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Improving drug retention in liposomes by aging with the aid of glucose

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ABSTRACT

This paper describes a novel method to improve drug retention in liposomes for the poorly water-soluble (lipophilic) model drug asulacrine (ASL). ASL was loaded in the aqueous phase of liposomes and the effects of aging conditions and drug loading levels on drug retention were investigated using an *in vitro* bio-relevant drug release test established in this study. The status of intra-liposomal drug was investigated using differential scanning calorimetry (DSC) and cryo-transmission electron microscopy (cryo-TEM). Pharmacokinetics and venous tolerance of the formulations were simultaneously studied in rabbits following one-hour intravenous infusion via the ear vein. The presence of glucose during aging was found to be crucial to accelerate drug precipitation and to stabilize the liposomal membrane with high drug loading (8.9% over 4.5% w/w) as a prerequisite. Although no drug crystals were detected, DSC showed a lower phase-transition peak in the glucose-assisted aged ASL-liposomes, indicating interaction of phospholipids with the sugar. Cryo-TEM revealed more 'coffee bean' like drug precipitate in the ASL-liposomes aged in the glucose solution. In rabbits, these liposomes gave rise to a 1.9 times longer half-life than the fresh liposomes, with no venous irritation observed. Inducing and stabilizing drug precipitation in the liposome cores by aging in the presence of sugar provided an easy approach to improve drug retention in liposomes. The study also highlighted the importance of bio-relevance of *in vitro* release methods to predict *in vivo* drug release.

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1. Introduction

Asulacrine (ASL), a topoisomerase II poison and an analogue of the clinical drug amsacrine, was developed at The University of Auckland (Baguley et al., 1984). ASL was 2–4 times more potent than amsacrine against solid tumors in experimental models (Baguley and Wilson, 1987), possibly due to its enhanced lipophilicity (Paxton and Jurlina, 1986). However, during the clinical trials of ASL (Fyfe et al., 2001), severe phlebitis in patients was observed after intravenous (i.v.) infusion, hampering further development. Our recent studies (See et al., 2014; Zhang et al., 2015a,b) showed that post-injection drug precipitation at the wall of veins was probably the main reason for the cause of phlebitis with ASL. And ASL-loaded PEGylated liposomes were developed to prevent these adverse events.

Furthermore, PEGylated liposomes with a size ranging from 100 to 200 nm (Li and Huang, 2008) were found to offer tumor-targeted drug delivery, controlled drug release (Charrois and Allen, 2004) and long circulation, as in the case of Doxil® (doxorubicin-loaded PEGylation liposomes). Surprisingly, although the area under the plasma concentration-time curve (AUC) significantly increased with the use of PEGylated ASL-liposomes, a similar elimination half-life to ASL solution was observed following i.v. infusion to rabbits, indicating that undesirable drug leakage from the carriers had occurred. This drug leakage would compromise the tumor targeting capacity of liposomes as well as their venous protection effects.

There are many literature reports of drug leakage from liposomes, even if they were actively loaded in the cores (Table 1). Drug release behaviors are in part related to their physicochemical properties (Lindner and Hossann, 2010). Drugs with high log P values (i.e. lipophilic) tend to diffuse through liposomal membranes easily, resulting in quick drug leakage (Table 1). In this case, permeability of the liposomal membrane is a determining factor for drug retention. However, the leakage does not apparently

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Table 1
Physicochemical properties (pK_a and log P) of different drugs that are reported to have 'leakage' from liposomes. All these drugs are anticancer agents apart from ciprofloxacin (an antibiotic).

Drug	pK_a	Reference	LogP	Reference	Leakage	Reference
Vinorelbine	5, 7.4	Owellen et al. (1977)	2.82	a	*In vitro* and *in vivo* (mice)	Zhigaltsev et al. (2005)
Vinblastine	7.4	Gaertner et al. (1998)	3.4	Etievant et al. (1998)	*In vitro* and *in vivo* (mice)	Zhigaltsev et al. (2005)
Paclitaxel	10.36, −1	c	3.2, 3.54	b and c	*In vivo* (Human)	Sharma et al. (1997),Soepenberg et al. (2004)
Ciprofloxacin	5.61–6.18	Pisal et al. (2004)	2.3	Botté et al. (2011)	*In vitro*	Maurer et al. (1998)
Idarubicin	8.2	Robert (2005)	1.69, 1.9	b and c	*In vitro* and *in vivo* (mice)	Dos Santos et al. (2002), Gubernator et al. (2010)
Asulacrine	6.7	See et al. (2014)	3.0 (pH 7)	See et al. (2014)	*In vivo* (rabbit)	Zhang et al. (2015a)

[a]MERCKINDEX (1996); [b]ALOGPS; [c]ChemAxon.

correlate to the drug pK_a, possibly due to the different intra-liposomal pH and solubility. For instance, liposomes containing an ammonium sulphate gradient for the loading of weakly basic drugs could have an intra-liposomal pH of 5.5 (Bolotin et al., 1994). A lower pH than the pK_a value is required to keep drug molecules ionized within the liposomes, reducing the drug efflux through lipid membranes (Maurer-Spurej et al., 1999).

Another important factor influencing drug stability in liposomes is the physical state of the drug inside the liposomes. A slow dissolution of intra-liposomal drug, when an insoluble complex or precipitate is formed, would give rise to better drug retention (Gubernator et al., 2010; Lasic et al., 1995; Zhigaltsev et al., 2006). For example, doxorubicin formed sulphate-based or citrate-based aggregates after being actively loaded and showed longer retention than that in liposomes without forming aggregates (Lasic et al., 1995; Li et al., 2000). The importance of the physical status of drug inside liposomes was also demonstrated with ciprofloxacin-loaded liposomes which leaked due to the lack of compact precipitate even if the intra-liposomal drug concentration exceeded its solubility by two orders of magnitude (Maurer et al., 1998). If the drug is in a solution state or forms amorphous precipitate, the neutral species will flow (or dissolve out) and be replenished according to the pK_a and intra-liposomal pH, in a similar manner to active drug loading. ASL, with a pK_a of 6.7, actively loaded with ammonium sulphate into liposomes, presumably falls into this category. This is supported by the fact that during drug loading (See et al., 2014), ASL with $<$10% unionized form could be rapidly loaded into the liposomes.

Forming a less-soluble drug complex inside liposomes (Gubernator et al., 2010; Taggar et al., 2006) has been a popular method to load the drug as well as to reduce drug leakage. However, it is a challenging task to find a suitable chemical to form a less soluble complex and effectively release at the tumor target. In addition, reducing permeability of the liposomal membrane is a widely-used strategy. This can be achieved by the use of cholesterol-free liposomes (Dos Santos et al., 2002), saturated phospholipids (Lindner and Hossann, 2010) and cross-linked lipid structures in the lipsomal membrane (Liu and O'Brien, 2002). Coating liposomes with chitosan (Mady and Darwish, 2010) or forming nanoshells by calcium phosphate (Thakkar et al., 2012) have also been employed to strenghen the liposomal membrane.

In the present work, we attempted to develop a simple approach to improve ASL retention in our previously developed liposome formulations (Zhang et al., 2015a). A novel method, called sugar assisted 'aging', was investigated with the aim of improving drug retention by changing the physical state of intra-liposomal drug and stabilizing the liposomal membrane via interaction between phospholipids and glucose. The optimal aging conditions and factors influencing the physical state of drug inside liposomes during aging were also studied using a bio-relevant *in vitro* release study. Pharmacokinetics and venous tolerance were simultaneously evaluated following a one-hour i.v. infusion in New Zealand White rabbits.

2. Materials and methods

2.1. Materials

Asulacrine isethionate salt (CI-921, 99% pure) was synthesized and kindly gifted by Auckland Cancer Society Research Centre. The phospholipids, 1, 2-dipalmitoyl-*sn-glycero*-3- phospocholinemonohydrate (DPPC), *N*-(carbonyl-methoxy-polyethyleneglycol2000)-1 and 2-distearoyl-*sn-glycero*–phosphoethanolamine (DSPE-mPEG 2000) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sigma-Aldrich Co., Ltd. Sulfobutyl ether β-cyclodextrin (Captisol®) was a gift sample from Captisol Technology (La Jolla, US). All other reagents used in this study were of analytical grade except methanol and acetonitrile which were of chromatographic grade.

2.2. Animals

New Zealand White rabbits weighing between 3.0 and 3.5 kg were obtained from the Vernon Jansen Unit (VJU) of The University of Auckland. Animals were maintained according to the standards relating to the care and management of experimental animals in New Zealand. The experiments were carried out in accordance with the guidelines for animal experimentation and approved by the Committee on Animal Experiments of The University of Auckland (Ethics Approval No. C00881).

2.3. Preparation and characterization of liposomes

The liposomes (empty) were prepared using a thin film hydration (TFH) method as described previously (Zhang et al., 2015a). Briefly, DPPC, DSPE-mPEG 2000 and cholesterol (6:1:3 mol ratios) were dissolved in organic solvent and dried under vacuum conditions. Then the thin film obtained was hydrated with 250 mM ammonium sulphate solution at 45 °C for 10 min, followed by a 7-cycle freeze and thaw. Size was controlled with extrusion through 0.1 μm-pore sized polycarbonate membrane filters (Whatman, UK) with a LIPEX™ Extruder (Northern Lipids Inc., Burnaby, Canada). A *trans*-membrane ion (H^+ and SO_4^{2-}) gradient was generated by removing extra-vesicular ammonium sulphate using dialysis method. The liposomes were then incubated with supersaturated drug solution in the presence of 5% sulfobutyl ether β-cyclodextrin (SBE-β-CD) at 37 °C for 1.5 h. The SBE-β-CD was employed to solubilize the drug by inclusion of the neutral species in its hydrophobic cavity, acting as a rich drug solution reservoir for drug loading. When drug loading was completed, free drug, ASL-CD complex and SBE-β-CD were removed by ultracentrifuge at 188,272*g* (4 °C) for 1 h (Zhang et al., 2015a). The obtained ASL-liposome (ASL-L) pellets were re-suspended in a glucose solution (5% w/v) and kept at 4 °C for further aging.

The particle sizes, polydispersity index (PDI) and zeta potentials of different ASL-Ls were determined using a Malvern Nano ZS

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