P-Glycoprotein-Dependent Disposition Kinetics of Tacrolimus: Studies in *mdr*1a Knockout Mice

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Purpose. This study was performed to evaluate the involvement of P-glycoprotein in disposition kinetics of tacrolimus (FK506), a substrate of P-glycoprotein, in the body.

Methods. The blood and tissue concentrations of FK506 after i.v. or p.o. administration (2 mg/kg) to normal and *mdr*1a knockout mice were measured by competitive enzyme immunoassay.

Results. The blood concentrations in knockout mice were significantly higher than those in normal mice. The value of the total clearance (CL_{tot}) for knockout mice (19.3 mL/min/kg) was about 1/3 of that for normal mice (55.8 mL/min/kg)($P < 0.001$), although there was no significant difference in the distribution volume at the steady-state (Vd_{ss}) (about 4.6 L/kg) between both types of mice. FK506 rapidly penetrated the blood-brain barrier and the brain concentration reached a maximum, which was about 10 times higher in knockout mice than in normal mice, 1 hr after administration. The brain concentration in normal mice thereafter decreased slowly, whereas in knockout mice, an extremely high concentration was maintained for 24 hr.

Conclusions. The pharmacokinetic behavior of FK506 in the tissue distribution is related with the function of P-glycoprotein encoded by the *mdr*1a gene. The brain distribution of FK506 is dominated by the P-glycoprotein-mediated drug efflux and presumably also by the binding to FK-binding proteins (immunophilins) in the brain.

KEY WORDS: tacrolimus; disposition kinetics; P-glycoprotein; *mdr*1a knockout mice; brain distribution.

INTRODUCTION

P-glycoprotein is encoded by the multidrug-resistance (*mdr*) gene and is expressed, not only in multidrug-resistant cancer cells, but also in various normal tissues such as the adrenal, kidney, liver, small intestine, colon, and capillary endothelium in the brain (1–3). Three *mdr* genes have been reported in rodents, while two have been identified in humans. In the mouse, *mdr*1a and *mdr*1b have been shown to confer multidrug resistance in cancer cells, whereas the *mdr*2 gene does not (4). Therefore, although P-glycoprotein is considered to play an important role in the tissue-distribution, clearance, and gastrointestinal absorption in animals, the contributions of individual gene products to the drug pharmacokinetics have not yet been clarified. Recently, Borst *et al.* (5) established *mdr*1a gene-deficient mice. Subsequently, Schinkel *et al.* (6, 7) reported the distribution of several P-glycoprotein substrates, such as ivermectin, vinblastine, digoxin, cyclosporin A, and dexamethasone, to the brain is significantly increased in the *mdr*1a knockout mice, when compared with normal mice. This evidence clearly indicates the P-glycoprotein encoded by *mdr*1a gene operates as a part of the blood-brain barrier in mice (8).

Tacrolimus (FK506) is a potent immunosuppressant having a macrolide lactone structure. It was isolated from *Streptomyces tsukubaensis* and is commonly used to prevent the rejection of organ transplants. FK506 has some adverse effects such as renal and hepatic toxicities (9), and there is also evidence of central nervous and heart toxicity (10). On the other hand, FK506 has recently been reported to have a neuroprotective effect in focal cerebral ischemia by inhibiting calcineurin in the brain (11,12). It has already been shown that FK506 is a substrate of P-glycoprotein, by means of *in vitro* studies using vincristine resistant mouse leukemia P388 cells (13) and LLC-PK1 cells transfected with human MDR1 cDNA (14). However, the involvement of P-glycoprotein in the *in vivo* tissue-distribution, absorption, and elimination of FK506 has not yet been clarified.

In this study, we examined the involvement of P-glycoprotein in the pharmacokinetics of FK506, especially its role in the characteristic behavior of FK506 in the brain, using *mdr*1a knockout mice.

MATERIALS AND METHODS

Materials

FK506, mouse anti-FK506 monoclonal antibody (FKmAb) and FK506-conjugated peroxidase (FK-POD) were gifts from Fujisawa Pharmaceutical Co. (Osaka, Japan). Mouse anti IgG polyclonal antibody (goat) was purchased from Incstar Co. (Minn, USA). All other chemicals were of reagent grade and were used without further purification.

Animal Experiments

Experiments were performed on male *mdr*1a (−/−) knockout mice (body weight 22–28 g, Taconic Farms Inc., NY, USA). We used male C57BL/6 mice (body weight 21–26 g, SLC, Hamamatsu, Japan) as the control mice because genetically compatible wild type (+/+) mice, F2 and F3 generations of 129/Ola × FVB mice (6), were not available in Japan. We confirmed in a preliminary study that male C57BL/6 mice showed similar disposition kinetics of several drugs, including tacrolimus, to the wild type (+/+) mice (data not shown).

FK506 (2 mg/kg) was injected via the jugular vein in a volume of 50 μl or was orally administered in a volume of 200 μl. Blood samples were collected from the intraorbital venous plexus using a heparinized capillary tube under light ether anesthesia, at designated time intervals. To determine the apparent tissue-to-blood concentration ratio ($K_{B,app}$), the mice

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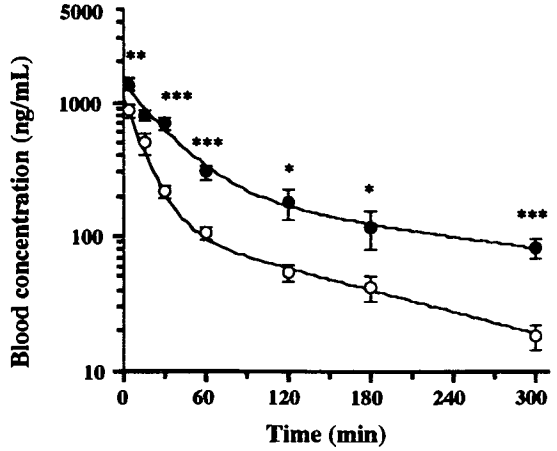

Fig. 1. Time courses of blood concentration of FK506 after i.v. administration (2 mg/kg) to normal (○) and knockout (●) mice. Each point with bar represents the mean ± SE. (n = 4–8). The lines were fitted to a biexponential equation by using the MULTI program. *, **, *** Significantly different from normal mice at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

were euthanized 5 hr after a single intravenous (i.v.) injection of FK506. The tissues were quickly excised, rinsed well with ice-cold saline, blotted dry, and weighed. The samples were homogenized in ice-cold saline (10%, w/v). For the biliary recovery of FK506, the bile duct was cannulated with polyethylene tubing (type sp-8 O. D. 0.5 mm, Natume, Tokyo, Japan) under light ether anesthesia. Cannulated mice were kept in a supine position on restraining plates. Blood, tissue, and bile samples were kept at −30°C until assay.

Assay for FK506

According to the method of Kobayashi *et al.* (15), FK506 was measured by competitive enzyme immunoassay with FKmAb and FK-POD. Briefly, FK506 in whole blood and bile was extracted with methanol. FK506 in tissue samples was extracted with n-hexane containing 2.5% isoamyl alcohol. The extraction solvent was evaporated and the residue was dissolved in FK-POD solution. The solution was added to a microtiter plate well, previously coated with FKmAb, to determine competitive binding of FK506 and FK-POD with FKmAb. POD activity was measured using o-phenylenediamine and hydrogen peroxide as cosubstrates. The reaction was stopped by addition of H_2SO_4, and the optical density was measured by a microplate reader (CS-9300PC, Shimadzu, Kyoto, Japan). FK506 content was determined by comparison with a standard curve.

Data Analysis

The steady-state distribution volume (Vd_{SS}) and the total body clearance (CL_{tot}) were estimated by means of model-independent moment analysis as described by Yamaoka *et al.* (16). The data were analyzed by using Student's *t* test for comparing the unpaired means of two sets of data. The number of determinations (N) is noted in each table and figure. A *P* value of 0.05 or less was used as the criterion of a significant difference between sets of data.

Table 1. Pharmacokinetic Parameters of FK506 in Normal and Knockout Mice After i.v. Administration (2 mg/kg)

	Normal mice	Knockout mice
AUC (ng · min/mL)[a]	35900 ± 3900	104000 ± 13800*
MRT (min)[a]	92 ± 22	205 ± 55*
Vd_{ss} (L/kg)[a]	5.14 ± 1.59	3.96 ± 1.40
CL_{tot} (mL/min/kg)[a]	55.8 ± 6.2	19.3 ± 2.6*
A_1 (ng/mL)[b]	937 ± 92	1178 ± 159*
λ_1 (min^{-1})[b]	0.0662 ± 0.006	0.0362 ± 0.0076*
A_2 (ng/mL)[b]	120 ± 14	223 ± 64*
λ_2 (min^{-1})[b]	0.00622 ± 0.00057	0.00332 ± 0.00122*

[a] Determined by model-independent moment analysis.
[b] Determined by applying the MULTI program to the biexponential equation:

$$C_b = A_1 \cdot \exp^{-\lambda 1 \cdot t} + A_2 \cdot \exp^{-\lambda 2 \cdot t}$$

Each value represents the mean ± SD. of four to eight mice.
* Significantly different from normal mice at $P < 0.01$ and $P < 0.001$.

RESULTS

Time Course of Blood Concentration of FK506

The time courses of blood concentration of FK506 after i.v. administration of FK506 (2 mg/kg) to normal and knockout mice are shown in Fig. 1. They are biphasic, with half-times for the distribution and elimination phases of 10.5 and 111 min in normal, and 19 and 209 min in knockout mice, respectively. The blood concentrations in knockout mice were significantly higher than those in normal mice.

As shown in Table 1, the value of the area under the blood concentration-time curve ($AUC_{(0\to\infty)}$) of FK506 from time zero to infinity for knockout mice was significantly larger than that for normal mice ($P < 0.001$). The value of the total clearance (CL_{tot}) for knockout mice was about 1/3 of that for normal mice ($P < 0.001$), though there was no significant difference in the distribution volume at the steady-state (Vd_{SS}) between them.

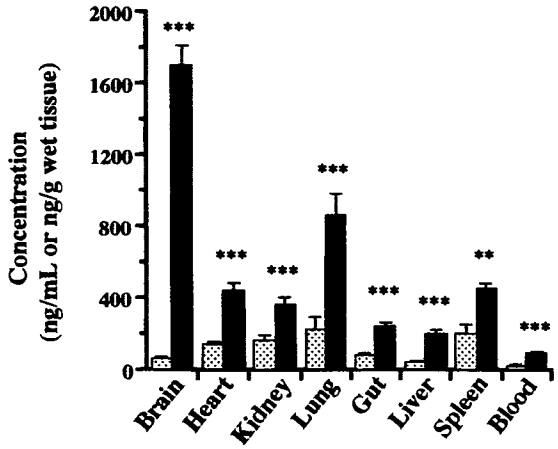

Fig. 2. Tissue and blood concentrations of FK506 at 5 hr after i.v. administration (2 mg/kg) to normal (▩) and knockout (■) mice. Each column with bar represents the mean ± SE. of four or five mice. **, *** Significantly different from normal mice at $P < 0.01$ and $P < 0.001$, respectively.

Table 2. Values of Apparent Tissue-to-Blood Concentration Ratio ($K_{B,app}$) of FK506 in Normal and Knockout Mice at 5 hr After i.v. Administration (2 mg/kg)

	Normal mice	Knockout mice
Brain	2.73 ± 0.58	16.4 ± 2.1*
Heart	7.09 ± 0.66	4.47 ± 0.60
Kidney	8.29 ± 1.55	5.35 ± 1.85
Lung	11.6 ± 3.6	11.9 ± 4.6
Gut	4.08 ± 0.47	3.99 ± 1.19
Liver	1.84 ± 0.28	3.20 ± 0.54*
Spleen	10.6 ± 2.3	7.77 ± 1.77

Each value represents the mean ± SE. of four to five mice.
* Significantly different from normal mice at $P < 0.05$ and $P < 0.001$, respectively.

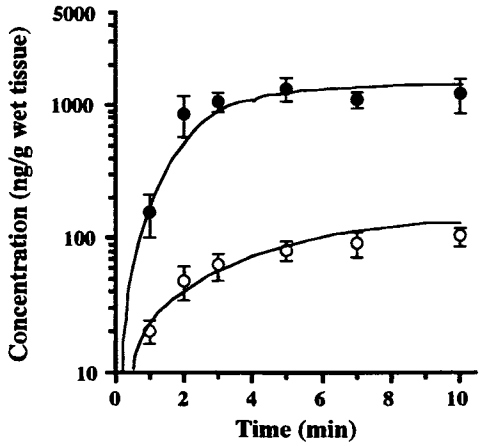

Fig. 4. Time courses of brain concentration of FK506 after i.v. administration (2 mg/kg) to normal (○) and knockout (●) mice. Each point with bar represents the mean ± SE. (n = 6).

Tissue Concentration of FK506

The concentrations of FK506 in various tissues at 5 hr after a single i.v. administration are shown in Fig. 2. The tissue concentrations of FK506 in normal mice were in the following order: lung > spleen > kidney > heart > gut > brain > liver. Those in knockout mice were significantly higher than those in normal mice and in particular, the concentration in the brain was 33-fold higher than that in normal mice.

The apparent tissue-to-blood concentration ratios ($K_{B,app}$) of FK506 at 5 hr after administration to normal and knockout mice are summarized in Table 2. The $K_{B,app}$ values of FK506 for tissues except for brain and liver in knockout mice were similar to those in normal mice. The values of brain and liver in knockout mice were about 6- and 2-fold greater than those in normal mice, respectively.

Time Course of Tissue Concentration of FK506

The time courses of FK506 concentration in the brain, heart, and liver after i.v. administration (2 mg/kg) to normal and knockout mice are shown in Fig. 3. The concentrations of FK506 in the heart and liver decreased approximately in parallel to that in blood in both types of mice. However, in the brain of normal mice, the concentration of FK506 reached the maximum within 60 min, then slowly decreased, whereas in the knockout mice, the brain concentration of FK506 was rapidly elevated and remained almost constant for 24 hr.

Figure 4 shows the time courses of brain concentration of FK506 for 10 min after a single i.v. administration of 2 mg/kg to normal and knockout mice. The uptake clearance (CL_{up}, calculated as FK506 amount in brain/AUC) was determined 1–2 min after administration. The CL_{up} values of FK506 for the brain in normal and knockout mice were estimated to be 0.022 and 0.117 mL/min/g, respectively, by using the integration plot method (17).

Biliary Excretion of FK506

The biliary excretion of FK506 within 30 min after i.v. administration of FK506 (2 mg/kg) to normal and knockout mice amounted to 202.2 ± 77.3 ng (n = 11) and 40.5 ± 8.9 ng (mean ± SD., n = 4), respectively. The biliary excretion in knockout mice was about 1/5 of that in normal mice ($P < 0.01$).

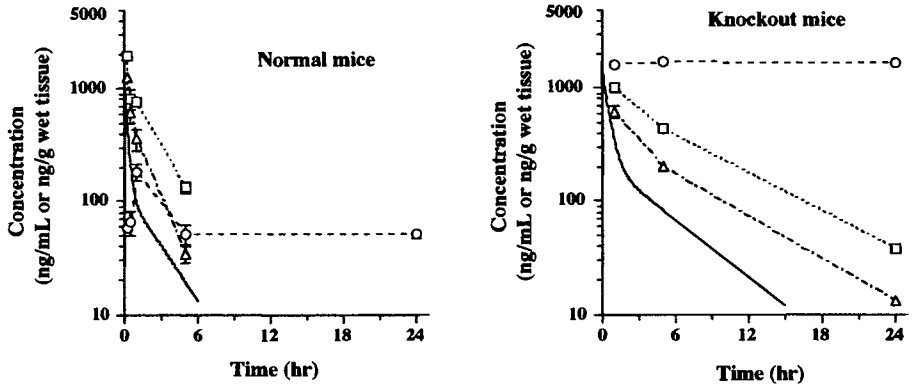

Fig. 3. Time courses of tissue concentration of FK506 after i.v. administration (2 mg/kg) to normal and knockout mice. Each point with bar represents the mean ± SE. of three or four mice. The solid line (blood) was calculated by applying the MULTI program to the biexponential equation. Key: brain, ----○----; heart, ----□-----; liver, —△—.

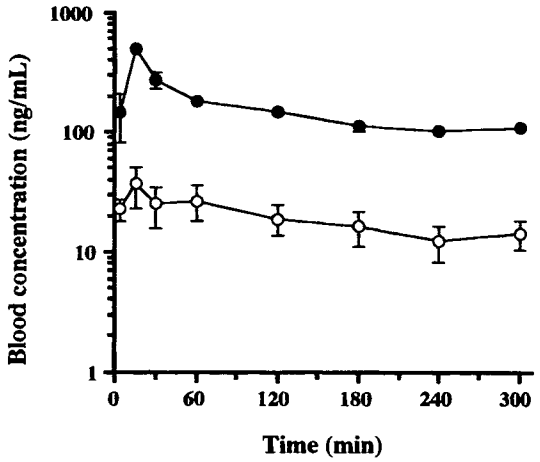

Fig. 5. Time courses of blood concentration of FK506 after p.o. administration (2 mg/kg) to normal (○) and knockout (●) mice. Each point with bar represents the mean ± SE. (n = 4).

Absorption of FK506 from Gastrointestinal Tract

The time courses of FK506 concentration in the blood after p.o. administration (2 mg/kg) to normal and knockout mice are shown in Fig. 5. The blood concentrations in both types of mice reached the maximum level within 15 min, however the concentration in knockout mice was significantly higher than that in normal mice ($P < 0.001$). As shown in Table 3, the value of $AUC_{(0\rightarrow5)}$ of FK506 from time zero to 5 hr after p.o. administration for knockout mice was about 8 times larger than that for normal mice ($P < 0.001$). The bioavailability in knockout mice was about 3 times higher than that in normal mice.

DISCUSSION

In this study, we used *mdr*1a gene-deficient mice to examine the influence of P-glycoprotein encoded by this gene on the *in vivo* disposition kinetics of FK506 and found that P-glycoprotein significantly affected the brain distribution, total body clearance, and oral bioavailability of FK506.

The blood concentrations of FK506 in *mdr*1a knockout mice after i.v. administration were significantly higher and decreased more slowly than those in normal mice (Fig. 1). It has been reported FK506 is mainly metabolized by cytochrome P450 3A in the liver to at least nine metabolites (18,19). Since the enzyme-linked immunoassay system used in this study could detect unchanged FK506 and three minor metabolites, but the major metabolite 13-O-demethyl FK506 and other metabolites are not immunoreactive with the antibody FKmAb (20), it is thought that the observed blood concentration closely reflected the behavior of unchanged FK506, as reported by Alak and Moy (21). It has also been reported that more than 95% of administered FK506 is excreted in bile as the unchanged form (less than 0.4%) and several metabolites (22,23), and less than 1% in urine (24). Cross-reactive substances with FKmAb in bile amount to less than 1%. In this context, we found the biliary excretion in knockout mice was about 1/5 of that in normal mice despite the higher liver concentration of FK506 in *mdr1a* knockout mice than that in normal mice (Fig. 2). P-glycoprotein is located on the bile canalicular surfaces of hepatocytes (1) and contributes to the biliary excretion of unchanged FK506 as well as cyclosporin A (25). However, because the excretion of FK506 is less than 1% in bile and urine, it is clear that its total clearance is essentially due to metabolism. The high blood concentration and low total clearance of FK506 in knockout mice may be due to the decreased metabolic activity of FK506 rather than the decreased biliary excretion. However, we could not clarifty the underlying mechanism for the alteration of hepatic clearance of FK506 in knockout mice.

Although the concentrations of FK506 in several organs of knockout mice at 5 hr after administration were higher than those in normal mice, as was the blood concentration (Fig. 2), only the $K_{B,app}$ values for the brain and liver in knockout mice were significantly higher than those in normal mice (Table 2). Since in both types of mice, the elimination half-life of FK506 in liver was comparable with those in the heart and blood (Fig. 3), the high $K_{B,app}$ value for the liver in knockout mice is not due to accumulation as a result of lowered liver-to-blood back flux of FK506, but may be based on the decreased biliary excretion clearance owing to the absence of P-glycoprotein. On the other hand, FK506 rapidly penetrated the blood-brain barrier (Fig. 4) and the CL_{up} value for knockout mice was clearly higher than that for normal mice. The value (0.117 mL/min/g) for knockout mice is comparable to the mouse brain blood flow rate (0.08 mL/min; estimated from the value for the rat (26)), suggesting that the uptake of FK506 into the brain may be blood flow rate-limited when P-glycoprotein is absent. The brain concentration in knockout mice reached the maximum level which was about 10 times that of normal mice, at 1 hr after administration (Fig. 3). The brain concentration in normal mice thereafter decreased slowly until 24 hr, whereas in knockout mice, an extremely high concentration was maintained for 24 hr. These results suggest that P-glycoprotein functions as a component of the blood-brain barrier and contributes substantially to the brain distribution of FK506. Moreover, efflux of FK506 by P-glycoprotein seems to be initiated immediately after the drug administration. In other tissues, no influence of P-glycoprotein was apparent, because the $K_{B,app}$ values for FK506 were not different between knockout mice and normal mice,

Table 3. Pharmacokinetic Parameters of FK506 in Normal and Knockout Mice After i.v. or p.o. Administration (2 mg/kg)

	Normal mice		Knockout mice	
	i.v.	p.o.	i.v.	p.o.
$AUC_{(0\rightarrow5)}$ (ng · min/mL)[a]	33800 ± 3800	5700 ± 1000	79400 ± 6700	46800 ± 2600
Bioavailability (%)	16.9		58.9	

[a] Area under the blood concentration-time curve of FK506 from time zero to 5 hr.

and the tissue concentrations decreased in parallel with the blood concentration. Despite the lack of the *mdr*1a gene in brain, heart and liver, the extents of influence of P-glycoprotein were different among these tissues. It is possible that some alternative mechanism operates in the *mdr*1a gene knockout mice.

Surprisingly, a high concentration of FK506 was maintained for a long time in the brain of knockout mice. Even in normal mice, the decrease in the brain concentration of FK506 from 5 hr after administration was small. This delayed elimination from brain might be related to the toxicity of FK506 on the central nervous system, as described elsewhere (10). The immunosuppressive action of FK506 arises when the drug-immunophilin complex associates with the calcium/calmodulin-dependent protein phosphatase, calcineurin, to inhibit its phosphatase activity in T cells (27). The level of immunophilins (FK-binding proteins, FKBP) in the brain is 10 times greater than those in other tissues (28,29). Therefore, high and sustained accumulation of FK506 in brain might be ascribed to tight drug binding to FKBP, although further study is needed to confirm this.

On the other hand, we previously demonstrated the P-glycoprotein plays a role as an absorption barrier by transporting acebutolol, vinblastine, and cyclosporin A out of the intestinal cells into the lumen (30). In this study, we found the bioavailability of FK506 in knockout mice was about 3 times higher than that in normal mice. Furthermore, in both types of mice the blood concentration of FK506 after p.o. administration reached rapidly the maximum level within 15 min (Table 3, Fig. 5). These results suggest the absorption of FK506 from gastrointestinal tract is limited in part by P-glycoprotein. Since the difference of bioavailability between normal and knockout mice may be caused by the variation of first pass effect due to hepatic and/or intestinal metabolic activity as well as efflux transport by P-glycoprotein, we could not determine whether the P-glycoprotein contributes to the intestinal permeability of FK506 or not.

In conclusions, we demonstrated the pharmacokinetic behaviors of tissue distribution, hepatic clearance, and oral bioavailability of FK506 are strongly influenced by the P-glycoprotein. The brain distribution of FK506 is dominated by the efflux via P-glycoprotein and presumably also by binding to FKBP in the brain.

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