DISPOSITION OF L-732,531, A POTENT IMMUNOSUPPRESSANT, IN RATS AND BABOONS


Department of Drug Metabolism (B.V.K., R.R.M., A.C., Y.S.T., M.L., R.A.S., S.-H.L.C., S.H.V.) and Laboratory Animal Resources (T.M.), Merck Research Laboratories, and the Southwest Foundation for Biomedical Research (K.D.C., T.H.)

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ABSTRACT:

L-732,531 is a semi-synthetic analog of the macrolide tacrolimus (Prograf®). Like tacrolimus, L-732,531 is a potent immunosuppressant. In this study, its absorption, distribution, metabolism, and excretion were studied in rats and baboons. In rats, its blood and plasma levels were similar, whereas in baboons, its blood levels were, on average, twice as high as those in plasma. This was consistent with the in vitro blood-to-plasma ratio of L-732,531, which in these two species, as well as in humans, was much lower than that of tacrolimus and showed a minimal concentration dependence. After iv administration to rats, the blood and plasma clearance of L-732,531 decreased from 60 ml/min/kg at 0.2 mg/kg to 30 ml/min/kg when dosed at 1 and 3 mg/kg. After oral administration, plasma area under the concentration vs. time curve (AUC) and maximal plasma concentration (C_{max}) increased more than proportionally to the dose. At 1, 5, and 15 mg/kg, plasma AUC was 29, 466, and 2832 ng/hr/ml, respectively, and C_{max} was 10, 129, and 304 ng/ml, respectively. Bioavailability, although compromised by nonlinear kinetics, was estimated to be between 8% and 18%.

In baboons, the clearance of L-732,531 was lower than that in rats, especially when calculated from blood concentrations (12 ml/min/kg at 0.2 mg/kg and 8 ml/min/kg at 1 mg/kg). After oral dosing, baboon plasma AUC and C_{max} were much lower than those in rats, but as in rats, they increased more than proportionally with increasing doses. The bioavailability of L-732,531 in baboons was estimated at 3%, 9%, and 24% when animals were dosed at 5, 15, and 26 mg/kg po, respectively. After oral administration of [3H]L-732,531 at 5 mg/kg, ~32% of the radioactivity was recovered in bile and urine of rats, compared with 9% in baboons. High-performance liquid chromatography profiles of rat and baboon plasma, bile, urine, and feces indicated that L-732,531 was metabolized extensively to a complex mixture of products. Some intact parent drug was observed in feces of orally dosed animals, indicating incomplete absorption. In vitro, L-732,531 was metabolized more extensively by baboon liver microsomes than rat or human microsomes. Its metabolism in human liver microsomes was shown to be catalyzed primarily by cytochrome P450 3A isoforms.

L-732,531 (fig. 1) is a semi-synthetic C_{32}-[1-(2-hydroxyethyl)-indol-5-yl]oxy derivative of the macrolide ascomycin (fig. 1), which is produced from a mutant of the microorganism Streptomyces hygroscopicus var. ascomycticus (MA6678). L-732,531 is a potent inhibitor of T cell proliferation with a mechanism of action similar to that of tacrolimus (Prograf®, Fujisawa USA Inc., Deerfield, IL; fig. 1) and cyclosporine (Peterson et al., 1998; Dumont et al., 1998). In the present studies, the absorption, distribution, metabolism, and excretion of L-732,531 were investigated in male rats and baboons, which were the two species used in the toxicological studies. The baboon was chosen as the primate species for toxicological studies because it was reported that the toxicity of tacrolimus in this species resembles that of cyclosporine. In vitro toxicity in humans (Ohara et al., 1996) in carbon tetrachloride was stirred at ~20°C with sodium borotritide and plasma concentrations in the pharmacokinetic studies in rats and baboons.

Materials and Methods

Chemicals. L-732,531 (fig. 1) was prepared at Merck Research Laboratories (Rahway, NJ) by chemical derivatization of ascomycin, as described (Sinclair et al., 1996), and was 93%–97% pure, as determined by high-performance liquid chromatography (HPLC<sup>1</sup>). Radiolabeled compound was synthesized with [3H] incorporated at C<sub>32</sub> as indicated in fig. 1. Tacrolimus and L-736,054 (fig. 1), used as internal standards in the LC-MS/MS assay, were prepared at Merck Research Laboratories by fermentation and chemical derivatization of ascomycin, respectively. Troleandomycin, enoxacin, quinidine, and 4-methylpyrazole were purchased from Sigma Chemical Co. (St. Louis, MO). Gestodene and sulfaphenazole were gifts from Dr. F. P. Guengerich (Vanderbilt University, Nashville, TN). Bufuralol was purchased from Gentest Corp. (Woburn, MA) and ketocazole from Research Diagnostics Inc. (Flanders, NJ).

Synthesis of [3H]L-732,531. A solution of 32-keto ascomycin (Sinclair et al., 1996) in carbon tetrachloride was stirred at ~20°C with sodium borotritide

<sup>1</sup>Abbreviations used are: HPLC, high-performance liquid chromatography; CYP or P450, cytochrome P450; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MS, mass spectrosopy; C_{max}, maximal plasma concentration; AUC, area under the concentration vs. time curve; FKBP12, FK506-binding protein; TFA, trifluoroacetic acid.
Liver microsomes were prepared by standard differential centrifugation methods from fresh rat and frozen human and baboon livers. Human liver microsomes were obtained from Dr. Judy Raucy (University of New Mexico, Albuquerque, NM). They were prepared from liver obtained from a 14-year-old boy with no drug history. Microsomes containing recombinant P450 isoforms were purchased from Gentest Corp. (Woburn, MA). Microsomes were prepared from metabolically competent derivatives of the human AHH-1 TK+/− cell line. The cells were transfected with cDNAs encoding human CYP1A1, CYP1A2, CYP2B6, CYP2D6, CYP2E1, and CYP3A4. Control cell lines did not contain any vector. The approximate specific activity of the catalytic assay for each of these microsomes were reported by the supplier to be as follows: CYP1A1, 7-ethoxyresorufin deethylase, 120 pmol/min/mg; CYP1A2, 7-ethoxyresorufin deethylase, 80 pmol/min/mg; CYP2B6, 7-ethoxy-4-trifluoromethylcoumarin deethylase, 400 pmol/min/mg; CYP2D6, bufuralol 1'-hydroxylase, 900 pmol/min/mg; CYP2E1, chlorozoxazone 6-hydroxylase, 100 pmol/min/mg; CYP3A4, testosterone 6β-hydroxylase, 500 pmol/min/mg; control, 7-ethoxycoumarin deethylase, 2 pmol/min/mg. These microsomes contained the necessary cytochrome P450 reductase. Therefore, incubations were supplied only with appropriate cofactors (NADPH or an NADPH-regenerating system) for activity measurements.

**Dosing Solutions.** For iv administration, L-732,531 was dissolved in polyethylene glycol 400 with 2%–4% ethanol and administered to animals at 0.2–1 ml/kg. The iv dose for the baboon studies was sterilized by filtration through a 0.2–µm filter. The oral dosing solutions for the tissue distribution, metabolism, and excretion studies in rats were 2–3 mg/ml suspensions of [3H]-L-732,531 in 0.5% methocel:ethanol (98/2). For po administration in all other studies (pharmacokinetic and biliary excretion studies in rats and baboons and biliary excretion studies in rats), the compound was dissolved at 2–4.5 mg/ml in polyethylene glycol 400, Tween 80, 10 mM citrate, pH 5.0 (5/5/90). The specific activities of the radioactive doses were determined by combustion or direct counting of two to three aliquots of each dosing solution and dividing by the amount of drug in the aliquot combusted or counted. For stability reasons, dosing solutions were either prepared fresh (rat studies) or transported over dry ice and stored at −20°C until use (baboon studies).

**Animal Studies.** All experiments were performed under protocols approved by the Institutional Animal Care and Use Committees, Merck Research Laboratories (Rahway, NJ, and West Point, PA).

**Rats.** Male Sprague-Dawley rats weighing ~160–220 g (Charles River Laboratories, Wilmington, MA) were used. They were housed under standard conditions and maintained under a 12-hr light/dark cycle with free access to food and water. Before oral dosing, animals were fasted (water ad libitum) overnight and for 4 hr after dosing. Oral dosing in all studies was by gavage, and iv dosing was via the femoral vein.

**Pharmacokinetic Studies.** Rats were dosed at 0.2, 0.3, 1, and 3 mg/kg iv and at 1.5, and 15 mg/kg po. Blood (~4 ml) was obtained by heart puncture under ether anesthesia from three rats at different time points between 5 min and 24 hr (0.2 and 1 mg/kg iv doses and all oral doses) or from a previously implanted cannula in the femoral artery (0.3 and 3 mg/kg iv doses). Plasma was obtained by centrifugation at room temperature after incubation at 37°C for 30 min and stored at −20°C until analyzed by LC-MS/MS using tacrolimus or L-736,054 as the internal standard.

**Tissue Distribution and Mass Balance Studies.** Rats were dosed with [3H]-L-732,531 (specific activity, 41–48 Ci/mg) at 2 mg/kg iv and 5 mg/kg po. Blood was obtained by cardiac puncture under ether anesthesia from three rats each at different time points between 5 min and 24 hr, and the rats were euthanized by cervical dislocation and pneumothorax, if needed. Tissues were removed at 5 min (iv only), 30 min (po only), and 2, 6, and 24 hr after administration, and all tissues, except adrenal glands, were homogenized with water. The adrenal glands, aliquots of the tissue homogenates, blood, and plasma were combusted for total radioactivity content.

To study excretion, rats were dosed with [3H]-L-732,531 at 2 mg/kg iv (specific activity, ~12 Ci/mg) or at 5 mg/kg po (specific activity, ~10 Ci/mg). Urine and feces were collected at 24-hr intervals for 3 days. Radioactivity in urine and aqueous fecal homogenates (1:5) was determined by combustion. Aliquots of the fecal homogenates were analyzed by HPLC for percentage of parent.

**Biliary Excretion.** Rats were implanted with catheters in the bile duct under pentobarbital anesthesia (50 mg/kg ip) 1 day before they were dosed with [3H]-L-732,531 (specific activity, ~8 Ci/mg) at 5 mg/kg po. Bile was collected at 1-hr intervals for the first 8 hr and at 8–24, 24–48, and 48–72 hr. Urine and feces were collected every 24 hr for 3 days. Radioactivity in bile and urine was determined by direct counting. Radioactivity in feces was determined by combustion of triplicate aliquots of 1:8 aqueous homogenates. Results were expressed as percentage of dose. Aliquots of fecal homogenates, urine, and bile were analyzed by HPLC for percentage of parent.

**Baboons.** The in-life phase of the iv pharmacokinetic study was conducted at Merck Research Laboratories (West Point, PA). All other studies were carried out at the Southwest Foundation (San Antonio, TX). Venous access was through the jugular vein. The animals were fasted overnight before iv and po dosing and for 8 hr after dosing (water ad libitum). Before dosing, the animals were sedated with ketamine (5 mg/kg iv), and they were generally awakened within 20–30 min. The iv dose was administered by a 2-min infusion.
into an indwelling catheter in the saphenous vein; the oral dose was given via an oral tube. There was a 3- to 4-week washout period between each dosing period. The doses used were 0.2 and 1 mg/kg iv, and 5, 16, and 26 mg/kg po. Blood (3-4 ml) was collected at selected time points from the contralateral saphenous vein (iv study) or from an implanted catheter in the iliac vein (oral study) into heparinized tubes. Plasma was obtained by centrifugation at room temperature after incubation of blood at 37°C for 30 min.

To study excretion, urine and feces were collected at 24-hr intervals from baboons dosed with [1H]-L-732,531 at 0.2 mg/kg iv and 1 mg/kg po. Radioactivity in feces (1:6 homogenates) and urine from the first 3 days was determined by combustion and direct counting, respectively.

**Blood-to-Plasma Ratio.** The *in vitro* distribution of L-732,531 between blood cells and plasma was studied by incubating different concentrations of [1H]-L-732,531 (specific activity, 0.1 mCi/mg) with fresh, heparinized rat, baboon, and human blood at 4°C, room temperature, or 37°C, for different periods of time, followed by centrifugation at 4°C, room temperature, or 37°C. The data were expressed as the ratio of radioactivity, determined by combustion, in equal volumes of blood and plasma, referred to as the blood-to-plasma ratio. The blood-to-plasma ratio of L-732,531 in baboons was determined using blood from control baboons as well as blood from the tethered baboons used in the *in vivo* studies, with essentially identical results.

**In Vitro Metabolism.** Incubations of [1H]-L-732,531 (10 µM) with liver microsomes (0.2–2 mg microsomal protein/ml) from humans, and from control and dexamethasone-induced rats, were carried out for 5–60 min in the presence of an NADPH-regenerating system. The incubation mixtures were quenched with three volumes of acetonitrile and the supernatants were evaporated to dryness under N₂. The residue was reconstituted in acetonitrile:water (50:50) and analyzed by HPLC using method 2 (rat and rabbit microsomal incubations) or 1 (human microsomal incubations), as described below. For identification of *in vitro* metabolites, liver microsomes (2.5 mg/ml) from dexamethasone-induced rats were incubated with 25 µM L-732,531 for 30 min in the presence of an NADPH-regenerating system at 37°C. The incubation mixtures were extracted with ethyl acetate, and the extracts were evaporated under N₂. The evaporated extracts were reconstituted in acetonitrile:water (1/1) and analyzed by HPLC using method 3.

**Identification of the Cytochrome P450 Isoenzyme Responsible for the *In Vitro* Metabolism of L-732,531 in Human Liver Microsomes.** Incubations of [1H]-L-732,531 (10 µM) were carried out at 37°C with human liver microsomes for 30 min in the absence or presence of cytochrome P450 inhibitors/substrates: ketoconazole for CYP3A (Maurice et al., 1992), enoxacin for CYP1A2 (Sarkar et al., 1990), 4-methylpyrazole for CYP2E1 (Feierman and Cederbaum, 1989), sulfaphenazole for CYP2C8/9 (Guengerich and Shimada, 1991), andbufuralol and quinidine for CYP2D6 (Zanger et al., 1988). The CYP3A4 mechanism-based inhibitors, troleandomycin (Guengerich, 1990) and gestodene (Pessayre et al., 1981), were preincubated with human liver microsomes for 30 min in the presence of an NADPH-regenerating system, [1H]-L-732,531 was added, and the incubation continued for an additional 30 min. [1H]-L-732,531 (10 µM) was incubated with microsomes containing recombinant CYP1A1, CYP1A2, CYP2B6, CYP2D6, CYP2E1, and CYP3A4 for 1 hr in the presence of an NADPH-regenerating system.

**Plasma Protein Binding.** Binding of [1H]-L-732,531 to rat, baboon, rhesus monkey, and human plasma proteins was determined by ultrafiltration. [1H]-L-732,531 (400 000 dpm, 0.1 µg) dissolved in ethanol was mixed with plasma and unlabeled L-732,531 at different total concentrations ranging from 0.1 to 10 µg/ml (rat, baboon, and human plasma) or 50 µg/ml (rhesus monkey plasma). After incubation at 37°C for 30 min, the plasma samples were transferred to Centrifree™ tubes (Amicon Co., Danvers, MA) and equilibrated at room temperature for 15 min. An aliquot from each tube was removed for liquid scintillation counting, and the remaining plasma was centrifuged at room temperature for 30 min. An aliquot of the filtrate was counted, and the unbound fraction was estimated from the ratio of radioactivity in equal volumes of filtrate and plasma.

**Analytical Procedures.** *Pharmacokinetic Calculations.* Established non-compartmental methods were used for calculating the various pharmacokinetic parameters. Blood and plasma AUC values were calculated using the UNICUE program (Yeh et al., 1987) with log-linear interpolation from t=0 to the last time point with plasma levels above the lower limit of quantification. Extrapolation to infinity was accomplished using the elimination rate constant calculated from the terminal phase of the blood or plasma concentration-time curve.

**Radioactivity Measurements.** Radioactivity in blood, plasma, tissue, and fecal homogenates was determined by combustion. Radioactivity in urine, bile, plasma, and plasma filtrate from the *in vitro* protein binding study and HPLC fractions was determined by direct counting in a Beckman LS 5000TD or LS 3801 Liquid Scintillation Spectrometer (Beckman Instruments, Columbia, MD). Quench correction was carried out using an external standard.

**LC-MS/MS Procedure for the Quantitation of L-732,531.** L-732,531 concentrations in rat and baboon blood and plasma were determined by LC-MS/MS. A SCIEX API III tandem mass spectrometer was used, interfaced via a SCIEX heated nebulizer (PE SCIEX, Concord, Ontario, Canada) to an LC system consisting of two Shimadzu LC-600 pumps, an SCL-6B controller, and an SIL-6B autoinjector (Shimadzu Scientific Instruments, Inc., Columbia, MD). The assay was developed using tacrolimus or L-736,054 as the internal standard, with similar results. Multiple reaction monitoring using the precursor/product ion combinations of 968/564, 821/576, and 982/564 were used to quantitate L-732,531, tacrolimus, and L-736,054, respectively. Blood and plasma samples (0.5 or 1 ml) were treated with 20 ng of tacrolimus or L-736,054 as internal standard, diluted with an equal volume of water, and deproteinized with three volumes of acetonitrile. The acetonitrile supernatant was diluted with water and subjected to solid-phase extraction using C18 cartridges (Worldwide Monitoring, Horsham, PA). Elution was achieved using methanol, and the eluates were evaporated to dryness and reconstituted in the mobile phase. Typically, 10–50 µl of the extract was analyzed by LC-MS/MS in the positive ion mode. Chromatography was on a Zorbax SB-CN C8 column (25 cm × 4.6 mm; Mac-Mod, Chadds Ford, PA) at 1 ml/min using acetonitrile:10 or 50 mM ammonium acetate:formic acid (70/30/0.1 v/v); L-732,531 concentrations were calculated using established standard curves of L-732,531 (8–10 concentrations ranging from 0.2 to 500 ng) and the internal standard (20 ng) in rat and baboon blood and plasma. The lower limit of quantitation was 1 ng (1 ng/ml for 1-ml sample or 2 ng/ml for 0.5-ml samples). The data were expressed as the ratio of radioactivity, determined by combustion, in equal volumes of blood and plasma, referred to as the blood-to-plasma ratio.

**HPLC Methods for Metabolite Profiles.** Three HPLC methods were used. In method 1, the HPLC system (Waters, Morrisstown, NJ) consisted of a multi-solvent delivery system (600E), a U6K injector, a photodiode array detector (Raytest-Ramona-LS, Wilmington, DE). Chromatography was performed on a Zorbax ODS column (25 cm × 4.6 mm; Whatman) maintained at 60°C and eluted isocratically with acetonitrile:water:TFA (60:40:0.1). One-minute fractions were collected, mixed with 5 ml Insta-Gel (Packard, Meriden, CT), and counted in a liquid scintillation counter. The HPLC system in method 2 consisted of two pumps (model 400; Applied Biosystems, Ramsey, NJ), a detector/gradient controller (Applied Biosystems, model 783A), and an on-line radioactivity detector (Raytest-Ramona-LS, Wilmington, DE). Chromatography was performed on a Zorbax ODS column (25 cm × 4.6 mm) at ambient temperature eluted at 1 ml/min with a linear gradient from acetonitrile:water (55/45) to 100% acetonitrile in 20 min. The UV absorption of HPLC eluate in both methods was monitored at 210 nm. Method 3 was used in identification of *in vitro* metabolites. Reconstituted extracts of microsomal incubations were chromatographed on a Zorbax ODS column, maintained at 50°C, and eluted with a linear gradient from 50% A (water with 0.1% TFA) to 100% B (acetonitrile with 0.1% TFA) in 50 min. Mass spectra were acquired by LC-MS on a SCIEX API III mass spectrometer (PE SCIEX, Concord, Ontario, Canada), using the heated nebulizer interface.

**Metabolite Profiles.** Aliquots of rat and baboon plasma and fecal homogenates were mixed with one to five volumes of acetonitrile and centrifuged. The supernatant was evaporated to dryness and reconstituted in water:acetonitrile (1:1). Urine and bile were diluted with an equal volume of acetonitrile and analyzed by HPLC method 1.
Results

Pharmacokinetics. Rats. Concentrations of L-732,531 in blood and plasma of iv-dosed rats were similar at all time points (fig. 2). The levels declined in a polyphasic manner with a terminal half-life of 5 hr. Blood and plasma AUC values were similar at both doses examined, 60 and 56 ng hr/ml, respectively, at 0.2 mg/kg and 488 and 506 ng hr/ml, respectively, at 1 mg/kg (table 1). The 8–9-fold increase in AUC was more than proportional to the increase in dose. Hence, blood and plasma clearance values decreased from 56 and 60 ml/min/kg, respectively, at 0.2 mg/kg to 34 and 33 ml/min/kg, respectively, at 1 mg/kg. Curiously, however, blood clearance did not decrease further when the dose was increased from 1 to 3 mg/kg. The volume of distribution of L-732,531 was 8–14 liters/kg at 1 to 3 mg/kg iv. The similarity in the blood and plasma levels and pharmacokinetic parameters of L-732,531 in the rat were consistent with its in vitro blood-to-plasma ratio, which ranged from 0.76 to 0.92 (see below).

Similar concentrations of L-732,531 in blood and plasma also were observed after po administration at 5 mg/kg (fig. 3). Thus only plasma was assayed in subsequent studies at 1 and 5 mg/kg po. Peak plasma concentrations were attained relatively early after the 1 and 5 mg/kg doses ($t_{\text{max}}$ of 0.5 and 1 hr) and at a later time after the 15 mg/kg dose ($t_{\text{max}}$ of 6 hr). The plasma AUC at the 5 mg/kg dose (466 ng hr/ml) was 16-fold higher than that at 1 mg/kg (29 ng hr/ml) and 6-fold lower than the AUC at 15 mg/kg, which was 2832 ng hr/ml (table 2). Because of nonlinear kinetics, bioavailability of the po doses could not be determined accurately. However, it can be inferred that by comparison of the AUC of the 1 and 5 mg/kg po doses with the AUC of the 0.2 and 1 mg/kg iv doses, the bioavailability was 8% and 18%, respectively.

Baboons. The blood-to-plasma ratio of L-732,531 in baboons was slightly higher than that in rats. After both iv and oral administration, concentrations of L-732,531 in blood were on the average 1.5–2 times those in plasma (figs. 4 and 5). The range of the in vivo blood-to-plasma ratio values was similar to that for the in vitro values (see “Blood-to-Plasma Ratio” below).

After iv administration, the clearance of L-732,531 in baboons was slower than that in rats, especially when calculated using blood concentrations. The blood and plasma clearance values were 12 and 24 ml/min/kg, respectively, at 0.2 mg/kg and decreased by 33% and 17%, respectively, to 8 and 20 ml/min/kg, when the dose was increased to 1 mg/kg iv (table 3). The terminal $t_{1/2}$ in baboons, 9–10 hr in plasma and 15–29 hr (mean value 20 hr) in blood, was longer than that in rats.

Blood and plasma concentrations of L-732,531 in baboons dosed orally at 5 mg/kg ($N = 5$), 15 mg/kg ($N = 2$), and 26 mg/kg ($N = 1$) are shown in fig. 5. Both blood and plasma concentrations at the 5-mg/kg dose were much lower than in rats. Plasma $C_{\text{max}}$ and AUC values were 9 ± 3 ng/ml and 72 ± 22 ng hr/ml (table 4) in baboons, as compared with 129 ± 68 ng/ml and 466 ng hr/ml, respectively, in rats. As in rats, the increase in AUC in baboons was more than proportional with dose, 15-fold between 5 mg/kg and 15 mg/kg and 67-fold between 5 mg/kg and 26 mg/kg. For all doses, the concentration vs. time profiles indicated a biphasic absorption phase. After the 5 mg/kg dose, the $C_{\text{max}}$ was reached at 1 hr, after which time there was a drop in L-732,531 levels at 4 hr, followed by an increase in levels up to 6 hr. After the 15 and 26 mg/kg doses, concentrations of L-732,531 exhibited a steady increase up to 1 hr, after which there was a drop in levels at 2 hr, followed by an increase, with the $t_{\text{max}}$
Plasma ratios measured at 5, 10, and 100 ng/ml were 1.17, 1.18, and 1.15, respectively. As was shown for tacrolimus (Beyens et al., 1991; Machida et al., 1991), the blood-to-plasma ratio of L-732,531 tended to be higher at 4°C. At 10 ng/ml, for example, the blood-to-plasma ratio of L-732,531 in humans was 1.65 at 4°C and decreased to 1.15 after incubation of blood at 37°C. Based on these data, it was concluded that precooled blood from dosed animals should be re-equilibrated at 37°C before centrifugation. Centrifugation can be carried out at room temperature, as the ratio was the same as when re-equilibrated blood was centrifuged at 37°C.

The in vivo blood-to-plasma ratio of L-732,531 was similar to that observed in vitro. In rats dosed at 0.2 and 1 mg/kg iv and 5 mg/kg po, the average blood-to-plasma ratios were 1.1, 1.0, and 0.9, respectively, over an 8-hr period, covering a concentration range from 2 to 700 ng/ml. In baboons, the in vivo blood-to-plasma ratio of L-732,531 was higher and more variable than in rats. In three baboons dosed at 0.2 and 1 mg/kg iv, the ratio was 2.0 ± 0.4 over a 24-hr period, covering a concentration range from 18 ng/ml to 10 µg/ml. The ratio in orally dosed baboons appeared to be less variable, at 1.6 ± 0.1 over a concentration range of 2–650 ng/ml.

### Table 4

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>AUC (ng·hr/ml)</th>
<th>Cmax (ng/ml)</th>
<th>tmax (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (N = 5)</td>
<td>72 ± 22</td>
<td>9 ± 3</td>
<td>0.5–6</td>
</tr>
<tr>
<td>15 (N = 2)</td>
<td>1043</td>
<td>121</td>
<td>6–8</td>
</tr>
<tr>
<td>26 (N = 1)</td>
<td>4786</td>
<td>437</td>
<td>6, 8</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (N = 5)</td>
<td>68 ± 28</td>
<td>20 ± 7</td>
<td>0.5–6</td>
</tr>
</tbody>
</table>

Table shows mean or mean ± SD values.

### Table 5

In vivo blood-to-plasma ratio of [3H]L-732,531 in rats, baboons, and humans

<table>
<thead>
<tr>
<th>Concentration in Blood</th>
<th>Rat</th>
<th>Baboon</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>ND*</td>
<td>1.70</td>
<td>1.17 ± 0.02</td>
</tr>
<tr>
<td>0.01</td>
<td>0.92</td>
<td>1.77</td>
<td>1.18 ± 0.03</td>
</tr>
<tr>
<td>0.05</td>
<td>0.85</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.1</td>
<td>0.82</td>
<td>1.58</td>
<td>1.15 ± 0.04</td>
</tr>
<tr>
<td>0.2</td>
<td>ND*</td>
<td>1.46</td>
<td>ND</td>
</tr>
<tr>
<td>0.5</td>
<td>0.8</td>
<td>1.09</td>
<td>ND</td>
</tr>
<tr>
<td>1</td>
<td>0.77</td>
<td>0.80</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>0.77</td>
<td>1.74</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>0.76</td>
<td>1.12</td>
<td>ND</td>
</tr>
<tr>
<td>100</td>
<td>ND*</td>
<td>0.88</td>
<td>ND</td>
</tr>
</tbody>
</table>

Fresh, heparinized rat, baboon and human blood was mixed with [3H]L-732,531 and incubated at 37°C for 1 hr prior to centrifugation at room temperature for 5 min. Radioactivity in blood and plasma was determined by combustion. * ND, not determined.
This was confirmed in a separate experiment, in which rats were dosed orally with [3H]L-732,531 at 2 mg/kg. The radioactivity in all tissues except large intestine amounted to 0.2 mg/kg iv (dose).

Concentrations of total radioactivity in tissues at 24 hr after the 14th dose were not substantially different from the concentrations after the first dose.

Activity remained in the stomach at 2 hr after administration, indicative of slow and variable absorption. Concentrations of radioactivity in most tissues were highest at 6 hr and were lower than after iv dosing.

The tissue-to-plasma ratios were in general similar after iv and oral dosing, and for some tissues, appeared to increase with time. This was especially true for the radioactivity in brain. The brain-to-plasma ratio of radioactivity increased steadily from 0.1 ± 0.02 (mean ± SD, N = 3) at 5 min to 0.2 ± 0.04 at 2 hr, 0.3 ± 0.05 at 6 hr, and 0.9 ± 0.2 at 24 hr after iv dosing.

At 24 hr after both iv and po administration of [3H]L-732,531, total radioactivity in all tissues except large intestine amounted to <5% of the dose, indicating that the potential for L-732,531 accumulation is low. This was confirmed in a separate experiment, in which rats were dosed orally for 14 days with [3H]L-732,531 at 5 mg/kg/day. Concentrations of total radioactivity in tissues at 24 hr after the 14th dose were not substantially different from the concentrations after the first dose.

Mass Balance and Biliary Excretion. Rats. The recovery of radioactivity in urine and feces of rats was determined after dosing at 2 mg/kg iv (N = 3) and 5 mg/kg po (N = 3) with [3H]L-732,531. Most of the radioactivity was excreted in feces (76% [54%–89%] of the iv and 89% [72%–100%] of the po dose) over a 3-day period. Excretion in urine was negligible (0.6%–1.1% of the iv and 0.1%–0.4% of the po dose).

In three bile duct–cannulated rats dosed at 5 mg/kg po with [3H]L-732,531, there was steady excretion of radioactivity into the bile for up to 6 hr. About 31% (17%–47%) of the dose was recovered in bile over a 3-day period, almost entirely as metabolites (see section on “In Vivo Metabolism”). The remainder of the dose (45%–93%) was recovered in feces. As in intact rats, recovery in urine was low (0.7%–1.7% of the dose).

Baboons. After administration of [3H]L-732,531 to three male baboons fitted with cannulae in their gallbladders, about 9% (range, 7%–13%) of a 1-mg/kg po dose was recovered in bile over a 3-day period (table 7). Most of the dose (80% [range, 55%–105%]) was recovered in the feces. After a 0.2-mg/kg iv dose of [3H]L-732,531, 76% (range, 47%–96%) of the radioactivity was recovered in the feces and 0.8%–1.3% in the urine.

In Vivo Metabolism. L-732,531 was the major component in plasma from iv-dosed rats up to 30 min, accounting for 48% of the radioactivity, after which time an increasing proportion of the radioactivity was a result of metabolites, which accounted for 75% at 2–8 hr and 90% at 24 hr. Plasma from rats dosed orally at 5 mg/kg contained mostly metabolites at all time points, with only 10%–40% of the radioactivity due to unchanged drug. HPLC analysis indicated that tissues such as brain, kidneys, spleen, and, to a lesser extent, liver contained a higher percentage of parent drug than did plasma (60%–70% at 2 hr and 30%–70% at 6 hr after iv dosing). Most of the metabolites in plasma and tissues were much more polar than L-732,531, eluting in the void volume of the HPLC column. HPLC analysis showed that feces and urine of iv-dosed rats contained only metabolites, whereas feces of po-dosed rats contained variable amounts of unchanged drug. A substantial amount of the radioactivity in feces from orally dosed rats was due to polar components, indicative of metabolism and/or degradation in the gastrointestinal tract.

In orally dosed baboons, parent compound accounted for 20%–30% of the radioactivity in blood and plasma at 15–30 min after dosing, and for <15% of the radioactivity at subsequent time points. As in rats, radioactivity excreted in baboon feces and bile was primarily composed of polar metabolites.

In Vitro Metabolism and Identification of Metabolites. [3H]L-732,531 was metabolized extensively when incubated with rat, human, baboon, and rhesus monkey liver microsomes, giving qualitatively similar metabolite profiles in all three species. Radiochromatograms from incubations for 60 min and longer times (not shown) contained a higher proportion of the more polar metabolites, similar to the in vivo metabolite profiles. The rate of metabolism of L-732,531, determined by incubating the compound (10 μM) with 0.2–2 mg microsomal protein/ml for 5–30 min, followed the rank order control rat (0.06 mmol/min/mg) < human (0.1 mmol/min/mg) < dexamethasone-induced rat (0.57 mmol/min/mg) < baboon (0.57 mmol/min/mg) < rhesus monkey (2.28 mmol/min/mg).

Information about the structure of the primary metabolites formed in vitro was obtained by LC-MS/MS analysis of ethyl acetate extracts of L-732,531 incubations with liver microsomes. Table 8 lists the characteristic fragment ions of the parent and major metabolites identified from incubations with liver microsomes from dexamethasone-induced rats. A representative ion current chromatogram (from m/z 650 to 1000) and UV chromatogram (220 nm) obtained on-line during analysis are shown in fig. 6. The parent compound (peak 14) has an (M+H)+ at m/z 951, a characteristic (M+NH4)+ at m/z 968.
This ion was used to determine if changes on the molecule have
by scission of the C1-O and C24-25 bonds (see fig. 1 for numbering).

A characteristic ion at m/z 564 is ascribable to the fragment generated
at m/z 564. As shown in table 8, 12 metabolites were identified as resulting from six different transformations on the C1-25 moiety of the molecule (fig. 1), namely 13- or 15-O-demethylation (major biotransformation), 13- and 15-O-di-demethylation, and hydroxylation with or without subsequent oxidation or loss of water. Thus, similar to the parent drug, the metabolites are represented by more than one separate chromatographic entity. Such behavior has been reported for tacrolimus (Nishikawa et al., 1993) and its metabolites (Christians et al., 1991; Chen et al., 1992; Vincent et al., 1992) and is believed to be due to ring opening to form the hemiketal at C-10 (Nishikawa et al., 1993) and, in the case of the 13-O-desmethyl metabolite, subsequent ring closure to form a five-membered ketal (Chen et al., 1992).

Identification of Cytochrome P450 Isozyme Responsible for the In Vivo Metabolism of L-732,531 in Human Liver Microsomes. The in vitro metabolism of L-732,531 in human liver microsomes was catalyzed mainly by cytochrome P450 3A4 with minor involvement by CYP2D6, as measured by substrate disappearance. Of all the cytochrome P450 inhibitors tested, only those of CYP3A4 (troleandomycin at 100 μM, gestodene at 100 μM, and ketoconazole at 1 μM) inhibited the metabolism of L-732,531 by 90%–100%, whereas the CYP2D6 inhibitor quinidine inhibited the metabolism by 16% at 100 μM (fig. 7). These results were further substantiated by incubations carried out with microsomes containing recombinant CYP3A4, which gave a metabolite profile of L-732,531 similar to that obtained with human liver microsomes. Of the other microsomes tested, only recombinant CYP2D6 metabolized L-732,531, but to a much smaller extent than CYP3A4 (6.7% vs. 19% metabolism in 1 hr).

Plasma Protein Binding. L-732,531 was bound extensively (≥99%) to proteins in rat, rhesus monkey, baboon, and human plasma. Binding to plasma proteins was measured by ultrafiltration after incubation of [1H]L-732,531 at 0.1–50 μg/ml. Nonspecific binding to the ultrafiltration unit was not determined in these experiments because of the low aqueous solubility of L-732,531 (<20 ng/ml).

Discussion

The disposition of L-732,531 was studied in rats and baboons after iv and oral dosing. Drug concentrations were determined in both blood and plasma because tacrolimus (Prograf®), a structural analog of L-732,531, is known to distribute preferentially into the cellular component of human blood in a concentration-dependent manner.

### Table 8

<table>
<thead>
<tr>
<th>Peak</th>
<th>[M + H+]⁺</th>
<th>Fragment ions</th>
<th>Mass Difference From Parent</th>
<th>Proposed Biotransformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>923</td>
<td>905, 887, 536</td>
<td>-28</td>
<td>Di-demethylated at 13 and 15 positions</td>
</tr>
<tr>
<td>2</td>
<td>951</td>
<td>933, 915, 564</td>
<td>0</td>
<td>Tautomer of L-732,531</td>
</tr>
<tr>
<td>3</td>
<td>923</td>
<td>905, 887, 536</td>
<td>-28</td>
<td>Didemethylated at 13 and 15 positions</td>
</tr>
<tr>
<td>4</td>
<td>937</td>
<td>919, 901, 550</td>
<td>-14</td>
<td>Demethylated at 13 or 15 position</td>
</tr>
<tr>
<td>5</td>
<td>937</td>
<td>919, 901, 550</td>
<td>-14</td>
<td>Demethylated at 13 or 15 position</td>
</tr>
<tr>
<td>6</td>
<td>967</td>
<td>949, 931, 580</td>
<td>+16</td>
<td>Addition of a hydroxyl group (on C1-25)</td>
</tr>
<tr>
<td>7</td>
<td>965</td>
<td>947, 578</td>
<td>+14</td>
<td>Addition of oxygen with subsequent oxidation (on C1-25)</td>
</tr>
<tr>
<td>8</td>
<td>937</td>
<td>919, 901, 550</td>
<td>-14</td>
<td>Demethylated at 13 or 15 position</td>
</tr>
<tr>
<td>9</td>
<td>965</td>
<td>947, 929, 578</td>
<td>+14</td>
<td>Addition of oxygen with subsequent oxidation (on C1-25)</td>
</tr>
<tr>
<td>10</td>
<td>965</td>
<td>947, 929, 578</td>
<td>+14</td>
<td>Addition of oxygen with subsequent oxidation (on C1-25)</td>
</tr>
<tr>
<td>11</td>
<td>949</td>
<td>931, 913, 562</td>
<td>-2</td>
<td>Desaturated or hydroxylated w/loss of H2O (on C1-25)</td>
</tr>
<tr>
<td>12</td>
<td>937</td>
<td>919, 901, 550</td>
<td>-14</td>
<td>Demethylated at 13 or 15 position</td>
</tr>
<tr>
<td>13</td>
<td>951</td>
<td>933, 915, 564</td>
<td>0</td>
<td>Tautomer of L-732,531</td>
</tr>
<tr>
<td>14</td>
<td>951</td>
<td>933, 915, 564</td>
<td>0</td>
<td>L-732,531</td>
</tr>
</tbody>
</table>

The peaks are labeled 1–14 according to their HPL retention times, as shown in fig. 6.
(Beyens et al., 1991; Machida et al., 1991; Kay et al., 1991; Nagase et al., 1994; Vincent and Karanam, 1996). As a result, tacrolimus concentrations in blood are higher than those in plasma, thus complicating the calculation and interpretation of pharmacokinetic parameters. Because there is no consensus as to whether blood or plasma levels of compounds such as tacrolimus should be measured (Beyens et al., 1991; Piekoszewski et al., 1993; Winkler et al., 1994; Legg and Rowland, 1988), we decided to collect data for L-732,531 in both blood and plasma. The blood-to-plasma ratio of L-732,531 also was measured after in vitro incubations with fresh heparynized human, rat, and baboon blood.

The results obtained from the present studies indicate that, unlike tacrolimus, L-732,531 does not partition extensively into erythrocytes. Its in vitro blood-to-plasma ratio was 0.8–0.9 in rats and slightly higher in baboons and humans. Moreover, it showed only a minimal concentration dependence. For example, in baboons, the blood-to-plasma ratio decreased from 1.7 at 5 ng/ml to 0.8 at 1 µg/ml. As a result, blood and plasma levels of L-732,531 were similar in rats, whereas in baboons, blood levels and pharmacokinetic parameters were, on the average, twice as high as those in plasma. As already mentioned, the relatively limited partitioning of L-732,531 into red cells is in contrast to what was observed for tacrolimus, whose blood-to-plasma ratio in some species, including baboons, can be as high as 20 or higher (Beyens et al., 1991; Machida et al., 1991; Kay et al., 1991; Nagase et al., 1994; Vincent and Karanam, 1996), and is consistent with the 10-fold lower affinity of L-732,531 than tacrolimus (Kd of 9 nM vs. 0.9 nM) for the immunophilin FKBP12 (Peterson et al., 1998; Dumont et al., 1998). FKBP12 has been shown to be present in higher amounts in erythrocytes from humans and other primates than rats (Vincent and Karanam, 1996).

The disposition studies indicate that, in rats, L-732,531 was cleared rapidly with plasma and blood clearance of ~60 ml/min/kg at 0.2 mg/kg iv and 30 ml/min/kg at 1–3 mg/kg iv. These data, taken together with results from mass balance and metabolism studies, which showed that the liver is the main organ of elimination of L-732,531, indicate that L-732,531 is subject to extensive extraction by the liver. The observations that blood clearance did not decrease further when the iv dose was increased from 1 to 3 mg/kg, and that at 2 mg/kg iv, intact compound accounted for only a small percentage of the radioactivity in plasma, suggest that the decrease in the clearance between 0.2 and 1 mg/kg iv may not be a result of saturation of metabolism. Saturation of processes other than metabolism such as active uptake into the liver may be contributing to the nonlinearity of the iv pharmacokinetics of L-732,531 in rats. Alternatively, the constant clearance between 1 and 3 mg/kg may be the result of opposing factors, such as saturation of metabolism and saturation of FKBP12 and other proteins in erythrocytes, which would essentially increase the concentration of drug available for uptake into the liver and subsequent metabolism. At the same time, sequestration of L-732,531 by baboon erythrocytes to a greater extent than that by rat erythrocytes may protect the compound from metabolism and result in blood and plasma clearance values that are much lower than expected, based on the in vitro metabolism data. The rate of metabolism of L-732,531 in baboon liver microsomes was found to be 9-fold higher than its rate in rat liver microsomes. Additionally, the decreased hepatic mass and blood flow relative to the body weight in baboons (Lin, 1995; Davies and Morris, 1993) may contribute to the lower clearance of L-732,531 in baboons, as compared with rats.

In both rats and baboons, the oral absorption of L-732,531 was low and variable, and the compound appeared to be subject to extensive presystemic metabolism, especially in baboons. In bile duct-cannulated rats, 31% of a radioactive 5 mg/kg oral dose was absorbed and recovered primarily as metabolites in the bile. The bioavailability of L-732,531 at this dose was estimated at 18%, suggesting that the remaining 13% of the absorbed radioactivity was metabolized presystemically either in the gut or the liver. In a similar experiment in baboons fitted with cannulae in their gallbladders, only 9% of a radioactive 5 mg/kg oral dose was found to be absorbed, suggesting that absorption was lower in baboons than rats. The bioavailability of L-732,531 at this dose in baboons was 3%, indicating that, as in rats, a substantial portion of the absorbed radioactive dose was subject to extensive presystemic metabolism. That the compound was metabolized in the GI tract, as well as the liver, was evidenced by the presence of highly polar metabolites along with intact parent in the feces of orally dosed bile duct-cannulated rats and baboons. Moreover, the lower metabolic stability of L-732,531 in baboon liver microsomes, as compared with the stability in corresponding preparations from the rat, undoubtedly contributed to its lower bioavailability in this species.

L-732,531 exhibited dose-dependent oral pharmacokinetic in both species. Although saturation of hepatic metabolism is a commonly invoked cause of nonlinear pharmacokinetics (Pond and Tozer, 1984), it is unlikely that this is the case here, as L-732,531 is not absorbed well and its concentrations in the liver and portal vein are not expected to be very high. The tissue distribution study in the rat indicated that after a 5 mg/kg oral dose, the highest concentration of total radioactivity in the liver was 10 µg eq/g, only 30% of which was accounted for by intact compound. When incubated with liver microsomes at similar or higher concentrations, L-732,531 was rapidly metabolized, especially in baboon liver microsomes. At 10 µM, the rate of metabolism was 0.57 nmol/min/mg protein in baboon vs. 0.06 nmol/min/mg protein in rat. Saturation of metabolism in the gastrointestinal tract and of multiple drug resistance–mediated efflux, rather than hepatic metabolism, may be contributing to the dose-dependent oral pharmacokinetics of L-732,531.

In summary, studies in rats and baboons showed that L-732,531 was rapidly cleared and underwent extensive metabolism in both species. It was poorly absorbed after oral administration and exhibited high interanimal variation, especially in the baboon. Its low bioavailability is most likely caused by both incomplete absorption and metabolism in the gastrointestinal tract and the liver.

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References