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Comparative Pharmacokinetics, Distributions in Tissue, and Interactions with Blood Proteins of Conventional and Sterically Stabilized Liposomes Containing 2′,3′-Dideoxyinosine

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The pharmacokinetics and distribution in tissue of 2′,3′-dideoxyinosine (ddI) encapsulated in sterically stabilized liposomes have been evaluated in rats. Most of the sterically stabilized liposomes concentrated in the spleen with a peak level at 24 h after their intravenous injection. An extended half-life in plasma was observed for sterically stabilized liposomes (14.5 h) compared with that of conventional liposomes (3.9 h). The systemic clearance of ddI incorporated in sterically stabilized liposomes was 180 times lower than that of the free drug. The levels of in vitro and in vivo protein binding on both conventional and sterically stabilized liposomes were also evaluated. Results suggest that the amount of proteins associated with liposomes might not be the only factor involved in the in vivo clearance of liposomes, as this process may also be influenced by the nature of the bound blood proteins.

Didanosine (2′,3′-dideoxyinosine [ddI]) is a nucleoside analog of adenosine which prevents replication of human immunodeficiency virus (HIV) by inhibiting reverse transcriptase (4, 18). This antiviral agent has been recommended by the Food and Drug Administration for the treatment of HIV-infected patients who are resistant or intolerant to zidovudine treatment. The most common dose-related adverse effects of ddI therapy are neuropathy and pancreatitis (8, 19, 25, 28). Moreover, the instability of ddI at gastric pH leads to a low drug bioavailability (approximately 40%) (8, 22). The half-life of ddI in plasma in humans is short (0.6 to 1.4 h), but its active metabolite 2′,3′-ddATP has a longer intracellular half-life (12 to 24 h) than does the active triphosphate metabolite form of zidovudine (3 h) (8). Encapsulation of drugs into liposomes represents a convenient approach to improve their pharmacokinetics and reduce their toxicity. We have recently reported that the encapsulation of ddI into conventional liposomes greatly reduced the drug systemic clearance and allowed efficient targeting of macrophage-rich tissues following intravenous administration in rodents (6, 12). As macrophages play a central role in HIV pathogenesis (10, 17), acting as reservoirs for dissemination of virus, the improved delivery of liposomal ddI into these tissues could improve the efficacy of the entrapped drug and reduce the toxic side effects associated with its administration.

The interaction of liposomes with blood proteins is known to play a major role in the biological fate of liposomes. Upon exposure to blood, liposomes become coated with different proteins which may destabilize the liposome membrane, resulting in a release of the entrapped drug that prevents realization of the desired therapeutic objective. Adsorption of opsonins on liposomes followed by phagocytosis is currently considered as the primary mechanism of liposome clearance from the bloodstream (11, 20, 24). It has been shown that immunoglobulins and complement fragments are directly implicated in opsonization mechanisms (3, 7, 15). These proteins can bind to the liposome membrane and modify its circulation time. Chonn et al. (3) have recently demonstrated that liposomes that have a small amount of proteins bound on their surface exhibit longer plasma half-lives than liposomes that have a large amount of bound proteins. They suggested that the C3b fraction of the complement could play an important role in the in vivo clearance of liposomes.

Many strategies have been developed in the past few years to increase the circulation time of liposomes by reducing their recognition by the mononuclear phagocyte system. Among them, the coupling of hydrophilic polyethylene glycol (PEG) chains to liposomes has been shown to extend blood circulation time compared with that of conventional liposomes because of the reduced interaction between these modified liposomes and plasma proteins (1, 2, 14, 26, 27). The presence of highly hydrated groups on the liposome surface is thought to inhibit both electrostatic and hydrophobic interactions with a variety of blood components. Such sterically stabilized liposomes are commonly used to target tissues outside the reticuloendothelial system. Although it may appear irrelevant to use such liposomes to target HIV reservoirs, we believe that the use of sterically stabilized liposomes could maintain an uptake of liposomes in tissues susceptible to HIV infection for longer periods than those associated with conventional liposomes and consequently prolong the therapeutic effect of the entrapped drug. In addition, the enhanced vascular permeability coefficient of the PEG-modified liposomes could allow a better penetration through capillaries (1). In the present study, the pharmacokinetic properties and tissue distribution of ddI encapsulated in PEG-modified liposomes have been investigated in rats. In addition, as lipid-protein interactions are a key factor affecting the distribution of liposomes, we have also evaluated the in vitro and in vivo levels of bound proteins on

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the surface of both conventional and sterically stabilized liposomes.

Distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG), and distearoylphosphatidylethanolamine (DSPE) were purchased from Avanti Polar Lipids (Alabaster, Ala.). The radioactive lipid tracers L-3-phosphatidylcholine 1,2-di[1-^{14}C]palmitoyl ([^{14}C]DPPC) and L-3-phosphatidyl [2-^{14}C]ethanolamine, 1,2-dioleoyl ([^{14}C]DOPE) were obtained from Amersham Canada Ltd. (Oakville, Ontario, Canada). Labeled 2′,[3′-^{3}H]ddI ([^{3}H]ddI) was obtained from Moravek Biochemicals Inc. (Brea, Calif.). DSPE-PEG was prepared as previously described (9, 13). Briefly, a mixture of methoxypolyethylene glycol succinimidyl succinate (PEG-OSu, molecular weight, 5,000; Sigma, St. Louis, Mo.), DSPE, and triethylamine in a molar ratio of 3:1:3.5 containing 0.01 μCi of [^{14}C]DOPE per μmol of lipids was incubated overnight at room temperature in $CHCl_3$. Solvent was then evaporated under a stream of nitrogen, and the dry mixture was hydrated with double-distilled water. The micellar solution was filtered through a column (32 by 2 cm) containing Bio-Gel A-1.5 M (50/100 mesh) to remove uncoupled PEG-OSu. Peak fractions containing DSPE-PEG were determined by both scintillation countings (for [^{14}C]DOPE-PEG) and absorbance readings at 225 nm (for free PEG-OSu). Fractions containing DSPE-PEG were pooled and dialyzed for 24 h against water by using a membrane with a nominal molecular weight cutoff of 300,000 (Spectra-Por; Spectrum Medical, Los Angeles, Calif.) and then lyophilized. Purity of DSPE-PEG was confirmed by thin-layer chromatography revealed with I_2.

Liposomes composed of DSPC-DSPG (10:3) and DSPC–DSPG–DSPE-PEG (10:3:1.45) were prepared by thin-lipid-film hydration. In brief, the lipid mixture was dissolved in chloroform-methanol (2:1, vol/vol) in the presence of a small proportion of [^{14}C]DPPC (<0.002%, mol/mol), and the solvent was then evaporated in a round-bottomed flask to form a thin lipid film. The lipid film was hydrated with a phosphate-buffered saline solution (PBS; 145 mM, pH 7.4) of ddI in a drug/lipid molar ratio of 2 to which a small proportion of [^{3}H]ddI (<0.001%, mol/mol) was added. Multilamellar vesicles were sequentially extruded by using polycarbonate membranes (Nuclepore, Cambridge, Mass.) with 1- and 0.2-μm pore diameters, generating liposomes with a diameter of 0.15 ± 0.01 μm (mean ± standard error of the mean) as determined by quasi-elastic light scattering measurements. Unencapsulated drug was removed by centrifugation (300 × *g* for 15 min at 4°C) of the liposomal preparation (1 ml) through a 10-ml coarse Sephadex G-50 column (Pharmacia LKB, Montreal, Quebec, Canada), and efficiency of drug entrapment was determined by radioactive countings. Drug retention was evaluated by incubating liposomes in PBS at 4 and 37°C and in 80% human activated serum at 37°C. At specific time points, 0.1 ml of the dispersion of liposomes was removed and centrifuged through a 1-ml Sephadex G-50 column (300 × *g* for 5 min at 4°C). Drug retention was evaluated by comparing radioactive countings of the centrifuged solution to the drug level at time zero.

For the tissue distribution and pharmacokinetic studies, a single dose of liposome-encapsulated ddI (3 mg of ddI per kg of body weight) was administered intravenously to female Sprague-Dawley rats (185 to 240 g) via a catheter inserted into the right jugular vein of animals as previously described (6). At defined time points (0.25, 5, and 30 min and 1, 2, 3, 4, 6, 12, 24, and 48 h) blood samples were collected in heparinized tubes and separated by centrifugation (3,000 × *g* for 10 min at 4°C). When the animals were sacrificed, selected tissues were collected, washed in saline, weighed, suspended in Triton X-100 (0.5%, vol/vol), and homogenized. Tissues and plasma were treated with a tissue solubilizer and decolored in H_2O_2. Total drug and lipid levels in each sample were determined by radioactivity countings. The pharmacokinetic parameters were determined by using a noncompartmental analysis. The elimination rate constant was estimated from the slope of the terminal elimination phase. The half-life in plasma of the terminal elimination phase was calculated as 0.693 divided by the elimination rate constant. Area under the plasma concentration-time curve ($AUC_{0-\infty}$) and area under the first moment of the concentration-time curve ($AUMC_{0-\infty}$) were calculated by using the log-trapezoidal rule with the terminal portion extrapolated to infinity. The systemic clearance was calculated as dose/$AUC_{0-\infty}$, while the mean residence time was calculated as $AUMC_{0-\infty}/AUC_{0-\infty}$. The steady-state volume of distribution (V_{ss}) was calculated as (dose × $AUMC_{0-\infty}$)/$(AUC_{0-\infty})^2$.

The amount of proteins bound to liposomes was evaluated after incubation of liposomes (7.5 μmol of lipids) in 1.5 ml of 80% activated human serum for 30 and 60 min at 37°C. At each incubation time, 500 μl of the mixture was eluted in a Bio-Gel A-15 M (100/200 mesh) column (25 by 1.5 cm) at an elution rate of 0.5 ml/min. Fractions containing liposomes were identified by radioactivity measurements of [^{14}C]DPPC and pooled. The amount of proteins associated with liposomes was evaluated with a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) in microtiter plates. For the in vivo experiments, liposomal ddI was administered intravenously to another group of animals. At specific time points (2 and 30 min and 24 h), animals were sacrificed and blood was collected in heparinized tubes and separated by centrifugation (3,000 × *g* for 10 min at 4°C). Five hundred microliters of plasma was then eluted in a Bio-Gel A-15 M column and processed as described above. Results were compared by a one-way analysis of variance. Group comparison was performed with the Fisher protected least significant difference test.

The encapsulation efficiency of ddI in sterically stabilized liposomes was 0.26 ± 0.04 μmol of ddI per μmol of lipid (n = 5). Over 90% of the ddI was still entrapped in liposomes after incubation for 24 h in PBS at 4°C (data not shown). This drug retention level was maintained for at least 2 weeks at 4°C. A reduced drug retention was observed for liposomes incubated at 37°C in PBS or activated human serum. In these cases, approximately 50% of the ddI remained in liposomes after a 24-h incubation time. These results are comparable to those obtained with conventional liposomes composed of DSPC-DSPG (10:3) under similar conditions (6). Figure 1 shows the tissue distribution in rats at different time intervals after the administration of a single intravenous dose of ddI (3 mg/kg) entrapped in sterically stabilized liposomes. Drug and lipid concentrations in all tissues were monitored with radioactive tracers ([^{3}H]ddI and [^{14}C]DPPC) without any discernment of potential metabolites, release of drug from liposomes, or exchange of labeled tracers which could have been produced during biodistribution. A steady decrease of drug levels in plasma was observed following the administration of liposomal ddI. On the other hand, a progressive accumulation of liposomal ddI was observed in the spleen in the first 3 h postinjection and was followed thereafter by a decrease in drug levels. Lower concentrations of the liposomal drug were observed in livers, lungs, and kidneys. Moreover, no significant levels of drug were detected in the brains and pancreases of animals at all time points (data not shown). The tissue distribution profile of labeled lipids showed a progressive uptake of phospholipid vesicles by the spleen up to 24 h after the injection, whereas the concentration of liposomal lipids within livers, lungs, and kidneys remained about the same at all time points. Moreover, in contrast to the entrapped drug, the labeled lipids remained for

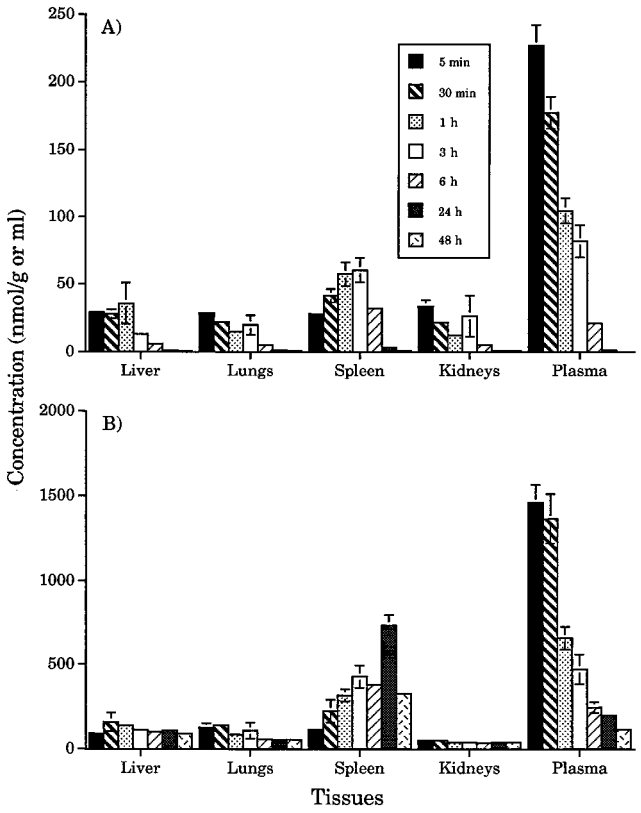

FIG. 1. Plasma and tissue distribution of ddI ([^{3}H]ddI) (A) and liposomal lipids ([^{14}C]DPPC) (B) from sterically stabilized liposome-encapsulated ddI after the administration a single intravenous dose (3 mg of ddI per kg) to rats. Values are the means (± standard error of the mean) obtained for four to six animals per group per time point.

a much longer time in plasma, where high levels of liposomal lipids could still be detected at 24 and 48 h postinjection. These results, which show a progressive reduction of the drug-to-lipid molar ratio in animal plasma as a function of time, indicate a release of ddI from liposomes.

Table 1 shows the pharmacokinetic parameters of ddI entrapped in sterically stabilized liposomes estimated from the concentration-time curves calculated for animal plasma. This table also presents for comparison the pharmacokinetic parameters of free ddI and of ddI encapsulated in DSPC-DSPG (10:3) conventional liposomes obtained from previous studies (6). As expected, the addition of 10% (mol/mol) DSPE-PEG to the conventional liposomes led to a marked reduction of the clearance of the phospholipid vesicles. The plasma half-life of the sterically stabilized liposomes was 3.7 times greater than that of the DSPC-DSPG formulation, resulting in a marked increase of the area under the plasma concentration-time curve. Improved pharmacokinetics were observed upon entrapment of the anti-HIV agent in PEG-modified liposomes. The plasma half-life of ddI in the sterically stabilized liposomes was 25 times greater than that of the free agent. Furthermore, the systemic clearance of the antiviral agent encapsulated in PEG-modified liposomes was 180 times lower than that of the unencapsulated drug.

The in vitro and in vivo protein binding levels of conventional liposomes and sterically stabilized liposomes have been evaluated. Results showed that after incubation in activated serum for 30 and 60 min, conventional liposomes bear 46.9 ±

TABLE 1. Pharmacokinetic parameters of ddI and liposomal lipids following the administration of a single intravenous dose of 3 mg of ddI per kg to rats[a]

Prepn	[^{3}H]ddI						[^{14}C]DPPC				
	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (nmol/ml/h)	k_{el} (h^{-1})	V_{ss} (ml/kg)	CL (ml/h/kg)	MRT (h)	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (nmol/ml/h)	k_{el} (h^{-1})	V_{ss} (ml/kg)	CL (ml/h/kg)	MRT (h)
Sterically stabilized liposomes	3.53 ± 0.17	950.3 ± 43.1	0.20 ± 0.01	70.9 ± 7.4	13.4 ± 0.1	5.31 ± 0.56	14.50 ± 0.90	17,420 ± 729	0.048 ± 0.003	76.8 ± 7.0	3.7 ± 0.1	21.04 ± 1.74
Conventional liposomes	2.64 ± 0.31	649.9 ± 59.8	0.26 ± 0.03	74.1 ± 9.2	19.5 ± 0.1	3.79 ± 0.46	3.92 ± 0.43	4,971 ± 276	0.18 ± 0.02	70.4 ± 4.4	12.8 ± 0.1	5.51 ± 0.33
Free ddI	0.14 ± 0.01	5.31 ± 0.21	4.97 ± 0.47	483 ± 23	2,391 ± 19	0.20 ± 0.04						

[a] Parameters were calculated from the plasma concentration-time curves obtained from 5 to 10 experiments per time point with a noncompartmental model. Values for free ddI and ddI entrapped in DSPC-DSPG (10:3) conventional liposomes were taken from our previous report (6). All values are means ± standard errors. Abbreviations: $t_{1/2}$, elimination half-life; $AUC_{0-\infty}$ area under the plasma concentration-time curve from zero to infinity; k_{el}, elimination rate constant; V_{ss}, steady-state volume of distribution; CL, systemic clearance; MRT, mean residence time.

6.2 and 75.6 ± 8.0 ng of protein per nmol of lipid compared with 26.1 ± 2.4 and 43.9 ± 6.1 ng/nmol for the PEG-modified liposomes, respectively (data not shown). Higher levels of bound proteins were also found in conventional liposomes (17.9 ± 6.1 ng/nmol) than in sterically stabilized liposomes (<10 ng/nmol) at 2 min after intravenous injection in rats. However, similar levels of bound proteins were found in conventional (105.9 ± 2.8 ng/nmol) and PEG-modified (112.7 ± 5.7 ng/nmol) liposomes at 30 min postinjection. The amount of blood proteins bound to the sterically stabilized liposomes (103.6 ± 8.4 ng/nmol) remained the same at 24 h postinjection. No protein binding assays could be done on conventional liposomes at 24 h postinjection because of the absence of significant traces of liposomal lipids in the plasma of animals.

We recently reported that conventional liposomes composed of DSPC-DSPG (10:3) mostly concentrate in the spleen after their intravenous injection in rats with a maximum peak level at 30 min postinjection (6). As expected, results reported here show that the uptake of PEG-modified liposomes by the spleen is reduced compared with the uptake of these conventional liposomes. However, the uptake of sterically stabilized liposomes progressively increased in the first 24 h postinjection. As the in vitro effective concentration of ddI required to achieve a 50% inhibition of virus replication is in the range of 4 to 10 μM (5), one may expect that the entrapment of ddI in PEG-modified liposomes may allow the delivery of such a concentration of ddI to the spleen for approximately 24 h after the injection. The large diameter of liposomes (150 nm) used in this study impedes, as previously demonstrated, the penetration of a high concentration of liposomes into the fenestrated capillaries (21, 23). On the other hand, the absence of liposomal ddI in the pancreas is of prime importance in the treatment of HIV disease, as pancreatitis is one of the most common adverse side effects of ddI therapy, and high levels of drug at this site have been postulated as a potential mechanism of toxicity. As previously observed after the administration of a single intravenous dose of free ddI (6), no drug was found in the brains of animals after the intravenous administration of ddI entrapped in PEG-modified liposomes. These results are due to the incapacity of liposomes to pass the tight intercellular junction of the blood-brain barrier after intravenous injection, as evidenced by the absence of liposomal lipids in the brain.

In the present study, 10% (mol/mol) DSPE-PEG was added to the DSPC-DSPG (10:3) conventional liposomes to improve their circulation times in vivo. As expected, a significantly extended plasma half-life was observed for sterically stabilized liposomes (14.5 h) compared with that of conventional liposomes (3.9 h) because of the reduced uptake of PEG 5000-containing liposomes by the mononuclear phagocyte system. Unfortunately, the plasma half-life of the entrapped drug (3.5 h) was much lower than that of the PEG-modified liposomes, indicating a release of the antiviral agent from the phospholipid vesicles as a function of time in blood. The development of a liposomal preparation allowing a better retention of the nucleoside analog should lead to a tissue distribution profile similar to that of the liposomal lipids. In this regard, incorporation of drugs into cyclodextrins and into liposomes may represent an attractive strategy to improve drug retention (16). Nevertheless, despite the drug leakage from PEG-modified liposomes, the elimination plasma half-life of the entrapped drug was much greater than that of the free drug (0.14 h).

It was recently demonstrated that there is a direct correlation between the complement-activating ability of liposomes and their residency time in vivo (7). Liposomes composed of short fatty acyl chains and having a high cholesterol content could better activate the complement in rat serum. The sterically stabilized and conventional liposomes used in the present study are composed of long fatty acyl chains and do not contain any cholesterol, which should reduce complement activation and increase circulation times. Even though both PEG-modified and conventional liposomes bind the same amount of proteins at 30 min postinjection, the nature of the proteins bound on the liposome membrane needs to be evaluated, as there might not be a direct correlation between the amount of proteins bound at this time and its influence on the circulation time of liposomes. A detailed description of the interaction of liposomes with blood proteins at the molecular level could lead to the preparation of specific liposomal formulations whose clearance and tissue distribution we could control, thereby improving the therapeutic potential of liposomes as a drug delivery system.

This study showed that entrapment of anti-HIV agents in liposomes can improve the pharmacokinetics of these agents and allow better targeting of some HIV reservoirs. Delivering a greater amount of antiviral agents to tissues susceptible to HIV infection and, at the same time, limiting the quantity of drugs at sites where they might be toxic should reduce the potential toxicity of such antiviral agents. In addition, the improved drug pharmacology could reduce the dose and the frequency of administration of drugs used in conventional therapy. The potential therapeutic advantages of liposomal formulations in the treatment of HIV disease deserve further attention, and studies are currently in progress to evaluate the antiviral efficacy of liposomal drugs in the murine AIDS model.

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