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Pharmacokinetics and tissue distribution of zidovudine in rats following intravenous administration of zidovudine myristate loaded liposomes

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Liposomes accumulating in the reticuloendothelial system (RES) appear to be a promising vehicle to improve the therapeutic index of anti-HIV drugs such as zidovudine (AZT). Since the entrapment efficiency of AZT in liposomes was found to be low and AZT leakage from liposomes is fast, zidovudine myristate (AZT-M) was synthesized as a prodrug, and AZT-M incorporated liposomes in a lyophilized form were prepared with an average diameter of 90 nm and an encapsulation efficiency of 98% after reconstitution. The pharmacokinetic profiles and tissue distribution of AZT after i.v. administration of AZT-M liposomes in rats were investigated, and the results were compared with those after i.v. administration of AZT solution. AZT levels in plasma were significantly higher following application of AZT-M liposomes compared with AZT solution, and $AUC_{0-\infty}$ increased from $5.0 \pm 0.7\ \mu mol \cdot min \cdot ml^{-1}$ to $8.2 \pm 1.7\ \mu mol \cdot min \cdot ml^{-1}$ accordingly. Tissue distribution studies also confirmed higher concentrations of AZT in organs of RES and brain, suggesting that AZT-M liposomes might be promising candidates for therapy of HIV infections.

1. Introduction

Zidovudine (AZT, 3′-Azido-3′-deoxythymidine) was the first reverse transcriptase (RT) inhibitor licensed for clinical use and remains an important component in highly active antiretroviral therapy (HAART). However, a significant dose-limiting toxicity and a relatively short plasma half life limit the effectiveness of AZT therapy.

The characterization of macrophages as infected cells able to spread virus material to bystander cells and to interfere with the homeostasis of the immune system and of the neural compartment, strongly supports the importance of inhibiting virus replication in such cells (Aquaro et al. 2002). It is well known that liposomes are readily taken up by phagocytic cells of the RES including macrophages. Therefore, they constitute a valuable carrier to control the progress of AIDS since cells of macrophage lineage represent a key target of human immunodeficiency virus (HIV) in addition to CD4-lymphocytes. In fact, liposomal encapsulation of AZT resulted not only in a significant reduction in toxicity, but also in a substantial enhancement of anti-viral activity (Phillips et al. 1991; Phillips and Tsoukas 1992).

However, it is not easy to obtain stable AZT-containing liposomes. AZT is an amphiphilic compound and tends to partition between the lipid bilayers and the aqueous milieu of liposomes, thus resulting in a low drug entrapment and significant drug leakage from the vesicles over time (Phillips and Tsoukas 1992). The use of lipophilic analogues would take advantage of an increased compatibility of the drug and the lipids to solve the formulation problems (Gulati et al. 1998). A variety of AZT prodrugs were synthesized and one of the most interesting compounds of this class is AZT myristate (AZT-M) (Parang et al. 1998a). Because of its high lipophilicity, AZT-M should be an excellent candidate for liposomal formulations, but so far there is no published data to support this idea.

In this study, AZT-M was synthesized and AZT-M loaded liposomes were prepared and characterized. In addition, the pharmacokinetic characteristics and tissue distribution of AZT after i.v. administration of AZT-M liposomes in rats were investigated, and the results were compared with those after i.v. administration of AZT solution.

2. Investigations, results and discussion

2.1. Preparation and characterization of AZT-M liposomes

AZT-M was synthesized according to the reported method, and its chemical structure was confirmed by IR, ^1H NMR, mass spectroscopy and element analysis (data not shown).

The AZT-M liposome suspension was prepared using a modified ethanol injection method followed by homogenization, filtration and lyophilization. The average diameters and AZT-M entrapment efficiency of the rehydrated liposomes were determined and the results are shown in Table 1. The average diameters of AZT-M loaded liposomes before and after lyophilization were 88.5 ± 4.5 nm and 89.6 ± 6.3 nm respectively, and the drug entrapment efficiency was $99.4 \pm 0.8\%$ and $98.3 \pm 1.2\%$ respectively,

Table 1: Characteristics of AZT-M liposomes[a]

AZT-M liposomes	Average diameter (nm)	Entrapment efficiency (%)
Before dehydration	88.5 ± 4.5	99.4 ± 0.8
After rehydration	89.6 ± 6.3	98.3 ± 1.2

[a] Each value represents the mean ± SD of three experiments

suggesting that the AZT-M incorporated liposomes were quite stable during lyophilization. This could largely be attributed to the lipophilicity of AZT-M and the lyoprotectant effect of trehalose.

Disaccharides have been proved to be very effective protectants during dehydration of proteins and liposomes by vitrification and water replacement mechanisms. In this study, trehalose was used and no significant changes were observed concerning mean diameters and encapsulation efficiency before and after freeze drying, indicating that no aggregation, fusion and drug leakage of AZT-M liposomes occurred during lyophilization.

As the presence of cholesterol in liposomes has been shown to be able to decrease bilayer fluidity, reduce the membrane permeability to water-soluble drugs and stabilize the membrane in biological fluids such as plasma, several liposomal formulations with and without cholesterol were tested in our preliminary tests. However, vesicle aggregation and size increase were observed after lyophilization and rehydration if cholesterol was incorporated in the lipid formulation. Furthermore, it has been reported that inclusion of increasing amounts of cholesterol into liposome bilayers resulted in a decrement in the uptake by macrophages (Allen et al. 1991). Therefore, AZT-M liposomes were prepared without cholesterol in this study.

2.2. Pharmacokinetics of AZT-M liposomes

The time courses of plasma concentration of AZT following i.v. administration of AZT-M liposomes and AZT solution in rats are shown in Fig. 1, and the calculated pharmacokinetic parameters are listed in Table 2. It can be seen from Fig. 1 that the plasma levels of AZT at each time point from 1 to 360 min were higher after administration of liposomes than those of AZT solution. The $AUC_{0-\infty}$ of AZT in the liposomal formulation increased from 5.1 ± 0.7 μmol · min · ml^{-1} to 8.2 ± 1.7 μmol · min · ml^{-1} in comparison with that of AZT solution, and total body clearance decreased from 16.6 ± 2.3 ml · min^{-1} to

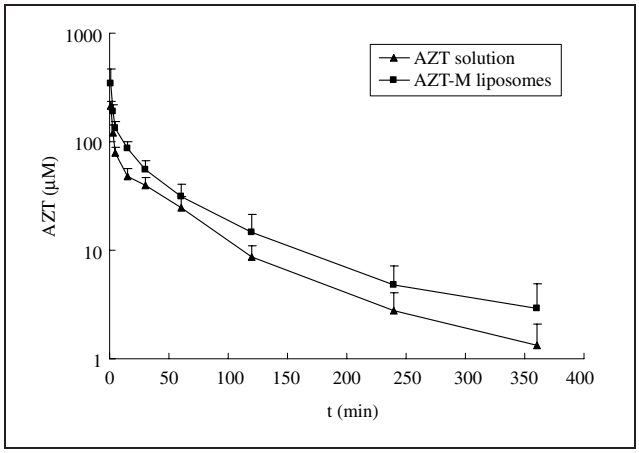

Fig. 1: Plasma concentration-time profiles of AZT after i.v. administration of AZT solution and AZT-M liposomes at equimolar doses of 0.084 mmol · kg^{-1} (n = 5)

Table 2: Pharmacokinetic parameters (mean ± SD) of AZT following i.v. administration of AZT-M liposomes and AZT solution in rats (n = 5)

Parameters	AZT solution	AZT-M liposomes
AUC_{0-t} (μmol · min · ml^{-1})	5.0 ± 0.7	7.8 ± 1.5*
$AUC_{0-\infty}$ (μmol · min · ml^{-1})	5.1 ± 0.7	8.2 ± 1.7*
$t_{1/2}$ (min)	73.5 ± 18.2	86.0 ± 20.4
CL_{tot} (ml · min^{-1})	16.6 ± 2.3	10.8 ± 2.2*
V_d (L)	1.8 ± 0.6	1.3 ± 0.3
MRT (min)	73.9 ± 17.4	83.1 ± 28.6

* $p < 0.05$ compared with AZT solution

10.8 ± 2.2 ml · min^{-1}. This suggested that incorporation of AZT into liposomes by a prodrug approach could significantly decrease the renal clearance rate and increase the retention time in the plasma compared with AZT solution.

2.3. Tissue distribution of AZT-M liposomes

The mean concentrations of AZT in different organs after i.v. administration of AZT solution and AZT-M liposomes are shown in Fig. 2. It is clear that AZT was rapidly distributed into different organs of rats such as kidney, spleen, lung, and liver. The administration of AZT-M liposomes resulted in significantly higher tissue levels of AZT in most of the tested organs in comparison with AZT solution. The AZT levels 1 h after injection of the liposomal

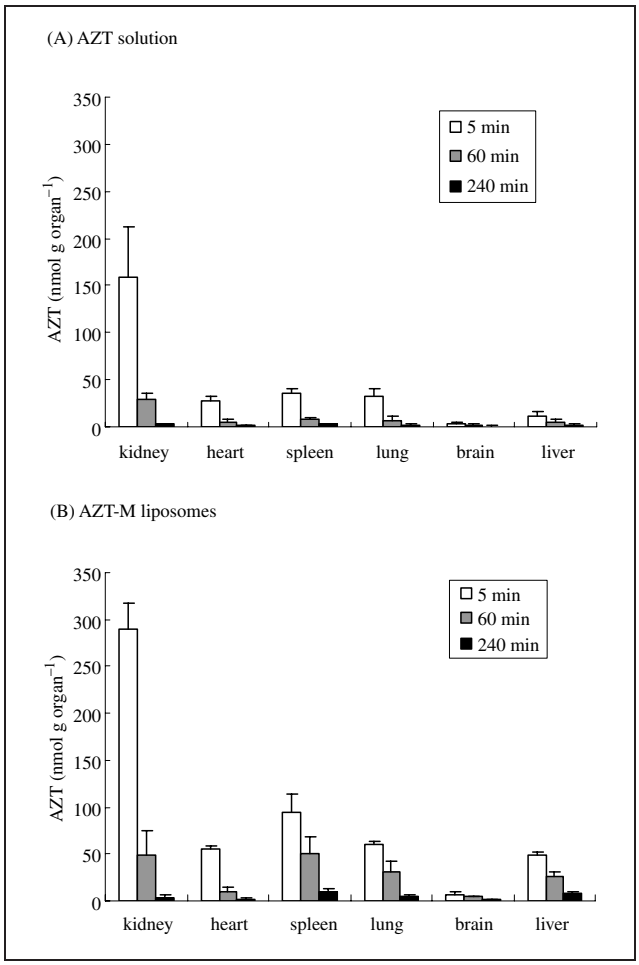

Fig. 2: Distribution of AZT in rat organs at different time point after i.v. administration of AZT solution (A) and AZT-M liposomes (B) (n = 5)

Table 3: The AZT levels (mean ± SD) after i.v. administration of AZT-M liposomes and AZT solution in rats (n = 5)

t (min)	AZT level in tissue (nmol · g^{-1})					
	Kidney	Heart	Spleen	Lung	Brain	Liver
AZT solution						
5	159.4 ± 53.0	28.1 ± 3.9	35.7 ± 4.2	32.6 ± 8.5	2.9 ± 1.5	10.7 ± 5.9
60	29.0 ± 6.3	4.9 ± 2.5	7.4 ± 2.8	7.2 ± 3.6	2.0 ± 0.8	5.5 ± 1.9
240	3.0 ± 0.5	1.3 ± 0.8	2.6 ± 1.3	1.5 ± 1.8	0.5 ± 0.3	1.9 ± 0.6
AZT-M liposomes						
5	290.0 ± 28.0[a]	55.8 ± 3.1[a]	95.2 ± 19.4[a]	60.3 ± 3.7[a]	6.7 ± 2.5[a]	48.9 ± 3.1[a]
60	48.2 ± 26.4	10.1 ± 5.3	51.0 ± 18.1[b]	31.2 ± 10.4[b]	4.5 ± 0.9[b]	26.2 ± 4.5[b]
240	3.7 ± 2.8	1.9 ± 1.6	9.6 ± 2.7[c]	4.8 ± 2.4[c]	1.3 ± 0.7[c]	8.5 ± 1.5[c]

[a] $p < 0.05$ at 5 min, [b] $p < 0.05$ at 60 min and [c] $p < 0.05$ at 240 min compared with AZT solution at the same time point respectively

formulation were 6.9, 4.7 and 4.3 times as great as those of AZT solution in spleen, liver and lung respectively (Table 3), indicating that when administered intravenously, AZT-M liposomes were rapidly phagocytosed by macrophages and localized to the organs of RES where a rapid hydrolyzation of AZT-M may occur. Surprisingly, higher concentrations of AZT were observed in brain at each determined time point after dosing with AZT-M liposomes than with AZT solution (e.g. 6.7 nmol · g^{-1} and 2.9 nmol · g^{-1} respectively after 1 min injection of equimolar dose; Table 3). This result is of considerable interest because a sufficiently high level of AZT penetrating into the central nervous system (CNS) is an essential requirement for its effectiveness in the treatment of acquired immunodeficiency syndrome (AIDS) dementia complex or AIDS encephalopathy (Sawchuk and Yang 1999).

The modified plasma pharmacokinetics and tissue distribution of liposomal AZT-M might be attributed to the combination effect of the liposome carrier and the high lipophilicity of AZT-M. It has been reported that AZT was almost completely eliminated by the kidney following injection of a free AZT solution while a third of the injected dose was eliminated via the feces when incorporated into nanoparticles (Löbenberg et al. 1998). Similarly, encapsulation of foscarnet in liposomes slowed down the renal elimination of foscarnet, improved drug levels in plasma and greatly enhanced the drug accumulation in brain and organs of RES (Dusserre et al. 1995). A prodrug of AZT, 3′-azido-2′,3′-dideoxy-5′-*O*-(2-bromomyristoyl) thymidine, resulted in higher concentrations of AZT in brain and liver after i.v. administration in mice compared with doses of AZT itself (Parang et al. 1998b).

What needs to be mentioned here is that AZT-M is quite unstable in the plasma and tissue samples although it is very stable in water and pH 4 to pH 7 buffer solutions. To get an accurate AZT-M concentration in plasma and tissues, these samples must be dissected out, prepared and analyzed as quickly as possible (Parang et al. 1998b). However, it is not easy to conduct that practically because there are too many samples in the pharmacokinetic and tissue distribution studies as it was reported by Kawaguchi that some prodrugs of AZT could not be detected in plasma samples even immediately after the administration (Kawaguchi et al. 1990). Therefore, in our preliminary experiments we established a standard sample preparation procedure during which a majority of AZT-M decomposed to AZT in both plasma and tissue samples. Considering that it is very difficult and inconvenient to simultaneously determine AZT and AZT-M by HPLC, the amount of AZT-M in the samples was ignored in the analysis in order to simplify the analysis procedure. Although further studies are needed to clarify the kinetics and distribution of the AZT-M liposomes, the simplified treatment would never weaken the significance of the prodrug liposome as a promising anti-HIV therapeutical approach.

In conclusion, the synthesized prodrug AZT-M, was successfully encapsulated into liposomes with high entrapment efficiency. The AZT-M liposomes were lyophilized in the presence of trehalose and the rehydrated liposomes retained the same characteristics as before lyophilization. Following i.v. administration, AZT-M loaded liposomes resulted in significant changes in the pharmacokinetic characteristics and tissue distribution, including higher levels of distribution in organs of RES and brain, a longer half life and a lower total clearance of AZT compared with AZT solution. Therefore, the prodrug liposomes approach might lead to a reduced toxicity and an increased efficiency of AZT-based HIV therapy.

3. Experimental

3.1. Reagents and chemicals

AZT and α-tocopherol (V_E) were obtained from Northeast General Pharmaceutical Factory. Myristoyl chloride was purchased from Shanghai Chemical Reagent Company. Soy phosphatidylcholine (SPC) was procured from Shanghai Taiwei Pharmaceutical Co. Ltd. Abs. ethanol was of analytical grade from Tianjin Bo Di Chemicals Co. Ltd. Methanol was of HPLC grade from Shandong Yuwang Co. Ltd. All other chemicals were of reagent grade and were used without further purification.

3.2. Synthesis of AZT-M

AZT-M was synthesized according to the procedure of Kawaguchi et al. (1990) with a slight modification. Briefly, AZT (0.5 g, 1.87×10^{-3} mol) was dissolved in a mixture of dry dichloromethane and dry pyridine. Myristoyl chloride (0.55 g, 2.23×10^{-3} mol) was added dropwise to the reaction mixture which was stirred at 40 °C and stirring was continued for 24 h. The crude product was washed with water to remove most of the pyridine hydrochloride. The organic phase was evaporated under reduced pressure at 45 °C, and the residues were purified using a silica gel chromatography column with a 97 : 3 (v/v) chloroform/ethanol mixture as mobile phase. The fractions containing AZT-M were collected, identified by TLC and concentrated by rotary evaporation. The residue on the flask wall was dissolved in a minimum amount of *t*-butanol, frozen at −45 °C and lyophilized under vacuum for 24 h using a LGJ-10 lyophilizer (Beijing Four-ring Scientific Instrument Factory, Beijing, China). The chemical structure of the resulting product, AZT-M, was supported by IR, ^{1}H NMR, mass spectroscopy and element analysis.

3.3. Preparation of AZT-M liposomes

AZT-M liposomes were prepared by the modified ethanol injection method (Yoshie et al. 2001) with a slight modification. SPC (400 mg), AZT-M (40 mg) and V_E (4 mg) were dissolved in warm abs. ethanol (5 ml) in a 100 ml round bottomed flask at about 55 °C on a thermostatic regulator magnetic force stirrer (DF101S, Yingyu yuhua Instrument factory, Gongyi, China). While stirring, 300 mM isotonic trehalose solution (50 ml) preheated at 55 °C was added rapidly to the lipid-ethanol solution. The aqueous phase immediately turned milky as the result of liposome formation. The ethanol

was then removed by rotary evaporation under reduced pressure at 40 °C under nitrogen, and the final volume was adjusted to 50 ml with water. The liposome suspension obtained was homogenized for six cycles at 14,000 psi using a high pressure homogenizer (M110L Microfluidizer Processor, Newton, MA, USA). After the last pass, the liposomes were filtered through a sterile 0.22 μm pore-size filter, then frozen at −45 °C for 6 h and dried in vacuum for 30 h, using a LGJ-10 lyophilizer. The lyophilized liposome powder was reconstituted with the corresponding amount of water under ambient conditions, immediately followed by vortexing for 5 s.

3.4. Measurement of entrapment efficiency

The liposome suspension was centrifuged at 30,000 × g (Hitachi GS-120, Japan) for 1 h at 4 °C. Under this conditions, the liposomes remained suspended and the free AZT-M precipitated. The amount of entrapped AZT-M was determined by lysis of the liposomes with abs. ethanol to give a clear solution and the AZT-M concentration was estimated by HPLC. The encapsulation efficiency was calculated as the ratio of AZT-M in liposome vesicles to the AZT-M of the total amount in the aqueous suspension.

3.5. Vesicle size determination

The size of AZT-M liposomes was determined using a Coulter LS 230 counter (Coulter, USA). Each sample was diluted to a suitable concentration with distilled water filtered through a 0.22 μm membrane.

3.6. Animal experiments

3.6.1. Pharmacokinetic studies

Animal experiments were performed according to the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University.
Wistar rats (220–260 g, male and female) were purchased from the animal laboratory of Shenyang Pharmaceutical University. The animals were kept under standard conditions, with free access to water and food. AZT-M liposomes and AZT solution were tested at equimolar dose (0.084 mmol · kg^{-1}; AZT-M, 40.0 mg · kg^{-1}; AZT, 22.4 mg · kg^{-1}), administered intravenously via the dorsal tail vein (groups of five animals). Blood samples (0.5 ml) were taken from the retro-orbital plexus at various time intervals (1, 3, 5, 15, 30, 60, 120, 240, 360, 480 min) after injection. Plasma (200 μl) was separated after centrifugation at 3000 × g for 15 min and then frozen to −20 °C until use. The AZT content in plasma samples was analyzed by HPLC.

3.6.2. Tissue distribution studies

The Wistar rats were injected with AZT-M liposomes or AZT solution as described above. Five rats in each group were sacrificed at 5, 60 and 240 min after injection and tissues (heart, kidney, liver, lung, spleen, and brain) were excised, washed with physiological solution and frozen to −20 °C until use.

3.6.3. Plasma and tissues samples preparation

Distilled water (100 μl) and 10% trichloroacetic acid (100 μl) were added to plasma samples (200 μl). The tube was vortex mixed for 30 s and centrifuged at 5000 × g for 15 min, and 50 μl of the supernatant was analyzed by HPLC. In the case of the tissue samples, a portion or whole of the tissue specimens was placed in a homogenizing tube with three times weight of abs. ethanol, and homogenization was done in an ice-bath for 15 min. The homogenate was centrifuged at 10,000 × g for 10 min, and 50 μl of the supernatant was analyzed by HPLC.

3.6.4. Analysis method

The HPLC assay method was established for determination of the concentration of AZT-M in liposomes in the *in vitro* experiments and AZT in plasma or tissues using an Agilent 1100 Series detector, an Agilent 1100 Series quaternaty pump, an Agilent auto sampling injector and the Agilent Chemstation. A Diamonsil C_{18} column (4.6 mm × 200 mm, 5 μm) was used. The UV detector was operated at 265 nm. The analysis was performed at a temperature of 25 °C and a flow rate of 1.0 ml · min^{-1}. In the analysis of AZT-M, the mobile phase was composed of methanol-H_2O (95 : 5, V/V), while for the assay of AZT in plasma and tissues, methanol-10 mM ammonium acetate (33 : 67, V/V) was employed as the eluent.
The pharmacokinetic parameters, AUC_{0-t} (area under the plasma concentration-time curve from time zero to the time of last measurable concentration), $AUC_{0-\infty}$ (area under the plasma concentration-time curve from time zero to infinity), $t_{1/2}$ (plasma half-life), CL_{tot} (total body clearance), V_d (apparent volume of distribution) and MRT (mean residence time) were computed by a non-compartmental model applying the program Topfit, version 2.0 (Thomae GmbH, Germany).

3.6.5. Statistical analysis

Statistical analysis was performed using Student's t-test, and the differences were considered significant at a probability level of 95% ($P < 0.05$).

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