Effect of TRX-Liposomes Size on Their Prolonged Circulation in Rats

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Newly formulated cationic liposomes (TRX-liposomes) with four different mean diameters were injected into twelve male rats *via* the lateral tail vein in order to evaluate the effect of liposomal size on pharmacokinetic parameters. TRX-liposomes disappeared from the blood according to the one-compartment model and demonstrated maximum and minimum half-lives of *ca.* 14 h (mean diameter of 114.3 nm) and *ca.* 5 h (mean diameter of 285.9 nm), respectively. This prolonged half-life tended to decrease at the boundary of 114.3 nm mean diameter. The optimal size (114.3 nm) for prolonged circulation of TRX-liposomes was consistent with that of pegylated liposomes such as Doxil®, however, the half-life was different among these liposomes. The electric charge of the TRX-liposomal surface is assumed to be responsible for this difference. The results of the present study will be very useful in the design of long-circulating cationic liposomes.

Key words cationic liposome; size distribution; half-life; TRX-20

Liposomal formulation has been a focus of attention since the late 1960s as an effective drug carrier system. In particular, many different applications of liposomes have been reported since the rapid sequestration by the reticuloendothelial system (RES) was resolved by modification of the liposomal surface with hydrophilic materials such as polyethylene glycol (PEG) derivative.[1] These liposomes are known as stealth liposomes,[2] and one of the liposomes successfully launched onto the world market is doxorubicin-encapsulated liposomes which have a half-life of 56.6 h in humans.[3] However, the fate of the liposome in the blood and tissues is not simply controlled by such physicochemical properties of the liposomal surface. The size of the liposome itself also plays a very important role in extended circulation.

Recently, liposomal formulations whose surface is modified with cationic moieties have been developed as useful carriers for gene delivery.[4] The surface potential and steric conditions of these liposomes are obviously different from PEG-modified liposomes so it is very likely that the pharmacokinetic behaviors of cationic liposomes are different with PEG-modified liposomes. We found that TRX-liposomes containing a newly synthesized cationic moiety (3,5-dipentadecyloxybenzenecarboxamidine hydrochloride (TRX-20)) had a specific interaction with a certain type of chondroitin moiety that appeared on the cell at the inflammation site.[5] However, no pharmacokinetic studies have been conducted thus far. In the present study, the effects of TRX-liposomes size on pharmacokinetic parameters, such as half-life, were investigated. Prednisolone sodium phosphate (PSLP), which has a relatively high solubility, was selected as a model drug to reduce its undesirable effect by encapsulation in the liposomes.

Experimental

Chemicals Hydrogenated soybean phosphatidylcholine (HSPC: SPC-3) and cholesterol (HP) were purchased from Lipoid KG (the Netherlands) and Solvay Pharmaceuticals B.V. (the Netherlands), respectively. TRX-20 (Fig. 1)[6] was synthesized at Joko-Yakuhin (Japan). *N*-(monomethoxy-polyethyleneglycolcarbamyl) distearoyl phosphatidylethanolamine (PEG5000-PE) with a purity of more than 95% was obtained from Genzyme Company (Switzerland). Lissamine rodamine B 1,2-dihexadecanoyl-phosphatidylethanolamine (rhodamine DHPS) and PSLP were purchased from Molecular Probes (U.S.A.) and Diosynth B.V. (the Netherlands), respectively. The other materials used in the study were of reagent grade.

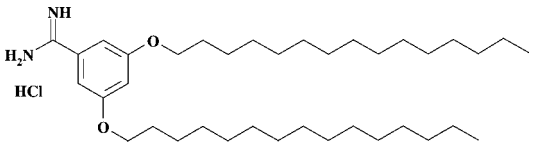

Fig. 1. The Chemical Structure of 3,5-Dipentadecyloxybenzenecarboxamidine Hydrochloride (TRX-20)

Preparation of TRX-Liposomes HSPC, TRX-20, cholesterol (363.7 mg, molar ratio of 50 : 42 : 8), and rhodamine DHPE (5 mg) were dissolved in *tert*-butyl alcohol and then freeze-dried to obtain the mixed-lipid. This mixed-lipid was dissolved in phosphate buffer solution (pH 7.2) containing PSLP and sonicated at 70 °C for 5 min. An extrusion process (Extruder T-10; Lipex Biomembranes, Canada) using the four different pore sizes of the membrane filters (0.4, 0.2, 0.1, 0.05 μm) (Whatman, U.S.A.) at 70 °C was employed to obtain the different sizes of TRX-liposomes. PEG5000-PE solution maintained at 60 °C was then added at 1.50 mol% (PEG5000-DSPE/HSPC) and unencapsulated PSLP was removed by gel filtration.

Determination of TRX-Liposome Size and Zeta-Potential Zetamaster S (Malvern Instruments, U.K.) was used to determine the size distribution and zeta-potential of the liposomes.

Animal Study Twelve male SD rats (Charles River Japan, Inc.) with an average body weight of 240 g were randomly classified into four groups. Two ml/kg of TRX-liposomes solution containing 2.4 mg HSPC was injected into the rats in each group *via* the lateral tail vein. Blood samples taken from the lateral tail vein at 1, 3, 6, 24, 48, and 72 h were refrigerated until analysis. During the study, the rats were given standard pellet food and water *ad libitum*.

Analysis of TRX-Liposomes in Blood TRX-liposomes in the blood were estimated by determining the fluorescent intensity of rhodamine DHPE contained in the liposomes according to the analytical method reported by Harigai *et al.*[5] An F-4000 spectrophotofluorometer (Hitachi, Japan) was used in the study.

Derivation of Pharmacokinetic Parameters The pharmacokinetic parameters of TRX-liposomes in the blood presented as HSPC concentration were calculated using the least-squares data fitting method based on the compartment model. Microsoft Excel 97 (Microsoft Corporation, U.S.A.) was used for the calculations.

Results and Discussion

Mean Diameter of TRX-Liposomes and Size Distribution The size distribution profiles of the five TRX-liposomes, as shown in Fig. 2, clearly indicated that the liposomal size distribution varies significantly depending upon their mean size. This is because the size distribution of TRX-

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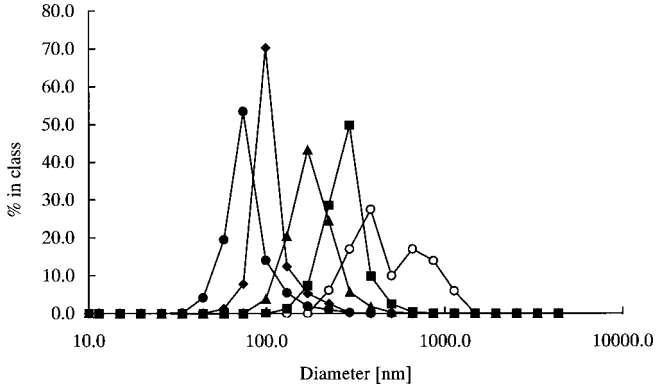

Fig. 2. Size Distribution Profiles of Five TRX-Liposomes

TRX-liposomes were prepared by extrusion with membrane filters with pore sizes of 0.05 μm (closed circles), 0.1 μm (closed ellipses), 0.2 μm (closed triangles), 0.4 μm (closed squares). Open circles indicate the size distribution profile of TRX-liposomes before the extrusion process.

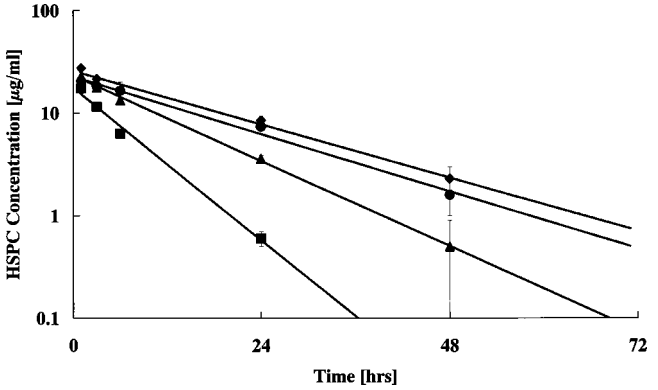

Fig. 3. Blood Concentration of HSPC in Rats Treated with 2.4 mg HSPC/kg TRX-20 Liposomes with a Mean Diameter of 84.9 nm (Closed Circles), 114.3 nm (Closed Triangles), 184.1 nm (Closed Ellipses), and 285.9 nm (Closed Squares)

Each value represents the mean±S.D. of three rats. Solid lines represent a monoexponential fit.

Table 1. Characteristics of TRX-Liposomes

Characteristic	0.05 μm	0.1 μm	0.2 μm	0.4 μm
Liposomes size	84.9 nm	114.3 nm	184.1 nm	285.9 nm
Zeta-potential	7.9 mV	5.2 mV	3.0 mV	2.4 mV
HSPC concentration	9.71 mg/ml	11.45 mg/ml	12.11 mg/ml	11.77 mg/ml
PSLP concentration	1.51 mg/ml	1.90 mg/ml	2.21 mg/ml	2.04 mg/ml

Table 2. Pharmacokinetic Parameters of TRX-Liposomes in Rats

Parameter	84.9 nm	114.3 nm	184.1 nm	285.9 nm
C_0 [μg/ml]	22.7±0.9	25.8±2.7	22.7±0.9	17.6±0.8
k_{el} [1/h]	0.054±0.008	0.051±0.008	0.076±0.008	0.145±0.010
$t_{1/2}$ [h]	13.1±1.9	14.0±2.3	9.2±0.9	4.8±0.3
AUC [μg h/ml]	444.5±43.3	507.7±30.3	292.3±17.7	118.2±5.7
MRT [h]	19.1±2.5	20.6±3.5	13.2±1.3	6.7±0.6
CL_{tot} [ml/min/kg]	0.091±0.009	0.079±0.005	0.137±0.008	0.339±0.016
V_{dss} [ml/kg]	103.0±3.6	97.0±13.9	108.2±4.0	135.9±6.9

Each value represents the mean±S.D. of three rats.

liposomes (open circle) formed before the extrusion process was broad and small liposomes with mean diameters of less than 200 nm had passed through the 0.4 and 0.2 μm pore membrane filters. On the other hand, the mean diameters were found to be 10 to 30 nm larger than the pore size of the membrane filter used in the extrusion process. The temperature (70 °C) employed in the extrusion process is higher than the phase transition temperature (Tc=55 °C) of HSPC and a high pressure of about 2 MPa was applied to extrude the liposomal suspension so that large TRX-liposomes might deform and pass through the membrane filter. However, as shown in Table 1, the ratio of HSPC and PSLP in TRX-liposomes is almost constant after the extrusion process, suggesting that TRX-lipsomes kept their original composition even if such deformation was presumed to have occurred during the process. Berger *et al.*[7] also observed a similar result, that is, liposomal size after the extrusion process was larger than the pore size of the filter membrane used in the process. A number of research papers examining the effect of liposomal size on pharmacokinetic parameters have been published, however no size distribution profiles were discussed in these reports. It should be noted that not only mean diameter but also the size distribution profiles should be carefully evaluated when considering the results of these studies.

Pharmacokinetic Behavior of TRX-Liposomes As shown in Fig. 3, the blood concentration profiles of four different TRX-liposomes could clearly be described by the one-compartment open model ($r^2>0.995$). The pharmacokinetic parameters calculated by the least-squares data fitting method are tabulated in Table 2. Tukey's multiple range test was used to evaluate the statistical significance of the parameters among the four TRX-liposomes. In all parameters, there was a significant difference ($p<0.01$) between TRX-liposomes with a mean diameter of 258.9 nm and the other three TRX-liposomes. However, no significant differences were obtained for the parameters between TRX-liposomes with mean diameters of 84.9 and 114.3 nm except for Co ($p<0.05$). The difference of four parameters (k_{el}, $t_{1/2}$, *AUC* and *MRT*) between TRX-liposomes with a mean diameter of 184.1 nm and the other two TRX-liposomes were significant

($p<0.05$). These results suggest there is an optimal TRX-liposomes size that provides prolonged circulation. In addition, TRX-liposomes with a mean diameter of 114.3 nm were found to give the largest *AUC* value and the longest *MRT*. Furthermore, smaller TRX-liposomes tended to have a longer half-life and this tendency stopped at the mean diameter of 114.3 nm.

PEG-modified doxorubicin encapsulated liposomes (Doxil®) with prolonged circulatory life are reported to have an average size of around 75 to 130 nm, with the half-life times in rats and humans reported to be around 20—30 h[8] and 56.6 h,[3] respectively. These data are consistent with our results from the viewpoint of optimal size (*ca.* 114 nm) for prolonged circulation. However, the half-life of TRX-liposomes was shorter than that of the PEG-modified liposomes mentioned above even though TRX-liposomes gave distinctly prolonged circulation. The electric charge and steric conditions of the liposomal surface are assumed to be critical factors which influence the interaction between the liposomes and the components of blood leading to prolonged circulation. In Doxil®, the molecular size of PEG (PEG2000-PE) formulated and its content (5.3 mol%) are significantly different from those of TRX-liposomes. Okabe *et al.*[9] suggested that PEG5000-PE at the concentration of 0.75 mol% would cause a significant reduction in the interaction between TRX-liposomes and blood components. In addition, the steric form of both PEG5000- and PEG2000-PE on the surface of the liposomes is considered to be the brush form at the above concentration. However, electric charge due to TRX-20 was not effectively masked at a PEG5000-PE concentration of 0.75 mol%. The zeta-potential of TRX-liposomes, as shown in Table 1, is positive and that of Doxil® was found to be negative (−31.2 mV). Rahman *et al.*[10] reported that large liposomes (diameter of about 500 nm) was easily taken up by Kupffer cells, but this uptake is less effective in small liposomes (diameter of about 80 nm). The mean diameters of both TRL-liposomes with the longest half-life and Doxil® are around 100 nm, therefore the effect of the liposomes-uptake in Kupffer cells would not be minimum. Based on the above comparisons and the results obtained by Okabe *et al.*,[9] we now believe that the differing electric charges of the TRX-liposomes are responsible for the difference in the half-life between TRX-lipsomes and Doxil® in rats. The results obtained in this study should be valuable for designing long-circulating cationic liposomes.

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