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EFFECTS OF THE IMMUNOSUPPRESSANT FK-506 AND ITS ANALOG FK-520 ON HEPATIC AND RENAL CYTOCHROME P450 MIXED-FUNCTION OXIDASE

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Abstract—The novel immunosuppressant FK-506 and its analog FK-520 were found to inhibit the hepatic microsomal mixed-function oxidase system in male Sprague–Dawley rats. At 5 and 10 mg/kg/day, s.c., for 6 days they caused 30–80% decreases in cytochrome P450 levels, NADPH–cytochrome P450 reductase, and benzphetamine *N*-demethylase activities. The metabolism of FK-506 itself was inhibited by 50%. FK-506 and FK-520 had a minimal effect on the renal cytochrome P450 levels unlike cyclosporin A which produced a 67% increase after six daily 25 mg/kg doses. A single dose of FK-506 (25 mg/kg, s.c.) had a minimal effect on the hepatic or renal metabolizing enzyme system. *In vitro*, addition of FK-506 and FK-520 to human and control rat liver microsomes resulted in a concentration-dependent inhibition of benzphetamine N-demethylation (10–20% at 50 μM, 60–75% at 250 μM). We suggest that in view of its potential to inhibit hepatic cytochrome P450-dependent mixed-function oxidase, resulting in the inhibition of its own metabolism, FK-506 should be administered with caution to transplant patients.

FK-506 (Fig. 1) is a novel immunosuppressant which is 10–100 times more active than cyclosporin A (CsA) when tested, *in vitro* as a suppressor of T cell activation [1–4], and *in vivo* in prolonging survival of allografts including kidney and liver in rats [5], monkeys [6] and humans [7]. However, like CsA, FK-506 exhibits hepato- and nephrotoxicity in the rat [8] by a mechanism(s) as yet unknown.

CsA has been shown to interact with cytochrome P450 and to inhibit rat liver microsomal cytochrome P450-dependent metabolism *in vitro* and *in vivo* after multiple administration to rats and mice [9–11]. Moreover, CsA administration to rats was reported recently to result in increased levels of renal cytochrome P450 and it was suggested that this effect may contribute to its nephrotoxicity [12]. Since FK-506 exhibits similar pharmacological and toxicological profiles as CsA, a study was undertaken to determine if FK-506 and its somewhat less active analog FK-520 (Fig. 1) have similar effects on the hepatic and renal cytochrome P450 enzyme system. Cytochrome P450 and NADPH–cytochrome P450 reductase levels as well as dealkylation activity, found to be decreased in liver microsomes by CsA treatment [9–11], were measured.

The results of the present study show that, like CsA, the immunosuppressant FK-506 and its analog FK-520 inhibit the hepatic cytochrome P450 metabolizing system of rats resulting in impairment of the metabolism of FK-506 itself. *In vitro*, FK-506 and FK-520 inhibited the benzphetamine *N*-demethylase activity of rat as well as human liver microsomes.

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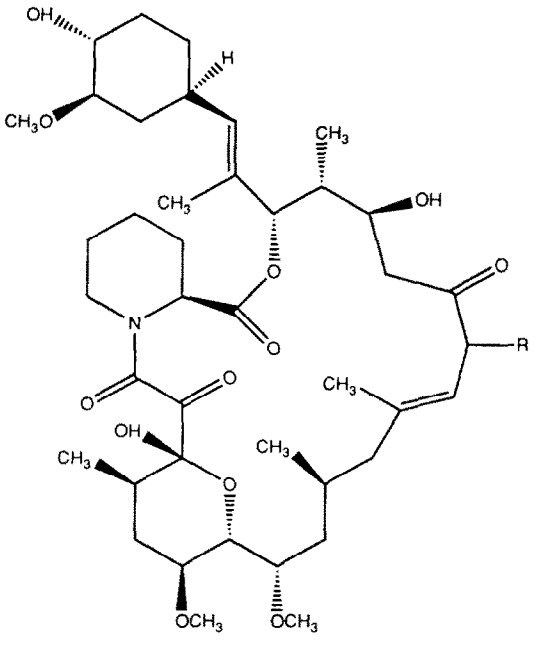
MATERIALS AND METHODS

Chemicals. FK-506 (98% pure) and FK-520 (95% pure) were isolated by Dr. G. Salituro at Merck Sharp & Dohme Research Laboratories from the fermentation broth of *Streptomyces tsukubaensis* supplied by the Fujisawa Pharmaceutical Co. (Ibaraki, Japan) through the Danish Patent Office. [^{14}C]FK-506 (96.5% pure) was isolated from fermentation medium enriched in [^{14}C]propionate by Dr. R. Ellsworth and S. O'Connor and purified by Dr. A. Jones at Merck Sharp & Dohme Research Laboratories. Cyclosporin A was purchased as Sandimmune (Sandoz, East Hanover, NJ). Cremophor was obtained from BASF (Parsippany, NJ). [*N*-methyl-^{14}C]Benzphetamine was synthesized by Dr. H. Mertel at Merck Sharp & Dohme Research Laboratories. Semicarbazide hydrochloride and all other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

Animals and drug administration. Male Sprague–Dawley rats weighing 230–250 g were divided into groups of six and were dosed s.c. daily for 6 days, unless otherwise specified, with one of the following compounds: CsA at 25 mg/kg of body weight injected as Sandimmune; FK-506 or FK-520 at 5 or 10 mg/kg injected as solutions in PEG-400 containing 3.5% ethanol. Four of the six rats dosed with FK-506 at 10 mg/kg died after the fifth dose. The remaining two rats did not receive the sixth dose. Control groups of four rats each received six daily s.c. injections of vehicle: Controls 1 and 3, 0.5 and 1.0 mL/kg of PEG-400 containing 3.5% ethanol respectively; Control 4, 0.5 mL/kg of 15% cremophor (the major vehicle in Sandimmune) diluted in normal saline. Another group of six rats received a single s.c. dose of 25 mg/kg FK-506. Control rats for this



FK-506 R=allyl
FK-520 R= ethyl

Fig. 1. Chemical structures of FK-506 and FK-520.

treatment (Control 2) were injected once with PEG-400 containing 20% ethanol.

Preparation of microsomes. Rats were killed 24 hr after the last injection by decapitation under CO_2 anesthesia. Livers and kidneys were excised, rinsed and weighed. Liver and renal cortex tissues from two rats were homogenized together so that in a group of six rats three different sets of liver and kidney microsomal preparations were made. In the FK-506 10 mg/kg group, tissues from the surviving two rats were homogenized separately. Livers from several untreated male Sprague–Dawley rats were used for the preparation of control liver microsomes. Human liver microsomes were prepared from frozen tissue.

Tissue homogenates were prepared in 50 mM Tris buffer, pH 7.5, containing 1.15% KCl and were centrifuged at 9000 *g* for 20 min at 4°. Supernatants were decanted and centrifuged at 100,000 *g* for 1 hr. Microsomal pellets were washed once with 10 mM EDTA, 1.15% KCl and resuspended in 10 mM KPO_4 buffer containing 0.25 M sucrose. Protein concentration was determined by the method of Lowry *et al.* [13] using bovine serum albumin (BSA) standards.

Quantitation of cytochrome P450 and NADPH–cytochrome P450 reductase. The cytochrome P450 content of liver and renal cortex microsomes was determined by the method of Omura and Sato [14]. NADPH–cytochrome P450 reductase was assayed by following the NADPH-dependent reduction of cytochrome *c* at 550 nm [15]. The activity (units/mg) was calculated assuming 1 unit of enzyme activity produces a change in absorption of 0.021/min.

The *in vitro* effects of FK-506, FK-520 and CsA on reductase activity were investigated using liver microsomes from untreated rats.

Binding spectra. The interaction of FK-506 with cytochrome P450 was investigated according to Schenkman *et al.* [16], by measuring the difference spectrum (350–550 nm) between a test cuvette containing 2.5 mg/mL of control rat liver microsomes and up to 300 μM FK-506 (in 100 μL ethanol) and a reference cuvette containing the microsomes and ethanol.

Benzphetamine N-demethylase activity. This was measured using the procedure of Thomas *et al.* [17]. The activity in microsomes from treated rats was determined by incubating 1 mM *d*-[*N*-methyl-^{14}C]benzphetamine at 37° for 10 min with microsomal protein (liver, 1 mg/mL; kidney, 3 mg/mL) in the presence of an NADPH-generating system. The incubation mixtures contained 4 mM semicarbazide hydrochloride for trapping *in situ* the [^{14}C]formaldehyde produced. The rate of benzphetamine metabolism (nmol/min/mg protein) was then determined by liquid scintillation counting of the [^{14}C]formaldehyde semicarbazone. The *in vitro* inhibitory effect was studied at 0–250 μM FK-506, FK-520 or CsA, using 1 mg/mL of human liver microsomes or liver microsomes from untreated rats and 0.1 mM [^{14}C]benzphetamine. The three inhibitors were added as ethanolic solutions; the final ethanol concentration was ≤2% and it had no effect on the activity. Results are expressed as percent inhibition relative to control incubation mixtures which contained everything (including 2% ethanol) except the inhibitors.

Ethoxycoumarin O-deethylase activity. The O-deethylation of ethoxycoumarin was measured fluorometrically by following the formation of 7-hydroxycoumarin according to the method of Ullrich and Weber [18] with some modifications [19]. Ethoxycoumarin (0.25 mM) was incubated at 37° for 10 min with liver and kidney microsomes (0.5 and 2.0 mg/mL protein respectively). The resulting 7-hydroxycoumarin was quantitated using a standard curve.

In vitro *metabolism of* [^{14}C]*FK-506.* [^{14}C]FK-506 (10 μM; sp. act. 3000 dpm/μg) was incubated in the presence of an NADPH-generating system at 37° for 30 min with liver microsomes (1 mg/mL microsomal protein) from FK-506-, FK-520- and CsA-treated rats. Ethyl acetate plus methanol extracts (recovery of radioactivity 95–100%) of 2-mL incubation mixtures were analyzed by reverse phase-HPLC on a Zorbax ODS column (50:50 acetonitrile:water for 10 min followed by a 30-min gradient to 100% acetonitrile. One-minute fractions were assayed for radioactivity by liquid scintillation counting. The percent metabolism was estimated as (96.5% − % FK-506) where 96.5% is the purity of [^{14}C]FK-506 and the percent FK-506 is the percent of radioactivity that elutes with the same HPLC retention time as [^{14}C]FK-506 standard.

RESULTS

In vivo *effects on hepatic and renal cytochrome*

Table 1. Cytochrome P450 levels in liver and kidney microsomes from rats treated with FK-506, FK-520, or cyclosporin A*

Treatment	Cytochrome P450 (nmol/mg protein) Liver	Kidney
FK-506, 5 mg/kg × 6 days	0.49 ± 0.10	0.10 ± 0.02
FK-520, 5 mg/kg × 6 days	0.60 ± 0.17	0.08 ± 0.01
Control 1	0.78 ± 0.34	0.12 ± 0.03
FK-506, 25 mg/kg × 1 day	0.73 ± 0.20	0.11 ± 0.02
Control 2	0.76 ± 0.21	0.09 ± 0.03
FK-506, 10 mg/kg × 5 days	0.32†	0.12
FK-520, 10 mg/kg × 6 days	0.25 ± 0.04†	0.11 ± 0.01
Control 3	0.54 ± 0.16	0.13 ± 0.01
Cyclosporin A, 25 mg/kg × 6 days	0.51 ± 0.06	0.15 ± 0.02
Control 4	0.51 ± 0.04	0.09 ± 0.04

* Male Sprague–Dawley rats (four to six rats per treatment group) were dosed s.c. and killed 24 hr after the last injection. Liver and kidney microsomes were prepared from fresh tissue and analyzed for protein and cytochrome P450 content. Mean values are listed (±SD when three preparations were analyzed).

† $P < 0.05$ compared to corresponding control (Student's two-sided *t*-test).

Table 2. NADPH–cytochrome P450 reductase activity of liver and kidney microsomes from rats treated with FK-506, FK-520 or cyclosporin A*

Treatment	NADPH–cytochrome P450 reductase (units/mg protein) Liver	Kidney
FK-506, 5 mg/kg × 6 days	89.7 ± 15.2†	29.3 ± 4.6
FK-520, 5 mg/kg × 6 days	140.5 ± 40.7	29.5 ± 4.9
Control 1	182.0 ± 72.1	45.5 ± 7.8
FK-506, 25 mg/kg × 1 day	146.0 ± 31.4	48.3 ± 5.7
Control 2	175.5 ± 30.4	49.5 ± 12.0
FK-506, 10 mg/kg × 5 days	114.2	40.4
FK-520, 10 mg/kg × 6 days	103.7 ± 22.2†	41.0 ± 0.8
Control 3	178.1 ± 52.6	59.3 ± 6.7
Cyclosporin A, 25 mg/kg × 6 days	108.7 ± 23.4†	52.9 ± 4.7
Control 4	209.4 ± 12.5	60.2 ± 7.0

* Experimental conditions were as described in Table 1. Mean values are listed (±SD when three preparations were analyzed).

† $P < 0.05$ compared to corresponding control (Student's two-sided *t*-test).

P450-dependent mixed-function oxidase in the rat. Repeated administration of the immunosuppressant FK-506 and its analog FK-520 to rats decreased the total cytochrome P450 content of liver microsomes (Table 1) as well as their NADPH–cytochrome P450 reductase (Table 2), benzphetamine *N*-demethylase (Table 3), and ethoxycoumarin *O*-deethylase (Table 4) activities by 30–80%. While CsA treatment did not change the total cytochrome P450 content of liver microsomes at the dose tested (Table 1), significant decreases in the NADPH–cytochrome P450 reductase (Table 2) and benzphetamine *N*-demethylase (Table 3) activities were observed. These findings with CsA, as well as the 67% increase it caused in renal cytochrome P450 (Table 1), are in agreement with reports of others [9, 10, 11]. Unlike CsA, FK-506 and FK-520 did not increase the total cytochrome P450 content of renal microsomes.

At a single dose of 25 mg/kg, FK-506 had no effect on the hepatic and renal cytochrome P450 enzyme system. It was also well tolerated, unlike the smaller multiple doses which caused several deaths, with four of six rats at 10 mg/kg and one of six rats at 5 mg/kg dying after the fifth dose. Of the six rats on CsA, one died after the sixth dose, whereas all the rats on FK-520 survived the treatments.

Metabolism of [^{14}C]FK-506 by liver microsomes from treated rats. The observation that the liver microsomal cytochrome P450 enzyme system of rats that received multiple doses of FK-506, FK-520 and CsA was inhibited markedly prompted us to examine the ability of the microsomes from these animals to metabolize FK-506 itself which is metabolized by cytochrome P450 to a complex mixture of metabolites (as shown in Fig. 2B). Not surprisingly, the NADPH-dependent metabolism of FK-506 by liver microsomes from rats that received multiple doses of FK-506, FK-520 and CsA was decreased. As shown in Table 5, a 48–69% decrease was observed, when compared

Table 3. Benzphetamine *N*-demethylase activity of liver and kidney microsomes isolated from rats treated with FK-506, FK-520 or cyclosporin A*

Treatment	Benzphetamine *N*-demethylase (nmol/min/mg protein) Liver	Kidney
FK-506, 5 mg/kg × 6 days	1.04 ± 0.17†	0.05 ± 0.02†
FK-520, 5 mg/kg × 6 days	2.51 ± 0.70	0.08 ± 0.01
Control 1	4.20 ± 2.02	0.09 ± 0.02
FK-506, 25 mg/kg × 1 day	3.66 ± 1.05	0.10 ± 0.02
Control 2	4.32 ± 0.76	0.08 ± 0.04
FK-506, 10 mg/kg × 5 days	0.55†	0.09
FK-520, 10 mg/kg × 6 days	1.11 ± 0.04†	0.08 ± 0.02
Control 3	2.77 ± 1.46	0.11 ± 0.02
Cyclosporin A, 25 mg/kg × 6 days	0.67 ± 0.17†	0.12 ± 0.04
Control 4	3.43 ± 0.01	0.14 ± 0.05

* Experimental conditions were as described in Table 1. Mean values are listed (±SD when three preparations were analyzed).
† $P < 0.05$ compared to corresponding control (Student's two-sided *t*-test).

Table 4. Ethoxycoumarin *O*-deethylase activity of microsomes from rats treated with FK-506 or FK-520*

Treatment	Ethoxycoumarin *O*-deethylase (nmol/min/mg protein) Liver	Kidney
FK-506, 5 mg/kg × 6 days	0.72 ± 0.22†	0.05 ± 0.02
FK-520, 5 mg/kg × 6 days	0.99 ± 0.32	0.08 ± 0.02
Control 1	1.50 ± 0.31	0.03 ± 0.003
FK-506, 25 mg/kg × 1 day	1.60 ± 0.10†	0.03 ± 0.004
Control 2	2.32 ± 0.55	0.03 ± 0.0001

* Experimental conditions were as described in Table 1. Mean values are listed (±SD when three preparations were analyzed).
† $P < 0.05$ compared to corresponding control (Student's two-sided *t*-test).

Table 5. [^{14}C]FK-506 metabolism by liver microsomes from rats treated with FK-506, FK-520 or cyclosporin A

Treatment	% Metabolism	% Inhibition
FK-506		
10 mg/kg × 5 days	24.8	54
25 mg/kg × 1 day	53.7	0
FK-520		
10 mg/kg × 6 days	28.0	48
Cyclosporin A		
25 mg/kg × 6 days	16.7	69

[^{14}C]FK-506 (10 μM, 1.08 μCi/μmol) was incubated at 37° for 30 min with microsomes (1 mg/mL microsomal protein) in the presence of an NADPH-generating system. Ethyl acetate plus methanol extracts were assayed by HPLC as described in Fig. 2. The results are expressed as percent metabolism and percent inhibition relative to the metabolism observed with microsomes from untreated rats. No inhibition corresponded to 53% metabolism or 5.3 nmol/mg protein/30 min.

with the metabolism by microsomes from control (untreated and vehicle-treated) rats. Representative profiles for the metabolism observed with microsomes from untreated and FK-506-treated rats are shown in Fig. 2. Microsomes from rats that received the single 25 mg/kg dose of FK-506 metabolized [^{14}C]FK-506 to the same extent as microsomes from control rats.

In vitro *effect on benzphetamine N-demethylation by rat and human liver microsomes and ethoxycoumarin O-deethylation by rat liver microsomes.* The N-demethylation of benzphetamine by control rat liver microsomes was inhibited by various concentrations of FK-506, FK-520 and CsA, as shown in Fig. 3 (top panel). At 50–250 μM, FK-506 and FK-520 were better inhibitors than CsA producing 20–30% inhibition at 100 μM and 70% at 250 μM. As shown in Fig. 3 (middle and bottom panels), FK-506 and FK-520 also inhibited the activity of liver microsomes from male and female humans (CsA was not included in this experiment).

FK-506 and FK-520 inhibited the ethoxycoumarin *O*-deethylase activity of liver microsomes from untreated rats by 30% at 20 μM, the highest concentration tested. In these experiments FK-506

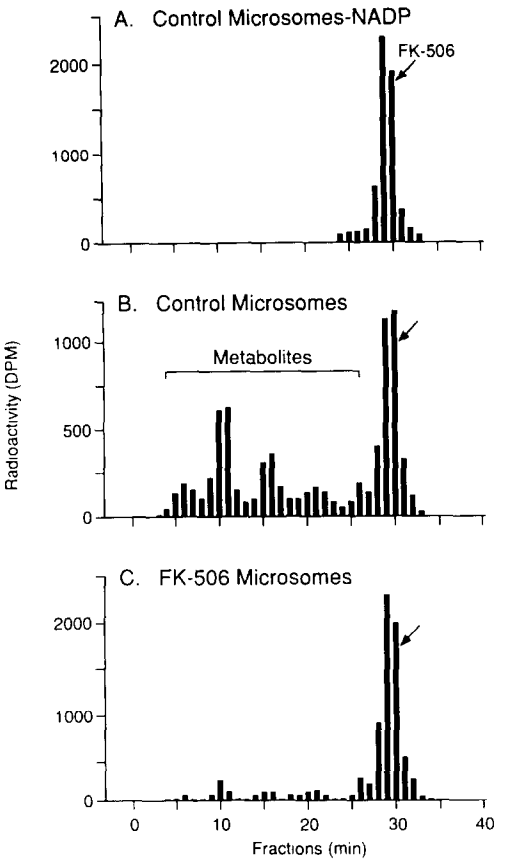


Fig. 2. HPLC profiles of radioactivity from incubation mixtures of [^{14}C]FK-506 with liver microsomes from untreated rats without (A) or with (B) NADP and from FK-506-treated rats with NADP (C). Incubations were at 37° for 30 min with 10 μM [^{14}C]FK-506 (sp. act. 1.08 μCi/μmol) and 1 mg/mL microsomal protein. Ethyl acetate plus methanol extracts were chromatographed on a Zorbax ODS column (0–10 min, acetonitrile:water 50:50; 10–40 min, linear gradient to 100% acetonitrile). One-minute fractions were collected and assayed for radioactivity by liquid scintillation counting. Radioactivity eluting between 24 and 34 min was considered as FK-506.

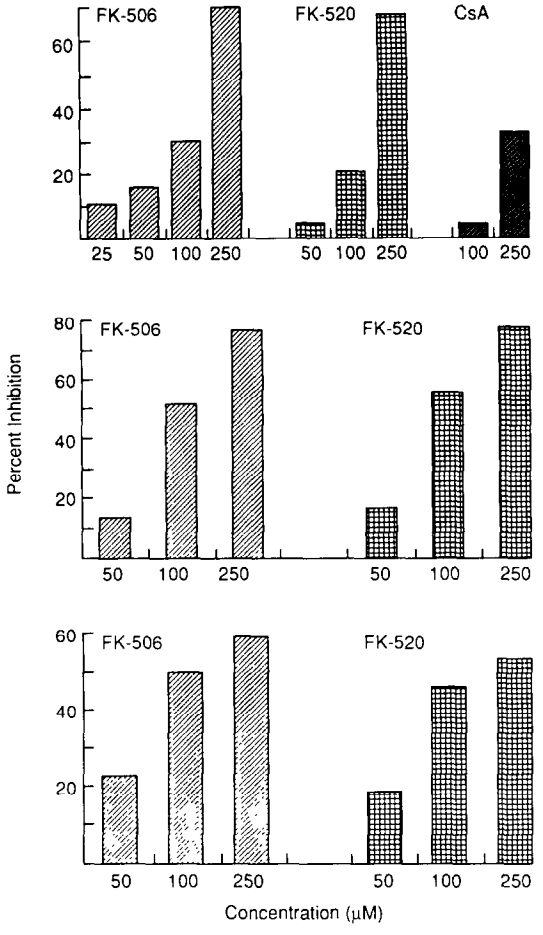


Fig. 3. *In vitro* inhibition of benzphetamine *N*-demethylase of rat and human liver microsomes by FK-506, FK-520 and cyclosporin A (CsA). Liver microsomes from untreated male Sprague–Dawley rats (top panel), a 59-yr-old male (middle panel), and a 21-yr-old female (bottom panel) were incubated at 1 mg/mL of microsomal protein with 0.1 mM [*N*-methyl-^{14}C]benzphetamine at 37° for 10 min in the presence of an NADPH-generating system and 0–250 μM FK-506, FK-520 or CsA. Benzphetamine metabolism was assessed by scintillation counting of [^{14}C]formaldehyde trapped as the semicarbazone. The results, expressed as percent inhibition, are the means of 2–4 separate determinations. No inhibition corresponded to a metabolism of 0.18 nmol/min/mg protein.

and FK-520 were added as methanolic solutions and the inhibition was expressed relative to methanol controls. At 1.6%, the highest amount used, methanol caused 26% inhibition. Other organic solvents, such as ethanol and propanol, caused greater inhibition. These solvents had no effect on benzphetamine N-demethylation.

Binding spectra and effect on NADPH–cytochrome P450 reductase. Addition of up to 300 μM FK-506 to control rat liver microsomes did not produce significant changes in the cytochrome P450 spectrum, as determined by difference spectroscopy. In addition, none of the three compounds had any direct effect on the NADPH–cytochrome P450 reductase of control rat liver microsomes.

DISCUSSION

FK-506 is a promising new immunosuppressant which has already been shown to be effective in liver, kidney and pancreas transplant patients with limited side-effects [7]. In the present study, FK-506 was shown to inhibit the cytochrome P450-dependent mixed-function oxidase after repeated administration to rats, resulting in inhibition of its own metabolism. *In vitro*, it was found to inhibit benzphetamine *N*-demethylase activity of human liver microsomes to the same extent as that of control rat microsomes.

These results indicate that FK-506 has the potential of inhibiting drug metabolism and as such it should be administered with caution during chronic treatment of transplant patients. Inhibition of its own metabolism as well as that of other drugs, as it is shown here to occur in rats, would lead to drug accumulation and drug-related toxicities.

The mechanism(s) by which FK-506 and its analog FK-520 inhibit the cytochrome P450-dependent mixed-function oxidase of the rat is beyond the scope of this study. However, it is interesting to note the following: (1) repeated administration of the compounds is needed (FK-506 had minimal effects after a single high dose), (2) NADPH–cytochrome P450 reductase, whose activity was greatly reduced after *in vivo* administration, was not inhibited *in vitro* by these compounds, and (3) CsA was reported recently to inhibit protein synthesis in rat hepatocytes [20]. These phenomena suggest a possible mechanism for the *in vivo* effects of CsA and perhaps also FK-506 and FK-520 on the cytochrome P450 enzyme system. It is also interesting to note that FK-506 and FK-520, unlike CsA, did not increase the cytochrome P450 levels of renal microsomes.

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