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Improved dissolution and pharmacokinetic behavior of cyclosporine A using high-energy amorphous solid dispersion approach

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ABSTRACT

The aim of the present investigation is to develop solid dispersion (SD) formulations of cyclosporine A (CsA) for improving the oral bioavailability of CsA. Amorphous SDs of CsA with eight hydrophilic polymers were prepared with wet-mill employing zirconia beads. The physicochemical properties were characterized with a focus on morphology, crystallinity, thermal behavior, dissolution, and interaction of CsA with co-existing polymer. Although CsA molecules were found to be amorphous in all wet-milled formulations, some SD formulations failed to improve the dissolution. Of all CsA formulations, SD using polymer with HPC(SSL) exhibited the largest improvement in dissolution behavior. Pharmacokinetic profiling of orally dosed CsA in rats was carried out using UPLC/ESI-MS. After the oral administration of HPC(SSL)-based SD, enhanced CsA exposure was observed with increases in C_{max} and AUC of ca. 5-fold, and the variation in AUC was ca. 40% less than that of amorphous CsA. Infrared spectroscopic studies suggested an interaction between CsA and HPC(SSL), as evidenced by the conformational transition of CsA. From the improved dissolution and pharmacokinetic data, the amorphous SD approach using wet-milling technology should lead to consistent and enhanced bioavailability, leading to an improved therapeutic potential of CsA.

1. Introduction

Cyclosporine A (CsA) is a nonpolar cyclic undecapeptide of fungal origin (Borel et al., 1976), and it has been used to prevent allograft rejection in various organ transplantations, such as kidney, liver, heart, lung, and pancreas (Calderon et al., 1992). These immunomodulatory actions result primarily from inhibition of signal transduction pathways in activated T lymphocytes, regulating the transcription of a number of genes including those for key pro-inflammatory cytokines and the interleukin-2 receptor (Underwood et al., 2001). In addition to the prevention of allograft rejection, CsA exhibits a variety of biological activities, including anti-fungal, anti-inflammatory, immunosuppressive, and anti-parasitic properties (Beauchesne et al., 2007). In clinical trials, inhaled CsA has also proved to be an effective treatment of chronic asthma (Alexander et al., 1992; Nizankowska et al., 1995). In spite of the great therapeutic potential of CsA as an immunosuppressive agent, its bioavailability after oral administration is low with a high variability (Lindholm et al., 1988). The incomplete bioavailability of CsA has been attributed to its high molecular weight, high lipophilicity, low intestinal permeability, and CYP3A-related biotransformation (Ismailos et al., 1991; Tjia et al., 1991). In particular, poor solubility has been thought to be as a critical factor for the low bioavailability of CsA, so that solubilization technology could be a key consideration for improving the bioavailability.

To improve the poor aqueous solubility of CsA, two types of approaches have mainly been proposed, which include the design of water-soluble prodrugs (Lallemand et al., 2005) and water-soluble formulations (Czogalla, 2009). Previously, UNIL088, a double ester of CsA, was designed as a prodrug with 25,000-fold higher solubility in water, and it could be converted to CsA through

Abbreviations: AUC, area under the curve of blood concentration vs. time; BCS, biopharmaceutics classification system; Bmt, (4*R*)-4[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine; 95% CI, 95% confidence interval; COPD, chronic obstructive pulmonary disease; C_{max}, maximum concentration; CsA, cyclosporine A; CV, coefficient of variation; CYP, cytochrome P450; DPI, dry powder inhaler; DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared; HCl, hydrochloric acid; HPC, hydroxypropyl cellulose; HPMC, hydroxypropyl methylcellulose; KBr, potassium bromide; MC, methylcellulose; PK, pharmacokinetic; PM, physical mixture; PXRD, powder X-ray diffraction; SEM, scanning electron microscopy; SD, solid dispersion; $T_{1/2}$, half-life; T_{max}, time to maximum concentration; UPLC/ESI-MS, ultra-performance liquid chromatography equipped with electrospray ionization mass spectrometry.

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hydrolysis (Lallemand et al., 2005). With respect to water-soluble formulations, emulsion-based liquid formulation systems were developed, including Sandimmune® (Johnston et al., 1986) and Neoral® (Kovarik et al., 1994). They were designed as a crude oil-in-water emulsion- or microemulsion-preconcentrate (Czogalla, 2009), showing improvements in dispersion in water and oral bioavailability. As the microemulsion formulations of CsA are far from optimized, various dosage forms have also been considered as potential alternative oral delivery systems (Czogalla, 2009), which include liposomes (Venkataram et al., 1990), nanoparticles (Beauchesne et al., 2007), and cyclodextrins (Miyake et al., 1999).

Previously, our group prepared an amorphous solid dispersion (SD) of CsA using newly developed wet-milling technology, as a novel dry powder inhaler (DPI) formulation of CsA for inhalation therapy in asthma (Onoue et al., 2009). The amorphous SD of CsA exhibited rapid dissolution in water and the onset of a pharmacological effect after the intratracheal administration of CsA DPI formulation in an experimental rat asthma model. These observations prompted us to optimize the amorphous SD formulation of CsA using various polymers and apply them to oral delivery systems, with the aim of enhancing the dissolution properties and oral bioavailability of CsA with low variation. In the present investigation, amorphous SDs of CsA with eight hydrophilic polymers were prepared with wet-mill employing zirconia beads. The SD formulations of CsA were characterized by scanning electron microscopy for morphology, powder X-ray diffraction (PXRD) for crystallinity, dissolution testing, and differential scanning calorimetry (DSC) for thermal behavior. The interaction between CsA and polymer was also assessed by Fourier transform infrared (FT-IR) spectral analysis on the basis of secondary structure of CsA dispersed in powder formulation. Pharmacokinetic (PK) profiling of CsA after the oral administration of amorphous SD was carried out using ultra-performance liquid chromatography (UPLC)/ESI-MS.

2. Materials and methods

2.1. Chemicals

Amorphous CsA was purchased from the Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd (Hangzhou, China), and the specification tests were carried out according to the Japanese Pharmacopeia (15th edition). The tetragonal crystal form of CsA was obtained by preparing a saturated solution of CsA in acetone and storing at −20 °C overnight. The crystals were collected by filtration from the cold acetone solution. Neoral® capsules (10 mg) were purchased from Novartis Pharma (Tokyo, Japan). Hydroxypropyl methylcellulose (HPMC) and methylcellulose (MC) were provided by Shin-Etsu Chemical (Tokyo, Japan), and polyvinylpyrrolidone (PVP) was provided by BASF Japan (Tokyo, Japan). Pullulan was supplied from Hayashibara Shoji (Okayama, Japan). Ethanol and acetonitrile (liquid chromatography grade) were purchased from Kanto Chemical (Tokyo, Japan). Potassium bromide (KBr), hydrochloric acid (HCl), hydroxypropyl cellulose (HPC), and ammonium acetate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tamoxifen was bought from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were purchased from commercial sources.

2.2. Wet-milled formulation of CsA

2.2.1. Preparation

The wet-milled CsA formulation was produced in accordance with the procedures reported previously (Niwa and Hashimoto, 2008), and several hydrophilic polymers, including HPC, HPMC, PVP, pullulan, and MC, were used. Briefly, 100 mg of CsA was weighed into the vessel of a rotation/revolution mixer (ARV-250, Thinky Co., Ltd., Tokyo, Japan). Zirconia (zirconium oxide) balls with a diameter of 0.5 mm (Nikkato Co., Ltd., Osaka, Japan) were put into the vessel and the indicated volume of polymer solution (3 mg/mL) was added. CsA suspension was micronized by 3-step wet-milling with the indicated pulverizing conditions as follows: the first step, 1000 rpm for 2 min with 0.3 mL of polymer solution; the second step, 2000 rpm for 2 min after addition of 9.7 mL of polymer solution (total volume: 10 mL); and the last step, 400 rpm for 1 min with the same polymer solution. After micronization with wet-milling, the CsA suspension, containing 100 mg of milled CsA and 30 mg of polymer, in a 20 mL vial was frozen with liquid nitrogen and freeze-dried using a FD-81 freeze-dryer (Tokyo Rikakikai, Tokyo, Japan).

2.2.2. CsA determination

The amount of CsA in the obtained dry powder was determined by an absolute calibration curve method using Waters Acquity UPLC system (Waters, Milford, MA), which included binary solvent manager, sample manager, column compartment and SQD connected with MassLynx software. An Acquity UPLC BEH C 18 column (particle size: 1.7 μm, column size: 2.1 mm × 50 mm; Waters) was used, and column temperature was maintained at 65 °C. Samples were separated using a gradient mobile phase consisting of acetonitrile (A) and 5 mM ammonium acetate (B) with a flow rate of 0.25 mL/min, and the retention time of CsA was 1.4 min. The gradient condition of the mobile phase was 0–1.0 min, 80% A; 1.0–3.0 min, 80–95% A; and 3.0–4.0 min, 95% A. Analysis was carried out using selected ion recording (SIR) for specific m/z 1203 for CsA $[M+H]^+$.

2.3. Scanning electron microscope (SEM)

Representative scanning electron microscopic images of CsA samples were taken using a scanning electron microscope, VE-7800 (Keyence Corporation, Osaka, Japan), without Au or Pt coating. For the SEM observations, each sample was fixed on an aluminum sample holder using a double-side carbon tape.

2.4. Dissolution properties

2.4.1. Dissolution test

Dissolution tests were carried out for 60 min in 100 mL of Milli-Q water or HCl solution, adjusted to pH 1.2, with constant stirring at 300 rpm using a magnetic stirrer SST-66 (Shimadzu, Kyoto, Japan) at ambient temperature. Each formulation and amorphous CsA powder was weighed to keep the total amount of CsA in the dissolution vessel constant at 3 mg. Samples were collected at the indicated times (1, 3, 5, 10, 15, 30, and 60 min) and filtered through a 0.20-μm filter. The filtrates were diluted with the same volume of ethanol immediately after filtration. To calculate the actual applied amount, 100 mL of ethanol was added to dissolve the drugs completely at the end of the dissolution test. The amount of CsA was determined by UPLC/ESI-MS, as described in Section 2.2.

2.4.2. Solubility test

To evaluate the solubility of amorphous CsA powder and the formulations, equilibrium solubility was determined. Equilibrium solubility measurement was carried out as follows: excessive amounts of amorphous CsA or the formulations were added to Milli-Q water to keep the CsA concentration constant at 1 mg/mL. Samples were put in a shaker and shaken for 72 h at 25 °C. The samples were then filtrated through a 0.20-μm filter and the CsA concentration was determined by the UPLC/MS method as described in Section 2.2.

Table 1
Physicochemical properties of CsA formulations.

	Polymer characteristics		PXRD	DSC (endothermic peak)	Dissolution rate[c] (h^{-1}; mean ± 95% CI)
	Molecular weight (Da)	Viscosity (mPa s)			
CsA (crystalline)	–	–	Tetragonal crystal	115 °C	0.059 ± 0.019
CsA (amorphous)	–	–	Halo pattern	128 °C	0.050 ± 0.017
Wet-milled CsA with					
HPC(SSL)	15,000–30,000	2.0–2.9[a]	Halo pattern	Not detected	6.9 ± 5.7
HPC(L)	55,000–70,000	6.0–10[a]	Halo pattern	Not detected	7.9 ± 5.8
HPC(H)	250,000–400,000	1000–4000[a]	Halo pattern	Not detected	1.3 ± 0.26
MC	ca. 300,000	4000[a]	Halo pattern	Not detected	0.74 ± 0.21
HPMC 60SH	ca. 100,000	50[a]	Halo pattern	Not detected	3.5 ± 1.4
PVP-K30	ca. 40,000	4–9[a]	Halo pattern	Not detected	0.26 ± 0.045
PVP-K90	ca. 360,000	50–90[a]	Halo pattern	Not detected	0.19 ± 0.029
Pullulan	200,000	100–180[b]	Halo pattern	Not detected	0.028 ± 0.0045

[a] 2% solution at 20 °C.
[b] 10% solution at 30 °C.
[c] Dissolution rate in deionized water.

2.5. Powder X-ray diffraction

The powder X-ray diffraction pattern was collected with D8 ADVANCE (Bruker AXS GmbH, Karlsruhe, Germany) with Cu Kα radiation generated at 40 mA and 35 kV. Data were obtained from 4° to 40° (2θ) at a step size of 0.014° and scanning speed of 4°/min.

2.6. Thermal analysis

Differential scanning calorimetry (DSC) was performed using a DSC Q1000 (TA Instruments, New Castle, DE, USA). The DSC thermograms were collected in an aluminum close-pan system using a sample weight of ca. 3 mg and a heating rate of 5 °C/min with nitrogen purge at 70 mL/min. The temperature axis was calibrated with indium (ca. 5 mg, 99.999% pure, onset at 156.6 °C).

2.7. Fourier transform infrared (FT-IR) spectroscopy

Changes in the secondary structure of CsA were studied with FT-IR to evaluate drug–polymer interaction. Briefly, powder samples were prepared by mixing approximately 2–3 mg of CsA and the physical mixture or SD formulation with approximately 300 mg KBr, and the mixture was pressed for preparation of the KBr disk. Spectra were recorded on IR Prestige-21 with IR solution software (Shimadzu, Kyoto, Japan), and 40 scans were performed with a resolution of 4 cm^{-1}. The measured spectra were smoothed with a 9-point smoothing function, and second derivative spectra were calculated. The derivative spectra were also smoothed with a 7-point smoothing function.

2.8. Animals

Male Sprague–Dawley rats (9–15 weeks of age; Japan SLC, Shizuoka, Japan), weighing 349 ± 16 g, were housed two per cage in the laboratory with free access to food and water, and maintained on a 12-h dark/light cycle in a room with controlled temperature (24 ± 1 °C) and humidity (55 ± 5%). All procedures used in the present study were conducted in accordance with the guidelines approved by the Institutional Animal Care and Ethical Committee of the University of Shizuoka.

2.9. Blood concentration of CsA after oral administration

CsA concentrations in whole blood were determined by UPLC/ESI-MS analysis according to the procedures reported previously with some modification (Chimalakonda et al., 2002). Animals were fasted for 12 h before oral administration. Blood samples were taken at a volume of 400 μL from the tail vein in the indicated periods after the oral administration of amorphous CsA and SD formulation (10 mg of CsA/kg) or intravenous injection of saline-suspended Neoral® (1 mg of CsA/kg). To 250 μL of blood in a glass tube were added 50 μL of internal standard solution (tamoxifen, 2.5 μg/mL), 1.75 mL of deionized water, and 200 μL of 1 M sodium hydroxide. The sample mixture was vortexed for 10 s. The CsA and internal standard were then extracted into 4 mL of a diethyl ether–methanol (95:5) solution by horizontal shaking for 10 min. After centrifugation at 2300 × *g* for 5 min, the organic layer was removed into glass tube. The extraction procedure was repeated, and the total organic layer was evaporated under a nitrogen stream at 50 °C. The residue was reconstituted in 200 μL of 50% acetonitrile, washed with 1 mL of *n*-hexane for 5 s, and centrifuged at 2300 × *g* for 1 min. After filtration of the underlayer using a 0.20 μm membrane filter, sample was analyzed by UPLC/ESI-MS to determine

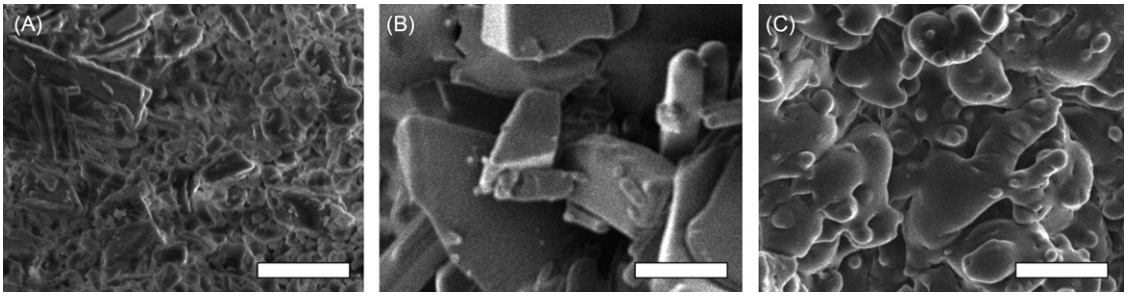

Fig. 1. Scanning electron microscopic images from cyclosporine samples, including (A) crystalline CsA, (B) amorphous CsA, and (C) solid dispersion of amorphous CsA with HPC(SSL) [SD-CsA/HPC(SSL)]. Each bar represents 5 μm.

the blood concentration of CsA. Column temperature was maintained at 65 °C, and the standard and samples were separated using a gradient mobile phase consisting of acetonitrile (A) and 5 mM ammonium acetate (B) with the flow rate of 0.25 mL/min. The gradient conditions of the mobile phase were 0–1.0 min, 60–70% A; 1.0–2.0 min, 70% A; 2.0–3.0 min, 84% A; 3.0–3.5 min, 95% A; and 3.5–4.0 min, 60% A. Peaks for internal standard and CsA were detected at the retention times of 2.06 and 2.90 min, respectively. Analysis was carried out using SIR for specific m/z 372 for tamoxifen $[M+H]^+$ and 1203 for CsA $[M+H]^+$.

3. Results and discussion

3.1. Dissolution behavior of wet-milled CsA

Several techniques have been used to improve dissolution and bioavailability of poorly water-soluble drugs, which include micronization, the use of surfactant and the development of SD formulation. Generally, SD formulation can be defined as a distribution of active ingredients in molecular, amorphous, and/or microcrystalline forms surrounded by inert carriers (Chiou and Riegelman, 1971). In the present investigation, amorphous SD formulations of CsA (SD-CsA) were prepared using various polymers, including HPC(SSL), HPC(L), HPC(H), MC, HPMC, PVP(K30), PVP(K90), and pullulan (Table 1). For preparation, CsA was micronized with wet-milling technology employing zirconia beads in various polymer solutions and freeze-dried to provide an SD formulation of CsA with an overall yield of 90% or higher. As shown in Fig. 1, SEM micrographs of crystalline/amorphous CsA and HPC(SSL)-based SD formulation revealed clear changes in the morphology of the powder particles after wet-milling owing to the evident formation of SD. Similar morphological transitions were also observed in other SD formulations with various polymers (data not shown).

The drug dissolution profiles of amorphous/crystalline CsA, the physical mixture of CsA and polymers, and SD-CsA formulations were examined in deionized water up to 60 min (Fig. 2A). Poor dissolution behaviors were seen in both crystalline and amorphous CsA at a dissolution rate of 0.05 h^{-1} (Table 1), and the physical mixture of CsA also exhibited the limited dissolution behavior in the same manner as crystalline CsA (data not shown). Generally, the amorphous form of pharmaceutical chemicals has received considerable attention since this form theoretically represents the most energetic solid state of a material, and thus it should provide the biggest advantage in terms of solubility and bioavailability. However, as observed in CsA, the limited solubility advantage for amorphous drug forms were also reported as compared to the crystalline form, for example, only 1.1- and 1.4-fold enhanced solubility for hydrochlorthiazide and griseofulvin, respectively (Hancock and Parks, 2000). According to the limited dissolution properties of the physical mixtures, polymer itself could not function as a potent solubilizer. Improved dissolution behavior was seen in most SD formulations of CsA prepared; however, the dissolution property of SD-CsA/pullulan was found to be equivalent to that of crystalline CsA. Of all the CsA formulations tested, SD-CsA with HPC, especially HPC(SSL) and HPC(L), showed the highest concentration of dissolved CsA compared with that of crystalline CsA, the dissolution rates of which were calculated to be ca. 150-fold higher than that of crystalline CsA. Interestingly, it was so challenging to determine the particle size distribution of both CsA suspension just after wet-milling and re-dissolved CsA particles by dynamic light scattering (DLS). These observations suggested that drug might be dispersed in solid water-soluble matrices molecularly. The equilibrium solubility of CsA formulations was determined after 72-h agitation in water at 25 °C, and the solubility of all CsA formulations in water was estimated to be ca. 20 μg/mL, almost corresponding to the equilibrium solubility of amorphous and crystalline CsA (20.6 μg/mL). On the basis of the equilibrium solubility and the dissolution of some CsA formulations reaching a plateau at ca. 60% (ca. 18 μg/mL), amorphous formation of CsA in these polymers led to the rapid dissolution, but not supersaturation phenomenon of CsA in water. According to the results from dissolution testing in acidic conditions (pH 1.2), SD-CsA with various polymers exhibited dissolution behaviors similar to those observed in water, and the fastest dissolution was observed for SD-CsA/HPC(SSL) (Fig. 2B). These observations were consistent with previous reports, showing that the formulation of poorly soluble drugs as SD could lead to a marked improvement in the dissolution properties (Ren et al., 2006). Typical mechanisms for the improvement of dissolution characteristics of drugs by the amorphous SD approach are reduction in particle size, the absence of crystallinity, and improved wettability (Leuner and Dressman, 2000). Thus, the presence of some hydrophilic polymers has a beneficial effect, possibly by a homogenous molecular interaction of drug and polymers or forming particles in which small amorphous powders of CsA are embedded in an SD.

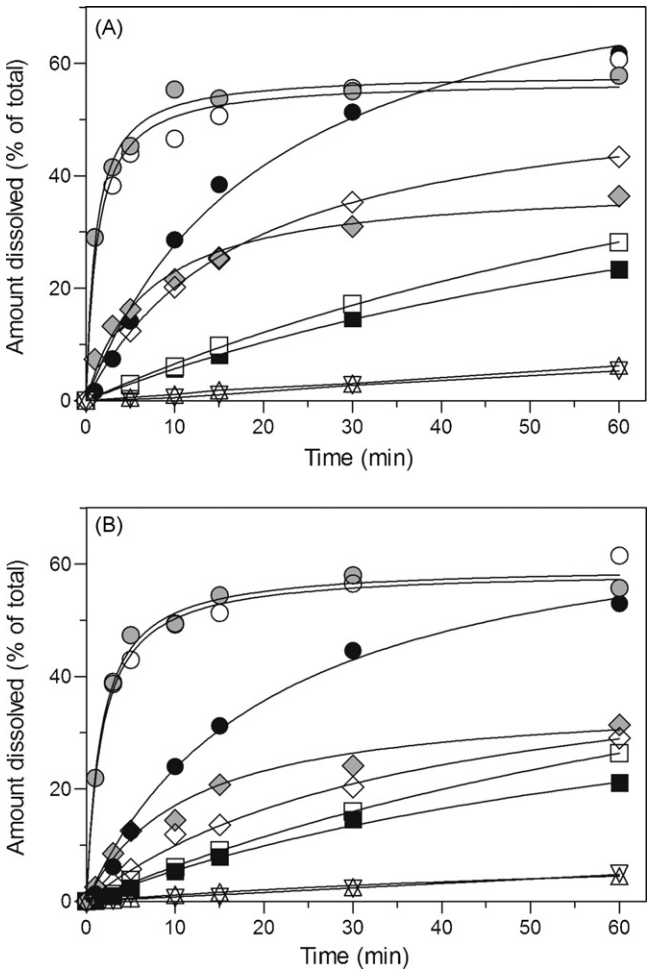

Fig. 2. Dissolution profiles of cyclosporine samples, differently treated in (A) deionized water and (B) acidic solution (pH 1.2). ▽, Amorphous CsA; △, solid dispersion of amorphous CsA with pullulan; ◯, HPC(SSL); ●, HPC(L); ●, HPC(H); □, PVP(K30); ■, PVP(K90); ◇, MC; and ◆, HPMC. Each bar represents mean ± SE of 3 independent experiments.

In general, dissolution is described by two rate processes: the rate of the interfacial or solid-solvent reaction leading to solubilization of the molecule, and the rate associated with the diffusion or transport process of the solvated molecule to the solution. According to the Stokes–Einstein equation, the diffusion coefficient is inversely proportional to viscosity (Parrott and Braun, 1973). In

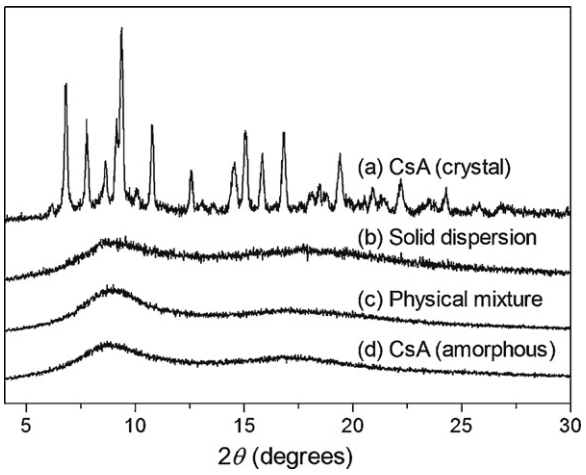

Fig. 3. Powder X-ray diffraction patterns of cyclosporine samples. (a) Crystalline CsA, (b) solid dispersion of amorphous CsA with HPC(SSL) [SD-CsA/HPC(SSL)], (c) physical mixture of amorphous CsA and HPC(SSL), and (d) amorphous CsA.

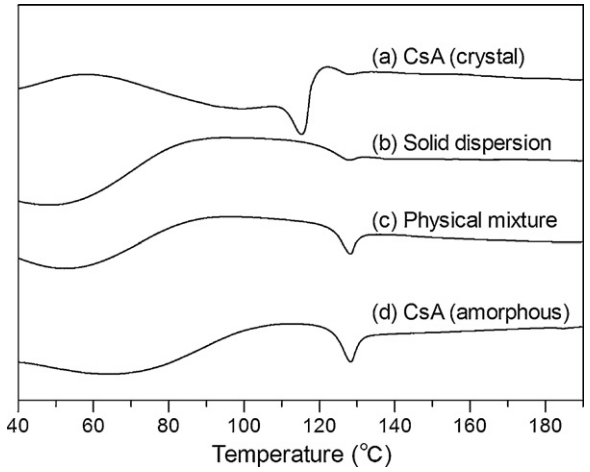

Fig. 4. Differential scanning calorimetry thermograms of cyclosporine samples. (a) Crystalline CsA, (b) solid dispersion of amorphous CsA with HPC(SSL) [SD-CsA/HPC(SSL)], (c) physical mixture of amorphous CsA and HPC(SSL), and (d) amorphous CsA.

practice, Tantishaiyakul et al. (1999) reported that the diffusion coefficient was largely decreased and the dissolution rate of drug became slow when the highly viscous PVP(K90) was used. Our dissolution data on SD-CsA with PVP and HPC series can be explained by their results, and better dissolution behavior was provided using polymers with lower molecular weight and viscosity.

3.2. Physicochemical characterization of wet-milled CsA

Since improved dissolution properties were observed in various SD-CsA formulations, the physical state of CsA formulations was further evaluated by PXRD and DSC. According to the electron microscopic images (Fig. 1), amorphous and crystalline CsA seemed to be rock-like crystal and amorphous powders, respectively. However, as shown in Fig. 3a, several intense peaks were observed in the PXRD pattern of crystalline CsA, and they were indicative of a tetragonal crystal form (Bertacche et al., 2006). Furthermore, amorphous CsA and wet-milled CsA formulations exhibited a halo diffraction pattern (Fig. 3b–d and Table 1). Not all of the characteristic peaks of crystalline CsA were found in the diffraction spectrum of SD-CsA. The amorphous form of CsA was kept during the wet-milling and freeze-drying processes. In addition to PXRD analysis, the thermal behaviors of CsA formulations were studied to ascertain the physicochemical status of the CsA trapped in the polymers. According to the DSC thermograms of several forms of CsA (Fig. 4), crystalline CsA produced a melting endotherm at 115 °C. Although amorphous CsA and the physical mixture of amorphous CsA and HPC(SSL) exhibited no thermal events at 115 °C, the endothermal peak was shifted to 128 °C, possibly due to a solid-to-liquid transition at over 120 °C (Lechuga-Ballesteros et al., 2003). Interestingly, SD-CsA/HPC(SSL) did not display any thermal events in the examined temperature range (Fig. 4b), and similar DSC thermograms were observed for all other SD formulations of CsA that were prepared (Table 1). These results suggested that the interaction between the CsA molecule and polymers in wet-milled SD prevented the phase transition at 128 °C. The lack of endothermal peaks of CsA in SD formulations also indicated that the wet-milling process, followed by freeze-drying, maintained CsA in a high-energy amorphous state. Amorphous solid dispersions can be classified according to the molecular interaction of drug and carriers in solid solutions, solid suspensions or a mixture of both (Vasconcelos et al., 2007). In particular, drug and carrier are totally miscible and soluble in amorphous solid solutions, originating a homogeneous molecular interaction between them. On the basis of DSC data, taken together with DLS analysis, the wet-milled CsA formulations we prepared might be amorphous solid solution. It is well established that the enthalpy, entropy, and free energy of an amorphous solid are much higher than those of its crystalline counterpart (Friesen et al., 2008). The excess free energy of an amorphous solid could provide a universal method to enhance the solubility of structurally diverse organic compounds. From these physicochemical characteristics of CsA formulations and dissolution profiles, wet-milling of a CsA formulation with several polymers, followed by freeze-drying, could provide an amorphous SD of CsA with improved solubility in water.

3.3. Interaction between CsA and polymer in SD

Although amorphization has been used to improve the dissolution properties of poorly water-soluble drugs, the excess free energy of an amorphous solid drives nucleation and crystallization of the drug. These drawbacks will often make it challenging to use an amorphous form in a commercial product (Bhugra and Pikal, 2008). However, the matrix polymers in the SD formulations trap the drug molecule in a metastable form and prevent precipitation or crystallization from the supersaturated state, by the formation of drug–polymer assemblies or by preventing or retarding nucleation and crystal growth (Serajuddin, 1999). In the present study, the interaction between CsA and polymer in SD-CsA/HPC(SSL), a highly water-soluble formulation, was assessed by FT-IR spectral analysis, and the bands were assigned according to previously published data (Zijlstra et al., 2007). Generally, the secondary structures of proteins and peptides display signature IR bands as a consequence of the polypeptide backbone amide bond arrangements (Onoue et al., 2006). As shown in Fig. 5A, there was no significant difference in IR spectral patterns between amorphous CsA and the physical mixture of CsA and HPC(SSL). In contrast, a slight transition of the bands at 1630–1700 cm^{-1} was observed in SD-CsA/HPC(SSL), in which amide I bands appear depending on the orientation of the amide groups in CsA. The variations in IR spectral patterns of CsA samples are considered to be indicative of different secondary structures or molecular environments among CsA samples. To clarify the conformational transition of CsA in SD-CsA/HPC(SSL), second derivative FT-IR spectra of CsA samples were superimposed (Fig. 5B). In the IR spectrum of CsA, β-sheet bands were observed at 1624 and 1636 cm^{-1}, a γ-loop band at 1647 cm^{-1}, a γ-turn band at 1661 cm^{-1}, a β-turn band at 1673 cm^{-1}, and a band for the β-OH

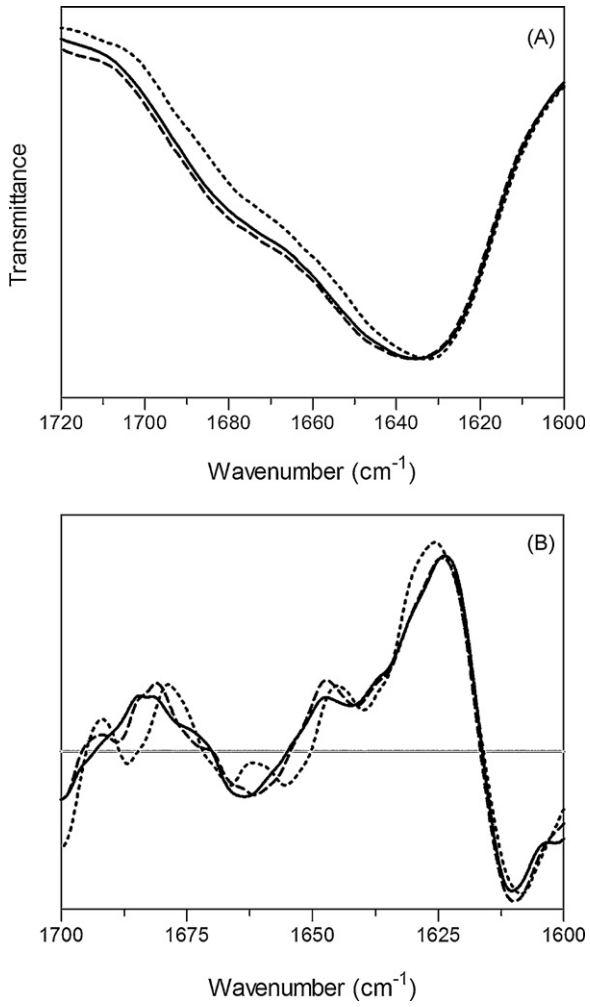

Fig. 5. FT-IR spectral analyses of cyclosporine samples. (A) Baseline-corrected and normalized amide I peak and (B) second derivative IR spectra. Solid line, CsA; broken line, physical mixture of amorphous CsA and HPC(SSL); and dotted line, solid dispersion of amorphous CsA with HPC(SSL) [SD-CsA/HPC(SSL)].

group in (4*R*)-4-[(*E*)-butenyl]-4,*N*-dimethyl-L-threonine (MeBmt) at 1684 cm^{-1}. There were no significant differences in spectral patterns between CsA and the physical mixture of CsA and HPC(SSL), suggesting similar secondary structures and molecular environments under negligible interaction with co-existing polymer. The second derivative FT-IR spectrum of SD-CsA/HPC(SSL) suggested conformational changes of the CsA molecule, as evidenced by the spectral variations: a β-sheet band at 1626 cm^{-1}, a γ-loop band at 1644 cm^{-1}, a MeBmt turn band at 1679 cm^{-1}, and the absence of clear β- and γ-turn bands. The slight conformational transition of CsA, indicated by the spectroscopic analysis of SD-CsA/HPC(SSL), might reflect interaction between CsA and polymer, consistent with the results from the thermal analysis. Theoretically, CsA could be molecularly dispersed in the matrix carrier in an amorphous SD formulation. The interaction with polymer might be attributed to

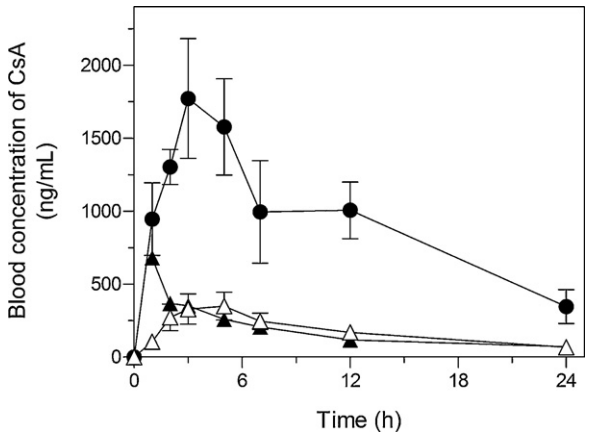

Fig. 6. Blood CsA concentrations in rats after oral or intravenous administration of CsA formulations. ▲, Neoral® (i.v., 1 mg CsA/kg); △, Amorphous CsA (p.o., 10 mg CsA/kg); and ●, solid dispersion of amorphous CsA with HPC(SSL) [SD-CsA/HPC(SSL)] (p.o., 10 mg CsA/kg). Data represent mean ± SE of 6 experiments.

the improved dissolution behavior of CsA and stabilization of the amorphous state.

3.4. Pharmacokinetic profiling of CsA SD

According to the biopharmaceutics classification system (BCS) defined by Amidon et al. (1995), CsA is categorized into BCS class II (Wu and Benet, 2005), characteristics of which are identified as low solubility and high permeability. Generally, the bioavailability of BCS class II drugs is rate-limited by its dissolution, and a small increase in dissolution rate sometimes results in a large increase in bioavailability (Lobenberg and Amidon, 2000). These previous findings, together with the improved dissolution properties of SD formulations of CsA, convinced us to clarify the pharmacokinetic behavior and enhancement of the bioavailability in rats. Fig. 6 shows the blood concentration–time profiles of CsA in rats after oral administrations of SD-CsA/HPC(SSL) (10 mg CsA/kg) and CsA (10 mg/kg), and relevant pharmacokinetic parameters including C_{max}, T_{max}, $T_{1/2}$, and AUC as listed in Table 2. The oral administration of CsA (amorphous, 10 mg/kg) resulted in a gradual elevation of blood CsA concentration up to C_{max} 404 ± 88 ng/mL, and the AUC value was calculated to be 4620 ± 993 ng h/mL. The blood concentration of CsA decreased gradually with an elimination half-life of 4.9 h. In contrast, blood CsA level was found to be high when the SD-CsA/HPC(SSL) (10 mg CsA/kg) was administered orally, and the C_{max} and AUC values were 2060 ± 312 ng/mL and 23,800 ± 3190 ng h/mL, respectively. Thus, there were ca. 5-fold enhancements in C_{max} and AUC of CsA using the wet-milling technology. On the basis of AUC value (5005 ng h/mL) of intravenously administered CsA (1.0 mg/kg), absolute bioavailability of amorphous CsA and SD-CsA/HPC(SSL) were calculated to be 9.2 and 47.5%, respectively. These findings were consistent with the results of a dissolution test, demonstrating that wet-milling technology using HPC(SSL) provided a marked increase in dissolution rate compared with that of amorphous CsA. There was a 40% reduction of inter-individual vari-

Table 2
Pharmacokinetic parameters of CsA formulations following oral or intravenous administration.

	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-inf} (ng h/mL)
SD-CsA/HPC(SSL) (p.o., 10 mg CsA/kg)	2060 ± 312	5.17 ± 1.42	2.74 ± 0.476	23800 ± 3190
CsA (amorphous) (p.o., 10 mg CsA/kg)	404 ± 88.0	4.17 ± 0.749	4.85 ± 1.69	4620 ± 993
Neoral® (i.v., 1 mg CsA/kg)	–	–	1.12 ± 0.182	5010 ± 1290

C_{max}: maximum concentration; T_{max}: time to maximum concentration; $T_{1/2}$: half-life; and AUC_{0-inf}: area under the curve of blood concentration vs. time from $t = 0$ to ∞ after administration. Values are expressed as means ± SE from 6 experiments.

ation in AUC from SD-CsA/HPC(SSL) (coefficient of variation, CV: 33%) compared with that from amorphous CsA (CV: 53%). Generally, efflux transporters would affect the extent of oral bioavailability and the rate of absorption of CsA and other BCS class II compounds. In addition, polymorphism in CYP3A, a major CsA-metabolizing enzyme, might account for variations in the oral bioavailability of CsA (Hesselink et al., 2003). The low solubility of CsA limits the concentration entering the enterocytes and prevents saturation of the efflux transporters and metabolism (Wu and Benet, 2005). However, as observed in some highly soluble formulations of CsA such as Neoral® and other emulsified forms (Czogalla, 2009), a marked increase in solubility might be attributable to improved and consistent absorption with low variation in bioavailability.

As well as the orally available form, our group previously proposed a novel DPI system of CsA, employing a methylcellulose-based SD approach, for inhalation therapy on asthma or other airway inflammations (Onoue et al., 2009). In experimental asthma/COPD model rats, inhalation of the novel DPI formulation of CsA resulted in marked attenuation of antigen-evoked inflammation in respiratory systems, whereas inhaled amorphous CsA powder itself was less effective. In the present study, the dissolution rate of SD-CsA/HPC(SSL) was found to be ca. 10-fold higher than that of SD-CsA/MC, although both formulations consist of amorphous CsA, as determined by PXRD and DSC. These observations suggested that the use of HPC(SSL)-based SD of CsA might contribute to the development of a more efficacious inhalation system, possibly leading to rapid onset and excellent clinical outcomes. *In vitro* dissolution and comparative pharmacokinetic evaluation against an amorphous CsA suspension suggest that rapid dissolution and enhanced exposure of CsA via the amorphous SD approach could be obtained using wet-milling technologies.

4. Conclusion

In the present study, several amorphous SD formulations of CsA were prepared using wet-milling technology, and their physicochemical and pharmacokinetic properties were characterized. There was significant improvement in dissolution/dispersion behavior of some SD formulations, especially CsA-loaded SD using water-soluble polymers with low molecular weight and viscosity, such as HPC(L) and HPC(SSL). FT-IR spectral data on SD-CsA/HPC(SSL) suggested the interaction of CsA with hydrophilic polymer, as evidenced by the slight transition in secondary structure, in particular, β-sheet structure. According to the pharmacokinetic profiles of SD-CsA/HPC(SSL), there was significant improvement in systemic exposure with increases in C_{max} and AUC by ca. 5-fold. From these observations of improved dissolution and pharmacokinetic behaviors, the amorphous SD approach employing wet-milling technology could be efficacious in enhancing the bioavailability of CsA, possibly leading to an enhanced therapeutic potential of CsA for the treatment of inflammatory diseases or management of post-transplantation.

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