Mechanistic understanding of the different effects of Wuzhi Tablet (*Schisandra sphenanthera* extract) on the absorption and first-pass intestinal and hepatic metabolism of Tacrolimus (FK506)

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ABSTRACT

We recently reported that the blood concentrations of Tacrolimus (FK506) in rats were markedly increased following the intake of a Chinese herbal preparation, Wuzhi Tablet (WZ, *Schisandra sphenanthera* extract). In order to identify the underlying mechanisms of the increase in FK506 level, we investigated the effects of WZ on the absorption and first-pass intestinal and hepatic metabolism of FK506 *in vitro* and *in vivo*. When co-administered with WZ, the $AUC_{0-\infty}$ value after oral FK506 dosing was increased by 2.1 fold, the oral bioavailability (F_{oral}) was increased from 5.4% to 13.2% ($p = 0.0002$), and the ($F_{abs} \times F_G$) was 111.4% ($p < 0.01$), much greater than that when FK506 was given alone. However, the F_H was only 21.2% greater than that when FK506 was given alone, which indicates that the reduction of intestinal first-pass effect was the major cause of the increased FK506 oral bioavailability when co-administered with WZ. In the Caco-2 cell transport study, the transport ratio of FK506 with WZ extract was significantly lower than that of FK506 alone, which suggested WZ extract inhibited P-gp-mediated efflux of FK506. Furthermore, 100 μM of WZ extract almost completely inhibited FK506 metabolism in rat and human liver microsomes, indicating WZ extract potently inhibited the CYP3A-mediated metabolism of FK506. In conclusion, WZ inhibited P-gp-mediated efflux and CYP3A-mediated metabolism of FK506, and the reduction of intestinal first-pass effect by WZ was the major cause of the increased FK506 oral bioavailability.

1. Introduction

Tacrolimus (FK506) is a well-known potent immunosuppressant agent for the prevention and/or treatment of graft rejection in solid organ transplantation patients (Mentzer et al., 1998;

Staatz and Tett, 2004; Bowman and Brennan, 2008). With its narrow therapeutic index, therapeutic drug monitoring is standard clinical practice to control carefully the blood level of the drug in the management of transplant recipients (Jusko et al., 1995; Venkataramanan et al., 1995). The metabolism of FK506 occurs in the liver and the small intestine via the cytochrome P450 3A (CYP3A), and its absorption is further limited due to the involvement of an efflux transporter P-glycoprotein (P-gp) (Jeong and Chiou, 2006; Iwasaki, 2007). Therefore, drug or compounds that inhibit or induce the CYP3A/P-gp may increase or decrease the blood level of FK506, respectively (van Gelder, 2002). Clinically and pre-clinically relevant interactions have been reported between FK506 and herbs such as St. John's wort, Pomelo, and grapefruit juice (Egashira et al., 2004; Herbert et al., 2004; Fukatsu et al., 2006; Peynaud et al., 2007).

Wuzhi Tablet (WZ) is a preparation of ethanolic herb extract of Wuweizi (*Schisandra sphenanthera*), which contains 7.5 mg Schisantherin A per tablet. Its major active chemical constituents

include Schisandrin A, Schisandrin B, Schisandrin C, Schisandrol A, Schisandrol B, Schisantherin A, Schisantherin B, etc. (Huyke et al., 2007). WZ is a prescribed drug (Registration number in China: Z20025766) rather than a herbal supplement in the clinical practice to protect liver function in chronic hepatitis and liver dysfunction patients (Loo et al., 2007). It is popularly prescribed for Chinese renal or liver transplant patients with FK506-induced hepatitis.

Recently, it has been reported in healthy Chinese human volunteers that concomitant administration of WZ Capsule (another preparation of *S. sphenanthera* extract (SchE), containing 11.25 mg of Schisandrin A per capsule, could enhance the *in vivo* whole blood concentration of FK506, which might be due to the inhibition of CYP3A and/or P-gp via substances in SchE (Xin et al., 2007). We observed similar phenomenon in renal transplant patients with about 2–3 folds increase in C_{max} and AUC after concomitant administration of WZ (data not shown). Our recent study in rats indicated that a concomitant dose of WZ could significantly increase the FK506 whole blood concentration with only a slight change in FK506 tissue distribution, suggesting WZ could be a promising FK506-sparing agent for transplant patients (Qin et al., 2010). However, to our knowledge, there is no published study that could clearly clarify the drug interaction between FK506 and WZ and the underlying mechanisms.

Therefore the current study aims: (1) to study the pharmacokinetics of FK506 in rats when concomitant administration of WZ; (2) to differentiate the effect of WZ on the intestinal FK506 first-pass effect from that of the liver metabolism; and (3) to examine the effect of WZ on the CYP3A-mediated FK506 metabolism and P-gp-mediated exsorption of FK506.

2. Materials and methods

2.1. Materials

FK506 with a purity of 98% as determined by HPLC with ultra-violet (UV) detection was synthesized and provided by Toronto Research Chemicals Inc. (Toronto, Canada). Ascomycin (FK520, as internal standard, IS) with a purity of 95% as determined by HPLC with UV detection was synthesized and provided by BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). Prograf® capsules (1 mg of FK506 per capsule) and FK506 injection (5 mg × 1 mL) were produced by Astellas Ireland Co., Ltd (Ireland). WZ (each tablet containing 7.5 mg Schisantherin A) was produced by Fang Lue Pharmaceutical Company (Guangxi, China). Verapamil, NADPH Na$_4$ (≥98%) and HEPES were purchased from Sigma ALDRICH Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, Hank's balanced salt solution (HBSS, pH 6.8), 0.05% trypsin–ethylenediaminetetra-acetic acid (EDTA) were obtained from Gibco BRL Life Technology (Grand Island, NY), penicillin and streptomycin were purchased from Hua Nan Pharmaceutical Company (Guangdong, China), and non-essential amino acids were obtained from Invitrogen (Carlsbad, CA, USA). Methanol and acetonitrile of HPLC grade were purchased from Tedia Inc. (Beijing, China). All other reagents were of analytical grade or HPLC grade when appropriate. Ultra-pure water was obtained from a Milli Q-plus system (Billerica, MA).

The extract used in *in vitro* studies was extracted from WZ. Briefly, 30 times (V:W) of ethanol (mL) was added into WZ powder (g) and vortexing-mixed for 1 min. The mixtures were then ultrasonic-extracted for 60 min and then centrifuged at $2500 \times g$ for 10 min. The residue was dissolved in 30 times of ethanol and repeated the above step again. The organic supernatants were combined and transferred to a clean tube and evaporated to dryness under vacuum. The residue was dissolved in ethanol as the stock solution (final concentration of the extract was 0.4 g/mL containing 20 mM Schisantherin A), and stored at −20 °C until use.

2.2. Animals

Male Sprague-Dawley rats (250–330 g) were supplied by the Laboratory Animal Service Center at Sun Yat-sen University (Guangzhou, China). The animals were kept in a room at 22–24 °C with a light/dark cycle of 12/12 h and 55–60% relative humidity. They had free access to standard rodent chow and clean tap water. The rats were fasted for 12 h before the experiments. All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China (http://www.most.gov.cn).

2.3. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured and used as previously described in our paper (Zhang et al., 2006).

2.4. Preparation of rat liver microsomes

Hepatic tissues were collected from healthy male Sprague-Dawley rats (230–310 g) and stored at −80 °C. Liver microsomes were prepared by differential centrifugation as previously described in our report (Bi et al., 2008). Human liver microsomes were purchased from BD Biosciences (NJ, USA). All liver microsomes were stored at −80 °C until use. The amount of protein of liver microsomes was measured using BCA Protein Assay Kit (http://www.beyotime.com/Compatibility Chart For BCA Kit.pdf).

2.5. Pharmacokinetic experiments in rats

On the day before the experiment, a light surgery for the rat was performed as described in our previously published paper (Bi et al., 2008). Briefly, a polyethylene catheter (0.4 mm i.d., 0.8 mm o.d., Portex Ltd, Hythe, UK) was inserted into the right jugular vein under light anaesthesia. Afterwards, the rats were placed individually in cage, allowed to recover and fasted for 12 h before the pharmacokinetic study.

For oral (p.o.) dosing, FK506 dissolved in pure water was given to rats by gavage at a dose of 1.89 mg/kg. WZ (0.25 g/kg) or Verapamil (30 mg/kg) dissolved in pure water was also given by gavage 1 min (for WZ) or 30 min (for Verapamil) before the administration of FK506. Rats receiving FK506 alone were given with an equivalent volume of vehicle. About 0.22 mL of blood samples were withdrawn via the right cannulated jugular vein before (0 h) and at 5, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h post-dosing.

For intravenous (i.v.) dosing, pilot studies indicated that dose-normalized FK506 AUC values were unchanged for 0.2, 0.756, and 1.89 mg/kg intravenous doses. The 0.2 mg/kg intravenous dose was chosen for detailed study. FK506 injection diluted in saline was injected via the right jugular vein at a dose of 0.2 mg/kg (2 mL/kg), followed by a 0.5 mL heparinized saline (50 U/mL) flush. WZ dissolved in pure water was also given by gavage at a dose of 0.25 g/kg 1 min before the administration of FK506. Rats receiving FK506 alone were given with an equivalent volume of vehicle. About 0.22 mL of blood samples were withdrawn via the right jugular vein before (0 h) and at 2, 5, 10, 15, 30, 45, 60 min, 1.5, 2, 3, 4, 6, 8, 12, and 24 h post-dosing.

After blood sampling, the cannula was flushed with an equal volume of heparinized saline solution (50 U/mL) to prevent coagulation and to replace the blood loss. 200 μL of the blood samples were immediately collected and stored at −20 °C until analysis.

2.6. Transport of FK506 in Caco-2 cells

The transport of FK506 across Caco-2 monolayers was investigated by the methods described previously (Zhang et al., 2006; Bi et al., 2008) with some modifications. In brief, Caco-2 cells were seeded at 1.0×10^5 cells/cm^2 on polycarbonate membrane Transwell inserts (Corning Co., Corning, NY, USA) and cultured for 21 days. At the beginning of the transport study, they were washed three times with HBSS, after the third wash, the plates were incubated at 37 °C for 30 min, then the TEER values were measured and the cells with TEER values exceeded 250 Ω cm^2 were used in the experiment.

Pre-incubation was carried out for 30 min in the presence or absence of WZ extract (1 mg/mL containing 50 μM Schisantherin A with a final ethanol concentration of 0.25%) or Verapamil (100 μM) in HBSS buffer at both the apical (AP) and the basolateral (BL) sides of the monolayers. The buffer was then replaced with fresh HBSS buffer on one side of the cell layer and FK506 solution (1 μg/mL) in HBSS buffer on the other side and incubation was performed at 37 °C. The bidirectional transport studies of FK506 were conducted by loading FK506 solution (1 μg/mL) to either the apical or basolateral side of the Caco-2 monolayers. 50 μL samples were taken from the receiver side at 30, 60 and 90 min, and an equal volume of HBSS buffer was replenished. All samples were stored at −20 °C until assay.

At the end of the transport experiment, the cell monolayers were replaced with fresh HBSS buffer, incubated for 30 min at 37 °C, and TEER values were measured. The TEER values were no significant different before and after the transport experiment.

2.7. Metabolism of FK506 by rat and human liver microsomes

The metabolism of FK506 was assayed by measuring the reduction of FK506. Briefly, the incubation system, with a total volume of 200 μL, contained 100 mM potassium phosphate buffer (pH 7.4), rat/human liver microsomes (final concentration 0.02 mg/mL), and FK506 (final concentration 5 ng/mL) with or without WZ extracts (containing 1, 10, 100 μM Schisantherin A). The reaction was started by addition of 20 μL of 10 mM NADPH to the system. The mixture was incubated for 5 min at 37 °C; then, 800 μL of ice-cold diethyl ether was added to the reaction mixture to stop the reaction and 10 μL of FK520 was added as an internal standard. FK506 was determined by LC–MS/MS method described below. The metabolism ratio was calculated by compared the initial drug concentration with the concentration after incubation.

2.8. Quantification of FK506 by LC–MS/MS method

FK506 in all samples was determined using our previously developed LC–MS/MS methods (Li et al., 2008; Qin et al., 2010). The blood samples were prepared using a single-step liquid–liquid extraction procedure described in our previously published paper (Qin et al., 2010). As for samples from transport study and microsomes metabolism experiment, 400 or 800 μL of extraction solvent ethyl acetate was added directly. After vortex-mixing for 1 min and standing at room temperature for 10 min, the mixtures were centrifuged at 2500 × g for 5 min. After centrifugation, the organic phase was then transferred to a clean centrifuge tube and evaporated to dryness. The residues were dissolved in mobile phase and an aliquot (10 μL) of the re-constituent solution was injected onto the LC–MS/MS for analysis.

The LC–MS/MS method was partially validated since the biological matrix was changed compared to our previously published methods (Li et al., 2008; Qin et al., 2010). The method had a chromatographic running time of 2 min and linear calibration curves over the concentrations of 0.5–300, 0.5–500 and 0.5–20 ng/mL

for FK506 in blood, HBSS buffer and microsomes buffer, respectively. The extraction recoveries were 56.1–62.4%, 55.1–60.4% and 67.6–74.3% for FK506, and 53.8%, 58.7% and 68.3%for FK520 in blood, HBSS buffer and microsomes buffer, respectively. The lower limit of quantification (LLOQ) of the analytical method was 0.5 ng/mL for FK506. The intra- and inter-batch precision and accuracy were less than 15% for all quality control samples in blood, HBSS buffer and microsomes buffer, respectively.

2.9. Data analysis

Pharmacokinetic analysis was performed using a non-compartmental analysis by the Pharmacokinetics and Bioavailability Program Package (Version 2.1, Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-set University, Guangzhou, China). Time of the peak blood concentration (T_{max}) and peak blood concentration (C_{max}) were read directly from the observed concentration versus time profiles. The initial drug concentration (the extrapolated concentration at zero time) of the drug following i.v. injection was calculated by back extrapolation of the blood concentration–time curve to the y-axis. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve to infinity ($AUC_{0-\infty}$) was calculated as: $AUC_{0-\infty} = AUC_{0-t} + C_t/\beta$, where C_t is the last measurable concentration. The blood clearance (CL) was estimated by dividing the total administered dose by the $AUC_{0-\infty}$. The volume of distribution (V_d) was calculated by dividing CL by β. The mean residence time (MRT) was calculated by $AUMC_{0-t}/AUC_{0-t}$. The absolute bioavailability of oral doses (F_{oral}) was calculated using the formula:

$$F_{oral} = \left(\frac{AUC_{0-\infty}, \text{p.o.}}{AUC_{0-\infty}, \text{i.v.}} \right) \times \left(\frac{\text{Dose, i.v.}}{\text{Dose, p.o.}} \right) \times 100\% \tag{1}$$

Since FK506 is known to be insignificantly excreted in urine as unchanged form after intravenous administration (Takada et al., 1991), the hepatic extraction ratio (ER_H) can be estimated as

$$ER_H = \frac{CL_{iv}}{Q_H} \tag{2}$$

where rat hepatic blood flow (Q_H) was assumed to be 55.2 mL/min/kg (or 3.312 L/h/kg) (Davies and Morris, 1993).

The oral bioavailability is a function of F_H [hepatic availability ($F_H = 1 - ER_H$)], F_G [gut availability ($F_G = 1 - ER_G$)], and F_{abs} [fraction absorbed], as given in Eq. (3) (Wu et al., 1995)

$$F_{oral} = F_{abs} \times F_G \times F_H \tag{3}$$

It is possible to estimate the product of $F_{abs} \times F_G$ as

$$F_{abs} \times F_G = \frac{F_{oral}}{F_H} \tag{4}$$

Comparison of $F_{abs} \times F_G$ calculated values gives an estimate of the effects of WZ on the gut processes affecting FK506 availability.

Total drug blood intrinsic clearance (fu × CL_{int}) was calculated based on the well stirred hepatic clearance model (Wilkinson, 1983),

$$fu \times CL_{int} = CL_{iv} \times \left(\frac{Q_H}{Q_H - CL_{iv}} \right) \tag{5}$$

The apparent permeability coefficient (P_{app}) in cellular monolayers is expressed in centimeters per second and calculated as in following equation:

$$P_{app} = \left(\frac{\Delta Q}{\Delta t} \right) \times \left(\frac{1}{60 \times A \times C_0} \right) \tag{6}$$

where $\Delta Q / \Delta t$ is the permeability rate (micrograms per second), A is the surface area of the membrane (square centimeters), and
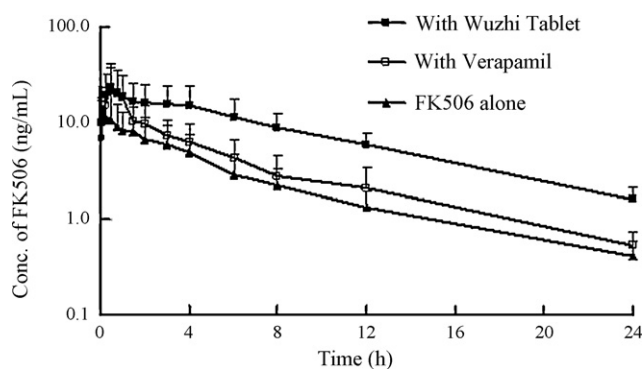
Fig. 1. Whole blood concentrations–time curves of FK506 after a single oral dose of FK506 (1.89 mg/kg) to rats with and without an oral dose of WZ (0.25 g/kg) or Verapamil (30 mg/kg). Each point represents the mean ± SD ($n \geq 8$). Data are depicted on a semi-logarithmic scale.
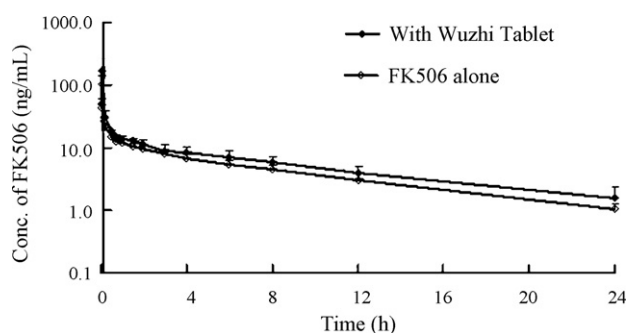
Fig. 2. Whole blood concentrations–time curves of FK506 after a single intravenous dose of FK506 (0.2 mg/kg) with and without an oral dose of WZ (0.25 g/kg). Each point represents the mean ± SD ($n \geq 4$). Data are depicted on a semi-logarithmic scale.

C_0 is the initial concentration in the donor chamber (micrograms per milliliter). Samples from the 90 min point were used for P_{app} calculations.

The net BL to AP efflux of FK506 (R_{net}, transport ratio) was determined by calculating the ratio of P_{app} in the BL to AP direction versus P_{app} in the AP to BL direction ($P_{app}(BL-AP)/P_{app}(AP-BL)$) as in Eq. (7) (Zhou et al., 2005; Zhang et al., 2006).

$$\text{Transport ratio} = \frac{P_{app}(BL-AP)}{P_{app}(AP-BL)} \qquad (7)$$

The results are expressed as the mean ± SD. Statistical significances were evaluated using Student's t-tests or Wilcoxon Two-Sample test. Statistical analyses were performed using SAS 8.1 software (SAS Institute, Inc., Cary, NC, USA). p value <0.05 was considered statistically significant.

3. Results and discussions

3.1. Effect of WZ on the pharmacokinetics of FK506 following oral and intravenous administration

The mean FK506 blood concentration–time curves obtained after oral or intravenous administration of FK506 with or without WZ are shown in Figs. 1 and 2. The pharmacokinetic parameters of FK506 are presented in Tables 1 and 2. When administered in combination with WZ (0.25 g/kg), the $AUC_{0-\infty}$ and C_{max} of oral administration of FK506 were increased by 209.9% ($p < 0.0005$) and 80.1% ($p < 0.05$), while the $AUC_{0-\infty}$ and C_{max} following an oral administration of FK506 were increased by 74.7% and 67.5% when Verapamil (30 mg/kg) were co-administered. The K_a values were almost the same in the WZ and Verapamil groups which were

Table 1
Pharmacokinetic parameters of FK506 after a single oral dose of FK506 (1.89 mg/kg) to rats with and without an oral dose of WZ (0.25 g/kg) or Verapamil (30 mg/kg). Data are the mean ± SD.

Group	FK506 alone ($n = 14$)	With WZ ($n = 14$)	With Verapamil ($n = 8$)
AUC_{0-24} (ng/mL h)	57.7 ± 31.1	188.3 ± 74.1***	85.4 ± 46.5
$AUC_{0-\infty}$ (ng/mL h)	67.7 ± 34.9	209.8 ± 80.1***	118.3 ± 39.3
MRT (h)	5.7 ± 1.4	7.3 ± 1.2*	5.2 ± 1.3
C_{max} (ng/mL)	15.1 ± 11.2	27.2 ± 16.7*	25.3 ± 17.0*
T_{max} (h)	0.9 ± 0.8	1.1 ± 1.1	0.5 ± 0.3
$t_{1/2}$ (h)	6.7 ± 1.9	7.0 ± 2.0	7.0 ± 2.5
CL/F (L/h/kg)	30.0 ± 11.9	10.6 ± 4.5***	25.2 ± 16.8
K_a (h^{-1})	0.21 ± 0.07	0.27 ± 0.07*	0.28 ± 0.07*

* $p < 0.05$ significantly different as compared with FK506 alone group.
*** $p < 0.0005$ significantly different as compared with FK506 alone group.

Table 2
Pharmacokinetic parameters of FK506 after a single intravenous dose of FK506 (0.2 mg/kg) with and without an oral dose of WZ (0.25 g/kg). Data are the mean ± SD.

Group	FK506 alone ($n = 4$)	With WZ ($n = 5$)	p
AUC_{0-24} (ng/mL h)	109.0 ± 13.5	141.2 ± 20.8	0.0410
$AUC_{0-\infty}$ (ng/mL h)	132.0 ± 14.2	167.7 ± 29.5	0.0856
CL_{iv} (L/h/kg)	1.5 ± 0.2	1.2 ± 0.2	0.0951
V_d (L/kg)	16.1 ± 1.5	11.2 ± 1.8	0.0152
$t_{1/2}$ (h)	6.8 ± 0.6	7.5 ± 1.3	0.3669
MRT (h)	6.5 ± 0.3	6.7 ± 0.7	0.5826

significantly higher than that of FK506 alone group ($p < 0.05$). Furthermore, the MRT of WZ group (5.7 h) was significantly delayed to 7.3 h compared to that of FK506 alone group ($p < 0.05$), the CL/F of WZ group (10.6 L/h/kg) was about 1/3 of that of the FK506 alone group (30.0 L/h/kg) ($p < 0.00001$), but there was no significant difference from that of the Verapamil group. On the other hand, WZ produced only a 27.0% increase in AUC after intravenous FK506 dosing, which was significantly lower than the effect of WZ on oral administration of FK506. The CL_{iv} and V_d of FK506 after intravenous administration were decreased in the WZ group.

In the current study, the dosages of FK506 and WZ were extrapolated from the clinical practice, and Verapamil was used as positive control. Verapamil is one of the most extensively characterized inhibitors of P-gp and is the first multidrug resistance (MDR)-reversal agents that reached clinical trial (Fisher and Sikic, 1995). AUCs of FK506 in rats were slightly increased over dose range of 10–60 mg/kg of Verapamil and reached the maximum increase at 30 mg/kg. Thus, the dose of Verapamil as a positive P-gp inhibitor was optimized at 30 mg/kg in rats. The results showed that oral concomitant administration of WZ could increase the whole blood concentration of FK506 over comparable time periods following oral or intravenous single dose of FK506, but the extent was much less through intravenous injection route (Figs. 1 and 2). When co-administered with WZ, the elimination of FK506 in the whole blood after oral or intravenous dosing was significantly slower than that of FK506 alone. When co-administered with Verapamil, the blood concentration of FK506 was sharply increased and then sharply decreased (Fig. 1). The C_{max} and K_a of the WZ and Verapamil group were significantly higher than that of control group, suggesting the absorption of FK506 was increased when co-administered with WZ or Verapamil (Table 1). The CL/F of FK506 was decreased significantly from 30.0 to 10.6 L/h/kg and the MRT was also extended to 7.3 h in the presence of WZ, but Verapamil had little effect on the CL/F and MRT of FK506 (Table 1). Decreases in the CL_{iv} and V_d of FK506 were also observed in the WZ group after intravenous administration of FK506. The $AUC_{0-\infty}$ of FK506 in the presence of WZ was 309.9% of that of FK506 alone, in contrast, the $AUC_{0-\infty}$ in the presence of Verapamil was only 174.7% of that of

Table 3
Summary of derived pharmacokinetic parameters of FK506 after a single oral dose (1.89 mg/kg) or intravenous dose (0.2 mg/kg) to rats with and without an oral dose of WZ (0.25 g/kg).

Group	FK506 alone	With WZ	p
$(fu \times CL_{int})$ (L/h/kg)	2.84 ± 0.54	1.99 ± 0.63	0.1042
F_{oral} (%)	5.40 ± 2.74	13.24 ± 5.05	0.0002
ER_H	0.46 ± 0.05	0.37 ± 0.07	0.0864
F_H	0.54 ± 0.05	0.63 ± 0.07	0.0864
$F_{abs} \times F_G$	0.10 ± 0.009	0.21 ± 0.025	0.0142

FK506 alone. These findings suggested that WZ-mediated absorption enhancement and elimination decrease caused the increase of FK506 bioavailability, while Verapamil also increased FK506 absorption but only had a moderate effect on the elimination and thus had a small effect on FK506 bioavailability.

3.2. Effect of WZ on the oral bioavailability, and gut/liver metabolic extractions of FK506

The derived pharmacokinetic parameters are presented in Table 3, the calculated total drug intrinsic clearance $(fu \times CL_{int})$ was decreased by 29.9% from 2.84 ± 0.54 L/h/kg to 1.99 ± 0.63 L/h/kg. The estimated ER_H of FK506 was 0.46 ± 0.05, therefore, from Eq. (4), the value of F_H was 0.54 ± 0.05 and then, $(F_{abs} \times F_G)$ was 0.10 ± 0.009, assuming that ER_H is the same following intravenous or oral dosing of FK506 (Takada et al., 1991). As compared with the FK506 alone group, F_{oral} of WZ group was significantly increased from 5.40% to 13.24% ($p = 0.0002$). The ER_H of WZ group was decreased by 19.6% compared with that of FK506 alone group. Calculating F_H and substituting into Eq. (4) allows estimation of $(F_{abs} \times F_G)$. The $F_{abs} \times F_G$ of WZ group was 111.4% greater than that of FK506 alone group ($p = 0.0142$), suggesting that oral concomitant administration of WZ in rats could markedly affect gut bioavailability of FK506.

As shown in Table 1, the CL/F of FK506 was far surpassed the rat hepatic blood flow (Q_H), which indicated that FK506 was also metabolized by extrahepatic tissues. Furthermore, high CL/F also suggested FK506 underwent significantly first-pass effect, which was in line with the reported results. The previous study showed that pre-systemic metabolism by gastrointestinal CYP3A was around 50% and was considered to be responsible for the limited bioavailability of FK506 (Undre et al., 1999). As shown in Table 3, the estimated gut availability $(F_{abs} \times F_G)$ of FK506 alone was only about one-fifth of the liver availability (F_H). Given that FK506 had a rapid and almost complete intestinal absorption (Tamura et al., 2002), the contribution of F_{abs} to the low estimated gut availability might be relatively minor as compared to that of F_G. Our study suggested that WZ could reduce the gastrointestinal and liver pre-systemic metabolism through CYP3A pathway. In the presence of WZ, the $(F_{abs} \times F_G)$ was 111.4% ($p = 0.0142$) greater than that of FK506 alone. On the other hand, WZ decreased hepatic first-pass
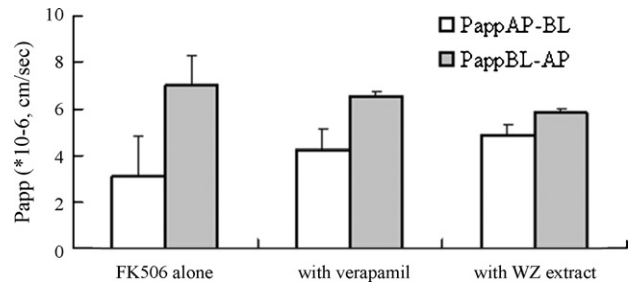

Fig. 4. Effect of Verapamil and WZ extract on the AP–BL and BL–AP transport of FK506 across Caco-2 monolayers. The monolayers were incubated for 90 min with 1 μg/mL FK506 in the absence or presence of Verapamil (100 μM) or WZ extract (1 mg/mL) added to both sides of the monolayers during pre-incubation (30 min). Each point represents the mean ± SD ($n = 3$).

metabolism (ER_H) from 45.7% to 36.9%, which could be calculated to an approximate 8.8% increase in F_{oral} if all other determinants of bioavailability were unaffected. However, the results showed that F_{oral} was significantly increased by 145.2% ($p = 0.0002$), indicating that the reduction of intestinal first-pass effect was the major cause of F_{oral} increase when co-administered with WZ. The significant gut metabolism was probably due to the high capacity of intestinal enzymes to metabolize FK506 and/or the increase of drug exposure to intestinal metabolism enzymes by the intestinal efflux transporters at the apical domain of the enterocyte.

3.3. Effects of WZ extract on the transport of FK506 in Caco-2 cells

The MTT assay indicated that WZ extract (range from 0.05 to 1 mg/mL) and FK506 (1 μg/mL) showed no cytotoxicity toward Caco-2 cells. The TEER values were not significantly different before and after the transport experiment. The time profile of the basal-to-apical (BL–AP) and apical-to-basal (AP–BL) transport of FK506 across Caco-2 cell monolayers showed that the transport of FK506 was linear growth with $R^2 > 0.9$ over 90 min (Fig. 3). The BL-to-AP transport of FK506 was 0.9 fold higher than that of the AP-to-BL transport with transport ratio of 1.9 (Fig. 4). Here, the monolayers were pretreated with WZ extract or Verapamil to prevent the direct interactions of FK506 with WZ extract or Verapamil. The WZ extract elevated the AP-to-BL transport of FK506 and reduced the BL-to-AP transport of FK506. In the presence of 1 mg/mL WZ extract, the P_{app} AP–BL was increased (from $3.1 \pm 1.7 \times 10^{-6}$ cm/s to $4.8 \pm 0.5 \times 10^{-6}$ cm/s), whereas the P_{app} BL–AP was decreased (from $7.0 \pm 0.5 \times 10^{-6}$ cm/s to $5.8 \pm 0.2 \times 10^{-6}$ cm/s), resulting in a mean transport ratio of 1.2. The positive control Verapamil increased P_{app} AP–BL (from $3.1 \pm 1.7 \times 10^{-6}$ cm/s to $4.2 \pm 0.9 \times 10^{-6}$ cm/s), and decreased P_{app} BL–AP (from $7.0 \pm 0.5 \times 10^{-6}$ cm/s to $6.5 \pm 0.2 \times 10^{-6}$ cm/s) and resulted in a mean transport ratio of 1.5. These findings suggested that both the WZ extract and Verapamil inhibited the P-gp-mediated efflux of FK506.
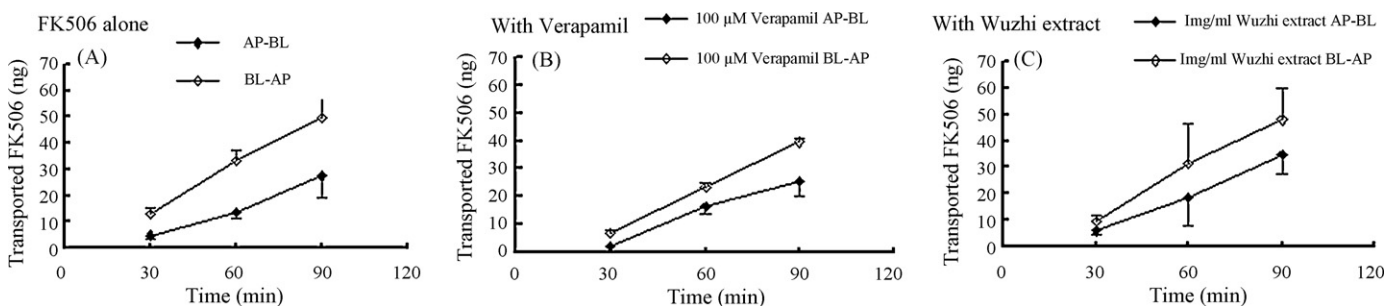
Fig. 3. The time profile of the AP–BL and BL–AP transport of FK506 across Caco-2 cell monolayers with 1 mg/mL WZ extract (B) or 100 μM Verapamil (C) ($n = 3$).
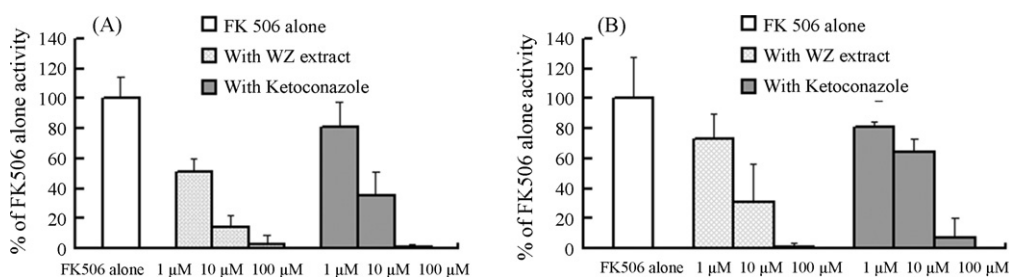
Fig. 5. Effects of different concentrations of WZ extract (1, 10, 100 μM) on the metabolism of FK506 (5 ng/mL) in the rat (A) and human (B) liver microsomes. Liver microsomes (0.02 mg protein/mL) were pre-incubated with WZ extract or Ketoconazole for 5 min in 100 mM potassium phosphate buffer (pH 7.4) containing 0.05 mM EDTA. The reaction was started by addition of 20 μL of 10 mM NADPH to the system. The mixture was incubated for 5 min at 37 °C. Each point represents the mean ± SD ($n = 3$).

It was reported that Caco-2 cells possessed only very slight CYP3A4 activity (Schmiedlin-Ren et al., 1997), indicating that the metabolism of FK506 in these cells would be almost negligible under the present experimental conditions. The recommended oral dose of FK506 was 0.15–0.3 mg/kg twice a day for renal transplant patients. Thus, for a 70-kg weight subject, taking the volume of gastrointestinal tract (3–5 L) into consideration (Egashira et al., 2004), the concentration of FK506 in the intestinal surface after taking an oral dose would be 1–3.5 μg/mL. Thus, the concentration of FK506 used in the Caco-2 cell transport experiment was set at 1 μg/mL. In the present transport study, the transport ratio of FK506 in the control group was only 1.9, which was considered to be relatively low as a good P-gp substrate. Partially, it might be possible that FK506 at the concentration 1 μg/mL (1.2 μM) used in the transport study inhibited the P-gp activity. Earlier study (Pawarode et al., 2007) suggested that 1 μM of FK506 could enhance cellular drug uptake in cells overexpressing P-gp, MRP-1 or BCRP. The transport ratio of FK506 in WZ extract pretreated group was lower than that of the control group, and lower than that of Verapamil-pretreated group. This finding suggested that WZ extract inhibited P-gp-mediated efflux of FK506 and the ability of WZ extract to reduce the active transport of FK506 was more potent than Verapamil, a well-known P-gp inhibitor. Previously published work has shown that several constituents in SchE could interact with P-gp. Yoo et al. reported that several lignans including deoxyschizandrin (Schisandrin A), angeloylgomisin M1, gomisin A (Schisandrol B), tigloylgomisin H, and gomisin C (Schisantherin A) exhibited statistically significant inhibitory effect on P-gp-mediated efflux of rhadamine-123 in Caco-2 cells (Yoo et al., 2007). Pan and Sun et al. reported that Schisandrin B bore strong potency to inhibit P-gp (Pan et al., 2005; Sun et al., 2007). Since the major active constituents in WZ were the lignans, the previous studies supported our current findings that WZ extract had inhibitory effect on P-gp and thus inhibited P-gp-mediated efflux of FK506.

3.4. Effects of WZ extract on the metabolism of FK506 in rat and human liver microsomes

The incubation time, protein concentration and the FK506 concentration were optimized by comparing the metabolized ratio of FK506 under different protein concentration (0.005–0.5 mg/mL), incubation time (1–15 min) and different substrate concentrations. The metabolized ratio of FK506 was linearly increased at the incubation time range of 3–15 min. The metabolized ratio of FK506 was increased from 6.1% to 58.3% over the protein concentrations range of 0.005–0.5 mg/mL, and the metabolism of FK506 showed a linear increase over the protein concentrations range from 0.005 to 0.02 mg/mL. The metabolism of different concentration of FK506 (1.25–20 ng/mL) in the rat liver microsomes was 17.0–24.0% with no significant difference. Taking the FK506 therapeutic range of 5–15 ng/mL (Venkataramanan et al., 1995) into considerations,

5 ng/mL of FK506 was chosen as substrate concentration in the metabolism study. Therefore, the incubation condition was set as 0.02 mg/mL of protein, 5 ng/mL of metabolism substrate (FK506) and 5 min of incubation time.

The effects of WZ extract and Ketoconazole on the metabolism of FK506 in rat and human liver microsomes were studied with the system described above. The effects of different concentrations of WZ extract on the metabolism of FK506 are shown in Fig. 5 and Table 4. The metabolism of FK506 was significantly decreased in the presence of Ketoconazole and WZ extract. The inhibition of metabolism of FK506 by WZ extract and Ketoconazole was concentration dependent. WZ extract had equivalent or even more potent inhibition than that of Ketoconazole. Metabolism of FK506 was almost completely inhibited by 100 μM of WZ extract and Ketoconazole [it was reported that 25 μM of Ketoconazole, a potent inhibitor of CYP3A, caused an 80% inhibition of FK506 metabolism (Lampen et al., 1996)], indicating a potent inhibition of FK506 metabolism by WZ and Ketoconazole.

Previously published work had shown that Schisandra fruit extract, gomisins B, C, G, and Schisandrin were potent inhibitors of CYP3A4 and the inhibitory effect of gomisin C (Schisantherin A) was stronger than that of Ketoconazole (Ki = 0.070 μM) (Iwata et al., 2004). Since Gomisin C was the quality control constituent and the most abundant lignan in WZ, it might play a very key role in the inhibitory effect of WZ on the CYP3A-mediated FK506 metabolism.

FK506 is a good substrate of CYP3A and P-gp. CYP3A is abundantly present both in liver and intestine and P-gp is expressed in many organs and tissues including intestine and liver. The overlap of physiological distribution of CYP3A and P-gp cause a complex interplay between CYP3A and P-gp during the metabolism and efflux process (Lemahieu and Maes, 2007). This dynamic enzyme–transporter interplay between CYP3A and P-gp in intestinal mucosa and liver together results in a robust first-past effect and can be applied to the regulation of oral FK506 exposure (Fig. 6). On the other hand, if tacrolimus is a substrate of other transporters

Table 4
Effects of different concentrations of WZ extract (1, 10, 100 μM) on the metabolism of FK506 (5 ng/mL) in the rat and human liver microsomes (0.02 mg/mL), $t = 5$ min ($n = 3$).

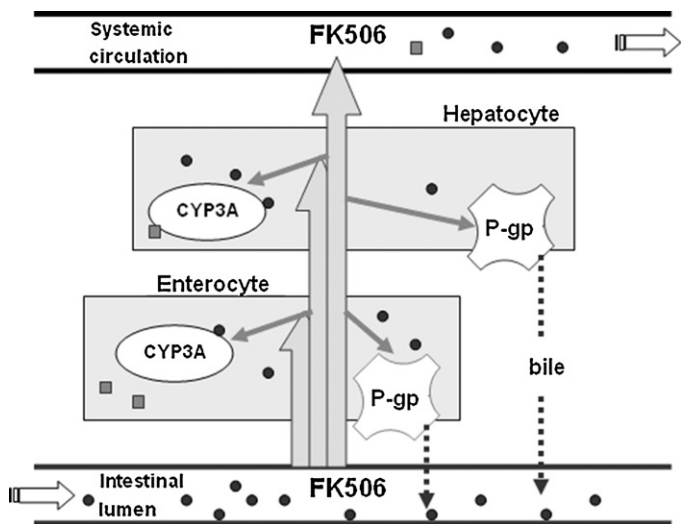| Groups	Metabolized ratio (%) in the rat liver microsomes	Metabolized ratio (%) in the human liver microsomes
Control	23.1 ± 3.3	20.7 ± 3.7
With WZ extract (μM)		
1	11.5 ± 4.1	15.4 ± 3.5
10	3.3 ± 1.9	6.3 ± 5.1
100	0.7 ± 1.4	0.3 ± 0.5
With Ketoconazole (μM)		
1	18.1 ± 3.7	17.0 ± 0.6
10	8.4 ± 3.6	13.2 ± 8.1
100	0.3 ± 0.4	1.6 ± 3.2

Fig. 6. Effect of interplay between CYP3A and P-gp in intestinal and liver on the regulation of oral FK506 exposure.

such as MRPs and BCRP, the involvement of these transporters in the WZ–FK506 interaction will make the enzyme–transporter interplay more complicated. The C_{max} and K_a of FK506 in the current study were increased significantly, and the transport ratio was decreased in Caco-2 cells, indicating the inhibition of P-gp. The CL/F was decreased markedly and the MRT was delayed, in addition, the FK506 metabolism was decreased by WZ, all these suggested the inhibition of CYP3A by WZ. For the potent inhibition of FK506 metabolism by WZ, we ever supposed that the inhibition of FK506 metabolism mostly contributed to the increase of oral FK506 exposure for the interplay of CYP3A and P-gp in liver. Interestingly, our data from *in vivo* pharmacokinetic study in rats suggested that the dramatic increase in oral bioavailability of FK506 in the presence of WZ was mostly due to the inhibition of intestinal first-pass extraction and only partially due to the inhibition of liver metabolism. However, the current study cannot fully differentiate the extent of hepatic/intestinal CYP3A/P-gp effect on the oral FK506 exposure with co-administration of WZ. And, it is still unknown whether the first-pass effect in the intestine is more related to CYP3A-mediated metabolism than to P-gp-mediated drug exsorption. Therefore, further studies are needed to elucidate the exact interaction of hepatic/intestinal enzyme and transporter during this WZ–FK506 interaction process. In addition, WZ contains multiple lignans and only a few of which have been investigated for their CYP3A/P-gp modulating activity, thus the exact effect of each lignan in WZ on the hepatic/intestinal CYP3A and P-gp activity is also needed to be further clarified.

4. Conclusions

In conclusion, the AUC of FK506 was significantly increased by 2 fold when co-administered with WZ, which is in line with our clinical observation on renal transplant patients and the previous reports. The results from transport and metabolism studies showed that WZ could significantly inhibit the CYP3A-mediated metabolism of FK506 and the P-gp-mediated FK506 efflux. The reduction of intestinal first-pass effect of FK506 by WZ was extensive and contributed a major part of the increase in FK506 bioavailability. Current findings from *in vitro* and *in vivo* studies consistently demonstrated that the reduced first-pass effect of FK506 by WZ was related to the inhibition of CYP3A-mediated FK506 metabolism and P-gp-mediated FK506 exsorption.

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