TITTLE: Transmembrane pH-Gradient Liposomes to Treat Cardiovascular Drug Intoxication: Making the Old New

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ABSTRACT: Injectable scavenging nanocarriers have been proposed as detoxifying agents when there are no specific antidotes to treat pharmacological overdoses. They act by capturing the drug *in situ*, thereby restricting distribution in tissues. In the clinic, the only systems used for that purpose are parenteral lipid emulsions, which are relatively inefficient in terms of uptake capacity. In this study, we investigated long-circulating liposomes with a transmembrane pH gradient as treatment for diltiazem intoxication. The unique ion-trapping properties of the vesicles towards ionizable compounds were exploited to sequester the drug in the bloodstream, and limit its pharmacological effect. After *in vitro* optimization of the formulation, the *in vivo* scavenging properties of the liposomes were demonstrated by examining the drug's pharmacokinetics. The reduced volume of distribution and increased area under the plasma-concentration-*vs*-time curve in animals treated with liposomes indicated limited tissue distribution. The vesicles exerted a similar but more pronounced effect on deacetyl-diltiazem, the principal active metabolite of the drug. This *in vivo* uptake of both drug and metabolite altered the overall pharmacological outcome. In rats receiving an intravenous bolus of diltiazem, the liposomes tempered the hypotensive decline, and maintained higher average blood pressure for 1 h. The detoxifying action of liposomes was even stronger when the rats received higher doses of the drug *via* perfusion. In conclusion, the present work provided clear evidence that liposomes with a transmembrane pH gradient are able to change the pharmacokinetics and pharmacodynamics of diltiazem and its metabolite and confirmed their potential as efficient detoxifying nanocarriers.

KEYWORDS: detoxification, lipid resuscitation, diltiazem, pharmacokinetics, scavenging nanocarriers

MANUSCRIPT TEXT

Over one million cases of drug intoxication are reported yearly in the United States. Cardiovascular drugs are among the substances most frequently encountered in overdoses, and within this class of therapeutic molecules, calcium channel blockers (CCB) are responsible for more than 30% of fatalities[1]. The clinical profile of CCB poisoning is erratic and dose-dependent. It usually involves peripheral vasodilatation and bradycardia, as well as arrhythmias and metabolic disorders, often followed by cardiogenic shock and death[2,3]. Moreover, because of the common availability of controlled-release CCB formulations, the prognosis is complicated by sustained absorption of the medication in the gastrointestinal tract over prolonged periods of time. Consequently, patient deterioration occurs gradually and leads to long-lasting and costly hospital stays in intensive care. Different treatment algorithms combine aggressive decontamination with supportive hemodynamic measures, and administration of calcium salts, insulin or glucagon. However, there are no therapeutic consensuses, and CCB overdoses remain difficult to treat, with morbidity and mortality as frequent outcomes[2,3].

Researchers have recently proposed an alternative treatment for CCB overdose. It consists of administering intravenous fat emulsions (IFE)[4-6]. These emulsions are composed of nanosized (~200-400-nm) droplets of soy bean oil stabilized with phospholipids (PL). Large doses of IFE are known to reverse the toxicity of local anaesthetics, tricyclic antidepressants, barbiturates, β-blockers, and CCB, in both animal models and human case reports[7]. Although the mechanism behind the effect of these emulsions has not been elucidated, the most commonly-accepted hypothesis is the *in situ* drug uptake in the lipid droplets, which reduces the available toxic concentration at different action sites[8]. Drug partitioning in the lipid emulsions has indeed been demonstrated *in vitro* for certain local anaesthetics[8-10]. However, this uptake depends on the drug's lipophilicity, and is limited by relatively fast clearance of the droplet from the bloodstream. Both these features are responsible for the use of very high IFE doses[6]. In addition to the unknown metabolic consequences of administering large amounts of lipids[11], other drawbacks to rescue with IFE have been recently brought to light. For example, possible toxicity

relapse after initial improvement[12], decreased efficiency of lipids in hypoxia[13,14], and probable interaction with other resuscitation medicines[15,16] are aspects that have mitigated the initial enthusiasm surrounding IFE deployment in CCB overdoses.

In the present work, long-circulating liposomes with a transmembrane pH gradient are proposed as surrogates to IFE to treat CCB intoxications. Vesicles with an acidic internal compartment possess ion-trapping properties for weak basic drugs (Fig. 1). This feature has been successfully applied to encapsulate various compounds with remarkable entrapment efficiencies[17]. The capture properties also appear to be maintained under *in vivo* conditions, as reported by Mayer *et al*., employing doxorubicin as model drug[18]. Surprisingly, there have been no attempts to adapt such systems as antidotes. Recently, we reported that transmembrane pH-gradient spherulites (a type of multilamellar vesicles) were highly efficient *ex vivo* in reversing amitriptyline-induced toxicity in isolated perfused hearts [19]. However, because of their modest stealth properties[20], they were not deemed ideal for *in vivo* application. In contrast, poly(ethylene glycol) (PEG)-stabilized liposomes of less than 200 nm are known to possess biological half-lives exceeding 15 h[21]. They are generally considered safe and biocompatible[22]. These characteristics, combined with the high efficiency of the pH-gradient-loading process, make them near optimal systems for drug detoxification. In this report, PEGylated liposomes with a transmembrane pH gradient were investigated to capture diltiazem (DTZ), a CCB frequently involved in life-threatening intoxications. The liposomal formulation was shown to modify the pharmacokinetics (PK) of DTZ and its principal active metabolite (deacetyl-diltiazem, DAD), and efficiently tempered the drug's hypotensive effect.

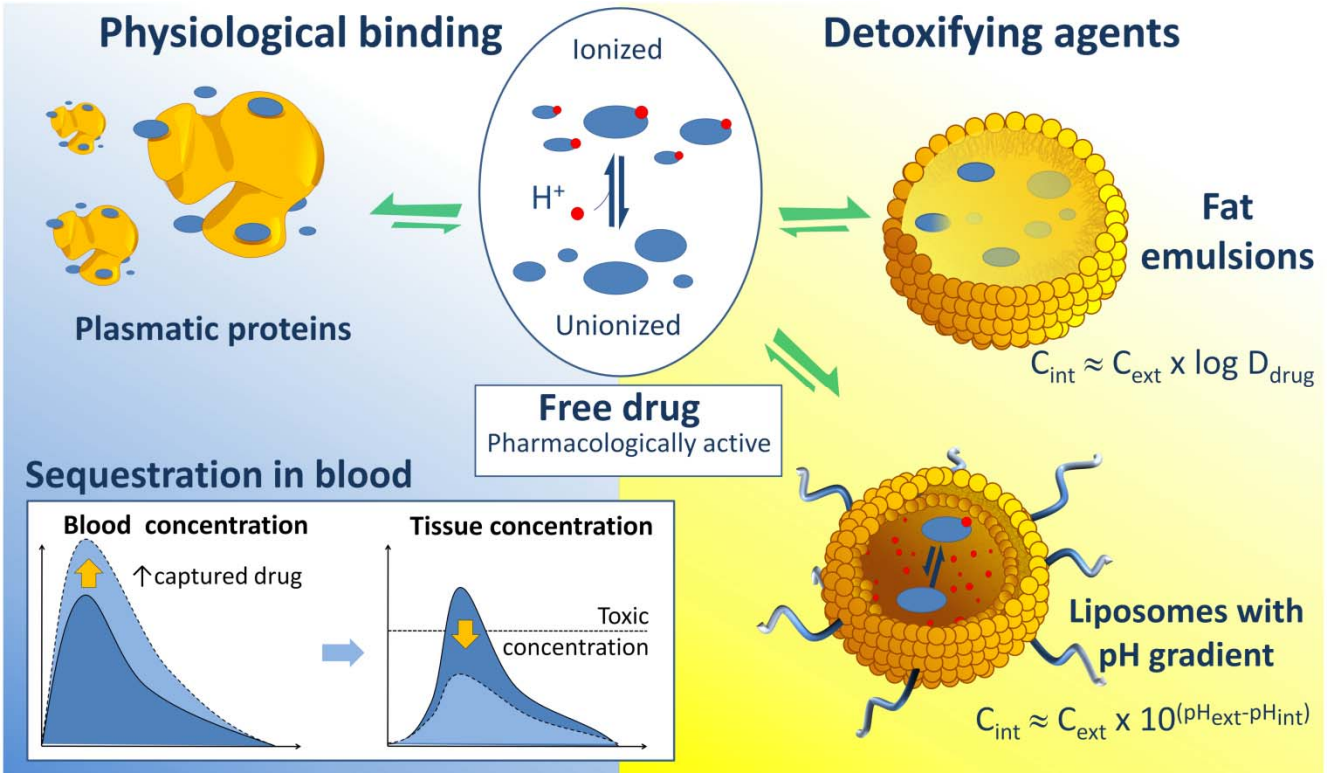

Figure 1. Rationale behind the use of transmembrane pH-gradient liposomes as antidotes. Similarly to IFE, the vesicles act as sinks to scavenge circulating free drugs. The transmembrane pH gradient maximizes the quantities that can be captured. Sequestration of the drug by vesicles in the blood decreases the amounts of pharmacologically-active free drug. In the equations, C_{int}, C_{ext}, D_{drug}, pH_{int}, and pH_{ext} represent the concentration of drug captured, the external drug concentration, the drug distribution coefficient and internal and external pHs, respectively.

RESULTS AND DISCUSSION

Preparation and characterization of liposomes. Liposomes were composed of egg phosphatidylcholine (Egg PC), cholesterol (Chol), *N*-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3 phosphoethanolamine (DSPE-PEG) and citric acid, ingredients that are already included in FDA-approved formulations. The structures of the lipid components and DTZ are presented in Fig. S1. Internal pH and citrate buffer concentration (Fig. 2 and Table S1) were identified as the main factors affecting drug uptake. Lowering the internal pH from 3 to 2 almost doubled the uptake capacity

(Fig. 2A). However, in the subsequent *in vivo* experiments, an internal pH of 3 was preferred in order to maximize stability and limit acid-catalyzed PL hydrolysis. Fig. 2B illustrates that at pH 3, capture capacity increased with citrate concentration until 200 mM. At this stage, the internal solution became iso-osmolar with the external milieu (300 mOsm/kg). Above 300 mOsm/kg, uptake decreased because of the opposing force of osmotic gradient pressure (Fig. S2) or the membrane instability caused by the high ionic strength[23]. The drug was mainly captured inside the vesicles and not adsorbed on their surface as indirectly revealed by the complexation of DTZ with encapsulated bromophenol blue inside the liposomes (Fig. S3), and the augmentation of uptake capacity with entrapped volume (Fig. S4). The liposomes were relatively stable in biological fluids with a minimal internal pH increase when incubated in 80% plasma at different concentrations (Fig. S5). The stability of the pH gradient comes from the limited permeability of charged molecules (*e.g.* protons) through the hydrophobic phospholipid membrane and from the high buffering capacity of the entrapped citrate. Similarly, a complementary experiment showed that the citrate buffer remained trapped in the liposomes *in vivo* after intravenous (i.v.) injection, as indicated by the parallel blood profiles of radiolabeled lipid and citrate (Fig. S6).

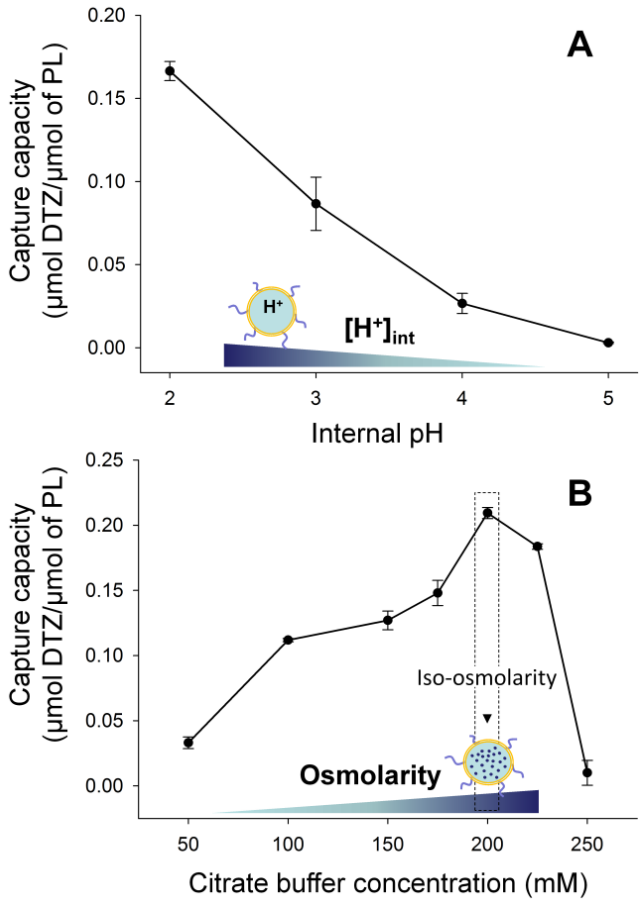

Figure 2. Internal buffer parameters influencing DTZ capture. **(A).** The greater the gradient between internal and external pHs, the more efficient the capture (buffer concentration 150 mM, values represent means ± SD, n=3). **(B).** Capture increases with buffer strength until iso-osmolarity is reached (buffer pH 3, values represent means ± SD, n=3). Values are normalized for the amount of PL in the formulation.

Uptake of DTZ and comparison with other formulations. Fig. 3 charts DTZ uptake in HEPES-buffered saline (HBS) and in the presence of 50% plasma by different colloidal formulations, including the commercially-available IFE Intralipid®. Transmembrane pH-gradient liposomes surpassed the IFE (Intralipid® 20%) by 40-fold in terms of capture capacity. They were also largely superior to neutral liposomes with an internal pH of 7.4 and negatively-charged liposomes (prepared with anionic 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) instead of EPC), which have recently been reported in the literature to be potential detoxifying systems, but never tested *in vivo*[24,25]. The presence of plasma

reduced the uptake capacity of transmembrane pH-gradient liposomes by about 35%. This was substantially better than IFE which experienced a loss of more than 3-fold DTZ uptake in plasma. The superior uptake capacity of the transmembrane pH-gradient liposomes over the IFE was confirmed with another, more hydrophilic basic drug, bupivacaine (Fig. S7). In a clinical context of drug overdose, this higher capture efficiency is of paramount importance because it would translate into much lower administered doses, shorter administration times, and faster onset of action. The smaller amounts injected also reduce the risks of metabolic disorders and other adverse effects potentially associated with lipid perfusion[11].

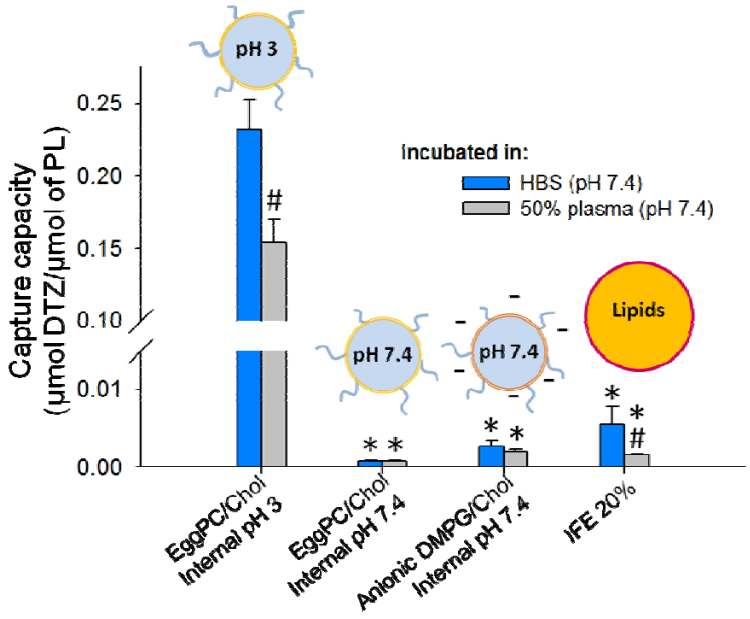

Figure 3. DTZ capture by different formulations in the presence of plasma. Values represent means ± SD, n=3. Values are normalized for the amount of PL in the formulation. # $p<0.05$ *vs.* same formulation in HBS, * $p<0.05$ *vs.* liposomes EPC/Chol internal pH 3 in plasma.

Impact of liposomes on the PK of DTZ and DAD. When given alone, DTZ has a high, non-physiological volume of distribution (V_d) ranging between 1.5 to 4 L/kg in rats[26], and slightly superior in humans[27]. This implies that DTZ is highly distributed in tissues and only a small fraction circulates in the blood. The DTZ metabolite DAD possesses around 25 to 50% of the pharmacological activity of the parent drug[28,29]. It is produced by lung, liver and blood esterases[30-32]. Its PK profile in rats is similar to

that of DTZ, with high V_d and rapid plasmatic clearance (Cl)[26,29,30]. The large V_d, combined with high plasma protein binding (70-80%) of DTZ and DAD, explains the inadequacy of conventional hemodialysis and hemoperfusion detoxification techniques[33]. It also justifies the need for effective circulating colloidal detoxifying agents to limit distribution toward the peripheral compartments[34].

Table 1. PK parameters for DTZ and DAD in the presence and absence of liposomes. Mean ± SD (n=7-8), ***** $p<0.05$ *vs.* control.

		Control	Liposomes	Difference (fold)
DTZ	C_{max} (ng/mL)	2421 ± 871	33,698 ± 8135*	14
	$AUC_{(0-6h)}$ (ng.h/mL)	1520 ± 445	9188 ± 1671*	6
	$t_{1/2}$ (h)	0.86 ± 0.48	0.92 ± 0.38	-
	V_d (mL/kg)	2141 ± 548	105 ± 37*	20
	Cl (mL/h)	1091 ± 276	180 ± 34*	6
DAD	C_{max} (ng/mL)	2430 ± 1032	30,583 ± 8045*	13
	$AUC_{(0-6h)}$ (ng.h/mL)	1213 ± 605	30,951 ± 13,459*	26
	$t_{1/2}$ (h)	0.59 ± 0.22	0.72 ± 0.75	-

Fig. 4, Table 1 and Table S2 show the PK profiles and parameters of DTZ (A) and DAD (B) in the absence and presence of a pre-injected bolus of transmembrane pH-gradient liposomes. In such cases, the antidote was administered prior to the drug in order to study the fundamentals of DTZ uptake by the liposomes, before introduction of confounding factors like absorption, distribution, metabolism and administration schedules. Moreover, this dosing sequence gave insights of the liposome ability to capture freshly-absorbed drug, in a context where DTZ absorption from the gastrointestinal tract would still be ongoing. In the control group, the PK of i.v.-injected DTZ and DAD was similar to that reported in the literature for similar doses[26,29,30]. The impact of liposome administration on the blood profile of DTZ was predominant in the early distribution phase of the drug, with a maximum plasma concentration (C_{max}) 14-fold higher than in the control group (Fig. 4A and Table 1). This resulted in a 20-fold lower

V_d, and a 6-fold increase in the area under the plasma concentration *vs.* time curve ($AUC_{(0\text{-}6h)}$). The increment of plasma drug exposure reflected early confinement in the blood compartment. The action of liposomes on DTZ PK declined over time until 2 h after the injection. After that time, the blood levels in both groups were superimposed. Hence, the terminal elimination half-lives ($t_{½}$) remained similar, and the decrease in Cl can be mainly explained by the reduced V_d.

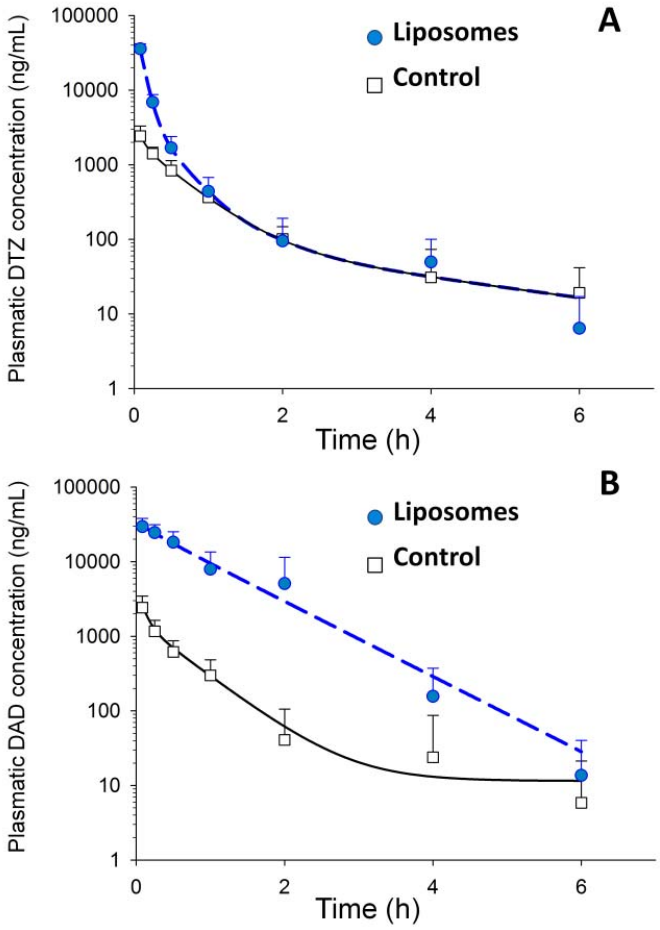

Figure 4. Plasma concentrations of DTZ and DAD in control and liposome-treated rats. **(A).** The influence of liposomes on DTZ is seen mostly during the distribution phase. **(B).** The liposomes also captured DAD, and the effect was more prolonged than with DTZ. Mean ± SD (n= 7-8), lines are used as eye-guides only.

Interestingly, the outcome of liposome pretreatment on DAD exposure was much more pronounced (Fig. 4B). Liposomes enhanced the C_{max} and $AUC_{(0\text{-}6h)}$ by 13- and 26-fold, respectively. Although V_d

could not be calculated because DAD is not the sole metabolite of DTZ, these findings confirm that the most important active metabolite of DTZ could also be captured and sequestered in the blood compartment. The effect of liposome pretreatment on the metabolite was maintained for more than 2 h after DTZ injection, resulting in the higher increase in $AUC_{(0\text{-}6h)}$ compared to the parent drug (6- *vs.* 26-fold increase for DTZ and DAD, respectively). This difference in effectiveness is difficult to rationalize as DTZ and DAD have the same pK_as (7.57 ±0.05 for DTZ and 7.68 ±0.05 for DAD), the same affinity for plasma proteins and their capture in liposomes *in vitro* was found to be identical (Fig. S8). However, it can be hypothesized that, by heightening the concentration of drug in the blood pool, liposomes may augment the DTZ exposure to the blood esterases, making the newly-formed metabolite readily available for capture by circulating liposomes. Indeed, Fig. S9 supports such a mechanism. In this experiment, DTZ was loaded into liposomes and the latter were incubated in whole blood for 4 h. During that period, DAD intravesicle concentration rose by 4-folds, while that of DTZ decreased. Such an effect was not observed for DTZ-loaded liposomes incubated in HBS.

Impact of liposomes on the pharmacological activity of DTZ. In subsequent experiments, the influence of liposomes on the hypotensive activity of DTZ was monitored for 60 min (Fig. 5 and Table S3). The first set of experiments examined the effect of an i.v. bolus of 5 mg/kg DTZ (Fig. 5A), while the second studied the hemodynamic response to a bolus (5 mg/kg) followed by continuous i.v. perfusion (8 mg/kg/h) (Fig. 5B). This second dosing schedule was chosen to evaluate if liposomes were able to maintain their efficacy when higher DTZ doses were administered. Fig. 5A, shows the relative variation of mean arterial blood pressure (MAP) after a single bolus of DTZ. A significantly lower maximum pressure drop (Δ Max) was seen when liposomes were injected into rats. Likewise, the mean hypotensive action of DTZ, assessed by the area under the relative-pressure *vs.* time curve ($AUC_{(0\text{-}60min)}$), also decreased significantly. After the initial drop in blood pressure, the recovery profiles were parallel, in agreement with the PK data, suggesting that liposomes exert most of their effect during the distribution phase, when DTZ concentrations are high. Fig. 5B illustrates the hemodynamic profile

when rats were continuously perfused with DTZ, following the administration of a bolus. These conditions allowed the administration of higher drug doses with a dosing schedule that could be related to the blood exposure observed with sustained DTZ oral absorption. Once again liposomes attenuated the hypotensive effect of DTZ, as revealed by 1.5- and 1.7-fold decreases in Δ Max and $AUC_{(0\text{-}60min)}$, respectively. These findings suggest that the sequestration of DTZ during the early phase of distribution, and of DAD over a prolonged time can partially abrogate the pharmacological activity of DTZ. Likewise, even when higher doses are administered in a sustained fashion, long- circulating liposomes maintain their detoxifying properties.

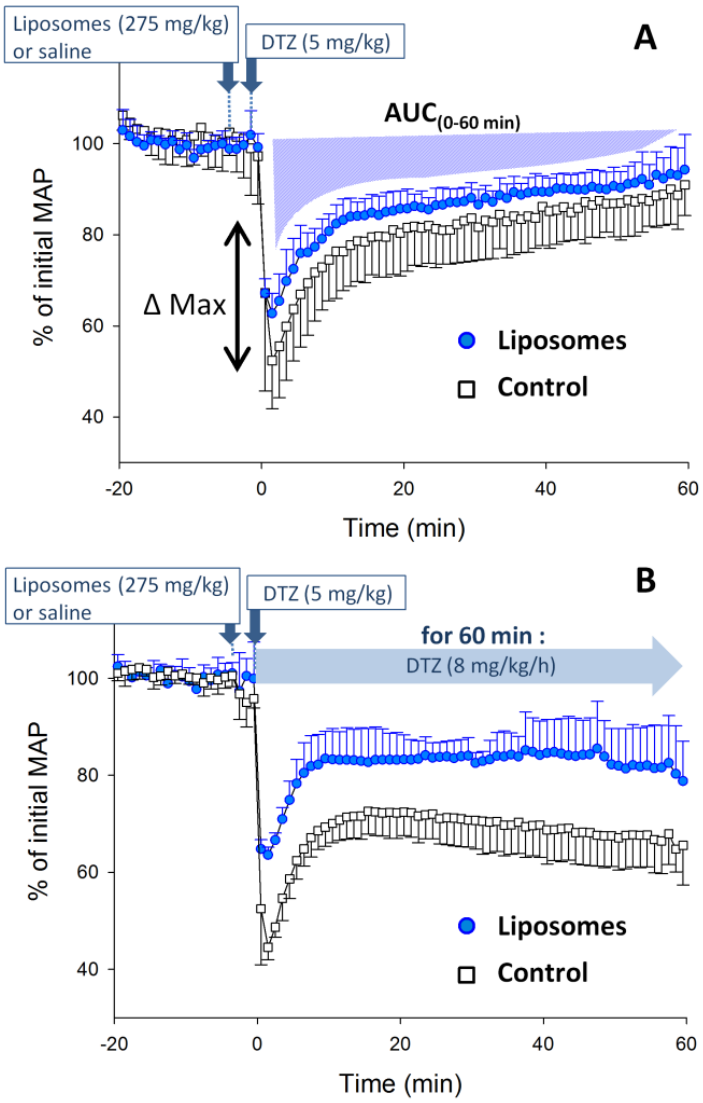

Figure 5. Influence of liposomes on the hypotensive effect of DTZ. **(A).** After a bolus injection of DTZ, the liposomes minimized the drop in blood pressure. **(B).** The effect of liposomes was more pronounced when higher DTZ doses were perfused over 1 h. Mean ± SD (n=5-6).

CONCLUSION

Stable liposomes with a transmembrane pH gradient were designed to trap DTZ and its active metabolite both *in vitro* and *in vivo*. In a simple i.v. model, the formulation was shown to diminish the pharmacological hypotensive effect of the drug after a single bolus injection and sustained perfusion. Based on this optimistic proof of concept, further studies are anticipated to focus on experimental designs closer to a clinical intoxication context. However, the unique properties of liposomes, which combine well-established innocuousness, long circulation properties, and efficient drug capture,

strengthen the belief that they could be a viable alternative to IFE, and other scavenging particulate systems in development[10,35,36] for the treatment of CCB overdose. Moreover, because the ion-trapping phenomenon applies to most ionisable molecules, it is believed that the versatility of the formulation could straightforwardly transpose to a variety of other drugs[34]. The latter feature is particularly important, given recent interest in developing pluripotent universal antidotes[37]. Finally, one important issue that will have to be addressed in the future is the necessity of PEGylating the liposomes. While PEG-phospholipids are useful to provide the liposomes with stealth properties, they have also been associated with complement-activated pseudoallergic reactions[38,39]. In the context of drug overdose, the relatively large dose of liposomes that may be given to treat the intoxication may obviate the need to PEGylate the liposomes given that the saturation of the MPS might suffice to increase their biological half-life[40].

METHODS

Preparation of PEGylated liposomes with transmembrane pH gradient

Liposomes were prepared by the film-hydration/extrusion method[41]. Briefly, Egg PC, Chol, and DSPE-PEG (58:38:4 mol%) (NOF Corporation, Tokyo, Japan) were dissolved in chloroform. After evaporation of the solvent, the dried film was hydrated for at least 1 h with a buffer solution of citrate and extruded through 400- and 200-nm polycarbonate membranes with a LiposoFast manual extruder (Avestin, Ottawa, ON, Canada) to yield < 200 nm vesicles (PdI < 0.2, as determined by dynamic light scattering). The pH gradient was generated by replacing external citrate buffer by isotonic HBS (20 mM HEPES, 144 mM NaCl) or normal saline solution (150 mM NaCl) either by size exclusion chromatography (SEC) on a Sephadex G-50 (Sigma, St Louis, MO) column (25-cm height, 1.5-cm width) or by dialysis for > 6 h on a Spectra/Por membrane with a molecular weight cut-off of 300 kDa (Spectrum Laboratories, Rancho Dominguez, CA). For the *in vivo* experiments, liposomes were concentrated by ultrafiltration on Amicon-4, 300-kDa centrifugal filter units (Millipore, Billerica, MA). Lipid concentration was quantified by the phosphorous assay[42].

In vitro uptake studies

Liposomes were incubated at 37˚C under horizontal shaking in buffer in the presence of [^{3}H]-DTZ (60-87 Ci/mmol, Perkin Elmer, Waltham, MA) (and [^{3}H]-DAD for experiments in the Supplementary Information). After 1 h of incubation, they were separated from the free drug by SEC on a Sephadex G-50 or Sepharose CL-4B (Sigma) column (20-cm height, 1.5-cm width) with HBS, pH 7.4, as mobile phase. Fractions of 1 or 2 mL were collected (up to 34-mL total volume), and radioactivity in each fraction was assessed by scintillation counting (Liquid Scintillation Analyzer, Tri-Carb 2100TR, Packard, Meridan, CT). The fraction of drug captured was determined by the ratio of the area under the radioactivity *vs*. elution volume curve for liposome-containing fractions (7 to 12 mL) over the total area under the curve. The quantity of drug captured was normalized by the amount of PL in solution. The default incubation buffer was isotonic HBS, pH 7.4, except when 50% rat plasma/HBS, pH 7.4, was included. The default drug concentration was 1 mM with a drug-to-PL molar ratio of 0.4 (liposome concentration of 2.5 mM of PL).

PK studies

All animal studies conducted were approved by the Animal Welfare and Ethics Committee of the University of Montreal in accordance with Canadian Council on Animal Care guidelines. Male Sprague-Dawley rats (300-350 g) (Charles River, Montreal, QC, Canada) were injected in the subclavian vein with liposomes (275 mg/kg), followed 2 min later by DTZ (5 mg/kg), under isoflurane (2%) anaesthesia. From each rat, 500-µL blood samples were collected in EDTA-coated Microtainer (BD, Franklin Lakes, CT) *via* the subclavian vein, under anaesthesia, at 5, 15, 30 min, 1, 2, 4 and 6 h. Plasma was separated immediately from blood cells by centrifugation at 2 000 *g* for 10 min, and samples were kept at -20 °C until further use. Seven and 8 animals were included in the control and treatment groups, respectively.

Calculations of PK parameters

PK data were treated by non-compartmental analysis of plasma concentration *vs.* time profiles. C_{max} corresponded to the maximum concentration measured while C_0 was extrapolated as the Y intercept of linear least-squares regression on the semi-log plot of the plasma concentration *vs.* time curve using the first 3 to 4 points of the curve. V_d was determined by dividing the injected dose by C_0. The apparent first-order terminal elimination rate (K_{el}) was estimated by linear least-squares regression on the semi-log plot of the plasma concentration *vs.* time curve with the last 3 to 4 points of the curve. $t_{1/2}$ was assessed as $\ln(2)/K_{el}$. $AUC_{0\text{-}6h}$ were calculated by the trapezoidal method from 0 to 6 h. Areas under the blood concentration-time curve from time zero to infinity ($AUC_{0\text{-}\infty}$) were calculated by adding $AUC_{0\text{-}6h}$ to the ratio of the last measurable concentration to K_{el}. Total Cl was quantified as the injected dose (5 mg/kg) divided by $AUC_{(0\text{-}\infty)}$.

DTZ extraction and high performance liquid chromatography (HPLC)

The extraction method was adapted from a previously published study[43]. Fifty µL of a 10-µg/mL solution of imipramine were spiked as internal standard to 220 µL of thawed plasma. After alkalinisation of the plasma (with 50 µL of NaOH 0.1 N), DTZ was extracted twice with 1 mL of diethyl ether. The organic phase was evaporated to dryness under mild heating (50-60 °C), dissolved in 100 µL of diethylether and back- extracted with 85 µL of acetate buffer, pH 5.5 (50 mM acetic acid). DTZ content in 60 µL of the aqueous phase was measured at 237 nm by reverse-phase HPLC in a system equipped with a 1525 binary pump, a dual wavelength absorbance detector, mounted with an Ascentis C18 column (15 cm x 4.6 mm, 3 µm) and Breeze chromatography software version 3.3 (Waters, Milford, MA). The flow rate was set at 1 mL/min for H_2O:acetonitrile (ACN) gradient elution of 30→70% ACN in 25 min, followed by a return to initial conditions over 2 min. Trifluoroacetic acid 0.1% (*v/v*) was added to the mobile phase as a charge stabilizer. Elution times of DAD, DTZ and the internal standard were 6.0, 8.8 and 10.7 min, respectively. Linearity of a 12-point calibration curve was achieved for concentrations ranging from 10 to 10,000 ng/mL with a quantification limit above 10

ng/mL for DTZ and 50 ng/mL for DAD. For mean calculations, values under the quantification limit were set at 0 ng/mL.

Pharmacological activity of DTZ

The pharmacodynamic study of DTZ was carried out on Sprague-Dawley rats with left femoral vein and artery catheterized with polyethylene-10 tubing (Folioplast SA, Sarcelles, France) under pentobarbital anaesthesia (65 mg/kg). Arterial access was connected to a pressure transducer for continuous measurement of systolic and diastolic blood pressures by Chart software version 5.5.6 (AD Instruments, Colorado Springs, CO). MAP was calculated as (2/3 diastolic + 1/3 systolic pressure). During hemodynamic monitoring, the animals were maintained under continuous isoflurane (2%) anaesthesia. After recording basal hemodynamic parameters for at least 20 min, venous access was used for the injection of identical volumes of either normal saline solution or pH-gradient liposomes (275 mg/kg), followed by DTZ (5 mg/kg) 2 min later. After each bolus injection, the catheters were rinsed with 300 µL of heparinized normal saline (100 UI/mL). In the perfusion experiment, DTZ 1 mg/mL solution was initiated at 8 mL/kg/h right after DTZ bolus. Pressures were measured for 1 h after DTZ dosing. Five rats were included in all groups except in the liposome-perfusion group where 6 animals were studied.

Statistical analysis

Statistics were computed with SigmaPlot 11.0 software (SPSS, Chicago, IL) (Table S4). Differences in group means were calculated by standard unpaired *t*-test, Mann-Whitney U test on ranks or Kruskal-Wallis test on ranks followed by Student-Neuman-Keuls test. Non-parametric tests were used when samples failed tests for equality of variance and/or normality with the Kolmogorov-Smirnov test. A value of $p<0.05$ was considered significant.

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SUPPORTING INFORMATION PARAGRAPH: Additional methods and results on the synthesis of DAD, *in vitro* uptake experiments, stability of the pH-gradient and citrate loading, and *in vitro* experiments on the conversion of DTZ to DAD. Tables of formulations used during experiments, detailed results of the PK and pharmacodynamic experiments, and statistical tests used in each figure and table are also reported. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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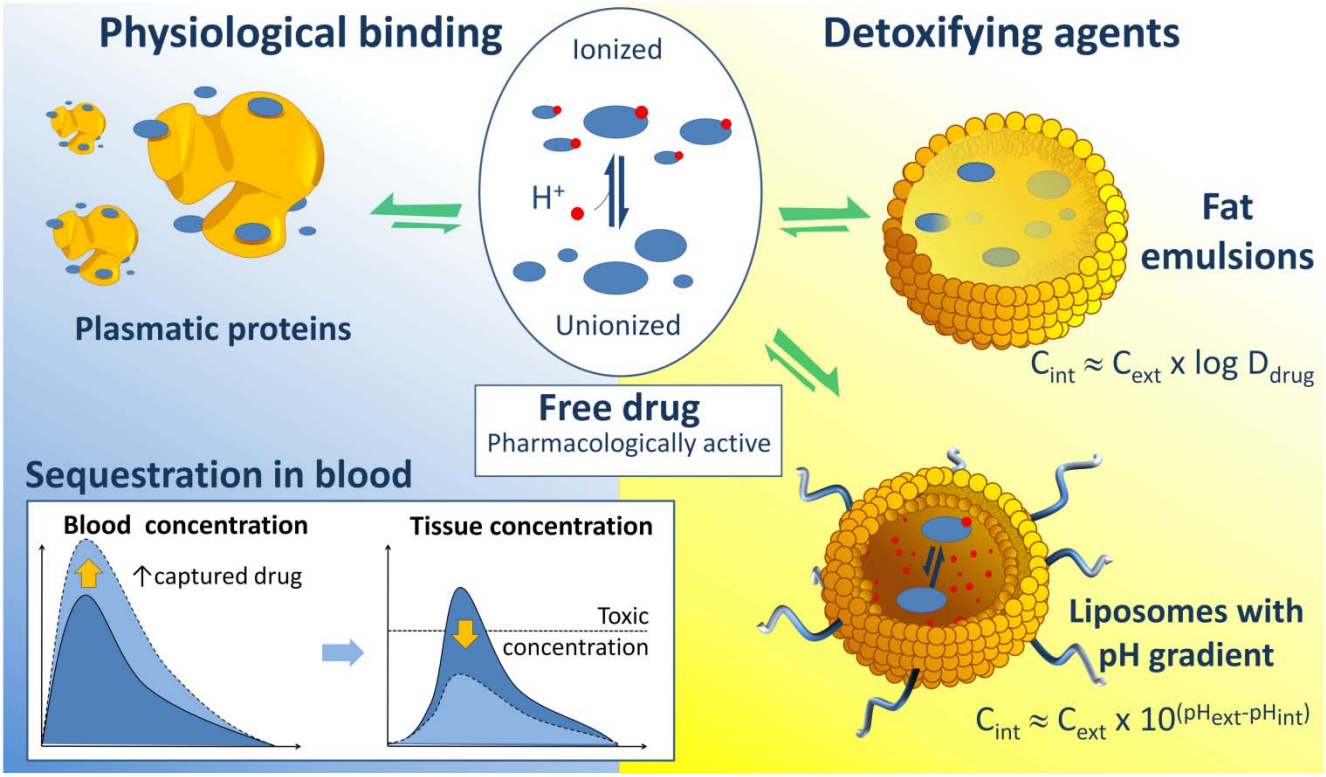
Physiological binding
Ionized
Detoxifying agents
H+
Plasmatic proteins
Unionized
Fat emulsions
$C_{int} \approx C_{ext} \times \log D_{drug}$
Free drug
Pharmacologically active
Sequestration in blood
Blood concentration
Tissue concentration
↑captured drug
Toxic concentration
Liposomes with pH gradient
$C_{int} \approx C_{ext} \times 10^{(pH_{ext}-pH_{int})}$