0090-9556/97/2508-0932–939$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 25, No. 8

ORALLY ACTIVE INHIBITORS OF HUMAN LEUKOCYTE ELASTASE. II. DISPOSITION OF L-694,458 IN RATS AND RHESUS MONKEYS

STYLIANI H. VINCENT, SUSAN K. PAINTER, DEBRA LUFFER-ATLAS, BINDHU V. KARANAM, E. MCGOWAN, CHRIS CIOFFE, GEORGE DOSS, AND SHUET-HING L. CHIU

Departments of Drug Metabolism (S.H.V., S.K.P., D. L.-A., B.V.K., E. McG., G.D., S.-H.L.C.) and Laboratory Animal Resources (C.C.), Merck Research Laboratories

(Received November 1, 1996; accepted April 7, 1997)

ABSTRACT:

The disposition of L-694,458, a potent monocyclic β-lactam inhibitor of human leukocyte elastase, was studied in male Sprague-Dawley rats and rhesus monkeys. After iv dosing, L-694,458 exhibited similar pharmacokinetic parameters in rats and rhesus monkeys. The mean values for its plasma clearance, terminal half-life, and volume of distribution at steady state were 27 ml/min/kg, 1.8 hr, and 4.0 liters/kg in rats and 34 ml/min/kg, 2.3 hr, and 5 liters/kg in rhesus monkeys. The bioavailability of a 10 mg/kg oral dose was higher in rats (65%) than in rhesus monkeys (39%). In both species, concentrations of L-694,458 in plasma increased more than proportionally when the oral dose was increased from 10 mg/kg to 40 mg/kg. In monkeys a protracted plasma concentration-time profile was observed at 40 mg/kg, characterized by a delayed T_{max} (8–24 hr) and a long terminal half-life (6 hr). [^{3}H]L-694,458 was well absorbed after oral dosing to rats at 10 mg/kg, as indicated by the high recovery of radioactivity in bile (83%) and urine (6%) of bile duct-cannulated rats. Only ~5% or less of the radioactivity in bile, urine, and feces was a result of intact L-694,458, indicating that the compound was being eliminated by metabolism, followed by excretion of the metabolites in feces, *via* bile. Demethylenation of the methylenedioxyphenyl group resulting in the catechol was the primary metabolic pathway in human and rhesus monkey liver microsomes. In rat liver microsomes, the major metabolite was the *N*-oxide of the methyl-substituted piperazine nitrogen. In rats dosed iv and orally with [^{3}H]L-694,458, concentrations of radioactivity were highest in the lung (the primary target tissue), adrenals, and liver. L-694,458 was unstable in rat blood and plasma, degrading *via* a pathway believed to be catalyzed by B-esterases and to involve cleavage of the β-lactam ring and loss of the methylpiperazine phenoxy group. *In vitro* studies indicated that in human liver, L-694,458 was metabolized by CYP3A and 2C isozymes, and in both monkey and human liver microsomes the compound acted as an inhibitor of testosterone 6β-hydroxylation.

Leukocyte elastase is a serine protease capable of proteolytic degradation of a variety of substrates, including elastin and collagen, which are components of connective tissue. Specific inhibitors of leukocyte elastase are being explored as potential therapeutic agents for the treatment of inflammatory diseases, such as cystic fibrosis and rheumatoid arthritis where high amounts of extracellular elastase, either free or bound to its natural inhibitors, α_1-proteinase inhibitor and secretory leukocyte proteinase inhibitor, have been detected extracellularly (1–3).

Several classes of inhibitors of elastase have been synthesized and evaluated to date (4–19). L-694,458 (*N*-[1(R)-(1,3-benzodioxol-5-yl)butyl]-3,3-diethyl-2(S)-[4-[(4-methyl-1-piperazinyl)carbonyl]phenoxy]-4-oxo-1-azetidinecarboxamide,[1,2] fig. 1) is a member of the monocyclic β-lactam series of elastase inhibitors, most of which are active after oral administration (8–14). It differs from the previously studied β-lactam inhibitors, L-680,833 (11) and L-683,845 (13, 14), in that it contains a weakly basic methyl piperazine amide moiety at the 4-position of the phenol at the azetidinone C-4, instead of an acetic acid group. Unlike the acidic inhibitors, L-694,458 can inhibit elastase in circulating leukocytes as well as elastase released extracellularly (13). Similar to L-680,833 and L-683,845, L-694,458 is highly selective for the human enzyme, being much less active against elastase and elastase-like enzymes from other species, including rhesus monkey. The present report describes its disposition in rats and rhesus monkeys, the two species used in the toxicological evaluation of this drug candidate. Studies on its stability in blood and plasma and its effect on cytochrome P450 also are reported.

[1] Abbreviations used are: L-694,458, *N*-[1(R)-(1,3-benzodioxol-5-yl)butyl]-3,3-diethyl-2(S)-[4-[(4-methyl-1-piperazinyl)carbonyl]phenoxy]-4-oxo-1-azetidinecarboxamide; Vd_{ss}, volume of distribution at steady state; TAO, troleandomycin; BNP, bis-p-nitrophenyl phosphate; PMSF, phenylmethylsulfonyl fluoride; HMQC, heteronuclear multiple quantum coherence.

[2] L-694,458 has been licensed to DuPont Merck, and is also referred to as DMP 777.

Send reprint requests to: Dr. Styliani H. Vincent, Merck Research Laboratories, RY 80L-109, P.O. Box 2000, Rahway, NJ 07065.

Materials and Methods

Chemicals. L-694,458 (fig. 1) and all other related chemicals, such as the monodemethylated metabolite of L-694,458, M5 and the monosubstituted urea, D1, were synthesized at Merck Research Laboratories (Rahway, NJ) according to published procedures ((19) and U.S. patents 183564 and 5591737). L-694,458 was supplied as the amorphous malate salt and the crystalline free base. All data presented in this report are expressed in free base equivalents. The radiolabeled compound, [butyl-3,4-^{3}H]L-694,458 (radiochemical purity ~98%) was prepared with ^{3}H at the positions shown in fig. 1. Pentadeuterated L-694,458, used as the internal standard in the LC-MS/MS assay, was synthesized with deuterium atoms at the positions indicated in fig. 1. Testosterone and its metabolites, including 6β-hydroxytestosterone and 4-androstene-3, 17-dione were obtained from Sigma Chemical Co. (St. Louis, MO).

Microsomes. Fresh rat liver and frozen human and rhesus monkey liver tissue were used for the preparation of microsomes. Microsomes containing

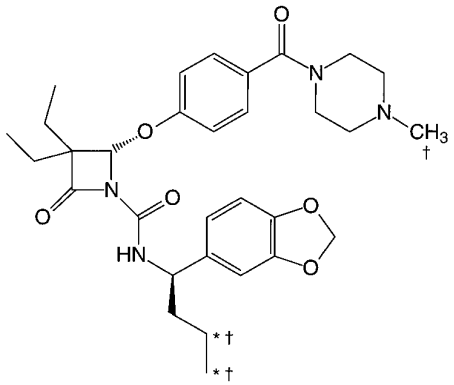

FIG. 1. *Chemical structure of L-694,458.*

recombinant human cytochrome P450 isozymes were obtained from Gentest Corp. (Woburn, MA). The approximate specific activities of the CYP3A4- and CYP2C9-containing microsomes were 500 and 130 pmol/mg/min, as determined by testosterone 6β-hydroxylation and diclofenac 4′-hydroxylation, respectively.

Animal Studies. All animal procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were pre-approved by the Merck Institutional Animal Care and Use Committee. The care and use of research animals at Merck Research Laboratories meets or exceeds all applicable local, national, and international laws and regulations.

Rats. Male Sprague-Dawley rats were used in all experiments. For the pharmacokinetic studies, rats weighing 240–270 g (N = 3–5/dose) were implanted with cannulas in the jugular and femoral veins for dosing and blood collection, respectively. They were dosed iv with the malate salt at 1 and 5 mg/kg and orally with the malate salt and the free base at 10 mg/kg and with the free base at 40 mg/kg. For tissue distribution, mass balance and biliary excretion studies, rats weighing 300–400 g (N = 3–5/dose) were dosed with [^{3}H]L-694,458 at 9 or 10 mg/kg po and 5 mg/kg iv. Intravenous dosing for the mass balance and tissue distribution studies was *via* the tail vein and for the biliary excretion studies *via* a previously implanted cannula in the femoral vein. Oral dosing was by gavage.

Rhesus monkeys. The studies were conducted in three periods using six male rhesus monkeys (4.5–7 kg). The animals were chaired without anesthesia and dosed iv *via* the saphenous vein and orally using a pediatric nasogastric tube. In the first period, three monkeys were dosed at 5 mg/kg iv, and another three monkeys were dosed orally at 10 mg/kg. In the second period, the first group of monkeys were dosed at 10 mg/kg po and the second group at 40 mg/kg po. The malate salt was used for both the iv and oral doses in the first and second periods. In the third period, the second group of monkeys were dosed orally with a suspension of the free base either at 30 mg/kg (N = 1) or 40 mg/kg (N = 2).

Dosing solutions. The malate salt of L-694,458 was dissolved in ethanol: saline (10:90) for iv dosing and in ethanol:0.5% methylcellulose (2:98) for oral dosing. The free base was prepared as a suspension in 0.5% methylcellulose. For iv studies with radiolabeled compound, [^{3}H]L-694,458 was diluted with the malate salt to a specific activity of 135 μCi/mg (tissue distribution) or 15 μCi/mg (mass balance and biliary excretion). For oral studies, [^{3}H]L-694,458 was diluted with the malate salt to a specific activity of 20 μCi/mg (tissue distribution) or with the free base to a specific activity of 15 μCi/mg (mass balance and biliary excretion).

Collection and handling of biological samples. In the pharmacokinetic studies, blood was collected from the femoral vein using a previously implanted cannula, and plasma, obtained by centrifugation at 4°C, was aliquoted and stored at −20°C until analyzed, within a week for the rat samples and within a month for the monkey samples. In the tissue distribution studies, blood was collected by heart puncture from three rats each at 30 min, 2, 6, and 24 hr after iv dosing, and at 2 and 6 hr after oral dosing. Tissues were removed and combusted either whole (cut into small pieces) or after homogenization. In the biliary excretion studies, bile was collected every hour for the first 8 hr and at 8–24, 24–48, and 48–72 hr from a previously implanted cannula in the bile duct. Urine and feces in the biliary excretion and mass balance studies were collected at 24-hr intervals for 3 days. Radioactivity in the dose, plasma, bile, urine, and HPLC fractions was determined by direct counting of aliquots. Radioactivity in the tissues and feces was determined by combustion of homogenates and counting of the resulting ^3H_2O. Acetonitrile extracts of plasma, liver, lung, bile, urine, and feces were analyzed by HPLC using method 1, as described in the section on HPLC analysis.

In Vitro Stability in Blood and Plasma. The *in vitro* stability of [^{3}H]L-694,458 (10 and 25 μM) in rat, monkey, and human blood and plasma was determined after incubation at 37°C for 1, 4, and 24 hr. Acetonitrile supernatants were analyzed by HPLC using method 2 and by LC-MS/MS. For the isolation of the degradation product D2, ethyl acetate extracts of incubations with plasma were chromatographed on a phenyl column eluted isocratically with acetonitrile:water (55:45). The isolated product was analyzed by NMR spectroscopy. Possible degradation of L-694,458 during storage and sample processing was investigated by storing samples of rat and monkey plasma containing known amounts of [^{3}H]L-694,458 at −20°C for periods up to 2 months and processing them by solid-phase extraction, as described for the pharmacokinetic studies, except that the reconstituted extracts were analyzed by HPLC methods 1 and 2.

***In Vitro* Metabolism of L-694,458.** L-694,458 was incubated at 37°C with liver microsomal preparations from male rats, rhesus monkeys, and humans in the presence of an NADPH-regenerating system. Incubations to determine the *in vitro* metabolite profiles were carried out at 10, 25, and 100 μM concentrations of L-694,458 using 0.5–2 mg (rat and monkey) or 1 mg (human) of liver microsomal protein/ml for 10 min to 1 hr. To determine the V_{max} and K_m, [^{3}H]L-694,458 was incubated with 0.2 mg of rhesus monkey liver microsomal protein/ml for 4 min. The rates of disappearance of L-694,458 and appearance of the three major metabolites, M1, M5, and M6, were determined at six substrate concentrations (0.25 to 15 μM), and the data were fitted to the Michaelis-Menten equation using nonlinear least squares. Incubations also were carried out with suspensions of freshly isolated rat hepatocytes (1×10^6 cells/ml), and with small segments of monkey jejunum (0.1 g of tissue cut into ~1 cm squares/ml of phosphate buffer). Acetonitrile extracts of incubation mixtures were analyzed using HPLC method 1, as described in the section on HPLC analysis.

Identification of the Major Cytochrome P450 Isozyme that Metabolizes L-694,458 in Human Liver Microsomes. The effect of specific P450 inhibitors and/or substrates on the metabolism of L-694,458 was determined by incubating [^{3}H]L-694,458 (10 μM) with human liver microsomes (1 mg protein/ml) at 37°C for 30 min in the presence of an NADPH-regenerating system. The reversible inhibitors, ketoconazole (inhibitor of CYP3A), sulfaphenazole (CYP2C), quinidine (CYP2D6), enoxacin (CYP1A2), and 4-methylpyrazole (CYP2E1) were added at the same time as L-694,458. The CYP3A suicide inhibitors, troleandomycin (TAO) and gestodene were pre-incubated with microsomal preparations in the presence of an NADPH-regenerating system for 30 min before [^{3}H]L-694,458 was added. Ketoconazole and sulfaphenazole were added at 1, 10, and 100 μM, and all other inhibitors at 100 μM. Incubations with microsomal preparations containing recombinant human P450 isozymes were conducted at 37°C for 1–4 hr using 2–4 mg microsomal protein/ml. Cytochrome c reductase was added to increase metabolism, except in incubations with CYP2C9-containing microsomes, which contained co-expressed cytochrome P450 reductase. Acetonitrile supernatants were analyzed by HPLC method 1. The concentrations of L-694,458 and its metabolites were determined from the percentage of radioactivity eluting in each peak.

Effect of L-694,458 on the *In Vitro* Metabolism of Testosterone. Human and monkey liver microsomal preparations were pre-incubated at 37°C for 30 min with L-694,458 (10 and 100 μM) or solvent (control) in the presence of an NADPH-regenerating system. Testosterone (100 μM) was added and aliquots were removed at 10, 20, and 30 min and analyzed by HPLC on a Zorbax Rx-C8 column eluted with water:acetonitrile:TFA (60:40:0.1). The identity of the testosterone metabolites was determined by co-elution with authentic materials.

***In Vitro* Interaction of L-694,458 with Rhesus Monkey Liver Microsomal Cytochrome P450.** Rhesus monkey liver microsomes were incubated with L-694,458 in the presence or absence of an NADPH-regenerating system

at 37°C for 0.5, 1, and 4 hr and aliquots were scanned from 500 to 400 nm on a Shimadzu UV160 UV-visible spectrophotometer against a reference cuvette containing microsomes and the NADPH-regenerating system.

Analytical Procedures. *Quantitation of L-694,458 in plasma by LC-MS/MS.* Concentrations of L-694,458 in plasma were determined by LC-MS/MS after solid-phase extraction. Aliquots of plasma (rat, 0.2 ml; monkey, 0.5 ml) were mixed with the internal standard (pentadeuterated L-694,458), diluted with water to 1 ml, and loaded onto CN cartridges. After a wash with water, the cartridges were eluted with methanol. The reconstituted extracts were analyzed by LC-MS/MS on a Spherisorb phenyl column using a mobile phase consisting of acetonitrile:50 mM ammonium acetate:formic acid (70:30:0.1). The column effluent was analyzed directly on a SCIEX API III mass spectrometer using the heated nebulizer interface. The precursor/product ion combinations of m/z 565/389 and 570/392 were used for selected reaction monitoring of L-694,458 and its internal standard, respectively. Quantitation was achieved using a standard curve generated from the mean of five replicates which were made by adding increasing amounts of L-694,458 (0.5–500 ng) and 20 ng of internal standard to control plasma. The lower limit of quantitation in most studies was 1 ng/ml for 0.2 ml rat plasma and 1 ng/ml for 0.5 ml monkey plasma.

Pharmacokinetic calculations. Pharmacokinetic parameters were determined using standard noncompartmental methods. Plasma AUC and AUMC were calculated using UNICUE with log-linear trapezoidal method for interpolation (20). Extrapolation to infinity was done using the terminal rate constant determined by linear regression from the slowest phase of the log plasma concentration-time curve in each animal and at each dose, except as indicated.

Radioactivity measurements. Direct liquid scintillation counting was carried out using a Beckman LS5000TD counter. Combustion was done in a Packard TriCarb Sample Oxidizer, Model B301, followed by counting of the resulting ^3H_2O. Quench correction was carried out by the external ratio method.

HPLC analysis. Two HPLC methods were used for the analysis of samples from the studies with L-694,458. In method 1, samples were analyzed on a Zorbax SB-CN column, eluted isocratically with a mixture of water:acetonitrile (60:40, v/v) with 10 mM ammonium acetate and 0.1% TFA in both solvents. In method 2, samples were analyzed on a Zorbax phenyl column eluted isocratically with acetonitrile:water:TFA (54:46:0.2,v/v). The UV absorbance of the eluate was monitored at 220 nm, and radioactivity was measured with an on-line radiometric detector. Parent and metabolite identification was accomplished by co-elution with authentic material and by LC-MS/MS analysis of extracts, as reported in the accompanying paper (21).

NMR analysis. Proton and HMQC (22) NMR spectra of D2, the plasma degradant of L-694,458, were recorded on a Varian Unity 500 MHz spectrometer in DMSO-d_6 and were referenced to the residual solvent signal at 2.49 ppm for ^1H and 49.0 ppm for ^{13}C.

Results

Pharmacokinetics. *Rats.* After iv dosing at 1 and 5 mg/kg, concentrations of L-694,458 in plasma declined rapidly within the first hour, and then more slowly with a terminal $t_{1/2}$ of ~1.5 hr (fig. 2 and table 1). The mean values of plasma clearance, mean residence time (MRT), and Vd_{ss} of L-694,458 were similar at the two doses studied, ~26 ml/min/kg, 2.5 hr, and 4 liters/kg, respectively (table 1).

After oral dosing at 10 mg/kg with a solution of the malate salt, peak drug concentrations in four rats ranged from 0.44 to 0.96 μg/ml and were achieved between 1 and 4 hr (fig. 2 and table 2). Similar drug concentrations were obtained when rats were dosed with a suspension of the free base in methylcellulose, with peak concentrations ranging from 0.71 to 0.94 μg/ml at 1 and 2 hr. Bioavailability of the 10 mg/kg dose was 65%. When the dose of the free base was increased to 40 mg/kg, peak concentrations of drug increased proportionally to a mean value of 3.4 μg/ml, while AUC increased by 9-fold from 4 μg/hr/ml to 38 μg/hr/ml. The greater than proportional increase in AUC was owing to sustained concentrations at the later time points. Because of the possibility of nonlinear kinetics at concentrations higher than those obtained with the iv doses studied, as indicated by the greater than proportional increase in AUC with increasing oral dose, accurate determination of the bioavailability at 40 mg/kg was not possible.

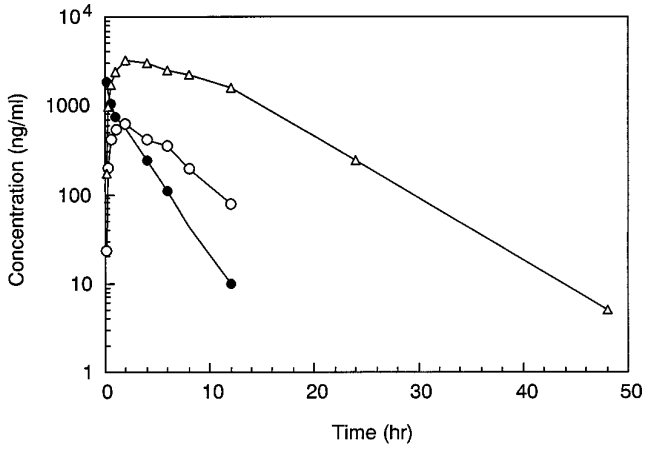

FIG. 2. *Mean plasma concentrations of L-694,458 in rats.*

●- 5 mg/kg iv; -○- 10 mg/kg po (free base); - △ - 40 mg/kg po (free base).

Rhesus monkeys. In rhesus monkeys dosed iv at 5 mg/kg, concentrations of parent compound in plasma (fig. 3) were similar to those measured in rats at the same dose. Plasma clearance, terminal $t_{1/2}$, and Vd_{ss} also were similar (table 1). In contrast, after oral dosing, L-694,458 concentrations were lower and more variable than in rats. Peak concentrations of L-694,458 in six monkeys dosed with the malate salt at 10 mg/kg ranged from 0.02 to 0.35 μg/ml (fig. 3 and table 2) and were reached at a later time than in rats (between 2 and 6 hr). The bioavailability of the 10 mg/kg dose was 39%. Drug concentrations also were variable when a solution of the salt was dosed at 40 mg/kg. At this dose, concentrations of L-694,458 in the plasma of two of the three animals were still increasing between 8 and 24 hr. In a third animal, peak concentrations were reached at ~8 hr. Similar results were obtained when the same monkeys were dosed with a suspension of the free base. In two monkeys dosed at 40 mg/kg, peak concentrations of L-694,458 in plasma were 0.53 and 0.66 μg/ml at 24 hr, declining with a much longer $t_{1/2}$ (6 hr) than after iv dosing, to 2 and 4 ng/ml at 72 hr. Delayed t_{max} also was observed in a third monkey dosed at 30 mg/kg, with peak concentrations of 0.39 μg/ml at 24 hr. As in rats, plasma AUC increased more than proportionally with the increase in the oral dose, and bioavailability of the higher doses could not be determined accurately.

Biliary Excretion and Mass Balance in Rats. [^{3}H]L-694,458 was well absorbed after oral dosing at 10 mg/kg, as indicated by the high recovery of radioactivity in bile and urine of bile duct-cannulated rats. About 83% of the dose was excreted in bile and another 6% in urine over a 72-hr period. Only 11% of the dose was recovered in the feces. When rats were dosed iv at 5 mg/kg, ~77% of the dose was excreted in bile, 9% in urine, and 3% in feces. In noncannulated rats, 92% of an iv dose and 75% of an oral dose were excreted in feces, with another 13% and 14%, respectively, recovered in urine (data not shown).

Tissue Distribution in Rats. The distribution of radioactivity to selected rat tissues was determined at 2 and 6 hr after a 9 mg/kg oral dose of [^{3}H]L-694,458. In all seven tissues examined (adrenals, heart, kidneys, liver, lung, pancreas, and spleen), radioactivity concentrations were much higher than in plasma with tissue to plasma ratios of 4–16 at 2 hr and 2–14 at 6 hr. Concentrations were highest in the liver (22.6 and 13.8 μg drug eq/g tissue at 2 and 6 hr, respectively), and lung (20.1 and 11.3 μg drug eq/g at 2 and 6 hr, respectively). A

TABLE 1

Pharmacokinetic parameters of L-694,458 in intravenously dosed rats and rhesus monkeys

Dose (mg/kg)	AUC (μg/hr/ml)	Clearance (ml/min/kg)	$t_{1/2}$ (hr)	MRT (hr)	Vd_{ss} (liters/kg)
Rats (N = 3–5)					
1	0.64 ± 0.09	26 ± 4	n.d.	2.7 ± 0.3	4.3 ± 0.4
5	3.18 ± 0.51	27 ± 4	1.8 ± 0.6	2.4 ± 0.6	4.0 ± 1.8
Rhesus Monkeys (N = 3)					
5	2.80 ± 1.06	34 ± 16	2.3 ± 0.3	2.5 ± 0.4	5.0 ± 2.4

Mean ± SD values; n.d. = Not determined.

TABLE 2

Pharmacokinetic parameters of L-694,458 in orally dosed rats and rhesus monkeys

Dose (mg/kg)	Form	AUC (μg/hr/ml)	C_{max} (μg/ml)	t_{max} (hr)	$t_{1/2}$ (hr)
Rats (N = 4)					
10	Malate	4.16 ± 1.42	0.74 ± 0.22	2.3 ± 1.3	2.2 ± 0.3
10	Free base	4.22 ± 0.93	0.82 ± 0.10	1.5 ± 0.6	2.7 ± 0.1
40	Free base	38.07 ± 4.87	3.39 ± 0.25	2.3 ± 1.3	4.3 ± 0.1
Rhesus monkeys (N = 1–6)					
10	Malate	1.60 ± 1.09	0.21 ± 0.14	3.0 ± 1.5	3.9 ± 0.8
30	Free base	8.81	0.39	24	6.3
40	Malate	19.26 ± 6.18	0.97 ± 0.25	19 ± 9	n.d.
40	Free base	12.88	0.60	24	6.0

n.d. = Not determined. Drug concentrations were still increasing at 24 hr, the last sampling time in this study. AUC extrapolations were performed using the terminal half-life obtained when the animals were dosed with the free base.

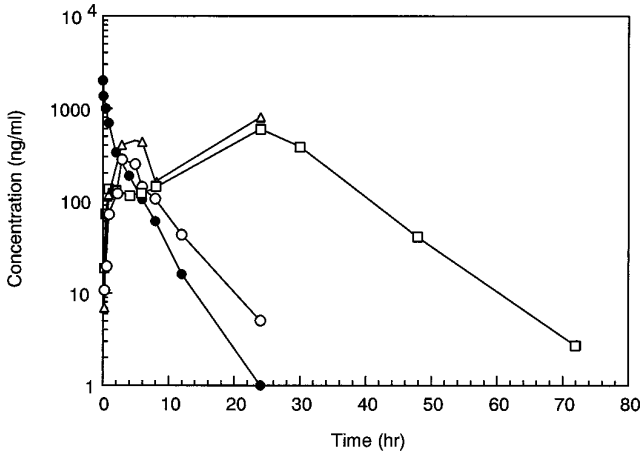

FIG. 3. *Mean plasma concentrations of L-694,458 in rhesus monkeys.*

(●), 5 mg/kg iv; (○), 10 mg/kg po (salt); (△) 40 mg/kg po (salt);(□) 40 mg/kg po (free base).

similar tissue distribution pattern was observed after iv dosing of [^{3}H]L-694,458 at 5 mg/kg. At 24 hr after iv dosing, concentrations were 0.3 μg eq/g in most tissues, except the liver (0.8 μg eq/g) and the small and large intestines (data not shown).

Metabolism. ***In vitro.*** L-694,458 underwent metabolism to several products in the presence of monkey and human liver microsomal preparations (fig. 4). The major metabolites were identified by LC-MS/MS and NMR (*N*-oxide only) analysis, as described in the accompanying paper (21), and were determined to be formed *via* three primary metabolic pathways: *O*-demethylenation of the methylenedioxyphenyl group to give the catechol product (designated as M1), *N*-demethylation of the methylpiperazine group to give M5, and addition of oxygen to the nitrogen of the piperazine ring to form the *N*-oxide (M6). The *N*-oxide was the major metabolite in rat liver microsomes, while the catechol was the major metabolite in monkey and human liver microsomes. Three other hydroxylated metabolites, M2, M3, and M4, were observed in rat and monkey but not human incubations and are discussed in the accompanying paper (21). Rat hepatocyte suspensions generated a similar metabolite profile for L-694,458 as rat liver microsomes, while monkey jejunum gave a similar metabolite profile as monkey liver microsomes. After a 4-hr incubation of [^{3}H]L-694,458 (25 μM) with segments of monkey jejunum (0.1 g tissue/ml), about 53% of the radioactivity eluted in the area of the parent, ~10% in the area of M5, and 22% in the area of M1.

Metabolism of L-694,458 was more extensive in monkey than rat and human liver microsomes. After incubation at 100 μM for 1 hr with 2 mg microsomal protein/ml, there was 55% metabolism in monkey microsomes, 44% in rat, and 32% in human liver microsomes. In a separate experiment, incubation of 25 μM L-694,458 with 0.5 mg of rat liver microsomal protein/ml for 10 min resulted in 8% metabolism (~0.4 nmol/min/mg), compared with 9% metabolism obtained after incubation with 0.2 mg of rhesus monkey liver microsomal protein/ml (~1.1 nmol/min/mg). The V_{max} and K_m values for the metabolism of [^{3}H]L-694,458 in liver microsomes from rhesus monkeys were determined to be 1.90 nmol/min/mg protein and 3.3 μM, giving an intrinsic clearance value of 0.58 ml/min/mg protein. Formation of the catechol metabolite, **M1** (V_{max} 0.96 nmol/min/mg and K_m 3.5 μM), comprised ~48% of the intrinsic clearance, while **M5** (V_{max} 0.42 nmol/min/mg and K_m 4.8 μM) and **M6** (V_{max} 0.90 nmol/min/mg and K_m 6.4 μM) contributed ~25% each.

In Vivo. L-694,458 was the major radioactive component in plasma, lung, and liver of rats dosed with [^{3}H]L-694,458 orally at 9 mg/kg, contributing to 50–60% of the radioactivity at 2 hr and 25–30% at 6 hr. LC-MS/MS analysis showed the presence of several metabolites, including M1, M5, and M6 (21). Rat bile, feces, and urine contained mostly metabolites, with <5% of the radioactivity eluting in the area of the parent compound (data not shown). More than 40 metabolites were identified from bile by LC-MS/MS analysis

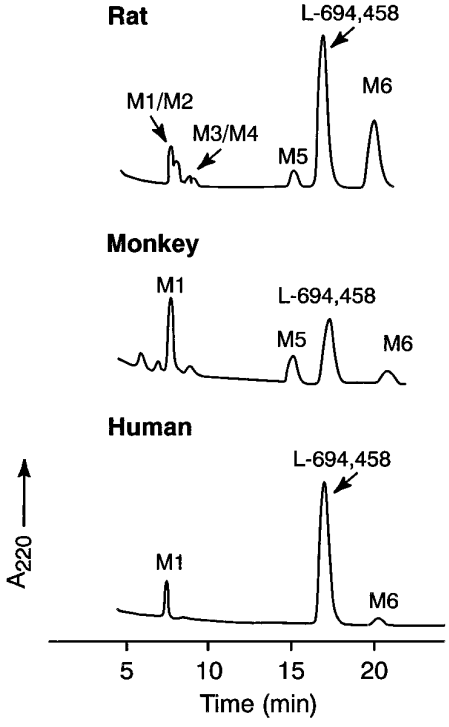

FIG. 4. *In vitro metabolism of L-694,458 in rat, monkey and human liver microsomes.*

Incubations of 100 μM compound were carried out at 37°C for 1 hr in the presence of an NADPH-regenerating system. The microsomal protein concentration was 2 mg/ml (rat and monkey) or 1 mg/ml (human). Acetonitrile extracts were analyzed by HPLC on a SB-CN column with UV detection at 220 nm.

(21). These data indicate that in rats, L-694,458 is eliminated by metabolism followed by excretion of the metabolites in feces *via* the bile.

Identification of the Major Cytochrome P450 Isozyme Responsible for the Metabolism of L-694,458 in Human Liver Microsomes. The major cytochrome P450 isozyme(s) catalyzing the metabolism of L-694,458 in human liver microsomes was identified as belonging to the CYP3A subfamily on the basis of results from three types of studies. These studies investigated i) the effect of isoform-specific P450 inhibitors and/or substrates on the metabolism of L-694,458 in human liver microsomes, ii) the correlation between the rate of metabolism of L-694,458 with different P450 isozyme activities of eight preparations of human liver microsomes, and iii) the capacity of recombinant human P450 isozymes to metabolize L-694,458.

Substrates and/or suicide inhibitors of CYP3A had the most inhibitory effect on the metabolism of L-694,458 in human liver microsomes (table 3). Ketoconazole caused 54% inhibition at 1 μM and 71% at 10 μM, while the CYP3A suicide inhibitors, TAO and gestodene, produced ~30% and 85% inhibition, respectively, at 100 μM. Of the other compounds tested, only sulfaphenazole, an inhibitor of CYP2C isozymes, inhibited L-694,458 metabolism by 18% at 1 μM and 48% at 10 μM. Furthermore, there was a good correlation between L-694,458 metabolism and CYP3A ($r = 0.93$) and CYP2C ($r = 0.93$) activities in the microsomal preparations as measured by testosterone 6β-hydroxylation and tolbutamide hydroxylation, respectively, implicating the involvement of these P450 isozymes in the metabolism of L-694,458. The metabolism of L-694,458 did not correlate with the activities of CYP2D6, 2E1, or 1A2 in the eight

TABLE 3

Effect of cytochrome P450 inhibitors on the in vitro metabolism of [^{3}H]L-694,458 by male human liver microsomes

Inhibitor	Concentration (μM)	P450 Isozyme Inhibited	% Metabolism	% Inhibition
I. Suicide inhibitors[a]				
(Control)			39.0	—
TAO	100	3A	27.2	30
Gestodene	100	3A	5.8	85
II. Competitive inhibitors[b]				
(Control)	—	—	32.8	—
Ketoconazole	100	3A	1.6	95
	10		9.4	71
	1		15.2	54
Sulfaphenazole	100	2C	15.8	52
	10		17.0	48
	1		26.8	18
Quinidine	100	2D6	23.4	29
Enoxacin	100	1A2	33.0	0
4-Methylpyrazole	100	2E1	33.1	0

[a] Liver microsomes were pre-incubated with the inhibitors in the presence of an NADPH-regenerating system before the addition of [^{3}H]L-694,458.

[b] Competitive inhibitors were added at the same time as [^{3}H]L-694,458.

microsomal preparations. The role of CYP3A4 was confirmed by showing that recombinant CYP3A4 can metabolize L-694,458 (~20% metabolism after 4 hr incubation) to M1 (catechol), M5 (*N*-demethylated), and M6 (*N*-oxide), as determined by LC-MS/MS. Metabolism by recombinant human CYP2C9 was much less (~6% in 4 hr). Thus, CYP2C isozymes may play a minor role in the metabolism of L-694,458 in human liver microsomes. L-694,458 was not metabolized by microsomal preparations containing recombinant CYP 1A1, 1A2, 2B1 or 2E1.

Effect of L-694,458 on Testosterone Metabolism. To investigate the possibility that metabolism of the methylenedioxyphenyl moiety of L-694,458 to the catechol (M1) involves the formation of a reactive intermediate, as has been shown for other methylenedioxyphenyl-containing compounds (23–25), the effect of L-694,458 on testosterone metabolism was determined. Testosterone was used as a model substrate for several cytochrome P450 isozymes, including CYP3A, the major isozyme that metabolizes L-694,458. Pre-incubation of human and rhesus monkey liver microsomes with L-694,458 in the presence of an NADPH-regenerating system resulted in inhibition of the CYP3A activity of the microsomes, as indicated by a decrease in the conversion of testosterone to its 6β-hydroxylated metabolite (table 4). Pre-incubation of human liver microsomes with L-694,458 at 10 μM and 100 μM resulted in ~83% inhibition of testosterone 6β-hydroxylation at both concentrations without affecting the formation of androstenedione, the other major metabolite. In rhesus monkey microsomes, testosterone 6β-hydroxylation was inhibited by 83 and 94% at 10 μM and 100 μM of L-694,458, respectively, while androstenedione formation was actually enhanced substantially.

Cytochrome P450 Difference Spectrum in Microsomes Incubated with L-694,458. Incubation of L-694,458 with rhesus monkey liver microsomes yielded a typical type II optical binding spectrum with a trough minimum at 412 nm and a maximum at ~424 nm (not shown). Addition of an NADPH-regenerating system gave a type III difference spectrum, with peaks at 427 nm and 455 nm, as shown in fig. 5 for the 1-hr incubation mixture.

***In Vitro* Stability in Blood and Plasma.** [^{3}H]L-694,458 was unstable in rat blood and plasma and relatively stable in monkey and human blood and plasma. About 30 and 50% degradation was ob-

TABLE 4

Metabolism of testosterone in liver microsomes pre-incubated with L-694,458 and NADPH

L-694,458 (μM)	Testosterone (nmoles remaining in 30 min)	6β-OH-Testosterone (nmoles produced in 30 min)	Androstenedione (nmoles produced in 30 min)
I. Rhesus monkey			
Control	27	47	9
10	51	8	19
100	65	3	25
II. Human			
Control	50	25	5
10	88	3	3
100	90	3	4

Microsomes (2 mg/ml) were pre-incubated with L-694,458 or solvent (control) in the presence of an NADPH-regenerating system at 37°C for 30 min. Testosterone (100 μM) was added and incubations continued for an additional 30 min. The amounts of testosterone remaining and metabolites formed were determined by HPLC analysis.

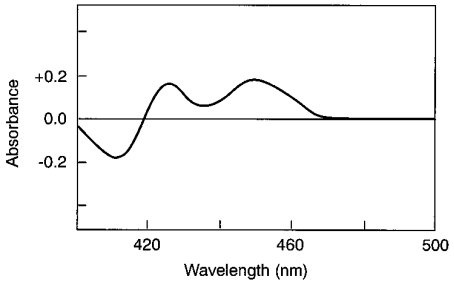

FIG. 5. *Type III optical difference spectrum recorded upon incubating L-694,458 with liver microsomes and NADPH.*

served after a 4-hr incubation at 37°C in rat blood and plasma, respectively. At the end of a 24-hr incubation with rat plasma, L-694,458 was almost completely degraded, as shown by HPLC analysis. Parent compound accounted for ~75, 58, and 15% of the total radioactivity in 24 hr incubations with human, monkey, and rat blood, respectively. No degradation was observed when L-694,458-containing rat and monkey plasma samples were stored at −20°C for up to 1 week or 2 months, respectively (data not shown).

The degradation of L-694,458 in rat plasma could be inhibited by bis-(p-nitrophenyl)phosphate (BNP) and phenylmethylsulfonyl fluoride (PMSF). At 1 mM, BNP inhibited the degradation of L-694,458 observed after a 4-hr incubation by 82%. PMSF was more effective, inhibiting degradation by 91%. These data suggest that the degradation of L-694,458 in rat plasma is mediated by a type B esterase (26).

A representative radiochromatogram of incubations with rat plasma and the structures of the degradants are shown in fig. 6. The identity of the degradants was determined by mass and NMR spectral analyses. D1 exhibited a similar mass spectrum as an authentic sample of the monosubstituted urea, while D2 showed a protonated molecular ion with *m/z* at 319 Da. The proton NMR spectrum of D2 indicated loss of the entire substituent at C-4 of the β-lactam ring. A key feature was a novel methine proton at 6.18 ppm (doublet, J = 10.8 Hz) coupled to an NH proton at 7.55 ppm. Such chemical shift is consistent with either the olefinic hydrogen in the proposed vinylurea structure in fig. 7 or a hemiaminal type structure. To distinguish between these two possibilities, a ^{1}H-^{13}C correlation (HMQC) was conducted on D2 (60 μg, ~50 hr acquisition). In this experiment, the proton with a chemical shift at 6.18 ppm was shown to be linked to a carbon at 117.5 ppm (fig. 7), clearly favoring the olefin structure

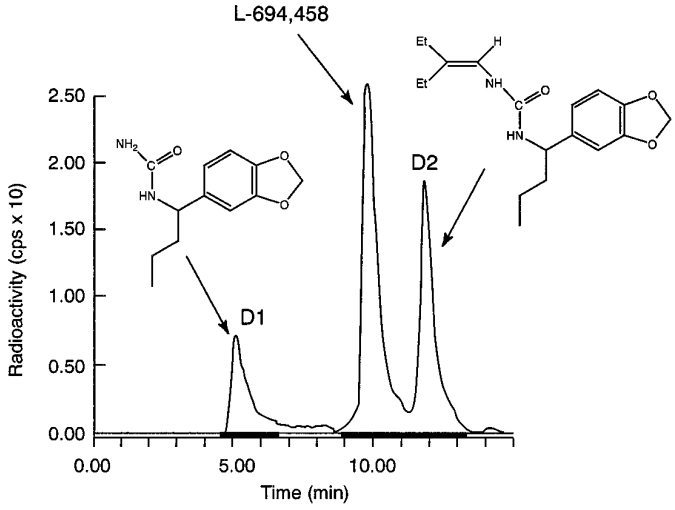

FIG. 6. *Radiochromatogram of an incubation mixture of [^{3}H]L-694,458 with rat plasma.*

The compound was incubated at 25 μM with fresh plasma for 4 hr at 37°C. Acetonitrile supernatants were analyzed by HPLC as described in *Materials and Methods.* The structures of the degradants were determined by MS and NMR spectroscopy.

shown. A similar structure was proposed for products formed by the base-catalyzed hydrolysis of other monocyclic β-lactam inhibitors of elastase (9).

Discussion

Disposition. L-694,458 exhibited similar pharmacokinetic parameters in rats and rhesus monkeys after iv dosing at 1 and/or 5 mg/kg, with mean values of plasma clearance, terminal half-life and Vd_{ss} of 26–34 ml/min/kg, ~2 hr, and 4–5 liters/kg, respectively. However, the apparently similar pharmacokinetics of L-694,458 in rats and rhesus monkeys may be serendipitous. On one hand, the higher affinity of L-694,458 for rhesus leukocyte elastase (13) and its lower metabolic stability in monkey liver would be expected to enhance its clearance in monkeys, while on the other hand, its greater instability in rat plasma would result in a higher clearance in rats.

The species differences in the disposition of L-694,458 in rats and rhesus monkeys were more apparent after oral dosing, with a higher bioavailability in rats than in rhesus monkeys. This finding is consistent with the lower metabolic stability of L-694,458 in liver microsomes from rhesus monkeys than rats and with the observation that L-694,458 is subject to metabolism in rhesus monkey jejunum. In both species, plasma AUC increased more than proportionally when the oral dose was increased from 10 to 40 mg/kg, suggestive of saturation of first-pass metabolism at the higher dose, and possibly resulting in part from saturation of elastase, especially in rhesus monkeys. Moreover, a protracted plasma concentration-time profile was observed in rhesus monkeys dosed at 40 mg/kg, with t_{max} at 8–24 hr, and a longer terminal half-life than that obtained after iv dosing (6 *versus* 2 hr). Although prolonged absorption could be a contributing factor to the delayed t_{max} and long $t_{1/2}$, these observations also are consistent with results from *in vitro* experiments which indicated that L-694,458 could act as an inhibitor of CYP3A, which catalyzes its metabolism. Pre-incubation of liver microsomal preparations from rhesus monkeys and humans with L-694,458 and NADPH resulted in a type III optical difference spectrum, characteristic of the formation of a cytochrome P450 inhibitory complex shown to occur with other methylenedioxyphenyl-containing compounds (23–25). In the case of

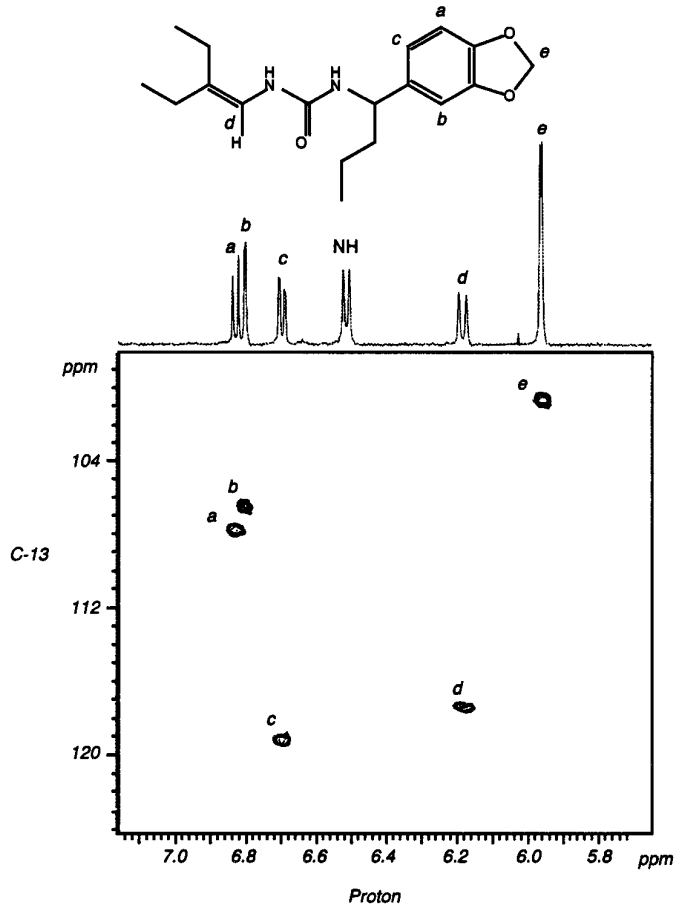

FIG. 7. *Low-field region of the two-dimensional ^{1}H-^{13}C correlation spectrum (HMQC) of the degradant D2 along with the corresponding region of the ^{1}H NMR spectrum (top).*

L-694,458, CYP3A-mediated cleavage of the methylenedioxyphenyl moiety to yield the catechol derivative is the major metabolic pathway in monkey and human liver microsomes. Moreover, L-694,458 seemed to be a specific inhibitor of CYP3A, inhibiting testosterone 6β-hydroxylation but not androstenedione formation, which is catalyzed primarily by isozymes other than CYP3A (27).

***In Vitro* Stability.** L-694,458 was unstable in rat plasma, degrading to two major products, whose structures are shown in fig. 6. The mechanism for the formation of D1 and D2 is probably similar to that involved in the inhibition of human leukocyte elastase by β-lactam compounds (11, 12), namely, an initial attack by a nucleophile on the lactam carbonyl and decarboxylation followed by loss of the phenoxy acetic acid moiety to give D2 or decomposition to the monosubstituted urea D1. D1 could also be formed by cleavage of D2, which was observed *in vitro* in the presence of acid. Based on the inhibitory effect shown by organophosphorus compounds such as BNPP, it can be postulated that the degradation of L-694,458 in rat plasma is mediated by a type B esterase as the catalyst (26).

In conclusion, substantial species differences were observed in several aspects of the disposition of L-694,458 in rats and rhesus monkeys. In addition to its higher affinity for monkey than rat elastase reported by others (13), this monocyclic β-lactam compound was inherently more unstable in rat than monkey plasma and was metabolized more extensively in monkey than rat liver microsomes. The net result of these effects is a similar pharmacokinetic profile in both species characterized by high clearance after iv dosing and a greater than proportional increase in the AUC when the oral dose was increased from 10 to 40 mg/kg. In addition, a protracted concentration-time curve was obtained in monkeys dosed orally at 40 mg/kg, possibly owing, at least in part, to inhibition by L-694,458 of its own metabolism which is catalyzed primarily by CYP3A isozymes. Inhibition of its own metabolism would be expected to be more apparent in rhesus monkeys than rats since monkey liver microsomes were more active towards demethylenation of the dioxymethylene moiety, the metabolic pathway believed to lead to cytochrome P450 inhibition.

Acknowledgments. We thank the following individuals of Merck Research Laboratories: Dr. P. E. Finke for the supply of L-694,458, pentadeuterated L-694,458, the monosubstituted urea and the olefin precursor for radiolabeling, as well as for helpful discussions; Dr. P. A. Krieter, Ms. A. E. Colletti, and Mr. R Alvaro for their assistance in the rat studies; Ms. S. Levy and B. Friscino for their assistance in the monkey studies; Dr. R. A. Stearns for support in the LC-MS/MS assays; Drs. A. Rosegay, F. Tang and A. Jones for synthesizing and analyzing the radiolabeled L-694,458; Mrs. R. Wang and Ms. D. Newton for determining the various P450 activities of human liver microsomes. We also thank Drs. T. Musick and M. Schrimpf of Hazelton Wisconsin, Inc. for determining the tissue distribution of [^{3}H]L-694,458 in iv-dosed rats.

References

1. A. Janoff: Elastase in tissue injury. *Annu. Rev. Med.* **36,** 207–216 (1985).
2. W. C. Groutas: Inhibitors of leukocyte elastase and leukocyte cathepsin G: agents for the treatment of emphysema and related ailments. *Med. Res. Rev.* **7,** 227–241 (1987).
3. G. L. Snider, P. J. Stone, and E. C. Lucey: The specific treatment of emphysema. *Eur. Respir. J.* **3(Suppl. 9),** 23s–28s (1990).
4. J. B. Doherty, B. M. Ashe, L. W. Argenbright, P. L. Barker, R. J. Bonney, G. O. Chandler, M. E. Dahlgren, C. P. Dorn, P. E. Finke, R. A. Firestone, D. Fletcher, W. K. Hagmann, R. Mumford, L. O'Grady, A. L. Maycock, J. M. Pisano, S. K. Shah, K. R. Thompson, and M. Zimmerman: Cephalosporin antibiotics can be modified to inhibit human leukocyte elastase. *Nature (Lond.)* **322,** 192–194 (1986).
5. J. B. Doherty, B. M. Ashe, P. L. Barker, T. J. Blacklock, J. W. Butcher, G. O. Chandler, M. E. Dahlgren, P. Davies, C. P. Dorn, P. E. Finke, R. A. Firestone, W. K. Hagmann, T. Halgren, W. B. Knight, A. L. Maycock, M. A. Navia, L. O'Grady, J. M. Pisano, S. K. Shah, K. R. Thompson, H. Weston, and M. Zimmerman: Inhibition of human leukocyte elastase. 1. Inhibition by C-7-substituted cephalosporin tert-butyl esters. *J. Med. Chem.* **33,** 2513–2521 (1990).
6. D. S. Fletcher, D. G. Osinga, K. M. Hand, P. S. Dellea, B. M. Ashe, R. A. Mumford, P. Davies, W. Hagmann, P. A. Finke, J. B. Doherty, and R. J. Bonney: A comparison of α_1-proteinase inhibitor methoxysuccinyl-ala-ala-pro-val-chloromethylketone and specific β-lactam inhibitors in an acute model of human polymorphonuclear leukocyte elastase-induced lung hemorrhage in the hamster. *Am. Rev. Respir. Dis.* **141,** 672–677 (1990).
7. P. E. Finke, S. K. Shah, B. M. Ashe, R. G. Ball, T. J. Blacklock, R. J. Bonney, K. A. Brause, G. O. Chandler, M. Cotton, P. Davies, P. S. Dellea, C. P. Dorn, D. S. Fletcher, L. A. O'Grady, W. K. Hagmann, K. M. Hand, W. B. Knight, A. L. Maycock, R. A. Mumford, D. G. Osinga, P. Sohar, K. R. Thompson, H. Weston, and J. B. Doherty: Inhibition of human leukocyte elastase. 4. Selection of a substituted cephalosporin (L-658,758) as a topical aerosol. *J. Med. Chem.* **35,** 3731–3744 (1992).
8. S. K. Shah, C. P. Dorn, P. E. Finke, J. J. Hale, W. K. Hagmann, K. A. Brause, G. O. Chandler, A. L. Kissinger, B. M. Ashe, H. Weston, W. B. Knight, A. L. Maycock, P. S. Dellea, D. S. Fletcher, K. M. Hand, R. A. Mumford, D. J. Underwood, and J. B. Doherty: Orally active β-lactam inhibitors of human leukocyte elastase 1. Activity of 3,3-diethyl-2-azetidinones. *J. Med. Chem.* **35,** 3745–3754 (1992).

9. W. B. Knight, B. G. Green, R. M. Chabin, P. Gale, A. L. Maycock, H. Weston, D. W. Kuo, W. M. Westler, C. P. Dorn, P. E. Finke, W. K. Hagmann, J. J. Hale, J. Liesch, M. MacCoss, M. A. Navia, S. K. Shah, D. Underwood, and J. B. Doherty: Specificity, stability and potency of monocyclic β-lactam inhibitors of human leukocyte elastase. *Biochemistry* **31,** 8160–8170 (1992).
10. W. K. Hagmann, A. L. Kissinger, S. K. Shah, P. E. Finke, C. P. Dorn, K. A. Brause, B. M. Ashe, H. Weston, A. L. Maycock, W. B. Knight, P. S. Dellea, D. S. Fletcher, K. M. Hand, D. Osinga, P. Davies, and J. B. Doherty: Orally active β-lactam inhibitors of human leukocyte elastase. 2. Effect of C-4 substitution. *J. Med. Chem.* **36,** 771–777 (1993).
11. J. B. Doherty, S. K. Shah, P. E. Finke, C. P. Dorn, W. K. Hagmann, J. J. Hale, A. L. Kissinger, K. R. Thompson, K. Brause, G. O. Chandler, W. B. Knight, A. L. Maycock, B. M. Ashe, H. Weston, P. Gale, R. A. Mumford, O. F. Andersen, H. R. Williams, T. E. Nolan, D. L. Frakenfield, D. Underwood, K. P. Vyas, P. H. Kari, M. E. Dahlgren, J. Mao, D. S. Fletcher,. P. S. Dellea, K. M. Hand, D. G. Osinga, L. B. Peterson, D. T. Williams, J. M. Metzger, R. J. Bonney, J. L. Humes, S. P. Pacholok, W. A. Hanlon, E. Opas, J. Stolk, and P. Davies: Chemical, biochemical, pharmacokinetic, and biological properties of L-680,833: A potent, orally active monocyclic β-lactam inhibitor of human polymorphonuclear leukocyte elastase. *Proc. Natl. Acad. Sci. USA* **90,** 8727–8731 (1993).
12. R. Chabin, B. G. Green, P. Gale, A. L. Maycock, H. Weston, C. P. Dorn, P. E. Finke, W. K. Hagmann, J. J. Hale, M. MacCoss, S. K. Shah, D. Underwood, J. B. Doherty, and W. B. Knight: Mechanism of inhibition of human leukocyte elastase by monocyclic β-lactams. *Biochemistry* **32,** 8970–8980 (1993).
13. P. E. Finke, C. P Dorn, A. L: Kissinger *et al.*: Orally active, intracellular inhibitors of human leukocyte elastase. Presented at American Chemical Society 210th National Meeting, Chicago, IL (1995).
14. S. H. Vincent, S. K. Painter, P. A. Krieter, A. E. Colletti, E. Lyszack, A. Rosegay, D. Dean, D. Luffer-Atlas, R. R. Miller, C. Cioffe, and S. H. L. Chiu: Orally active inhibitors of human leukocyte elastase. I. Disposition of L-683,845 in rats and rhesus monkeys. *Drug Metab. Dispos.* **24,** 1369–1377 (1996).
15. M. Alpegiani, P. Bissolino, R. Corigli, S. Del Nero, E. Perrone, V. Rizzo, N. Sacchi, G. Cassinelli, G. Franceschi, and A. Baici: Cephem sulfones as inactivators of human leukocyte elastase. 5. 7α-methoxy- and 7α-chloro-1,1-dioxocephem 4-ketones. *J. Med. Chem.* **37,** 4003–4019 (1994).
16. P. Warner, R. C. Green, B. Gomes, and A. M. Strimpler: Non-peptidic inhibitors of human leukocyte elastase. 1. The design and synthesis of pyridone-containing inhibitors. *J. Med. Chem.* **37,** 3090–3099 (1994).
17. J. R. Damewood, P. D. Edwards, S. Feeney, B. C. Gomes, G. B. Steelman, P. A. Tuthill, J. C. Williams, P. Warner, S. A. Woolson, D. J. Wolanin, and C. A. Veale: Nonpeptidic inhibitors of human leukocyte elastase. 2. Design, synthesis, and *in vitro* activity of a series of 3-amino-6-arylopyridin-2-one trifluoromethyl ketones. *J. Med. Chem.* **37,** 3303–3312 (1994).
18. D. J. Hlasta, C. Subramanyam, M. R. Bell, P. M. Carabateas, J. J. Court, R. C. Desai, M. L. Drozd, W. M. Eickhoff, E. W. Ferguson, R. J. Gordon, J. A. Johnson, V. Kumar, A. L. Maycock, K. R. Mueller, E. D. Pagani, D. T. Robinson, M. T. Saindane, P. J. Silver, and S. Subramanian: Orally bioavailable benzisothiazolone inhibitors of human leukocyte elastase. *J. Med. Chem.* **38,** 739–744 (1995).
19. R. J. Cvetovich, M. Chartrain, F. W. Hartner, C. Roberge, J. S. Amato, and E. J. J. Grabowski: An asymmetric synthesis of L-694,458, a human leukocyte elastase inhibitor, via novel enzyme resolution of β-lactam esters. *J. Org. Chem.* **61,** 6575–6580 (1996).
20. K. C. Yeh, G. A. Winchell, J. S. Barrett: A program for calculating AUC in pharmacokinetics using stable piecewise cubic polynomial interpolants. *J. Pharm. Sci.* **76,** S104 (1987).
21. D. Luffer-Atlas, S. H. Vincent, S. K. Painter, B. H. Arison, R. A. Stearns, and S. H. L. Chiu: Orally active inhibitors of human leukocyte elastase. III. Identification and characterization of metabolites of L-694,458 by liquid chromatography-tandem mass spectrometry. *Drug Metab. Dispos*., **25,** 940–952 (1997).
22. A. Bax and S. Subramanian. Sensitivity-enhanced two-dimensional heteronuclear shift correlation NMR spectroscopy. *J. Magn. Reson.* **67,** 565–569 (1986).
23. C. F. Wilkinson, M. Murray, and C. B. Marcus: Interactions of methylenedioxyphenyl compounds with cytochrome P-450 and effects on microsomal oxidation. *Rev. Biochem. Toxicol.* **6,** 27–63 (1984).
24. K. F. Zhang, G. Lepage, G. Cuvier, J. Astoin, M. S. Ashed, and T. A. Baillie: The metabolic fate of stiripentol in the rat. *Drug Metab. Dispos.* **18,** 794–803 (1990).
25. L.-S. Yu, C. F. Wilkinson, and M. W. Anders: Generation of carbon monoxide during the microsomal metabolism of methylenedioxyphenyl compounds. *Biochem. Pharmacol.* **29,** 1113–1122 (1980).
26. W. N. Aldridge: Serum esterases. 1. Two types of esterase (A and B) hydrolyzing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination. *Biochem. J.* **53,** 110–117 (1953).
27. M. P. Arlotto, J. M. Trant, and R. W. Estabrook: Measurement of steroid hydroxylation reactions by high-performance liquid chromatography as indicator of P450 identity and function. *Meth. Enzymol.* **206,** 454–462 (1991).