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Self-assembled drug delivery systems Part 3. *In vitro*/*in vivo* studies of the self-assembled nanoparticulates of cholesteryl acyl didanosine

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ABSTRACT

Self-assembled drug delivery systems (SADDS) are defined as the self-assemblies of amphiphilic prodrugs, integrating prodrugs, molecular self-assembly and nanotechnology for drug targeting and controlled release. Cholesteryl-succinyl didanosine (CSD) and cholesteryl-adipoyl didanosine (CAD) nanoparticulate systems in water were previously prepared and optimized. In this paper, the *in vitro* and *in vivo* behavior of them was investigated. Precipitation occurred when they were mixed with acid solutions due to rapid production of hypoxanthine and subsequent disruption of supramolecular structures. They showed pH-dependent degradation and kept relatively stable in the neutral pH range. CSD is more stable than CAD due to the shorter spacer and poloxamer protection. CSD showed different degradation rates in various plasma with the descending order of rat, mouse, rabbit, dog and human. The half-life ($t_{1/2}$) of CSD is 9 days in rat plasma, and 5.9 days in rat liver homogenates. CAD has a faster degradation than CSD though the $t_{1/2}$ in rat liver homogenates is long to 23 h. CSD nanoparticulates showed no significant anti-HIV effect in MT4 cell model because of very slow degradation. CSD nanoparticulates showed the distribution $t_{1/2}$ of 7.6 min after bolus intravenous (i.v.) administration to rats, and the site-specific distribution in liver, lung and spleen with the high $t_{1/2}$ of 10 days in liver. The factors affecting achievement of successful SADDS are discussed.

1. Introduction

Self-assembled drug delivery systems (SADDS) are defined as the self-assemblies of amphiphilic prodrugs, firstly developed in our lab (Jin et al., 2006). Three technologies, prodrug, molecular self-assembly and nanotechnology are integrated into SADDS. Compared to drug carriers, such as liposomes and nanoparticles, the unique advantages of SADDS include high drug loads, no drug leakage and controlled drug release in targets because the colloidal SADDS almost or wholly consist of amphiphilic prodrugs (Jin, 2008; Jin et al., 2006). In our previous research, a long chained glyceride derivative of acyclovir was prepared and self-assembled into stable rod-like nanoparticles in water (Jin et al., 2005). After intravenous (i.v.) administration of the self-assembled nanoparticles to rabbits, targeting to the mononuclear phagocyte system (MPS) and then controlled release of parent drugs were achieved (Jin et al., 2006).

Didanosine (ddI) is one first-line anti-HIV agent, approved by FDA in 1991. Its bioavailability is reported to range from 20 to 40% depending on the formulation used. Didanosine is metabolized intracellularly to the active antiviral metabolite dideoxyadenosine triphosphate. The plasma elimination half-life is reported to be about 1–2 h (Faulds and Brogden, 1992). Didanosine was modified to various lipidic derivatives to improve the bioavailability for direct lymphatic delivery against HIV (Lalanne et al., 2007a, 2007b; Manouilov et al., 1997). Peroral bioavailability of didanosine was improved with its octanoate and benzoate prodrugs (Hasegawa and Kawaguchi, 1994). The sterically stabilized liposomes of didanosine were prepared to achieve long circulating *in vivo* (Dipali et al., 1997; Harvie et al., 1996). The mannosylated gelatin nanoparticles of didanosine were delivered to the targeted organs (Jain et al., 2008).

We perform a series of researches on SADDS currently and in future. In our previous paper, a series of cholesteryl derivatives of antiviral nucleoside analogues, involving acyclovir, didanosine and zidovudine, were prepared, and after injecting their solutions into water they self-assembled to form highly dispersed aggregates (Jin et al., 2008b). Two derivatives, cholesteryl-succinyl didanosine

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(CSD) and cholesteryl-adipoyl didanosine (CAD) were further investigated in details on the self-assembly behavior in water (Jin et al., 2008a). The morphologies of CSD self-assembled nanostructures are relevant to the amount of incorporated solvent tetrahydrofuran (THF) into bilayers. After removal of THF, only the short nanotubes of average 200 nm in size were remained, also as the most stable form. The optimal formulation of CSD self-assembled nanoparticulate systems was screened out, wherein the concentration of CSD in organic solutions and the additional amount of stabilizer poloxamer 188 were the key factors. After removing solvents and part of water, a stable concentrated nanoparticulate system was achieved, containing more than 10 mg/ml CSD, looking like milk, and totally consisting of short nanotubes. The formulation is ready for i.v. administration with the small injected volume containing an appropriate dose. However, another didanosine lipid derivative, CAD, only formed the nanoscale vesicles of average 175 nm in size, without morphological changes when further processing or storage. The relative long spacer of CAD contributed to the formation of flexible tails to further keep the vesicle form. The self-assembled nanoparticulate system of CAD could also be concentrated to a high concentration of more than 10 mg/ml CAD, maintaining stable for more than 1 month partially due to the high zeta potential of −44.5 mV.

Self-assembled nanoparticulates could preferentially distribute in the MPS due to their colloidal characteristic, as shown in our previous study (Jin et al., 2006). The MPS-targeted distribution benefits to anti-HIV therapy because macrophages are the main reservoirs of HIV (Aquaro et al., 2002). In this study, the *in vitro* and *in vivo* studies of the self-assembled nanoparticulates of CSD and CAD were performed. Chemical stability of them was investigated. Anti-HIV action on cell model was detected. Site-specific distribution and sustained release were achieved after i.v. administration of CSD nanoparticulates. The key factors determining successful SADDS are discussed, and the prospect of SADDS is described at the end of the paper.

2. Materials and methods

2.1. Materials

Two cholesteryl acyl derivatives of didanosine, i.e. cholesteryl-succinyl didanosine ($C_{41}H_{60}N_4O_6$) and cholesteryl-adipoyl didanosine ($C_{43}H_{64}N_4O_6$) were prepared according to the previous research (Jin et al., 2008b). Analytical reagents were used otherwise specially indicated. Chromatographic reagents were used in high-performance liquid chromatography (HPLC). Distilled water was always used. A polyoxyethylene–polyoxypropylene copolymer, poloxamer 188 (P188) was supplied by Shenyang Jiqi Pharmaceutical Co. Ltd., China. The pig liver carboxylester enzyme (PLCE, Sigma) was dissolved in sterilized Tris–HCl buffer solutions (20 mM, pH 7.4) before use. MT4 cells and human immunodeficiency virus type-1 (HIV-1_{IIIB}) virus were from the Center of AIDS, Beijing Institute of Microbiology and Epidemiology. The plasma from BALB/c mice, Sprague-Dawley rats and albino rabbits was prepared in our lab. The plasma from beagle dogs and healthy human was donated by Prof. G. Dou of Beijing Institute of Transfusion Medicine.

Sprague-Dawley rats from Laboratory Animal Center of Beijing Institute of Radiation Medicine (BIRM) were used. Principles in good laboratory animal care were followed and animal experimentation was in compliance with the Guidelines for the Care and Use of Laboratory Animals in BIRM. The rats were sacrificed by euthanasia to remove tissues. The rat tissue homogenates used in the experiments of chemical stability and tissue distribution were prepared in tissue/water (1:1, w/w).

2.2. Preparation of nanoparticulates

The self-assembled nanoparticulates of CSD and CAD were prepared according to our previous researches (Jin et al., 2008a, 2008b). In brief, a CAD solution (5 mg/ml) in THF was slowly injected into the vortexed water containing 5% (v/v) THF by a microsyringe. The injecting processes were repeated for several times until to obtain a homogeneous and slightly opalescent suspension. When preparing CSD self-assembled nanoparticulates, the injection solution containing 5 mg/ml CSD and 1 mg/ml P188 in the THF/ethanol (4:1, v/v) mixture solvent was injected as above. We usually mixed CSD stock solution in THF and P188 stock solution in ethanol before injection. After removing solvents and partial water, a stable concentrated nanoparticulate system was obtained, containing more than 10 mg/ml CSD or CAD. Both kinds of nanoparticulate systems can keep stable for more than 1 month at room temperature.

2.3. HPLC determination of didanosine and its derivatives

HPLC experiments were performed on a Shimadzu 10Avp HPLC system (Japan) at room temperature, consisting of LC-10Avp pump, SPD-10Avp UV detector, SCL-10Avp controller, and Shimadzu CLASS-VP 6.02 chromatographic workstation software. Diamonsil™ C18-ODS HPLC columns (5 μm, 250 mm × 4.6 mm) and EasyGuard™ C18-ODS HPLC guard columns (5 μm, 8 mm × 4 mm) were purchased from Dikma (China). A manual injection valve and a 20-μl loop (7725i, Rheodyne, USA) were used. UV detector was fixed at 249 nm.

Didanosine and its cholesteryl derivatives were separately determined with different mobile phases due to their significant differences of polarity, and the samples from different resources, including as the primitive nanoparticulate systems and the samples in various environments for the stability measurement, were also determined using different mobile phases. The details of HPLC are referred to in Table 1.

2.4. Degradation of didanosine derivatives

2.4.1. Degradation in buffer solutions

Aliquots of 800 μl of CSD or CAD self-assembled nanoparticulate systems, were diluted with 2.4 ml of various buffers including 20 mM phosphate buffers (pH 5.0 and 7.4) and 20 mM Tris–HCl buffers (pH 9.0 and 12.0), and the dilutions were incubated in a 37 °C bath. At predetermined time intervals, aliquots of 20 μl were removed, dissolved with 180 μl methanol, and assayed with HPLC. The chemical stability of derivatives in acid solutions (pH 1.0 and 2.0, HCl solutions) was also detected as above.

2.4.2. Degradation in enzyme solutions, plasma and tissue homogenates

The effects of PLCE solution, animal and human plasma, and rat tissue homogenates on the chemical stability of derivatives at 37 °C were investigated as above. CSD or CAD self-assembled nanoparticulate systems of 200 μl were mixed with the enzyme solutions (20 U/ml) of 800 μl, and 300 μl of nanoparticulate systems were mixed with 600 μl of plasma or tissue homogenates. For the determination of CSD, aliquots of 20 μl were withdrawn and deproteinized with isopropanol of 80 μl, followed by vortex for 2 min and centrifugation at 5000 × *g* for 10 min. The deproteinizing reagent for CAD determination was methanol with the same dilution fold as above. The extracting reagent of didanosine was methanol/5% $ZnSO_4$ (1:3, v/v) followed by centrifugation as above. The supernatants were determined with HPLC.

Table 1
HPLC determination of didanosine and its cholesteryl derivatives.

Resources	Components[a]	Mobile phases	Rate (ml/min)	t_R (min)
Primitive suspensions and in buffers	ddI	Methanol/20 mM ammonium acetate (25:75, v/v), pH 5.5	1.0	6.8
	CSD	Methanol/isopropanol (90:10, v/v)	1.2	10.4
	CAD	Methanol/isopropanol (95:5, v/v)	1.0	17.3
In enzyme solutions, plasma and tissue homogenates	ddI	Methanol/20 mM ammonium acetate (25:75, v/v), pH 5.5	1.0	6.8
	CSD	Methanol/isopropanol (80:20, v/v)	1.2	8.5
	CAD	Methanol/isopropanol (80:20, v/v)	1.2	8.2

[a] ddI: didanosine; CSD: cholesteryl-succinyl didanosine; and CAD: cholesteryl-adipoyl didanosine.

2.5. Anti-HIV effect on cell model

MT4 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. The cultural medium was the RPMI 1640 solution supplemented with 10% calf serum, 1% glutamine and 1×10^5 unit penicillin and streptomycin. MT4 cells were cultured in 96-well plates at a concentration of 2×10^4 cells per well. HIV-1 viruses were diluted to 1×10^{-3} $TCID_{50}$/ml, and the dilutions were separately added into the above wells with 100 μl per well. Negative controls were the cell wells without viruses, and the positive controls were the cell wells without detected drugs. Sterilized CSD nanoparticulate systems and didanosine solutions were diluted with the no-serum-containing cultural medium for two folds each time continually, and then added to the above wells. The concentration of CSD in wells ranged from 9.38 to 300 μg/ml, divided in six grades and performed in triplicates per grade. The concentration of didanosine in wells ranged from 3.12 to 100 μg/ml, also in six grades and in triplicates per grade. After 72 h of incubation at 37 °C, the cytopathic effect (CPE) assay was performed with a light microscope. The CPE in this study was defined as (a) '−', no syncytium formation; (b) '±', no syncytium formation but in abnormal cell status; and (c) '+', syncytium formation. The 50% effective concentration (EC_{50}) was deduced based on anyone of the following standards: (a) one well was given '+', and the other two '−'; (b) three wells '+', '−' and '±', respectively; (c) all the three wells '±'. In addition, when three wells were given '+' and the next higher-grade concentration drug-containing wells were given '−', the median concentration was also regarded as EC_{50}.

2.6. Pharmacokinetics and tissue distribution in rats

Pharmacokinetics and tissue distribution of CSD were studied after bolus i.v. administration of CSD nanoparticulates to rats. The sterilized nanoparticulate system containing ~20 mg/ml CSD was administered to rats with CSD dose of 40 mg/kg through tail vein. About 0.25 ml of blood sample was collected into heparinized centrifuge tubes at 0, 1, 3, 5, 8, 10, 15, 20, 25, 30, 40, 50, 60 min after medication. Plasma was separated by centrifugation at 3000 rpm for 5 min. Plasma samples were stored at −20 °C until HPLC analysis (see Section 2.4.2). In the tissue distribution experiments, rats were administered with CSD nanoparticulate systems as above followed by raising them in a standard animal room. They were sacrificed after 0.5, 2, 4, 7, 14, 28 days, and the tissues were removed, weighted and disrupted to homogenates. The sterilized didanosine aqueous solutions of ~7.5 mg/ml were also administered by i.v. injection as controls with didanosine dose of 7.5 mg/kg. CSD and didanosine were determined as above.

The long-term toxicity of CSD nanoparticulates was investigated through raising the administered rats in a standard animal room. The body weight of them was recorded every day. Healthy rats were also investigated as controls.

3. Results

3.1. Stability of didanosine derivatives

3.1.1. Stability in buffers and acid solutions

CSD nanoparticulates maintained physical stability in buffers and weakly acidic solutions except for the pH 1.0 acid solution wherein precipitates began to appear after mixing for 5 h. It is well known that didanosine is sensitive to acidic environments, transforming to hypoxanthine due to disruption of the bonds between the purin moiety and the aglycone moiety (Bekers et al., 1993). A preliminary HPLC determination of CSD nanoparticulate systems diluted with acidic solutions significantly showed the production of hypoxanthine (data not shown). Therefore, CSD as didanosine derivative is also sensitive to acidic environments. The produced hypoxanthine could rapidly leave out of the supramolecular structures of nanoparticulates, and then the nanostructures could become unstable and dissociate followed by precipitation. For CAD nanoparticulates, the pH 2.0 solution already damaged the supramolecular structure due to the same reason as above. Poloxamer 188 coating around CSD nanoparticulates is a stabilizer to protect the nanostructures from attacking of hydrogen ions. And it is known that CAD is more sensitive to acidic environments than CSD according to the following researches. Therefore, the poloxamer-coating CSD nanoparticulates show stronger physical stability than the naked and sensitive CAD nanoparticulates in acidic solutions.

Chemical stability of CSD and CAD nanoparticulates in buffers is dependent on pH (Fig. 1). The degradation processes follow pseudo-first order kinetics over the entire pH range. The most stable environment of CSD nanoparticulates appears near pH 5.0 according to the relationship of degradation kinetic constants (k) *vs.* pH, though the degradation rates in neutral and weak alkaline solutions is almost comparable to that in pH 5.0 (Fig. 2). The most stable pH is near 8.0 or higher for CAD nanoparticulates. Significantly, CAD shows higher sensitivity to acidic environments than CSD, and the degradation profile of CAD is similar to that of didanosine (Bekers et al., 1993). The degradation difference between CSD and CAD should result from the difference of molecular structures and the shielding effect of poloxamers on CSD nanoparticulates. It is also reported that P188 stabilizes rabeprazole sodium in aqueous solutions (Ren et al., 2008).

CSD has the very long degradation half-lives ($t_{1/2}$) in buffers, long to 2310 and 1733 h at pH 5.0 and 7.4, respectively. The degradation $t_{1/2}$ of CAD are shorter than those of CSD in the entire pH range (Fig. 2), to 67 and 462 h at pH 5.0 and 7.4, respectively. In the whole stability experimental process, little didanosine was detected in all the samples of CSD nanoparticulates although a small amount of hypoxanthine was found in acidic solutions. The possible degradation products of CSD include didanosine, succinyl didanosine and hypoxanthine. According to the HPLC determination, the production rate of hypoxanthine is much higher than those of didanosine

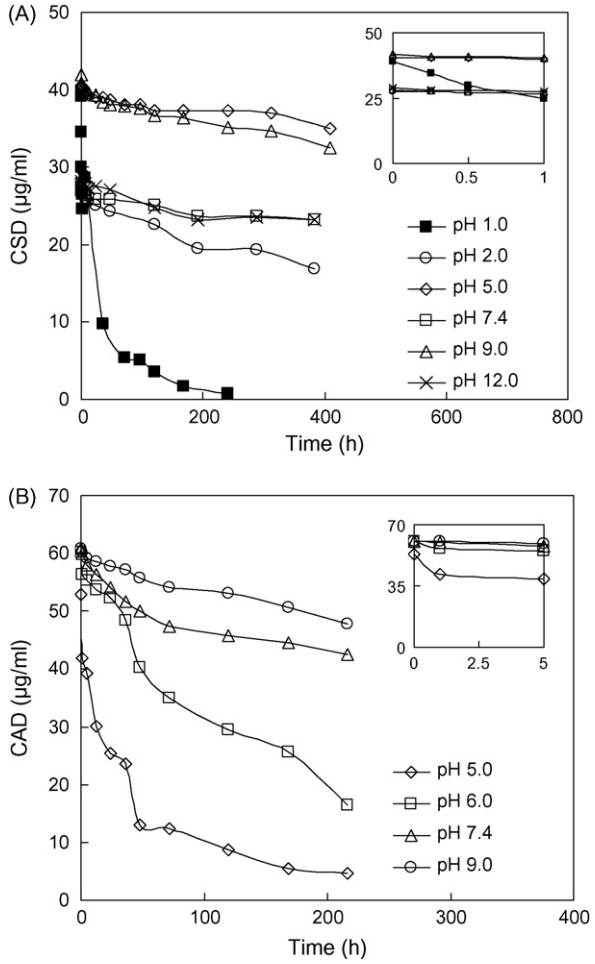

Fig. 1. Degradation of cholesteryl acyl didanosine in nanoparticulates in buffers and acid solutions at 37 °C ($n = 3$). (A) CSD and (B) CAD.

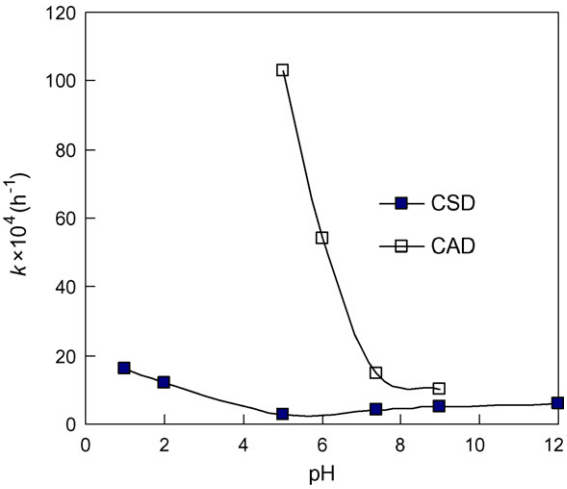

Fig. 2. The relationship between pH and degradation rates of cholesteryl acyl didanosine.

and succinyl didanosine in acidic environments, i.e. k_2 is much higher than k_1 and k_3 shown in Fig. 3. In the neutral and alkaline environments, CSD degradation in the various routes should be very slow.

Compared to the previously synthesized prodrug (stearylglycero-succinyl-acyclovir, SGSA) and its self-assembled nanoparticulates (Jin et al., 2006), CSD and CAD in nanoparticulates have a very low degradation rate in the entire pH range, indicating that the prodrugs would degrade *in vivo* not well. The following experimental results further demonstrate the degradation rule of the didanosine derivatives in biological environments.

3.1.2. Stability in enzyme solutions

There are a great number of hydrolysis enzymes in our body, and carboxylesterases are ubiquitous and important. Extracted animal ester hydrolases are usually used in the stability investigation, and PLCE is frequently used (Bodor and Buchwald, 2000). CSD showed a higher degradation rate in enzyme solutions than in pH 7.4 buffer solutions, the $t_{1/2}$ of which was 301 h (12.5 days), about 1/6 of that in the buffer. A little didanosine was detected in the samples though the production rate was low. The ester bond between the didanosine moiety and succinyl in CSD molecules is one possible action site of PLCE. After CAD nanoparticulate systems were incubated in the PLCE solution at 37 °C for 2 h, about 90% of CAD had been degraded, and more didanosine was produced than CSD. Besides the factor of molecular structure, the high concentration of enzyme in solutions is also one primary reason of rapid degradation of CAD.

3.1.3. Stability in plasma

Five kinds of plasma were used to degrade CSD, separately from rats, mice, rabbits, dogs and human. The profiles of degradation and parent drug production depended on types of plasma (Fig. 4), and the difference between rodents and human was largest. Degradation rates descended in the order of rats, mice, rabbits, dogs and human, and the $t_{1/2}$ were 217, 277, 495, 866 and 990 h, respectively, based on the pseudo-first order kinetics, i.e. 9.0, 11.5, 20.6, 36.1, 41.2 days. A little didanosine was produced though the molar amount was much lower than that of disappeared CSD. CSD could degrade to other products than didanosine in plasma, such as succinyl didanosine and hypoxanthine, and succinyl didanosine might further degrade to other products, as shown in Fig. 3. Rat plasma

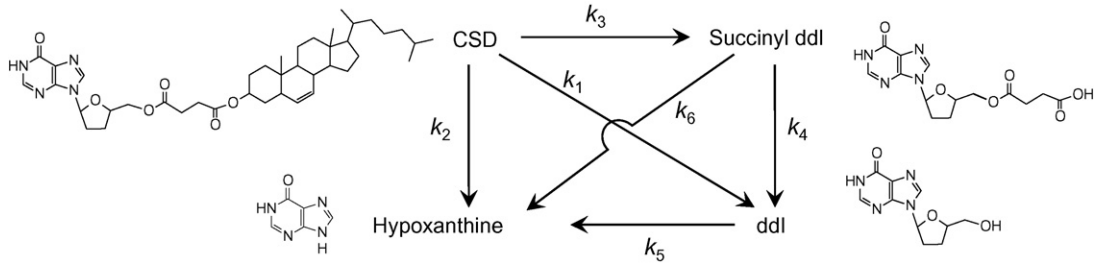

Fig. 3. Schematic illustration of possible degradation routes of CSD.

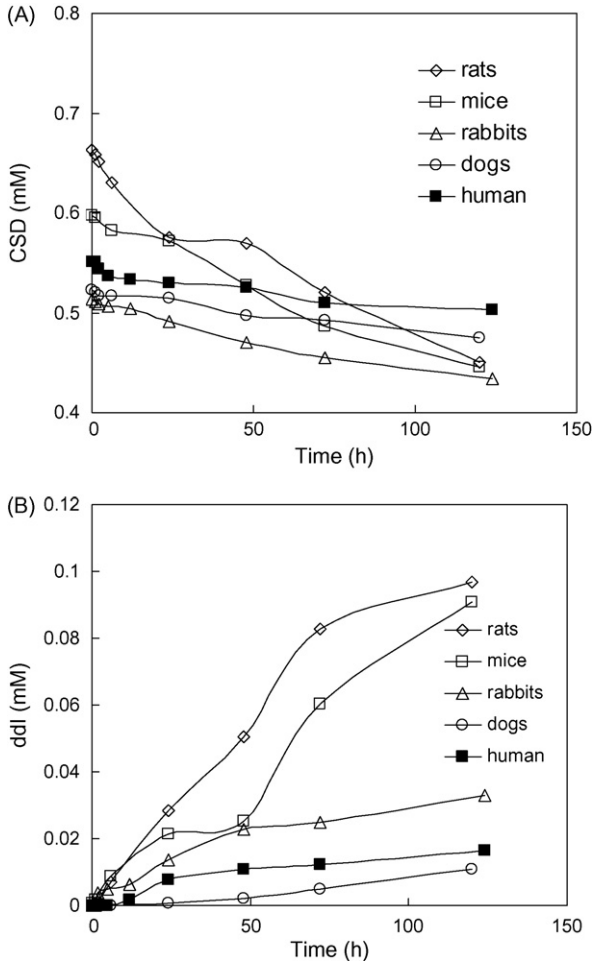

Fig. 4. Degradation of CSD in nanoparticulates in plasma at 37 °C ($n = 3$).

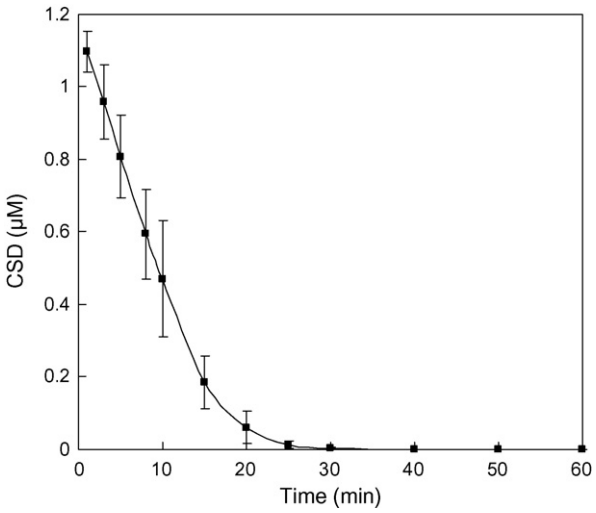

Fig. 5. The time profile of CSD concentration in plasma after bolus i.v. administration of CSD nanoparticulates to rats ($n = 5$).

was chosen to degrade CAD in nanoparticulates due to the highest degradation capability. The degradation of CAD in rat plasma was faster than that of CSD, the $t_{1/2}$ of which was 70 h (2.9 days). The poloxamer coating of CSD nanoparticulates could protect CSD molecules from attacking of plasma enzymes, as mentioned above. The degradation rates of CSD and CAD seem to be too slow to become prodrugs because a little parent drugs could not be preferred for pharmacotherapy.

3.1.4. Stability in rat tissue homogenates

Human plasma has the very weak capability to degrade CSD whereas rat plasma shows the strong degradation activity. Therefore, rats were selected as models to further explore the *in vivo* fate of nanoparticulates. It is well known that the MPS is the target of nanoparticulates *in vivo* due to the opsonization effect (Ishida et al., 2002). The MPS mainly includes liver, lung and spleen. Therefore, rat liver and lung were selected as the experimental tissues to degrade prodrugs. Unfortunately, the degradation of CSD in rat liver and lung homogenates was also very slow, the $t_{1/2}$ of which reached 5.9 and 40.3 days, respectively. Therefore, it is easily predicted that CSD is not satisfied when administration to rats because a very low concentration of didanosine hardly achieves sufficient anti-HIV therapy in targets. In contrast, CAD showed a relatively short degradation $t_{1/2}$ of 23 h in rat liver homogenates. But this rate is also slow for antiviral therapy because the released parent drugs hardly reach to the minimum inhibitory concentration (MIC). More importantly, the released didanosine would also be rapidly eliminated from the targeted tissue, i.e. liver (Harvie et al., 1996; Kang et al., 1994).

3.2. In vitro antiviral activity of CSD

The anti-HIV EC_{50} of didanosine on the MT4 cell model was 25 μg/ml (106 μM) in this study. However, the EC_{50} of CSD nanoparticulates was more than 300 μg/ml (426 μM), i.e. beyond the highest one of all experimental concentrations. In fact, it may be concluded that CSD hardly shows anti-HIV activity in the experimental range. Significantly, this result may also be predicted in the light of the very slow degradation of CSD in enzyme systems, and little production of active parent drug didanosine. This undesired result reminds us that the degradation or metabolism of prodrugs of self-assembled systems must be carefully considered when designing and preparing SADDS.

3.3. Pharmacokinetics and tissue distribution of CSD nanoparticulates

CSD nanoparticulates showed rapid elimination from circulation after bolus i.v. administration to rats (Fig. 5), resulting from the targeting effect of colloidal systems. Absolute elimination occurred at 1 h. The field of 0–10 min was considered as the distribution phase with the $t_{1/2\alpha}$ of 7.6 min based on the first-order kinetics. The pharmacokinetic behavior of CSD nanoparticulates is similar to that of the other nanoparticulate systems such as liposomes and nanoparticles (Torchilin, 2006). However, compared to the previous acyclovir self-assembled nanoparticles (Jin et al., 2006), CSD nanoparticulates have a relatively longer circulating time. The $t_{1/2\alpha}$ of the former is only 1.5 min and the plasma concentration of acyclovir prodrug SGSA has descended for about 90% after 5 min of i.v. administration. Long circulating time of CSD nanoparticulates should result from poloxamer coating. It is known that hydrophilic polymers including poloxamers prevent nanoparticulates from opsonization in blood, further prolonging the circulating time (Göppert and Müller, 2005; Moghimi et al., 2001; Redhead et al., 2001; Stolnik et al., 2001). The parent drug didanosine was not found in blood after administration. One reason may be the rapid distribution of nanoparticulates into the MPS. The other reason may be the very slow degradation of CSD in blood based on the above stability results. In addition, it was shown in this study that the elimination of free didanosine from circulation was also very fast

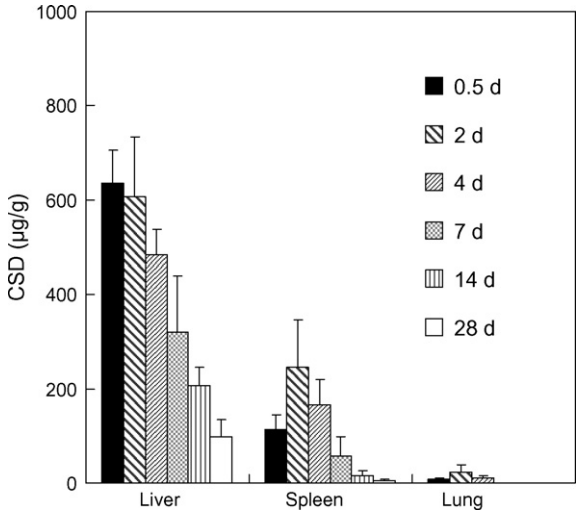

Fig. 6. The tissue distribution of CSD after bolus i.v. administration of CSD nanoparticulates to rats ($n = 4$).

with the $t_{1/2}$ of 6.4 min in rats after i.v. administration of didanosine aqueous solutions, in accordance with other reports (Harvie et al., 1996; Kang et al., 1994).

The *in vivo* fate of CSD nanoparticulates was further demonstrated through measuring drug distribution in tissues. CSD was determined in the main tissues including liver, spleen, lung, heart, kidney and brain of rats. The MPS, including liver, spleen and lung, is the main distribution site of CSD (Fig. 6), agreeing with the effect of site-specific distribution of other colloidal systems (Ishida et al., 2002; Moghimi et al., 2001). No CSD was detected in heart, kidney and brain. The concentration of CSD in liver achieved to a high level of 634 μg/g after 12 h, and descended very slowly along with time wherein the first kinetic $t_{1/2}$ was long to 10 days! This value is comparable to the degradation rate in rat liver homogenates (see Section 3.1.4). The amount of CSD accumulated in liver was equal to about 70% of the total administered dose after 12 h, and 30% even after 14 days. No didanosine was detected in the entire experimental process.

When administering didanosine aqueous solutions to rats, didanosine distributed in kidney, liver, lung, spleen, heart and brain, whereof the drug concentration in kidney was highest. The elimination of didanosine from tissues was also very fast. There was no drug detected after 1 h of i.v. administration. These results agree with other reports (Harvie et al., 1996; Kang et al., 1994).

Summarily, CSD nanoparticulates give a very strong targeting effect to the MPS including liver, spleen and lung. However, very unfortunately, it is impossible to achieve the satisfied therapeutic effect due to the very slow degradation of CSD. Furthermore, the heavy accumulation of CSD in targets such as liver, did not lead to toxicity because the body weight of all animals had no difference between the administered rats and the blank ones within the long experimental time range, but gave a significant increase.

4. Discussion

It is reported that 5′-position fatty acyl derivatives of didanosine are degraded in rat tissue homogenates and plasma, involving acetate, octanoate, stearate and hemisuccinate (Kawaguchi et al., 1992). Didanosine hemisuccinate has the lowest degradation rate though it possesses the best water solubility. Another research of the authors also show the similar result for hydrolysis of a series of 4′,5′-diesters of 5-fluoro-2′-deoxyuridine in rat plasma and liver homogenates, wherein the longer the acyl chain, the higher the susceptibility of hydrolysis is (Kawaguchi et al., 1985). Shao et al. (1994) prepared a series of 2′-ester prodrugs of acyclovir, including butyrate, pivalate, valerate and hexanoate, and explored their hydrolysis in rat plasma. It was shown that increases in the side-chain length and lipophilicity led to facilitated cleavage of the ester bond in the order hexanoate > valerate > butyrate. The authors regard possibly enhanced binding of the substrate to a hydrophobic pocket at the active center of carboxylesterase. Branching of the side-chain, on the other hand, results in much slower hydrolysis of acyclovir pivalate due to steric hindrance of the substituent.

The degradation of CSD is also slower than that of CAD in all kinds of media although CAD has just two more methylene groups than CSD. The different degradation profiles and stable pH of CSD and CAD should primarily result from the different influences of cholesteryl on the degradation rates of the sensitive bonds, especially the bond between didanosine and aglycone. The long spacer of CAD leads to the influence of cholesteryl weak, resulting in a similar degradation profile to that of didanosine in aqueous solutions. Didanosine degradation is acid catalyzed. In neutral and alkaline media didanosine is very stable (Bekers et al., 1993). It is well known that poloxamers can protect nanoparticulates from protein adsorption (Göppert and Müller, 2005). The degradation difference between CSD and CAD in biological environments should result from the shielding effect by poloxamers on CSD nanoparticulates and the effect of molecular structures. Both effects make the degradation of CSD much slower than that of CAD.

The above reported prodrugs, didanosine hemisuccinate and acyclovir butyrate have the degradation $t_{1/2}$ in the level of minutes (Kawaguchi et al., 1992; Shao et al., 1994). Compared to them, CSD and CAD have the very long $t_{1/2}$ in the level of days. Therefore, the cholesteryl moiety in prodrug molecules could make a great hindrance to carboxylesterases binding. Cholesteryl ester hydrolases (CEH) are a diverse group of serine esterases which catalyze the reversible hydrolysis of cholesteryl esters to free cholesterol and fatty acids (Natarajan et al., 1996). Significantly, the close-packed molecular structures of CSD and CAD could hinder the CEH, and the longer spacer of CAD weakens the steric hindrance so that CAD has a shorter degradation $t_{1/2}$.

It is interesting that the degradation capability of plasma from different species varies greatly in this study. Examples of interspecies differences in drug biotransformation are numerous. They may involve the same route, but differ in the rate or they may adopt different pathways (Ionescu and Caira, 2006). It is reported that the esterase activity of rat plasma is 10 folds higher than human plasma (Jemal and Hawthorne, 1997), even up to several hundreds folds for some certain esters (Minagawa et al., 1995). In our previous research, the acyclovir prodrug SGSA also has a higher hydrolysis rate in mouse and rat plasma than human plasma (data not shown). It is reasonable to select an animal as the model, which occupies a comparable plasma degradation rate to human plasma, in the research involving prodrugs. However, in this study, the degradation rates of didanosine derivatives in all kinds of plasma are very low, so that we have to select rats as models due to the relative rapid degradation.

The basis of SADDS is amphiphilic prodrug molecules that have to transform to the corresponding parent drugs to perform therapy. Therefore, it is very important to control the transformation or degradation rate and extent of prodrugs in targets for SADDS. Because the biotransformation rate of a prodrug is determined by the enzymes in targets and the unique molecular structure of prodrugs, understanding of enzyme systems *in vivo* especially in targeted tissues and designing of prodrug structures are the key points besides the self-assembling property of prodrugs. A pre-

liminary stability study in the *in vitro* environments is simple and useful to predict the *in vivo* fate. More importantly, different diseases have their specific therapeutic strategies, i.e. different desired drug concentration ranges in a certain time period. Generally, in an antiviral therapy, the rapid and sufficient drug release or accumulation in target cells or tissues is necessary to achieve the MIC to inhibit or kill viruses. However, in some hormone therapies, a very long sustained drug release is preferred, even to 4 months (Periti et al., 2002). Therefore, we must know the types of treated diseases and the required concentration of released drugs in targets before designing a prodrug structure for SADDS, and try our best to satisfy this goal though it may be hard to achieve.

It is very difficult to judge whether a SADDS would be successful just from the structures of prodrugs. A great deal of work has to be done, including design and synthesis of prodrugs, preparation of self-assemblies, analysis and *in vitro*/*in vivo* studies. However, we try our best to achieve a successful SADDS, and we believe the more understanding of SADDS, the closer access to success.

5. Conclusions

It has to be admitted that CSD and CAD nanoparticulates as SADDS is unsuccessful in the light of pharmacokinetics, metabolism and therapy, although the self-assembly of amphiphilic prodrugs and the stability of self-assembled nanoparticulates is satisfied. Significantly, the molecular structure consisting of one nucleoside head, one shortly chained hydrocarbon spacer and one cholesteryl moiety shows good molecular self-assembly, but it simultaneously prevent degradation in the physiological environment. Because the ultimate goal of SADDS is to provide an ideal therapy, only the achievement of self-assembly and good stability is not enough if active parent drugs are difficult to release with a desired mode in targets. Therefore, a prodrug used for preparing SADDS must be designed and modified carefully to fulfill all the aspects of self-assembly, stability and degradation.

The length of spacer becomes the key to obtain a susceptibly degradable prodrug. The longer the spacer, the faster degradation of cholesteryl acyl derivatives of didanosine would be. Moreover, an alternative spacer would lead to satisfied degradation. For example, the phosphatase-sensitive phosphoryl spacer is well degraded *in vivo*, and the phosphorylation of nucleoside drugs is an effective approach to address the problem of antiviral resistance (Aquaro et al., 2000; Hecker and Erion, 2008; Lalanne et al., 2007b; Morris-Natschke et al., 2003). Cholesteryl phosphoryl zidovudine (CPZ) has been prepared and shows self-assembly in aqueous media (Jin et al., 2008b), which will be as the next investigated subject. Certainly, both very quick and very slow degradation should be avoided, and the release rate of parent drugs should be modulated to a suitable extent according to the desired drug concentration profile in targets.

Summarily, the most optimal prodrugs for preparing SADDS should have the self-assembling capability to form stable and highly dispersed aggregates, in nanoscale at best, and they had better degrade to parent drugs only in targeted cells or tissues with a controllable rate. The followings are the key points in the research of SADDS:

(a) choice of suitable parent drugs and design of amphiphilic prodrugs with the good self-assembling potential;
(b) synthesis of the designed amphiphilic prodrugs;
(c) self-assembly of amphiphilic prodrugs in aqueous media;
(d) physical stability of self-assemblies to ensure storage for a relatively long time;
(e) suitable or desired degradation of prodrugs in targets.

6. Perspectives

SADDS as a novel drug delivery approach have been focused on by us for several years. In the beginning of research, the most focused on aspect is the self-assembling ability of amphiphilic prodrugs in aqueous media and the stability of self-assemblies. However, after this study, we know that only satisfied self-assembly and stability is not enough to produce an ideal SADDS. It must be known that whether the prodrug degrades in targets, and the extent and rate of active parent production. Fortunately, there are much more choices of prodrug structures beyond the types in this paper. A phosphoryl derivative of anti-HIV nucleoside analogue zidovudine has been obtained (Jin et al., 2008b), and other hopeful amphiphilic prodrugs are being synthesized. Anyway, a deep understanding of mechanism of drug action benefits to the ideal design and the preparation of practical drug delivery systems. Self-assembly, stability and degradation will simultaneously be considered, and a relatively optimal SADDS will probably be found in the future based on a lot of new knowledge about SADDS from this study. Moreover, one of difficulties of SADDS research is the integrated technique, just as same as its advantages. Multi-techniques or multi-disciplines are involved in SADDS research, including medicinal chemistry, physical chemistry, supramolecular chemistry, nanochemistry, pharmaceutics, pharmacokinetics and drug metabolism, and even computational chemistry used for molecular dynamic simulation. All aspects must be carefully considered when performing the research, and this is a unique way to ultimately obtain a successful SADDS.

It needs very long time from the original research of one novel drug delivery system to a successful marketed product. For example, the first liposomal drug, Ambisome® (amphotericin B liposome for injection) went into market in 1992, when 27 years had passed from the liposome discovery (Bangham et al., 1965). Paclitaxel protein-bound nanoparticles (Abraxane®) were launched in 2005, though the research of nanoparticles as drug delivery systems had been focused on for three decades. Therefore, it is hard to predicate when to make a marketed SADDS since this novel research has only passed for several years.

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