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Research paper

Pharmacokinetics of intravenously administered stealth liposomal doxorubicin modulated with verapamil in rats

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Abstract

Treatment of cancer through co-administration of anticancer drugs and multidrug resistance (MDR) modulators as a strategy to overcome drug resistance has been extensively explored. However, success has been limited by pharmacokinetic interactions because of non-specific blockade of P-glycoprotein (P-gp) in normal tissues or inability to reach relevant concentrations clinically. We hypothesized that stealth liposomal co-encapsulation of doxorubicin (DOX) with a P-glycoprotein inhibitor, verapamil (DARSLs), may overcome these limitations. Using intravenous (i.v.) administrations, the effects of verapamil (VER) either free (FV) or liposome co-encapsulated with DOX (DARSLs) on the pharmacokinetics and tissue distribution characteristics of DOX either as free (FD) or liposome-encapsulated (LD) were evaluated in normal rats. FV increased ($P<0.05$) the plasma AUC of free DOX (FD). Preparations containing LD had significant prolonged systemic exposure and slow tissue distribution of DOX. LDFV (liposomal DOX with free verapamil) and DARSLs shared similar DOX pharmacokinetics but the latter showed slower DOX distribution in most tissues studied and slower ($P<0.05$) DOX biliary transport. The addition of VER into LD in these two preparations significantly increased the AUC ($P<0.01$) and reduced the clearance ($P<0.01$) of DOX when compared to LD. Specifically, DARSLs reduced initial DOX distribution to the heart ($P<0.05$) corresponding to initial alleviation ($P<0.05$) of bradycardia when compared to other DOX with VER preparations. In conclusion, liposomal co-encapsulation of DOX with VER has promise of significant therapeutic advantages, and should be explored further in therapeutic studies with animal tumor xenograft models.

Keywords: Doxorubicin; Verapamil; Stealth liposomes; Pharmacokinetics; Biodistribution

1. Introduction

P-glycoprotein (P-gp) is a member of the ATP-Binding Cassette superfamily proteins involved in the transport of a wide variety of substrates. It is widely distributed in the intestine, lung, kidney, liver, adrenal gland, blood–brain barrier, and placenta [1,2]. When over-expressed by tumors, P-gp-mediated efflux of drugs with resultant multidrug resistance (MDR) tumor cells is a major obstacle to successful clinical cancer chemotherapy. Therefore, attempts to circumvent this problem through pharmacological inhibition of P-gp during cytotoxic drug administration, for example, using verapamil, cyclosporine, valspodar, GF120918 or LY357739 to enhance intracellular drug accumulation into these MDR cells [3,4] have been studied. However, several possible disadvantages of this approach were anticipated, namely pharmacokinetic interactions arising from a considerable overlap of P-gp and CYP3A4 co-expressed in the excretion and metabolism organs [5–8], toxicity of the MDR modulators at doses required for P-gp inhibition and enhanced accumulation of cytotoxic drugs in normal tissues, causing more toxicity. Increased toxicity has

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been reported in neoplastic patients when doxorubicin (DOX) and verapamil (VER) were co-administered to overcoming multidrug resistance [9,10]. High plasma concentrations (2–6 μM) of VER were required for MDR reversal, much higher than for cardiovascular therapy (0.4–1.2 μM), and as a consequence, had a high potential for cardiotoxicity [11,12]. More specific P-gp modulators with less pharmacological effects on normal tissue have limited success as well.

Stealth liposome-based anticancer chemotherapy offers therapeutic advantages of reduced systemic toxicity combined with selective tumor localization. This is due to liposome longevity in circulation providing sustained release of lower concentrations of cytotoxic drug and to liposome extravasations through the abnormally permeable microvasculature of systemic tumors. It has also been demonstrated that the selective tumor localization of doxorubicin encapsulated in stealth liposomes (DOXIL®) is associated with superior therapeutic activity over free drug activity in various systemic tumor models [13–15]. Given the relative tumor specificity of liposomal encapsulation, less undesired pharmacokinetics interactions with P-gp modulators are expected.

Previous studies have demonstrated that liposomal doxorubicin has less potential for pharmacokinetic drug interaction with P-gp inhibitors like valspodar (PSC833) [16,17]. We hypothesized that co-encapsulation of both cytotoxic and P-gp inhibitor might lead to even greater specificity in drug delivery and intracellular retention of cytotoxic drug in the tumor, thereby enhancing therapeutic index. In this study, we formulated stealth liposomal preparations, one of which is co-encapsulated DOX with VER stealth liposomes (DARSLs or *d*oxorubicin *a*nti*r*esistant *s*tealth *l*iposome*s*) [26], and studied doxorubicin pharmacokinetics and cardiotoxicity of these preparations in rats and compared them with free DOX and VER.

2. Materials and methods

2.1. Materials

Egg phosphatitylcholine (EPC), cholesterol (CHOL), polyethylene glycol–distearoylphosphatitylethanolamine (PEG2000-DSPE) were purchased from NOF (Tokyo, Japan); ammonium sulfate, (±)verapamil hydrochloride were purchased from Sigma (USA); polycarbonate membrane filters were purchased from Millipore Corporation (USA). Doxorubicin (DOX) was obtained from Woo-Shin Med. Co. (Korean); all other chemicals were of the commercially available grade. HPLC system with fluorescence detector was from Waters (USA).

2.2. Liposomes and drug preparation

LD and DARSLs (stealth liposomes co-encapsulated with both DOX and VER) were prepared according to Haran et al. [18]. DARSLs were prepared by incorporation of both DOX with VER into stealth liposomes to form DARSLs according to the following process.

EPC (100 μmol), CHOL, PEG2000-DSPE (50:45:5) were dissolved in chloroform. The chloroform was evaporated to dryness under vacuum with a rotary evaporator (IFQ-85A), and the lipid film was hydrated with 10.0 ml of ammonium sulfate solution (150 mM) by sonication in water bath at 60 °C for 30 min, and a suspension containing blank liposomes was obtained. The liposomes were then extruded five times through each polycarbonate membranes (Nucleopore, USA) of pore sizes 0.6, 0.4, 0.2 μm consecutively to make smaller size of liposomes. The resulting liposomes were dialyzed (MWCO 12,000–14,000; Servapor™, Serva Germany) for 40 h at 4 °C against 100 mM phosphate buffer (pH 7.4).

Only DOX or DOX plus VER were encapsulated in the liposomes using the ammonium sulfate gradient loading procedure (Remote loading). The optimum weight ratio of drug:lipid for LD and DARSLs was 1:5 (DOX:Lipid) and 1:0.11:10 (DOX:VER:Lipid), respectively. The resultant DOX liposomes exhibited a mean particle size ranging between 100 and 130 nm as determined using a Zetasizer (Particle sizing systems, Malvern Instruments Ltd, UK), operating at a wavelength of 633.0 nm. Unencapsulated DOX and VER were removed by centrifugation through sephadex-G50 mini columns; DOX and VER concentrations encapsulated in liposomes were measured by HPLC after disruption of the liposomes with methanol. Encapsulation efficiencies of DOX and VER were more than 90 and 70%, respectively.

Free DOX (FD) formulation was administered with sterile saline. Verapamil hydrochloride (for animal studies) was dissolved in sterile saline to form free VER (FV) formulation and administered via intravenous injection. Liposomal DOX preparations (including LD, LDFV and DARSLs) were diluted with sterile saline to appropriate concentrations as necessary prior to intravenous administration.

2.3. Animal experiments

Animal experimental protocols were designed according to the guidelines of International Guiding Principles for Animals Research (WHO Chronicle, 39 (2): 51–56, 1985; A CIOMS Ethical Code for Animal Experimentation). The protocols were approved by the National University of Singapore Animal Ethics Committee. Male SD rats, weight 200–250 g, were housed under standardized conditions of temperature (22 °C) and exposed to a regular light/dark schedule (7:00–19:00/19:00–7:00). Food and water were provided ad libitum. All animals were kept for 1 week prior to drug administration and were fed a standard rat chow.

The animals were randomly divided into experimental groups (varying from 3 to 15 rats per group) for treatment with saline and formulations of FD, FV, FDFV, LD, LDFV

and DARSLs. Saline and formulations at doses of DOX (5 mg/kg) and VER (1 mg/kg) were administered via the tail vein (administration rate 0.4 ml/min).

After injection, blood was serially sampled from retro orbital sinus at 1, 5, 15, 30 min, 1, 2, 4, 8h, 24h, 48, 72 and 96 h. Blood samples (300 μl) were collected in heparinized tubes, and then centrifuged at 2000 rpm for 10 min at 4 °C to separate the plasma and stored at −20 °C until assayed for DOX.

In the biodistribution study, rats in groups of 3–4 were injected in the same manner and with the same preparations and doses of DOX and VER. At 30 min, 2, 24, 96 h after injection, the rats were anaesthetized with 7% chloral hydrate (0.35 mg/kg, intraperitoneally) and the bile was collected via capillary tubes. The rats were then sacrificed and blood withdrawn. Heart, liver, spleen, lung, kidney, brain were immediately removed, weighed and homogenized with 1:15 parts of 1 M phosphate buffer (pH 7.4 ± 0.1) and made up to 100 mg/ml for heart, spleen, lung, kidney and 200 mg/ml for liver and brain. Tissue homogenates were stored at −20 °C until assayed.

2.4. HPLC analysis for DOX

The HPLC assay of Andersen et al. [19] was used to analyze DOX with minor modification. Briefly, 1 ml acetonitrile was added to each 200 μl sample to precipitate proteins and extract DOX. The organic phase (800 μl) was transferred into another tube, dried with nitrogen gas, and reconstituted in 100 μl mobile phase prior to injection into a C18 reverse phase column (ODS ThermoHypersil, 150 mm × 4.6 mm, 5.0 μm, Thermo Electron Corp, USA). Detection was via DOX intrinsic fluorescence at 580 nm on the HPLC system (Waters Alliance 2690 module) with a fluorescence detector (Waters 484). The mobile phase consisted of 20 mM phosphate buffer (pH 3.8, 10% triethylamine):acetonitrile:methanol (45:20:35) delivered at a rate of 1.0 ml/min. The limit of quantitation (LOQ) was 0.005 μg/ml. In plasma and bile, coefficient of variation (CV) of precision and accuracy was $<10\%$ and recovery was $>90\%$. In tissues, CV of precision and accuracy was $<15\%$ in the concentration range of 0.005–1.0 μg/ml of DOX. Recoveries were 91, 76, 68, 63, 57 and 51% for brain, heart, kidney, liver, lung and spleen, respectively.

2.5. Electrocardiogram study

Considering the inherent pharmacological activity and cardiotoxicity properties of free DOX and VER, the rat ECG was monitored from the beginning of i.v. injection. Rats were anaesthetized with 7% chloral hydrate (0.35 mg/kg, intraperitoneally). The various formulations (saline, FD, FV, FDFV, LD, LDFV and DARSLs) at DOX dose of 5 mg/kg and VER dose of 1 mg/kg were administered as single bolus doses via the tail vein (administration rate 0.4 ml/min) into groups of 10–15 rats. The electrocardiogram (ECG) was recorded using an electrocardiography (ADInstrument Power Lab) after injections.

2.6. Data analysis

The plasma concentration data were fitted using Kinetica™ version 4.0 pharmacokinetic software (Innaphase Corp., USA), to calculate the area under the curve (AUC), terminal elimination half-life ($T_{1/2\beta}$), plasma clearance (CLp) and elimination rate constant (Lz). Appropriate models fitting the plasma concentrations data were evaluated by criteria according to the goodness of fit for each model. These included the objective function, visual assessment of distribution of residuals, and Akaike's Information Criterion (AIC). Data are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Post Hoc Tukey HSD test, and statistical significance was set at $P<0.05$.

3. Results

3.1. Plasma pharmacokinetics

Fig. 1A and B illustrate the plasma DOX concentration–time profiles of FD, FDFV, LD, LDFV and DARSLs. DOX plasma concentration–time data with free DOX, both in the presence and absence of VER, were best fitted with a two-compartment model, characterized by an initial rapid phase of drug concentration decrease, and a slower terminal elimination phase (Fig. 1A). At 8 h, the plasma concentrations were way below 1 μg/ml. The pharmacokinetic parameters are shown in Table 1. The pharmacokinetics of free DOX was characterized with an $AUC_{0-8\ h}$ of 2.29 ± 0.11 μg h/ml, a terminal half-life ($T_{1/2}$) of 2.75 ± 0.25 h, and a plasma clearance (CLp) of 485.6 ± 26.1 ml/h. In the presence of VER, significant 1.8-fold increase in $AUC_{0-8\ h}$ and two-fold increase in $T_{1/2}$ were observed (Table 1). The increase in AUC was consistent with earlier reported clinical observations [7].

When formulations containing liposomal DOX were administered, the plasma concentration–time profiles were entirely different from those of free DOX. These data were best fitted with a one-compartment model characterized by a prolonged monophasic elimination profile (Fig. 1B) requiring 96 h before plasma concentrations decreased to less than 10 μg/ml. It showed that the addition of free or liposomal VER significantly ($P<0.01$) altered some pharmacokinetic parameters of DOX when compared to LD without VER (Table 1) where VER increased significantly the AUC_{0-t} and reduced the V_{dss} and CLp of DOX. Compared with FDFV treatment, a C_{max} of 88.8 ± 9.6 μg/ml and an $AUC_{0-96\ h}$ of 2515 ± 230 μg h/ml obtained from the DARSLs treatment represented a very significant 590-fold increase in AUC, confirming slower DOX removal from the plasma compartment of liposome-encapsulated

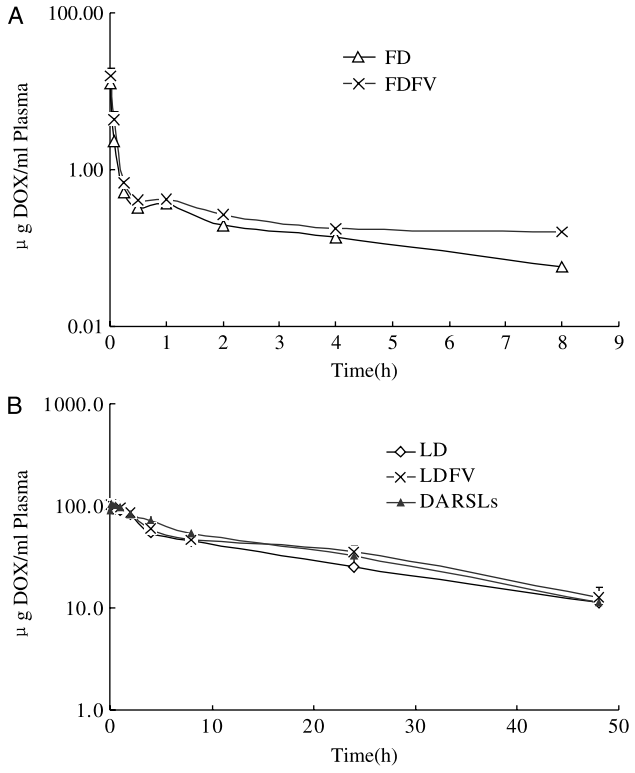

Fig. 1. Plasma DOX concentration–time profiles of (A) FD (free DOX) and FDFV (free DOX with free VER); and (B) LD (liposomal DOX), LDFV (liposomal DOX with free VER) and DARSLs (co-encapsulated liposomal DOX with liposomal VER) at a DOX dose of 5 mg/kg and VER dose of 1 mg/kg i.v. administered. Data are represented as means ($n=5$–6 rats/group). Error bars, SD.

DOX. An elimination $T_{1/2}$ of 19.8 ± 2.17 h was 3.7-fold higher and a CLp of 0.41 ± 0.03 ml/h was 617-fold slower when compared to FDFV. But V_{dss} for FDFV was 125-fold more than that of DARSLs. However, with LDFV, the pharmacokinetic parameters were not significantly different from that with DARSLs (Table 1), suggesting that VER either free or liposome-encapsulated had similar effects on the pharmacokinetics of liposomal DOX (LD).

3.2. Tissue distributions

The distribution characteristics of DOX to the various tissues are shown in Table 2. At 0.5 h after administration of the preparations of free DOX (FD), the highest DOX concentration was found in kidney > liver > heart > lung > spleen > brain. Distribution of DOX at 0.5 h time point to the kidney, liver and heart was significantly ($P<0.05$) reduced by intravenously co-administered free VER (FDFV).

It was very obvious that at 0.5 h and some including 2 h, the distribution of DOX to heart, liver and kidneys for liposomal DOX preparations (LD, LDFV, DARSLs) was very significantly reduced when compared to the free DOX preparations (FD, FDFV). The 24 and 96 h distribution patterns were not so much changed giving the appearance of a slower and prolonged process of distribution for the liposomal DOX preparations. It is interesting to note that at 0.5 h, significant increase of DOX contents in heart and brain tissues was observed for the combination with free VER (LDFV) compared to that of LD only. Liposomal VER in DARSLs lowered (statistically significant for the heart but not for most tissues) the DOX concentrations in all tissues studied when compared to LDFV where VER was not encapsulated. Distribution of DOX for DARSLs was, however, quite similar to that of LD without VER. Importantly, DARSLs reduced ($P<0.05$) DOX in heart significantly at 0.5 h and in spleen ($P<0.01$) at 2 h when compared to LDFV. These results suggested that unlike free VER, liposomal VER did not change the uptake of DOX into cardiac muscle cells (initially) or spleen (2 h).

In the lung and spleen, distribution data did not show any significant differences in DOX distribution with the different preparations at 0.5 h except for significant lower DOX content for DARSLs in lung when compared to FD. However, at 2 h, the spleen seemed to display the highest ($P<0.01$) concentration with LDFV when compared to all the rest of the preparations. For preparations containing liposomal DOX, DOX concentrations in the spleen remained high up to 96 h.

Table 1
Summary of the pharmacokinetic parameters (mean ± SD, $n=5$–6/group) for FD, FDFV, LD, LDFV, DARSLs at DOX i.v. dose of 5 mg/kg in the absence and presence of verapamil (i.v. dose of 1 mg/kg)

Parameter	Time (0–t (h))	AUC_{0-t} (μg h/ml)	MRT (h)	Lz (1/h)	C_{max} (μg/ml)	$T_{1/2}$ (h)	V_{dss} (ml)	CLp (ml/h)
FD	0–8	2.29 ± 0.11	2.81 ± 0.45	0.25 ± 0.02	18.8 ± 5.12	2.75 ± 0.25	1371 ± 274	485.6 ± 26.1
FDFV	0–8	4.26 ± 0.45	5.83 ± 0.98	0.13 ± 0.02‡	20.5 ± 5.50	5.39 ± 0.70	1460 ± 175	252.8 ± 27.9‡
LD§	0–96	1774 ± 114$^{\|\|}$	25.97 ± 2.00	0.039 ± 0.003	99.5 ± 1.67	18.03 ± 1.42	15.29 ± 0.63$^{\|\|}$	0.59 ± 0.03$^{\|\|}$
LDFV*,***	0–96	2375 ± 256	25.96 ± 3.70	0.039 ± 0.006	91.9 ± 4.31	18.0 ± 2.56	11.4 ± 0.78	0.44 ± 0.05
DARSLs**†	0	2515 ± 230	28.51 ± 3.12	0.035 ± 0.004	88.8 ± 9.60	19.8 ± 2.17	11.7 ± 0.70	0.41 ± 0.03

*$P<0.01$ (between FD and LDFV), **$P<0.01$ (between FD and DARSLs), ***$P<0.01$ (between FDFV and LDFV), $^{\dagger}P<0.01$ (between FDFV and DARSLs), $^{\ddagger}P<0.01$ (between FD and FDFV), $^{\S}P<0.01$ (between LD and FD/FDFV), $^{\|\|}P<0.01$ (between LD and LDFV/DARSLs). FD is free DOX; FDFV is free DOX with free VER; LD is liposomal DOX; LDFV is liposomal DOX with free VER; DARSLs is co-encapsulated liposomal DOX with liposomal VER.

Table 2
Tissue distribution of DOX after i.v. administration of FD, FDFV, LD, LDFV, DARSLs at doses of 5 mg/kg of DOX in the absence and presence of VER (1 mg/kg)

Tissue	Group	Time (h)			
		0.5	2	24	96
Heart	FD	1310±147	919±185	242±8	88±7
	FDFV	1047±150§	938±100	195±89	96±6
	LD	363±32$^{\|\P\P}$	382±15$^{\|\P\P}$	321±42¶¶	142±24¶
	LDFV	485±25* *,*** ***	423±45* *,*** ***	345±86***	149±29**
	DARSLs	371±16** **,††,‡	386±52** **,††	369±24†	196±91†
Liver	FD	1968±333	578±166	149±22	54±6
	FDFV	1048±347§§	503±79	102±20	64±5
	LD	688±92¶¶	621±84	574±42¶¶	234±83¶
	LDFV	729±126* *,***	633±167	597±98* *,*** ***	243±113
	DARSLs	568±68** **,††	678±93	534±39** **,††	256±178**,†
Kidney	FD	2844±340	1372±82	454±63	136±19
	FDFV	2100±307§§	1066±115§	327±88	156±12
	LD	571±81¶¶	482±24¶¶	604±41¶¶	174±34
	LDFV	579±182* *,*** ***	501±64* *,*** ***	629±50*** ***	199±71
	DARSLs	551±103****,††	317±66****,††	668±131**,††	248±32****,†
Brain	FD	20±9	14±2	7.2±1.3	3.3±0.8
	FDFV	26±13	11±4	7.3±1.2	7.4±6.2
	LD	55±12$^{\|\P\P}$	52±5¶¶	24±4¶¶	3.5±1.9
	LDFV	74±8* *,*** ***	62±12* *,*** ***	25±4* *,*** ***	4.3±1.5
	DARSLs	59±13** **,††	56±5** **,††	28±5** **,††	4.0±0.7
Lung	FD	677±175	768±136	226±75	85±19
	FDFV	552±140	531±117	232±100	101±38
	LD	438±57	312±45¶	206±28	142±22
	LDFV	453±152	333±144*	202±27	159±92
	DARSLs	392±103**	292±75**	229±11	172±88
Spleen	FD	68±6	105±56	116±86	68±21
	FDFV	68±22	79±19	62±22	58±23
	LD	94±34	144±59$^{\|\|}$	236±53¶¶	205±68¶¶
	LDFV	114±52	373±84* *,*** ***	273±76* *,*** ***	255±118* *,*** ***
	DARSLs	86±41	161±25‡‡	229±11** **,††	198±71**†

Data (nanogram DOX/100 mg tissue) are represented as mean±SD (n=3–4rats/group). *$P<0.05$ (between FD and LDFV), **$P<0.05$ (between FD and DARSLs), ***$P<0.05$ (between FDFV and LDFV), $^{\dagger}P<0.05$ (between FDFV and DARSLs), $^{\ddagger}P<0.05$ (between DARSLs and LDFV), $^{\S}P<0.05$ (between FD and FDFV), $^{\|}P<0.05$ (between LD and LDFV), $^{\P}P<0.05$ (between LD and FD /FDFV). Single marker represents $P<0.05$; double marker represents $P<0.01$. FD is free DOX; FDFV is free DOX with free VER; LD is liposomal DOX; LDFV is liposomal DOX with free VER and DARSLs is co-encapsulated liposomal DOX with liposomal VER.

Distribution of DOX into the brain was increased ($P<0.01$) with preparations containing liposome DOX and VER, especially when VER was free (Table 2).

Fig. 2 shows the biliary concentrations of DOX after intravenous administration of various preparations. The free DOX preparations showed extremely high DOX concentrations in bile. The combination with free VER (FDFV) resulted in a significant decrease ($P<0.05$) in DOX concentrations in bile compared to free DOX only (Fig. 2A). This might have arisen from the inhibition of P-gp expressed in biliary canaliculi. Considering that liposomes do not permeate across hepatocyte membranes directly [20] and encapsulated DOX has to be released from the liposomes to enter these cells, the DOX excretion from bile was very significantly ($P<0.01$) reduced (Fig. 2A and B) following the administration of the liposomal DOX preparations. Among these preparations, biliary excretion of DOX with DARSLs was even lower ($P<0.05$) than with LD and LDFV at 0.25 and 0.5 h (Fig. 2B).

3.3. Bradycardia evaluation

There were no significant differences in the ECG patterns except for heart rate in the ECG recording of the various preparations during i.v. bolus injection. Fig. 3 shows bradycardia changes (%reduction of heart rate from normal to lowest point in heart rate) after injection. The results showed that firstly, LD produced significantly less ($P<0.05$) bradycardia than FD and secondly, most of the bradycardia effect was due to the inclusion of VER. FDFV produced the highest bradycardia in rats, showing the highly significant increase ($P<0.01$) in cardiotoxicity when FV was added to FD. For the liposomal preparations, bradycardia effect became significantly ($P<0.01$) less severe when compared to the FDFV group. The combination with free VER (LDFV) did result in significant increase ($P<0.01$) and significant decrease ($P<0.05$) in bradycardia when compared to LD and FDFV, respectively. It was important to note that DARSLs with

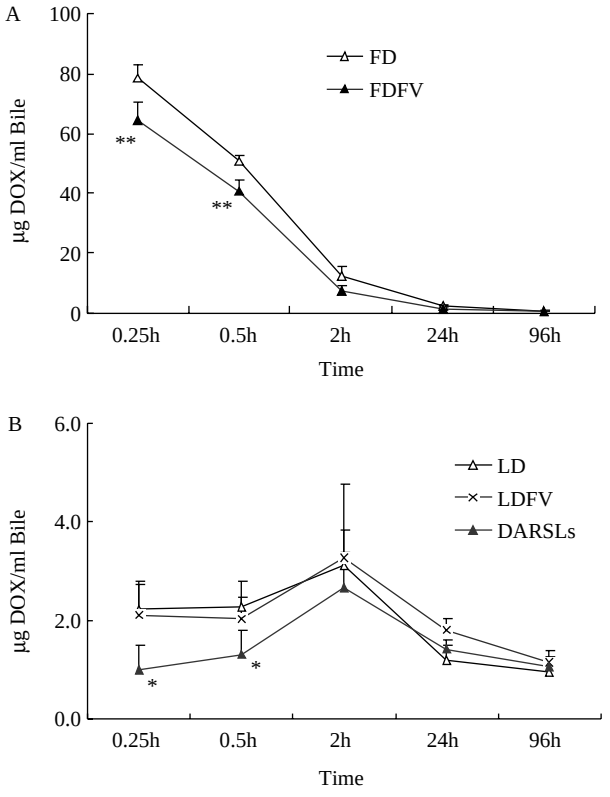

Fig. 2. (A) Biliary DOX concentration–time profiles after i.v. administration of FD (free DOX), FDFV (free DOX with free VER) at DOX doses of 5 mg/kg of and VER dose of 1 mg/kg. Data are represented as mean ± SD. (n = 5–6 rats/group). $^{**}P < 0.01$ (between FD and FDFV). (B) Biliary DOX concentration–time profiles after i.v. administration of LD (liposomal DOX), LDFV (liposomal DOX with free VER) and DARSLs (co-encapsulated liposomal DOX with liposomal VER) at DOX doses of 5 mg/kg and VER dose of 1 mg/kg. Data are represented as mean ± SD (n = 5–6 rats/group). $^{*}P < 0.05$ (between DARSLs and LDFV&LD).

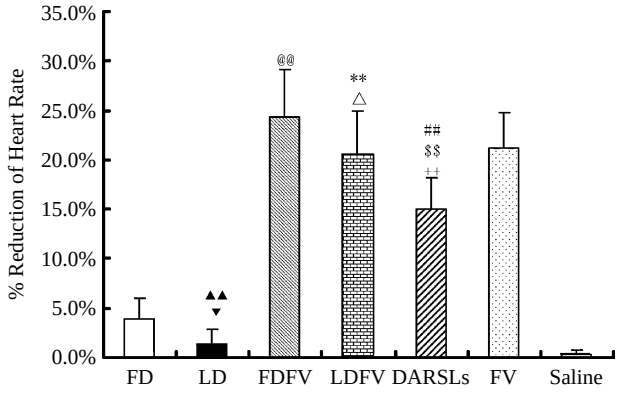

Fig. 3. Percentage reduction of heart rate during i.v. bolus injection (0.4 ml/min) of various formulations. Data are represented as means (n = 10–15 rats/group). Error bars, SD. $^{*}P < 0.05$ (between FD and LDFV), $^{\#}P < 0.05$ (between FD and DARSLs), $^{\Delta}P < 0.05$ (between FDFV and LDFV), $^{\$}P < 0.05$ (between FDFV and DARSLs), $^{+}P < 0.05$ (between DARSLs and LDFV), $^{@}P < 0.05$ (between FD and FDFV), $^{\blacktriangledown}P < 0.05$ (between LD and FD), $^{\blacktriangle}P < 0.05$ (between LD and LDFV/DARSLs). Single marker represents $P < 0.05$; double marker represents $P < 0.01$.

VER-encapsulated further reduced the bradycardia ($P < 0.01$) when compared to LDFV. These results suggested that liposome co-encapsulation of VER with DOX exerted a further protection for acute cardiotoxicity arising from the combination preparations like FDFV and LDFV during i.v. bolus injection.

4. Discussion

We have demonstrated that liposomal-encapsulated VER did reduce P-gp-mediated interaction with the pharmacokinetics and distribution of liposomal DOX preparations, and resulted in more favorable pharmacodynamics in terms of reduced acute cardiotoxicity. This would improve the therapeutic index of DOX, and is a proof of principle in support of administering liposomally co-encapsulated drug and drug resistance modulator.

As our study involved liposomal preparations, all drugs were administered intravenously where the dose of P-gp inhibitor used was lower than that for oral or intraperitoneal routes [5,7,16].

Consistent with previous reports [21,22], VER interfered with DOX pharmacokinetics when both drugs were not encapsulated (Table 1 and Fig. 1A). Clearance was reduced resulting in increased AUC and prolongation of half-life. Encapsulation of DOX and its co-administration with VER (free or co-encapsulated) have tremendous effects on reducing DOX clearance and increasing DOX AUC very significantly (Fig. 1A and B). Reduction in DOX transport across the biliary canaliculi (Fig. 2A and B) might also have contributed to this effect of extended systemic exposure to DOX. There was also increased access of liposomal DOX into the central nervous system (Table 2) which may be attributed to the membrane permeability of liposomes [23], as well as P-gp inhibition at the blood–brain barrier [5]. As shown in Table 2, the concentrations of DOX binding to heart tissue with the free DOX formulations were much higher than the liposomal DOX formulations up to 2 h after injection. Since both DOX and VER are cardiotoxic, the co-encapsulation of VER into stealth liposomal DOX as in DARSLs producing the least bradycardia during i.v. administration of this drug combination (Fig. 3) in accordance to the lower initial distribution of DOX to the heart (Table 2) demonstrated the novel application of this formulation.

Although other investigators [16,20] had established that administering P-gp inhibitors like PSC833 with liposomal DOX (LD) did not significantly alter DOX pharmacokinetics and tissue distributions of DOX, we have in this study shown that DARSLs with co-encapsulated VER did increase the AUC and reduce the CLp of DOX significantly when compared to LD. This could be due in part to the P-gp inhibitory action of liposomal VER in the biliary transport of LD resulting in significant reduction of DOX in bile in

the earlier samples before the 2-h sample (Fig. 2B). These results suggested that the effects of encapsulated VER on membrane transport of LD might be transient and not sustained, probably due to the inadequate stability of the liposomal VER in DARSLs in the in vivo system. In vitro, the release of VER from encapsulated VER in rat plasma was about 10 and 60% occurring within 30 min and 12 h of incubation, respectively, while the corresponding release of DOX from DARSLs of 0 and 20%, respectively, supported the much lower stability of liposomal VER in DARSLs [26]. Nonetheless, the transient reduction in permeation of DOX resulting in less bradycardia compared to LDFV (Fig. 3) did demonstrate better cardiac safety profile with encapsulated VER. This provided the impetus for pursuing a more stable formulation of co-encapsulation of DOX with VER.

Another strategy of liposomes carrying monoclonal antibody against P-gp, MRK-16 (MRK-Lip) suggested that the interaction between liposomes and multidrug resistance cells increased by the modification of liposomes with MRK-16 in in vitro cytotoxicity experiments [24]. But, concern in the clinical in vivo use of MRK-Lip is the problem of side effects resulting from its non-specific binding to the P-gp in normal tissues especially the blood–brain barrier [1,2]. Thus, this approach could induce more adverse effects in normal tissues rather than target specifically on MDR tumor. Doxorubicin antiresistant stealth liposomes (DARSLs) as a unit would in principle provide both a better targetable and inhibitory effect for the active drug efflux system than MRK-Lip.

In conclusion, this study has shown that the co-encapsulation of DOX with VER into stealth liposome (DARSLs) resulted in higher systemic exposure (AUC) of DOX but yet with better safety profile and therapeutic index than using the two non-capsulated free drugs (FDFV) or LDFV. Though the stability of encapsulated VER might be a likely problem, the promise of better therapeutic index warrants further efforts to develop more stable co-encapsulated liposomal VER or an alternative P-gp inhibitor. It would be interesting to determine the efficacy of DARSLs in the treatment of tumor with over-expressed P-gp. Theoretically, the availability of both encapsulated DOX and VER at the site of the tumor, where leaky capillaries allowing selective concentrations of both agents [25], would facilitate maximum therapeutic index of DOX. This proof of principle might be applicable to a variety of other cytotoxics and P-gp inhibitors as well to improve safety in delivering chemotherapy.

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