0)/ (F_{max}-F_0)*100$

Where F_0 and F_t are the fluorescence intensities of a given liposome sample prior to and after a given duration (t) of US respectively, and F_{max} is the fluorescence intensity after liposome solubilisation with surfactant (Triton-X 100). The diluted liposome samples were solubilised with Triton-X 100 at a ratio of 100:1 (v/v). Fluorescence intensity measurements were performed using a fluorescence spectrometer from Ocean Optics (model QE65000. Duiven, Netherlands). The excitation and emission wavelength for DXR were 488 and 595 nm, respectively. Triplicate samples were measured.

2.5 Serum stability assay

Liposome stability *in vitro* was studied using a well-established serum-induced leakage assay as previously described by Evjen *et al.*, 2010. Liposome dispersions, diluted 1:125 (v/v) with sucrose/HEPES solution containing 20% serum were incubated up to 24 h at 37 ºC. Time-dependent leakage of DXR was quantified by fluorescence measurements, by further diluting the samples 1:4 (v/v) with sucrose/HEPES solution, according to Equation 1. Triplicate samples were measured.

2.6 Pharmacokinetic and biodistribution studies in non-tumoured mice

Male atymic nude Balb/c mice were provided by the Department of Comparative Medicine (animal facility), the Norwegian Radium Hospital. The mice were housed in transparent boxes with bedding material, fed *ad libitum* and kept under specific pathogen-free conditions. The temperature and relative humidity were kept constant at 20-21°C and 60%, respectively. At the end of the experiments all animals were euthanized by cervical dislocation. All procedures were performed according to protocols approved by the National Animal Research Authority and carried out in compliance with the European Convention for the Protection of Vertebrates Used for Scientific Purposes.

The administered DXR dose for animals receiving the various DOPE-based liposomes was 7 mg/kg body weight. The DXR dose for animals receiving standard liposomal DXR was 14 mg/kg. The doubled DXR dose administered was due to the doubled drug-to lipid-ratio of the standard liposomes (1:8 drug/lipid) versus DOPE-based liposomes (1:16 drug/lipid). Thus, the dosing was based on an identical lipid dose for all investigated liposome

formulations, as the lipid dose (i.e. liposome dose) is generally known as a key factor influencing blood pharmacokinetics (Drummond *et al.*, 1999).

The liposomes were injected intravenously (i.v.) into the tail vein under anaesthesia induced by subcutaneous administration of 0.1 ml anaesthetic agent. Animals were sacrificed in groups of three at different time points post injection (0.5, 1, 3, 8 12, 24, 48 h). Blood samples were obtained by cardiac puncture using heparinized syringes and stored in heparinized tubes. Liver, spleen and kidneys were excised and their weight registered. All blood and tissue samples were kept on ice bath until storage at -80 °C.

Quantification of DXR was performed similarly as described by Gabizon *et al.*, 1989. In brief, 0.1 ml of whole blood samples (lysed due to freezing) was mixed with 1.9 ml acidified ethanol (0.3 M HCl in 50% ethanol) giving a final 1:20 (v/v) dilution. Duplicate samples were prepared for further processing. Tissue samples were added 50% acidified ethanol in a final 1:10 (v/v) dilution and homogenized using a Homogenizer PRO 200® (PRO Scientific, Monroe, CT, USA). The digested blood and tissue samples were incubated for 24 h at 4 °C in the dark. Following incubation the precipitate was removed by centrifugation (20000 *g*, 20 min, 4 °C) and the supernatant (containing extracted DXR) stored at -20°C until fluorescence measurements were performed. The extracted DXR was quantified by fluorescence measurements as previously described using multipoint standard curves. The latter were generated by adding known amounts of standard liposomal DXR to blood and tissue (liver, spleen and kidney) homogenate and incubated and centrifuged as described above. All calibration curves had regression coefficients (R^2)>0,99.

2.7 Statistics

All data are reported as mean values with standard deviation. Statistical comparison of the DXR concentration in blood and organs for the different liposome formulations was performed at each time point post injection using a student t-test with $p<0.05$.

3. RESULTS

3.1 Blood pharmacokinetics of prototype DOPE-based liposomes

DOPE-based liposomes with membrane composition DOPE:DSPC:DSPE-PEG 2000:CHOL 62:10:8:20 mol% were previously demonstrated to release their DXR load almost completely during 4 minutes of US exposure *in vitro* (Evjen *et al.*, 2011). Furthermore these liposomes showed negligible leakage of DXR during 24 h *in vitro* incubation in sucrose/HEPES solution containing 20% serum at 37 °C (Evjen *et al.*, 2011).

Hence, this DOPE-based formulation was first selected for assessment of blood pharmacokinetics in mice. Blood levels of DXR provide a good indication of liposomal drug retention, since DXR released in the bloodstream is rapidly distributed to other tissues and organs (Gabizon *et al.*, 1989).

Fig 1 shows the blood clearance profiles of DXR for DOPE-based liposomes in comparison to standard liposomal DXR. Both formulations showed exponential declines in blood concentrations, starting with a rapid decline in DXR blood concentrations followed by a slower elimination phase. In the first distribution phase, DXR concentrations were comparable between the two formulations. In the slower elimination phase, starting approximately at 3 h post injection, a significantly lower blood concentration was observed for the DOPE-based liposomes as compared to standard liposomal DXR. At 24 h post injection only 5% of the injected DXR dose remained in blood for the DOPE-based liposomes versus 20% for standard liposomal DXR (Fig 1).

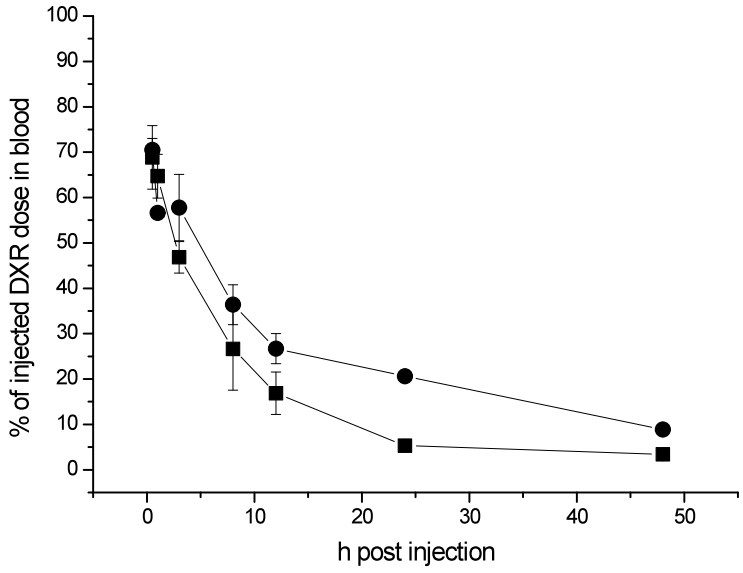


Fig 1. Blood clearance kinetics of i.v. injected liposomal DXR in mice, ■ DOPE-based liposomes (DOPE:DSPC:DSPE-PEG 2000:Chol; 62:10:8:20 mol%) in comparison to ● standard liposomal DXR (HSPC:DSPE-PEG 2000:Chol; 57:5:38 mol%). Bars represent the SD of the mean (n=3).

3.2 Effect of lipid composition on DXR blood pharmacokinetics of DOPE-based liposomes

Further, both blood clearance kinetics and *in vitro* sonosensitivity for a range of DOPE-based formulations with different lipid composition were investigated. Unsaturated phospholipids, like DOPE, fluidize the liposome membrane thereby increasing the membrane permeability at physiological temperature due to their low gel-to-liquid-crystalline phase transition temperature (T_c) (Demel *et al.*, 1976). Previous studies have shown that addition of cholesterol to liposomes rigidifies the bilayer and reduces leakage of entrapped material at temperatures above the T_c of the membrane (Gregoriadis, 1988; Damen *et al.*, 1981; Ishida *et al.*, 2006).

In an attempt to prolong blood circulation time of DOPE-containing liposomal DXR, membrane composition was altered by both increasing the level of cholesterol and reducing the level of DOPE. An overview of the different formulations produced is presented in Table 1.

Note that all formulations were comparable in terms of mean size and drug-to-lipid ratio, and differed only in membrane composition. The comparable liposome size was essential to better assess the influence of the membrane composition *per se* on blood circulation time and biodistribution. *In vitro* leakage studies showed no detectable leakage of DXR from the liposomes during the course of 24 h incubation in 20% serum at 37 ºC.

Table 1. Overview of DXR-containing DOPE-liposomes.

no	**DOPE:DSPC:DSPE-PEG:Chol (mol%)**	**Mean size, nm (P.I)**	**% DXR in plasma 24 h post inj.**	**% DXR released 6 min post US**
1	62:10:8.20	86 (0.14)	5 ± 1	91± 2
2	52:5:8:35	86 (0.12)	3 ± 1	79 ± 1
3	52:20:8:20	80 (0.10)	4 ± 1	78 ± 6
4	52:0:8:40	86 (0.10)	2 ± 0	74 ± 1
5	32:20:8.40	86 (0.10)	20 ± 3	87 ± 2
6	25:27:8:40	87 (0.10)	17 ± 1	71 ± 4
7	12:40:8:40	83 (0.10)	NA	11 ± 1
8	Standard liposomal DXR	76 (0.11)	21 ± 1	3 ± 1

At a constant DOPE level of 52 mol% no change in blood clearance were observed for liposomes containing different cholesterol levels up to 40 mol% (formulation no 1-4) (Fig 2). In contrast, liposomal DXR containing an intermediate level of DOPE (32 and 25 mol%, respectively), and consequently an increased level of DSPC (20 and 27 mol%, respectively), showed a significant prolonged circulation time (Fig 3). The blood clearance profiles of DXR

for these intermediate DOPE formulations were approximately similar to standard liposomal DXR, where about 20% of the injected DXR dose remained in blood circulation 24 h after injection (Fig 3).

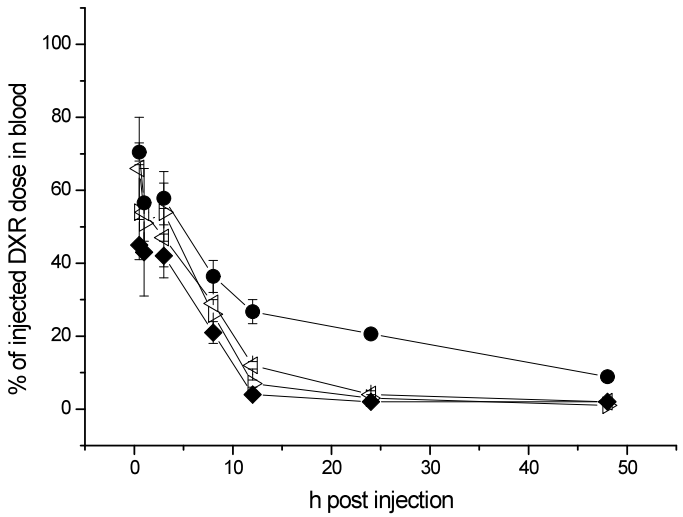


Fig 2. Blood clearance kinetics of i.v. injected liposomal DXR in mice of liposomes containing the following cholesterol levels; ◁ 20 mol%, ▷ 35 mol%, ♦ 40 mol%. (DOPE and DSPE-PEG 2000 levels: 52 and 8 mol%, respectively. DSPC level co varies with cholesterol level). ● standard liposomal DXR (HSPC:DSPE-PEG 2000:Chol; 57:5:38 mol%) is included for comparison. Bars represent the SD of the mean (n=3).

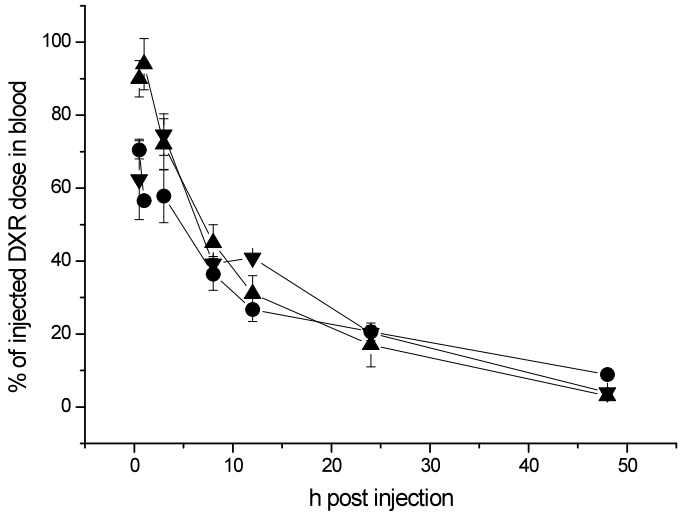


Fig 3. Blood clearance kinetics of i.v. injected liposomal DXR in mice of liposomes containing the following DOPE levels; ▲ 25 mol% and ▼32 mol%. (Cholesterol and DSPE-PEG 2000 levels: 40 and 8 mol%, respectively. DSPC level co varies with DOPE level). ● standard liposomal DXR (HSPC:DSPE-PEG 2000:Chol; 57:5:38 mol%) is included for comparison. Bars represent the SD of the mean (n=3).

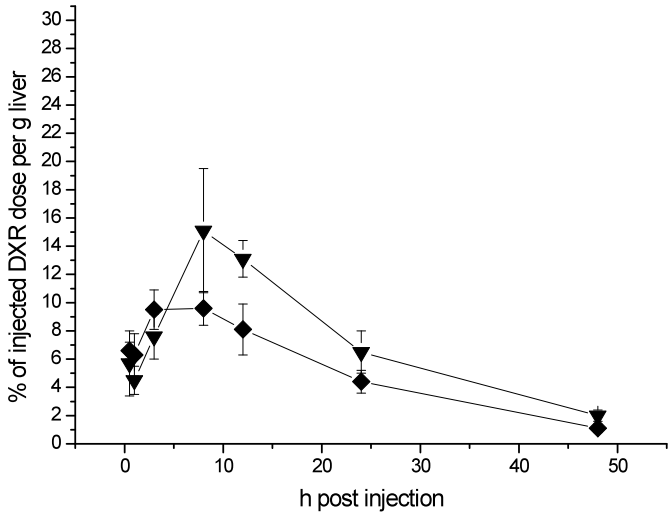
3.3 Effect of lipid composition on biodistribution of DOPE-containing liposomal DXR

If the relatively faster blood clearance of DXR for DOPE-rich liposomes was a consequence of carrier recognition by the mononuclear phagocyte system (MPS), then a corresponding increased accumulation in liver and spleen would be expected. Hence, DXR levels in liver, spleen and kidneys were investigated for two formulations comprising 32 and 52 mol% DOPE, respectively (no 4 and 5). These two formulations, herein termed intermediatelevel and highlevel DOPE-containing liposomes, showed a relatively slow and fast blood clearance of DXR, respectively (Fig 2 and 3).

No significant differences in DXR concentration in spleen could be observed for the intermediate and highlevel DOPE formulations (Fig 4B). The data revealed a slightly lower DXR concentration in liver for the highlevel DOPE-containing liposomes (Fig 4A). However, the difference was only significant at 12 h and 48 h post injection, $p<0.05$ (Fig 4A). Thus, the faster blood clearance profile of DXR for this formulation most likely reflected leakage of DXR from the liposome carrier, and not an increased uptake by the MPS.

Moreover, for the highlevel DOPE-containing liposomes, DXR levels in kidneys reached a maximum 12 h after injection. The intermediatelevel DOPE-containing liposomes showed sustained levels of DXR throughout the course of the experiment (Fig 4C). This pattern may reflect slow release of DXR from the intermediatelevel DOPE-containing liposomes in blood circulation, as indicated in Figs 2 and 3, resulting in higher DXR concentrations in the kidneys at the later time-points (Fig 4C).

A



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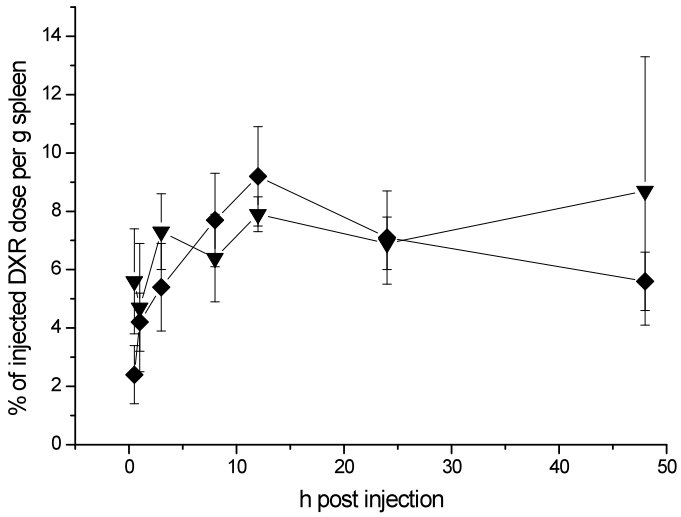

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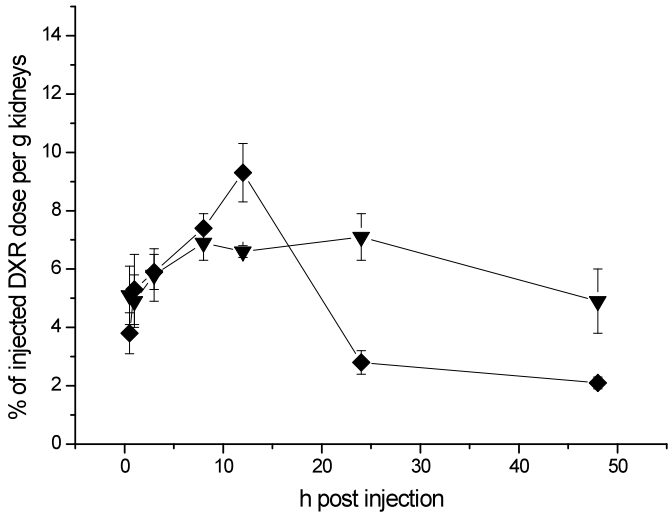

Fig 4. % DXR of injected dose in (A) liver, (B) spleen and (C) kidneys after i.v. injection of DOPE:DSPC:DSPE-PEG 2000:Cholesterol liposomes in mice: ♦ 52:0:8:40 mol%, ▼32:20:8:40 mol%. Bars represent SD of the mean (n=3).

3.4 Effect of DOPE level on sonosensitivity

Variations in lipid membrane composition are shown to influence sonosensitivity of liposomes (Pong *et al.*, 2006; Schroeder *et al.*, 2009; Evjen *et al.*, 2010a; Evjen *et al.*, 2010b; Lin *et al.*, 2001). We recently demonstrated that incorporation of DOPE into liposome bilayers drastically enhanced sonosensitivity compared to DSPE-based liposomes and standard liposomal DXR, respectively (Evjen *et al.*, 2011a). The minimum amount of DOPE required for maintaining an acceptable sonosensitivity *in vitro*, however, was not explored.

Fig 5 compares the US mediated DXR release profiles of liposomes containing different amounts of DOPE, during exposure to 40 kHz US in sucrose/HEPES solution containing 20% serum. High sonosensitivity was observed for formulations comprising between 25 and 52 mol% DOPE (Fig 5). These formulations showed up to a 7-fold increase in DXR release extent when compared to standard liposomal DXR after 6 min US exposure. Poor sonosensitivity was observed for liposomes composed of 12 mol% DOPE, as evidenced by a significant reduction in DXR release (Fig 5).

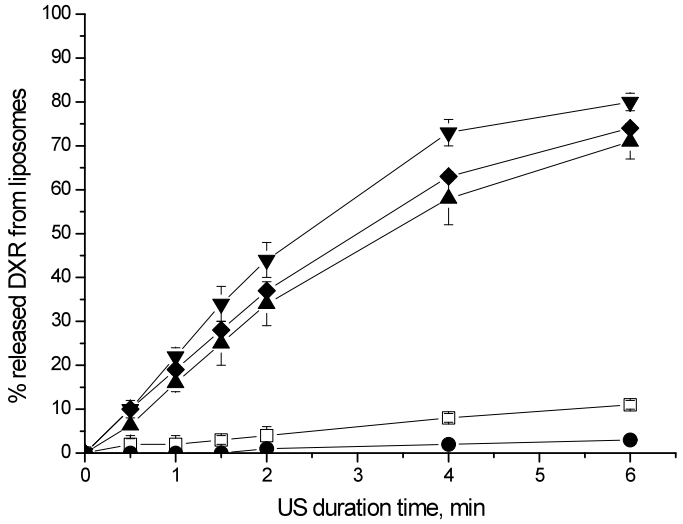

Fig 5. Effect of DOPE level on US mediated DXR release from liposomes in HEPES/sucrose solution containing 20% serum (40 kHz US). DOPE-levels: ▼ 32 mol%, ◆ 52 mol%, ▲ 25 mol%, □ 12 mol%. (Cholesterol and DSPE-PEG 2000 levels: 40 and 8 mol%, respectively. DSPC level co varies). ● standard liposomal DXR (HSPC:DSPE-PEG 2000:Chol; 57:5:38 mol%) is included for comparison. Bars represent the SD of the mean of triplicate measurements.

DISCUSSION

Application of sonosensitive liposomes for US mediated drug delivery requires that the liposomes retain their drug contents in blood circulation until they reach the tumour tissue. The results of the current study showed that DOPE-containing liposomes combining long blood circulation time of DXR with sufficient sonosensitivity might be obtained by altering liposome membrane composition.

The results demonstrated that lipid composition significantly influenced the blood clearance kinetics of DOPE-based liposomal DXR. The *in vivo* blood clearance of liposomal drug is mainly determined by two factors: (1) uptake of intact liposomes by the MPS, (2) leakage of the entrapped drug from the liposomes in blood circulation. In general, small PEGylated liposomes below 100 nm in diameter, as described in the current study, are to a certain extent able to escape the MPS, which in turn provides prolonged blood circulation time of the liposome carrier and consequently increased accumulation within tumour tissue. For reviews see Drummond *et al.*, 1999; Gabizon *et al.*, 2003. For successful drug delivery, it is required that the liposomes are able to retain the entrapped drug in blood circulation *en route* to the tumour tissue.

The liposomes comprising high levels of DOPE (62 and 52 mol%) showed relatively faster blood clearance of DXR than standard liposomal DXR (Fig 1). This finding may reflect leakage of DXR from the liposome carrier due to high membrane permeability and consequent distribution to other tissues, and/or premature uptake of the DOPE-rich liposomes by the MPS. The DOPE-based liposomes showed no *in vitro* DXR leakage in 20% serum. However, the stability *in vitro* may not always reflect liposomal drug retention *in vivo*, due to e.g. differences in the composition of bovine serum and mice blood. Discrepancy between *in vitro* and *in vivo* leakage results has previously been observed (Charrois and Allen., 2004; Mayer *et al.*, 1990).

Previous studies have demonstrated faster blood clearance kinetics of liposomal drugs composed of fluid lipids as a consequence of drug leakage (Charrois *et al.*, 2004, Gabizon *et al.*, 1988; Gabizon *et al.*, 1993., Ishida *et al.*, 2006). The permeability of liposome bilayers is to a great extent modulated by the T_c of the phospholipids and the presence of cholesterol (Gabizon *et al*., 2003). DOPE has a T_c of -16 °C (Cullis *et al.*, 1978), resulting in highly fluid membranes at physiological temperature that are more permeable to molecules and ions. A partial loss of transmembrane ion-gradient might account for an elevated percentage of non-ionized DXR in the liposome interior and subsequently DXR leakage.

Furthermore, a correlation between liposome permeability, lipid phase transition and cholesterol content has been established previously (Papahadjopoulos, 1996; Demel *et al.*, 1976). In general, cholesterol is known to improve the physical stability of PC-based liposomes by tightening the lipid membrane, reducing its permeability and consequently leakage of entrapped drug (Demel *et al.*, 1976). It was therefore interesting to note that no prolonged blood circulation time was obtained by increasing the cholesterol level from 20 to 40 mol% in DOPE-rich liposomes. The lack of any cholesterol stabilizing effect might be related to membrane packing constrains such as inhomogeneous distribution of cholesterol within the lipid membranes and/or poor cholesterol-phospholipid interactions, leading to high membrane permeability (Demel *et al.*, 1976).

When the DOPE level in liposomes was reduced to 32 mol% or below, the amount of DSPC in the membrane was consequently increased. The significantly prolonged blood circulation time of DXR for these formulations was probably both a result of reduced levels of DOPE and increased levels of DSPC. Previous studies have shown that saturated phosphatidylcholine provides order and rigidity to fluid-like lipid membranes and prolongs the blood residence time of liposomal drugs due to reduced leakage from the carrier (Demel *et al.*, 1976; Charrois *et al.*, 2004; Gabizon *et al.*, 1988; Ishida *et al.*, 2006; Senior *et al.*, 1982).

The biodistribution studies indicates that the faster blood clearance for the DOPE-rich liposomal DXR was not the result of enhanced uptake by the MPS but due to increased leakage of DXR from the liposome carrier. This could be assumed as no increased uptake of DXR in liver and spleen was observed for the DOPE-rich liposomes, which displayed the lowest blood concentrations. On the contrary, slightly higher levels of DXR in liver and predominantly in kidneys were observed for the long circulating intermediatelevel DOPE-containing liposomes. Considering slower release of DXR from the intermediatelevel DOPE-containing liposomes in blood circulation, increased delivery of the liposome-encapsulated DXR to tissues would be expected. The significantly higher DXR concentrations in the kidneys at the latest time points after injection might reflect a sustained release of DXR in blood circulation. It should not be excluded, though, that the different blood clearance of liposomes comprising intermediate and high levels of DOPE also could be explained by different extent of uptake or accumulation in other organs or tissues not investigated here, such as the skin or paws.

There is a well-established correlation between long blood circulation time and increased liposome accumulation in tumours (Gill *et al.*, 1995). Hence, the prolonged blood circulation time for the 25 and 32 mol% DOPE-containing liposomes should provide a good basis for efficient tumour accumulation. To enable full availability of DXR to tumour cells, the DOPE-containing liposomes should be sufficiently destabilized by US at the tumour site. Interestingly, the *in vitro* sonosensitivity of liposomes was largely maintained down to a level of 25 mol% DOPE. This particular formulation, comprising DOPE:DSPC:DSPE-PEG:cholesterol 25:27:8:40 mol%, showed approximately 70% release of DXR after 6 min US exposure in 20% serum. The results suggest that DOPE is a very potent sonosensitivity modulator, as only small amounts of the lipid were required to achieve efficient US mediated drug release. The DOPE-liposomes represented a several fold improvement in sonosensitivity compared to DSPE-based liposomal DXR and standard liposomal DXR, respectively (Hagtvet *et al.*, 2010; Evjen *et al.*, 2011).

The US regime used in the current study (4 min 40 kHz US) is recently reported to induce a significant tumour regression in mice receiving DSPE-based liposomes, without inducing any acute toxicity (Hagtvet *et al.*, 2010). However, for further preclinical and clinical studies of DOPE-containing liposomes a more sophisticated US device enabling increased focusing ability to tumours may be preferred. This will be accomplished by using high frequency focused US probes. However, previous studies have shown that sonosensitivity of the liposomes are comparable using 40 kHz and 1.13 MHz US (Evjen *et al.*, 2011b).

In conclusion, novel sonosensitive and long-circulating DXR-liposomes containing DOPE were developed by optimising the membrane composition. Low or moderate amounts of DOPE were required to obtain a sufficiently long blood circulation time after i.v. injection in mice. This reduction in DOPE level did not adversely affect sonosensitivity. Further studies are required to establish whether the novel formulations in combination with US improve DXR delivery to tumour cells enhancing the therapeutic index.

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