

IN VITRO METABOLISM STUDIES ON THE ISOXAZOLE RING SCISSION IN THE ANTI-INFLAMMATORY AGENT LEFLUNOMIDE TO ITS ACTIVE α -CYANOENOL METABOLITE A771726: MECHANISTIC SIMILARITIES WITH THE CYTOCHROME P450-CATALYZED DEHYDRATION OF ALDOXIMES

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ABSTRACT:

The 3-unsubstituted isoxazole ring in the anti-inflammatory drug leflunomide undergoes a unique N–O bond cleavage to the active α -cyanoenol metabolite A771726, which resides in the same oxidation state as the parent. In vitro studies were conducted to characterize drug-metabolizing enzyme(s) responsible for ring opening and to gain insight into the mechanism of ring opening. Under physiological conditions, leflunomide was converted to A771726 in rat and human plasma (rat plasma, $t_{1/2}$ = 36 min; human plasma, $t_{1/2}$ = 12 min) and whole blood (rat blood, $t_{1/2}$ = 59 min; human blood, $t_{1/2}$ = 43 min). Human serum albumin also catalyzed A771726 formation, albeit at a much slower rate ($t_{1/2}$ = 110 min). Rat and human liver microsomes also demonstrated NADPH-dependent A771726 formation (human liver microsomes, $V_{\max}$ = 1,797 pmol/min/mg and K_m = 274 μ M). Leflunomide metabolism in microsomes was sensitive to furafylline treatment, suggesting P4501A2 involvement. 3-Methylleflunomide, which contained a

3-methyl substituent on the isoxazole ring, was resistant to ring opening in base, plasma, blood, and liver microsomes. In microsomes, two monohydroxylated metabolites were formed, and metabolite identification studies established the 3- and the 5-methyl groups on the isoxazole ring as sites of hydroxylation. These results indicate that the C3–H in leflunomide is essential for ring opening. Although A771726 formation in human liver microsomes or recombinant P4501A2 required NADPH, its formation was greatly reduced by oxygen or carbon monoxide, suggesting that the isoxazole ring opening was catalyzed by the P450Fe(II) form of the enzyme. A mechanism for the P450-mediated ring scission is proposed in which the isoxazole ring nitrogen or oxygen coordinates to the reduced form of the heme followed by charge transfer from P450Fe(II) to the C=N bond or deprotonation of the C3–H, which results in a cleavage of the N–O bond.

N-[(4-Trifluoromethyl)phenyl]-5-methylisoxazole-4-carboxamide (leflunomide³) is currently approved in the United States, the European Union, and 43 other countries around the world as an orally active disease-modifying anti-inflammatory agent for the treatment of advanced rheumatoid arthritis. After oral administration to humans, leflunomide is rapidly metabolized such that circulating concentrations of leflunomide are mostly below the

limit of detection (Lucian et al., 1995; Rozman, 2002). The principal biotransformation pathway of leflunomide in humans involves an N–O bond cleavage on its isoxazole ring, leading to the formation of the 2-cyano-3-oxo-*N*-[(4-trifluoromethyl)phenyl]butyramide (A771726) metabolite that resides in the same oxidation state as the parent drug (Fig. 1) (Lucian et al., 1995; Davis et al., 1996; Bertolini et al., 1997; Silva and Morris, 1997; Prakash and Jarvis, 1999; Rozman, 2002). In the case of leflunomide, the isoxazole ring opening to A771726 represents an important biochemical consequence since this metabolite is responsible for the anti-inflammatory and disease-modifying properties of leflunomide (Rozman, 2002). Despite the pharmacological importance of this metabolic fate, there exist no details in the primary literature on the characterization of drug-metabolizing enzymes responsible for the leflunomide conversion to A771726. Therefore, an immediate objective of this investigation was to conduct a detailed in vitro characterization of the enzyme system(s) involved in the biotransformation sequence leflunomide $\rightarrow$ A771726.

From a mechanistic standpoint, the “nonreductive” isoxazole ring opening in leflunomide to A771726 appears to contrast the general “reductive” N–O bond cleavage in isoxazole rings. For instance, in the

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³ Abbreviations used are: leflunomide, *N*-[(4-trifluoromethyl)phenyl]-5-methylisoxazole-4-carboxamide; A771726, 2-cyano-3-oxo-*N*-[(4-trifluoromethyl)phenyl]butyramide; 3-methylleflunomide, *N*-[4-(trifluoromethyl)phenyl]-3,5-dimethylisoxazole-4-carboxamide; P450, cytochrome P450; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; CID, collision-induced dissociation; R_t , retention time; LC/MS-MS, liquid chromatography-tandem mass spectrometry; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline.

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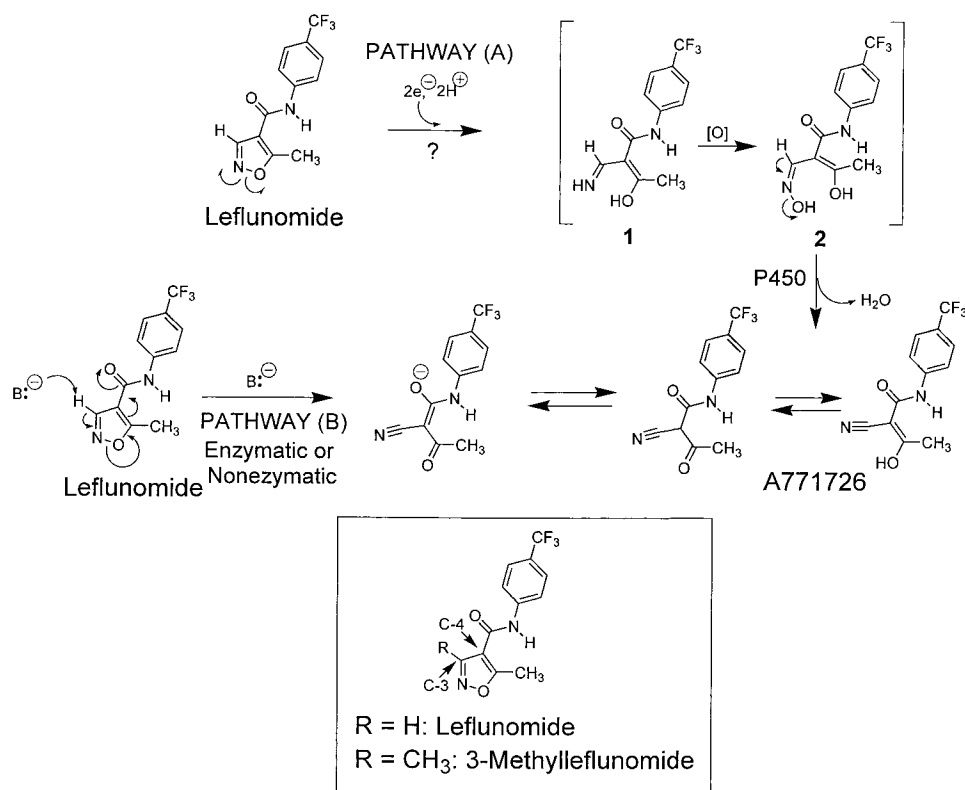


FIG. 1. Proposed mechanisms for the metabolic isoxazole ring opening in the anti-inflammatory agent leflunomide.

case of the anticonvulsant agent zonisamide, P450, aldehyde oxidase, and intestinal bacteria have been implicated in the reductive ring scission of the 1,2-benzisoxazole moiety leading to the 2-(sulfamoylacetyl)phenol metabolite (Stiff and Zemaitis, 1990; Nakasa et al., 1992, 1993a,b; Kitamura et al., 1996; Sugihara et al., 1996). The proposed mechanism for the formation of 2-(sulfamoylacetyl)phenol involves a two-electron reductive cleavage of the N–O bond to an imine intermediate that is hydrolyzed to the phenol (Fig. 2). The facile reductive cleavage of the N–O bond has been attributed to the greater electronegativity of the oxygen atom adjacent to the nitrogen in the isoxazole ring, a feature that is absent in the isomeric oxazole ring system that exclusively undergoes oxidative ring opening (Dalvie et al., 2002). Additional examples of reductive N–O bond cleavage in 1,2-benzisoxazole rings are also discernible in the P450 or gut microflora-mediated biotransformation of the antipsychotic agents risperidone and iloperidone (Fig. 2) (Mannens et al., 1993; Mutlib et al., 1995).

Because of these differences, additional *in vitro* metabolism studies in human liver microsomes and recombinant enzymes were conducted to examine the mechanism of “nonreductive” ring opening. Based on the proposed reductive ring opening mechanism for 1,2-benzisoxazoles, it is conceivable that leflunomide can also undergo the obligatory two-electron reduction to the imine intermediate **1**. A mechanism consistent with the subsequent conversion of the imine **1** to A771726 involves further oxidation of **1** to the aldoxime intermediate **2** followed by its P450-catalyzed dehydration to A771726 in a manner similar to that observed with alkyl- or arylaloximes (Boucher et al., 1994; Mathews et al., 1998) (Fig. 1, pathway A). Alternately, a concerted β -elimination pathway involving enzymatic (or nonenzymatic) deprotonation of the C3–H on the isoxazole ring followed by N–O bond cleavage to A771726 (Fig. 1, pathway B) is also viable, considering that in the presence of base, 3-unsubstituted isoxazoles readily undergo ring scission to intermediates similar in structure to

A771726 (Wakefield and Wright, 1979). The viability of pathway B was examined by comparing the *in vitro* metabolism of *N*-[4-(trifluoromethyl)phenyl]-3,5-dimethylisoxazole-4-carboxamide (3-methylleflunomide, a leflunomide analog containing a C-3 methyl substituent on its isoxazole ring; see Fig. 1) to that of leflunomide.

Materials and Methods

Materials. All chemicals and solvents used in synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI). 3,5-Dimethylisoxazole-4-carbonyl chloride was obtained from Maybridge Chemicals (Trevillet, UK). 1H NMR spectra in $CDCl_3$ or $DMSO-d_6$ were recorded on a Varian Unity M-400 MHz spectrometer (Varian, Inc., Palo Alto, CA); chemical shifts are expressed in parts per million (ppm, δ) calibrated to the deuterium lock signal for $CDCl_3$ or $DMSO-d_6$. Spin multiplicities are given as s (singlet), d (doublet), and m (multiplet). Coupling constants (*J*) are given in hertz (Hz). Negative ion electrospray ionization (ESI) and collision-induced dissociation (CID) mass

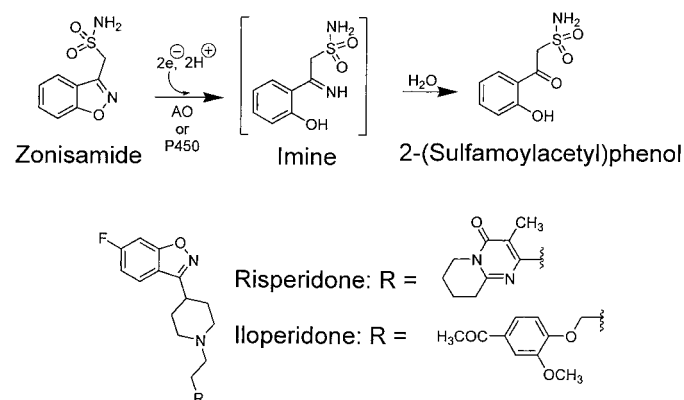


FIG. 2. Reductive ring scission reactions of the 1,2-benzisoxazole-containing anticonvulsant zonisamide.

spectra were obtained on a Sciex API model 2000 triple quadrupole mass spectrometer (Thornhill, ON, Canada).

Synthesis of 2-Cyano-3-oxo-N-[(4-trifluoromethyl)phenyl]butyramide (A771726). The synthesis was conducted via modifications of procedures reported for analogous compounds (Sjogren et al., 1991; Kuo et al., 1996). 2-Cyano-N-[(4-trifluoromethyl)phenyl]acetamide (1 g, 9.38 mmol) (Sjogren et al., 1991) in 10 ml of anhydrous tetrahydrofuran was added dropwise at 0°C to a solution containing sodium hydride (60% dispersion in mineral oil, 0.385 g, 9.64 mmol) in freshly distilled tetrahydrofuran (30 ml). This mixture was stirred at room temperature for 30 min and then treated with acetyl chloride (0.37 ml, 5.26 mmol) over a period of 5 min. Upon stirring at room temperature for 4 h, the mixture was quenched with glacial acetic acid (1 ml) and poured over ice water (50 ml) containing 1 N HCl (2 ml). A yellow precipitate was formed, filtered, and dissolved in methylene chloride (50 ml). The organic solution was washed with water (two times 25 ml), dried (MgSO₄), filtered, and concentrated under reduced pressure to afford A771726 as a pale yellow solid. Recrystallization from toluene afforded white needles (0.95 g, 40%). m.p. = 226°–228°C. ¹H NMR (DMSO-d₆) δ 7.69–7.72 (m, 2H, ArH), 7.60–7.64 (m, 2H, ArH), 2, 15 (s, 3H, CH₃). ESI analysis in the negative ion mode revealed a single peak (*R*_f = 12.7 min) with a deprotonated molecular ion at 269 and a base fragment ion at 82.

Synthesis of 3-Methylleflunomide. To a stirred solution of 4-trifluoromethylaniline (2 ml, 15.9 mmol) in methylene chloride (4 ml) was added 3,5-dimethylisoxazole-4-carbonyl chloride (0.77 g, 4.83 mmol), and the mixture was allowed to stir at room temperature overnight. The reaction mixture was diluted with water and extracted with ethyl acetate (two times 10 ml). The combined organic solution was washed with brine and water, then dried (MgSO₄) and filtered. The solution was concentrated under reduced pressure, and the residue was crystallized from ethanol/water to afford the desired product as a white crystalline solid (0.75 g, 55%). m.p. = 184°–185°C. ¹H NMR (CDCl₃) δ 7.65 (d, 2H, *J* = 8.71 Hz, ArH), 7.60 (d, 2H, *J* = 8.71 Hz, ArH), 7.31 (s, 1H, NH), 2.67 (s, 3H, CH₃), and 2.50 (s, 3H, CH₃). ESI analysis in the negative ion mode revealed a single peak (*R*_f = 16.5 min) with a deprotonated molecular ion at 283 and base fragment ions at 160 and 96.

Enzymology. Microsomal fractions were prepared from male Sprague-Dawley rats (pool of 10–15 livers) (Charles River Laboratories, Inc., Wilmington, MA) and human livers (International Institute for the Advancement of Medicine, Exton, PA) using standard procedures. Protein concentrations were determined using the bicinchoninic acid assay method (Pierce Chemical, Rockford, IL). Total P450 content was measured according to published protocols (Omura and Sato, 1964), and human microsomes were characterized using P450-specific marker substrate activities. Human liver microsomes were isolated from 56 individual livers, and aliquots from the individual preparations were pooled on the basis of equivalent protein concentrations to yield a representative pool of human liver microsomes. Ketoconazole, sulfaphenazole, quinidine, ticlopidine, human and bovine serum albumin, and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). Furfurylline, human liver cytosol, and recombinant human P450 isozymes, coexpressed with NADPH-P450 oxidoreductase in baculovirus-insect cells, were purchased from BD Gentest (Woburn, MA).

Incubations with Liver Microsomes. *Microsomal stability.* Leflunomide stock solutions were prepared in methanol. The final concentration of methanol in the incubation media was 0.2% (v/v). Leflunomide *t*_{1/2} in microsomes was determined in triplicate after its incubation (2 μM) with rat or human liver microsomes (P450 concentration, 0.25 μM) in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C. The total incubation volume was 0.6 ml. The reaction mixture was prewarmed at 37°C for 2 min before adding NADPH (1.2 mM). Aliquots (75 μl) of the reaction mixture at 0, 5, 15, and 30 min (time period associated with reaction linearity) were added to acetonitrile (200 μl) containing indomethacin (0.05 μg/ml) as internal standard, and the samples were centrifuged at 2,500*g* for 5 min before liquid chromatography/tandem mass spectrometry (LC/MS-MS) analysis of leflunomide disappearance. For control experiments, NADPH was omitted from these incubations.

Metabolite identification. For the purposes of metabolite identification studies, the concentration of leflunomide in the microsomal incubations was raised to 50 μM and that of P450 in rat and human liver microsomes was raised to 0.5 μM. The duration of the incubation time was increased from 30 min to 45 min. After quenching the incubation mixtures with 2 volumes of acetoni-

trile, the solutions were centrifuged (3,000*g*, 15 min) and 400-μl aliquots of the supernatants were analyzed for metabolite formation by LC/MS-MS. Metabolite identification studies with 3-methylleflunomide were conducted in a similar fashion. For hydrogen/deuterium exchange studies, incubations of leflunomide (final concentration, 50 μM) were conducted with recombinant P4501A2 in 0.1 M sodium phosphate buffer (pH 7.4) prepared in 99.996% D₂O (Cambridge Isotope Laboratories, Inc., Andover, MA).

Anaerobic incubations. Separate reaction vials containing human liver microsomal or recombinant P4501A2 membranes in a volume of 445 μl and a 10 mM NADPH stock solution in 0.1 M potassium phosphate buffer (pH 7.4) were crimp-sealed with rubber septa and made anaerobic over a period of approximately 1 h, on ice, by repeated cycles (>10) of vacuum at 35 mm Hg and pressurization (10–15 psi) with highly purified oxygen-free nitrogen. For anaerobic reactions with a carbon monoxide atmosphere, carbon monoxide replaced the nitrogen in the final two cycles. To initiate the reaction, leflunomide (final concentration, 100 μM) was injected with a gas-tight syringe into the reaction vials followed by transfer of the NADPH solution (final concentration of NADPH was 1.3 mM). The reaction vials were then transferred to a water bath at 37°C while the atmosphere of nitrogen or carbon monoxide was maintained. Identical reactions were also carried out aerobically. Reactions were terminated by adding acetonitrile (500 μl) via syringe. An additional 4.5 ml of acetonitrile was then added to the reaction mixtures, and the precipitate was removed by centrifugation at 3,000*g* for 15 min. The acetonitrile solution was evaporated to dryness in a vacuum centrifuge. The residue was dissolved in 200 μl of the initial HPLC mobile phase and equal aliquots were then subjected to LC/MS analysis.

***V*_{max} and *K*_m Determination in Human Liver Microsomes.** Leflunomide (0–500 μM) was incubated with pooled human liver microsomes (P450 concentration, 0.25 μM) and NADPH (1.2 mM) in 200 μl of 0.1 M phosphate buffer (pH 7.4) in duplicate. The reaction mixtures were prewarmed at 37°C for 2 min before adding NADPH, then incubated for 20 min.

Identification of the Human P450 Isozymes Responsible for the Conversion of Leflunomide to A771726. *Chemical inhibition studies.* For the mechanism-based inhibition studies on P4501A2, human liver microsomes (P450 concentration, 0.25 μM) were preincubated with NADPH (final concentration, 1.2 mM) at 37°C in the presence of the P4501A2 inactivator furafylline at a final concentration of 20 μM for 10 min. Leflunomide (final concentration, 2 μM) in methanol was then added, and the reaction mixture was further incubated for 30 min at 37°C. For competitive P450 inhibition studies, human liver microsomes (P450 concentration, 0.25 μM) were incubated with leflunomide (2 μM), NADPH (1.2 mM), a P450 inhibitor, quinidine at a final concentration of 1 μM (P4502D6), sulfaphenazole at a final concentration of 5 μM (P4502C9), ticlopidine at a final concentration of 10 μM (P4502C19), and ketoconazole at a final concentration of 2 μM (P4503A4/5) for 30 min at 37°C. Incubations were conducted in duplicate. The final concentration of methanol in the incubation media was 0.2% (v/v), and the total incubation volume was 0.6 ml. Periodically (0–30 min), aliquots (75 μl) of the reaction mixture were added to acetonitrile (200 μl) containing indomethacin (0.05 μg/ml) as internal standard and the samples were centrifuged at 2,500*g* for 5 min and analyzed for leflunomide disappearance.

Metabolism by heterologously expressed P450 isozymes. Leflunomide (final concentration, 2 μM) in methanol was incubated with microsomes from cells containing human recombinant P450s 1A2, 2B6, 2C9, 2C19, 2D6, 3A4, and 3A5 (P450 concentration, 0.01 μM) in the presence of NADPH (1.2 mM) at 37°C. Reactions were conducted in 0.1 M potassium phosphate buffer (pH 7.4) and were prewarmed at 37°C for 2 min before the addition of NADPH. All incubations were conducted in triplicate. The final concentration of methanol in the incubation media was 0.2% (v/v), and the total incubation volume was 0.6 ml. Periodically (0–60 min), aliquots (75 μl) of the reaction mixture were added to acetonitrile (200 μl) containing indomethacin (0.05 μg/ml) as internal standard, and the samples were centrifuged at 2,500*g* for 5 min and analyzed for leflunomide disappearance.

Cytosol Incubations. Cytosol incubations were conducted as described in previous reports (Sugihara et al., 1996). Reductive metabolism of leflunomide (2 μM concentration for substrate disappearance and 50 μM concentration for metabolite identification) or 3-methylleflunomide (50 μM concentration for metabolite identification) was carried out in nitrogen similar to the procedure described for anaerobic microsomal incubations, whereas aerobic metabolism

was conducted in test tubes open to air, with vigorous shaking. Standard incubations contained human liver cytosol (2 mg) or human liver cytosol (2 mg) + NADPH (final concentration, 2 mM) or human liver cytosol (2 mg) + 2-hydroxypyrimidine (final concentration, 100 μ M) in a final volume of 1 ml of 100 mM phosphate buffer (pH 7.4). Flasks were preincubated for 5 to 7 min in air or nitrogen as appropriate, and the reaction was started by the addition of leflunomide or 3-methylleflunomide in methanol. The reaction mixtures were incubated at 37°C for 40 min. Periodically (0–40 min), aliquots (75 μ l) of the reaction mixture were added to acetonitrile (200 μ l) containing indomethacin (0.05 μ g/ml) as internal standard, and the samples were centrifuged at 2,500g for 5 min and analyzed for leflunomide disappearance. In metabolite identification studies, the reaction mixtures were quenched by the addition of acetonitrile (2 volumes). The reaction mixtures were centrifuged (3,000g for 15 min) and 500- μ l aliquots of the supernatants were analyzed for metabolites by LC/MS-MS.

Incubations in Plasma, Whole Blood, and Albumin. *Plasma stability.* Fresh human blood and plasma was obtained by venipuncture of a healthy adult volunteer. Plasma was prepared by centrifugation, and blood products were maintained on ice until use (<1 h postcollection). Rat blood and plasma was obtained by abdominal aortic artery puncture of male Sprague-Dawley rats. Leflunomide (final concentration, 2 μ M) in methanol was incubated with fresh human or rat plasma (0.5 ml) in the presence or absence of 20 mM sodium fluoride at 37°C in a shaking water bath. Periodically (0–60 min), aliquots (75 μ l) of the reaction mixture were added to acetonitrile (200 μ l) containing indomethacin (0.05 μ g/ml) as internal standard and were analyzed for leflunomide disappearance. Incubations were conducted in duplicate.

Blood stability. Leflunomide (final concentration, 2 μ M) in methanol was incubated with fresh human or rat blood (2 ml) at 37°C in a shaking water bath. Periodically (0–60 min), aliquots (500 μ l) of the leflunomide/blood mixture were centrifuged (3,000g for 5 min) to harvest plasma. Aliquots of the harvested plasma (75 μ l) were immediately added to acetonitrile (200 μ l) containing indomethacin (0.05 μ g/ml) as internal standard and were analyzed for leflunomide disappearance. Incubations were conducted in duplicate.

Albumin stability. Leflunomide (final concentration, 2 μ M) in methanol was incubated with 2% human or bovine serum albumin in Dulbecco's PBS (pH 7.4) at 37°C in a shaking water bath. The volume of the incubation was 0.6 ml. Periodically (0–60 min), aliquots (75 μ l) of the leflunomide/albumin mixture were added to acetonitrile (200 μ l) containing indomethacin (0.05 μ g/ml) as internal standard and were analyzed for leflunomide disappearance. Incubations were conducted in duplicate.

Temperature and pH Stability of Leflunomide. Leflunomide (final concentration, 2 μ M) in methanol was incubated with 100 mM potassium phosphate buffer (1 ml) at a pH range of 4.0 to 10. The pH of the buffer was adjusted with 1 N HCl or dibasic potassium phosphate. Incubations were conducted at 25°C and at 37°C for 3 h in a shaking water bath. Periodically (0–3 h), aliquots (75 μ l) of the reaction mixture were added to acetonitrile (200 μ l) containing indomethacin (0.05 μ g/ml) as internal standard, and the samples were analyzed for leflunomide disappearance. Incubations were conducted in duplicate.

Data Analysis. Substrate saturation data were plotted on Eadie-Hofstee plots to check for linearity or nonlinearity of data. After this examination, data were plotted on substrate saturation curves and fitted to the Michaelis-Menten equation using SigmaPlot (version 8.0, SPSS Science, Chicago, IL) to yield apparent K_m and V_{max} values.

For $t_{1/2}$ determinations in leflunomide depletion experiments, leflunomide/internal standard peak height ratios were determined and normalized to the value obtained at time $t = 0$. The percentage of leflunomide remaining versus time was fitted to first-order decay functions to yield *in vitro* $t_{1/2}$ values. If leflunomide demonstrated nonlinearity on log percentage remaining versus time curves, only those initial time points wherein log-linearity was observed were used to determine $t_{1/2}$ values.

LC/MS-MS Assay for Leflunomide Quantitation. Leflunomide depletion was monitored on a Sciex API model 3000 LC/MS-MS triple quadrupole mass spectrometer. Leflunomide and A771726 were detected using ESI in the negative ionization mode by monitoring their precursor-product combination in multiple reaction-monitoring mode. The ion spray interphase temperature was 400°C and nitrogen was used as the nebulizing and heating gas. The detecting channels for both leflunomide and A771726 were m/z 269 $\rightarrow$ m/z 82.

In Fig. 3, the product ion spectra of leflunomide (Fig. 3B) and A771726 (Fig. 3C) are shown. In the case of leflunomide and A771726, this reaction follows the deprotonated parent mass at 269 to its CID fragment at m/z 82, which corresponds to the 5-methylisoxazolyl moiety in leflunomide and the cyanomethyl methyl ketone group in A771726, respectively.

Since both leflunomide and A771726 displayed similar mass spectral properties, for the purposes of accurate quantitation it was crucial that the two analytes were easily separable by LC. A reasonable separation of leflunomide and A771726 was observed under reversed-phase LC conditions using a binary gradient consisting of a mixture of 95% water/5% acetonitrile with 0.1% acetic acid (solvent A) and 95% acetonitrile/5% water with 0.1% acetic acid (solvent B). The flow rate was 0.7 ml/min with initial conditions of 90% solvent A and 10% solvent B. After injection, the percentage of solvent B was increased linearly from 10 to 95% over 1.0 min and held there for 3.0 min before rapidly returning to the initial conditions. The total run time was 4.0 min. Under these conditions, leflunomide and A771726 eluted at 2.2 min and 1.8 min, respectively (Fig. 3A).

LC/MS-MS Assay for Metabolite Identification. Qualitative assessments of leflunomide and 3-methylleflunomide metabolism were conducted on a Sciex API model 2000 LC/MS-MS triple quadrupole mass spectrometer in conjunction with an LDC Analytical SpectroMonitor 3200 variable wavelength UV detector (LDC Analytical, Riviera Beach, FL). Leflunomide and its ring-opened metabolite A771726 were chromatographically separated using a Hewlett Packard Series 1100 HPLC system [Zorbax Eclipse XDB-C8 4.6 $\times$ 150 mm column (Agilent Technologies, Palo Alto, CA)] using a binary gradient consisting of a mixture of 10 mM ammonium formate, 0.1% formic acid (solvent A), and acetonitrile (solvent B) at a flow rate of 1 ml/min. The LC gradient was programmed as follows: solvent A to solvent B ratio was held at 100:0 (v/v) for 3 min and then adjusted from 100:0 (v/v) to 10:90 (v/v) for 20 min and from 10:90 (v/v) to 100:0 (v/v) from 20 to 25 min. Postcolumn flow was split such that mobile phase was introduced into the mass spectrometer via an ion spray interface at a rate of 50 μ l/min. The remaining flow was diverted to the UV detector positioned in line to provide simultaneous UV detection at $\lambda = 254$ nm and total ion chromatogram. Ionization was conducted in the negative ion mode at the ion spray interface temperature of 150°C using nitrogen as a nebulizing gas. Ion spray voltage was 4.5 kV, and the orifice voltage was 30 eV. Under these conditions, leflunomide and A771726 eluted at 16.5 and 12.7 min, respectively. The compounds were detected in a negative ion mode using precursor ion scanning of the fragment ion m/z 82. Metabolite identification studies on 3-methylleflunomide ($R_t = 16.5$ min) were also conducted in the negative ion mode. Initial Q1 scans were performed between m/z 50 and 500. 3-Methylleflunomide metabolites in rat and human liver microsomes were detected by comparing samples at initial time 0 with 30-min incubation samples (with or without NADPH). Structural information was generated from CID fragmentation of the corresponding molecular ions.

Results

pH and Temperature-Dependent Decomposition of Leflunomide to A771726. Since isoxazoles readily undergo base-catalyzed ring scission reactions, leflunomide stability was determined at various pH values by monitoring the disappearance of leflunomide (and the concomitant formation of A771726) by LC/MS-MS. The pH stability experiment was conducted at ambient temperature ($\sim 25^\circ\text{C}$) and at 37°C. At 25°C, leflunomide was resistant to isoxazole ring opening at acidic (4.0) and neutral (7.4) pH, but decomposed to A771726 at basic pH (10.0) with an estimated $t_{1/2}$ of ~ 6.0 h (Fig. 4A). Although leflunomide was also stable at 37°C in acidic pH buffer, conversion of leflunomide to A771726 was noticeable as the solution became more basic. The apparent $t_{1/2}$ for leflunomide decomposition at 37°C (pH 7.4) was 7.4 h and the observed $t_{1/2}$ at 37°C (pH 10) was 1.2 h (Fig. 4B). Thus, base-catalyzed ring opening in leflunomide was considerably faster at 37°C than at ambient temperature. A LC/MS-MS chromatogram of leflunomide degradation to A771726 after a 2-h incubation in pH 10 buffer at 37°C is also depicted in Fig. 3A. In contrast with these results, 3-methylleflunomide was stable in buffer (pH 4.0–10.0) at 25 or 37°C.

Stability of Leflunomide in Human and Rat Plasma and Whole Blood. To examine the metabolic stability of the isoxazole ring in leflunomide in plasma and whole blood, leflunomide at a concentration of 2 μM was incubated with fresh rat and human plasma and blood at 37°C for 60 min. The $t_{1/2}$ for leflunomide decomposition to A771726 in rat and human plasma was 36 and 12 min, respectively, whereas $t_{1/2}$ in rat and human whole blood was 59 and 43 min, respectively. Pretreatment of human plasma with the nonspecific hydrolase inhibitor sodium fluoride (Kozak et al., 2001) at a concentration of 20 mM did not prevent isoxazole ring opening ($t_{1/2} \sim 13$ min). The stability of leflunomide toward isoxazole ring opening was also assessed in human and bovine serum albumin. Although leflunomide was stable in bovine serum albumin, human serum albumin catalyzed the conversion of leflunomide to A771726 with an apparent $t_{1/2}$ of 110 min. Representative results for leflunomide decomposition in human plasma, blood, and albumin are shown in Fig. 5. Under the present assay conditions, 3-methylleflunomide was stable in rat and human plasma and blood.

Leflunomide Stability in Liver Microsomes and Cytosol. To assess the conversion of leflunomide to A771726 in liver microsomes, leflunomide (2 μM) was incubated with rat and human liver microsomes in the presence of NADPH cofactor for 30 min at 37°C. In control experiments, NADPH was omitted from the microsomal incubations. The estimated $t_{1/2}$ for leflunomide disappearance in rat and human liver microsomes in the presence of NADPH was 40 and 22

min, respectively. Some leflunomide conversion to A771726 was also observed in human and rat liver microsomal incubations lacking NADPH (~ 5 –6% decomposition in a 30-min period). Metabolite identification studies on rat and human liver microsomal incubations containing leflunomide (50 μM) also revealed the formation of A771726 in incubations lacking NADPH (Fig. 6B). However, the yield of A771726 from microsomal incubations lacking cofactor was significantly lower than the yield obtained in the presence of cofactor. Complete attenuation of A771726 formation in boiled human liver microsomes (data not shown) suggested that some non-NADPH-dependent protein(s) in human liver microsomes could also catalyze isoxazole ring opening in leflunomide. Finally, no ring opening was discernible in leflunomide incubations in NADPH alone. Under the present experimental conditions, no other metabolites besides A771726 were detected in rat and human liver microsomal incubations upon analysis of the initial Q1 scans. Figure 6 illustrates LC/MS-MS chromatograms of leflunomide-human microsomal incubations at time 0 (Fig. 6A) and at 30 min in the absence (Fig. 6B) or presence of NADPH (Fig. 6C). In contrast with the findings in liver microsomes, leflunomide (2 or 50 μM) was stable in human liver cytosol in the presence or absence of NADPH or an electron donor (2-hydroxypyrimidine) during the length of the incubation ($t_{1/2} > 120$ min).

V_{max} and K_m Determination for Leflunomide Metabolism in Human Liver Microsomes. The steady-state Michaelis-Menten con-

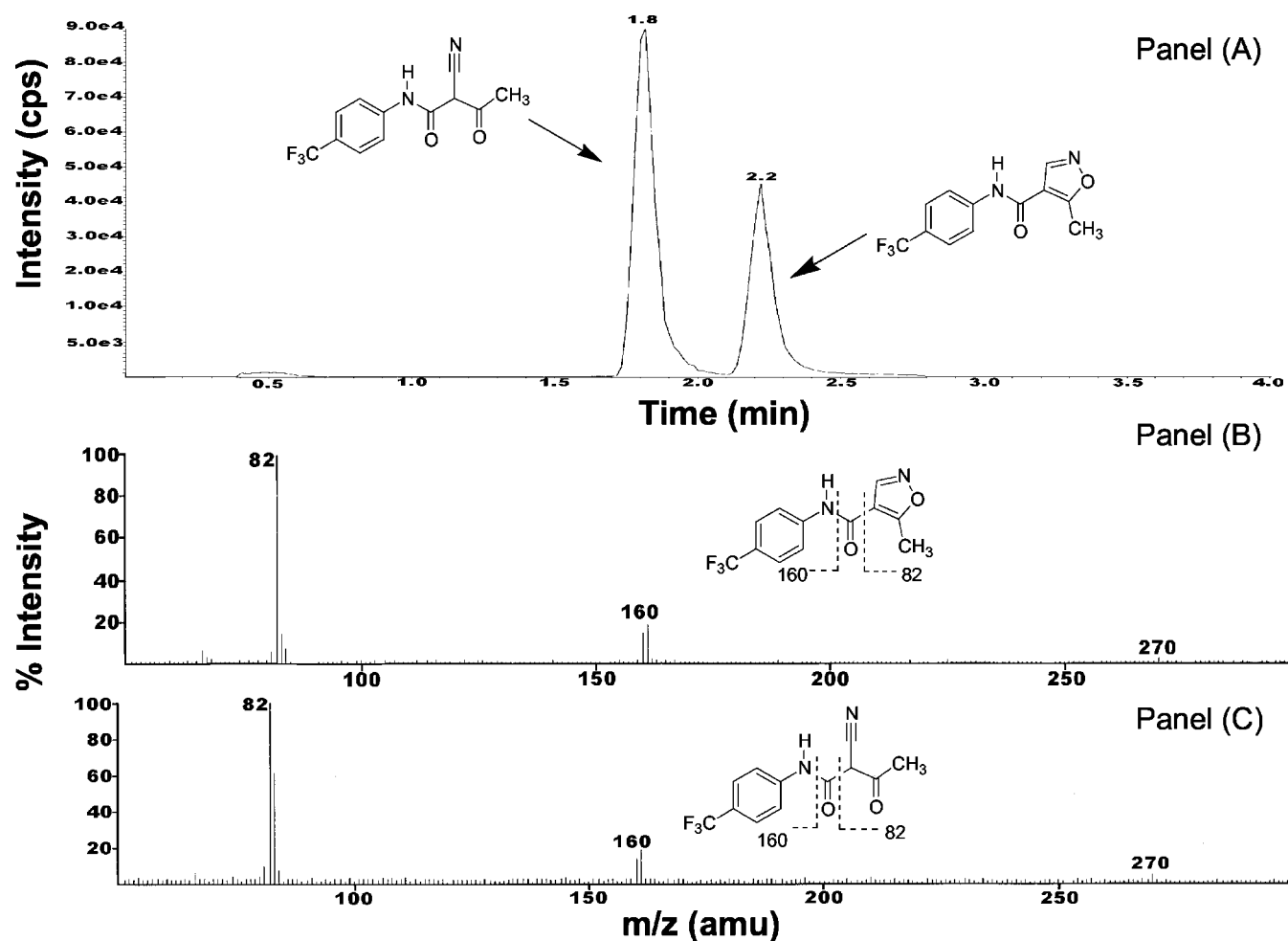


FIG. 3. LC/MS-MS chromatogram (multiple reaction monitoring of m/z 269 $\rightarrow$ m/z 82) in the negative ionization mode of an incubation mixture containing leflunomide (2 μM) in pH 10.0 buffer at 37°C for 2 h.

The product ion spectra of leflunomide and A771726 are also shown.

stants $V_{\max}$ and K_m for A771726 formation in pooled human liver microsomes were 1,797 pmol/min/mg and 274 μ M, respectively (Fig. 7). Furthermore, substrate saturation experiments suggest the presence of two kinetically distinguishable activities as observed on an Eadie-Hofstee plot (Fig. 7, inset).

Identification of the P450 Isozymes Responsible for the Metabolism of Leflunomide to A771726. Data were also obtained to address the identity of human P450 isozymes involved in the metabolism of leflunomide to A771726. Leflunomide (2 μ M) was incubated with human liver microsomes containing NADPH in the absence or presence of isozyme-selective P450 inhibitors at 37°C. Isoxazole ring opening was markedly inhibited in human liver microsomes pretreated with the P4501A2 inactivator furafylline ($t_{1/2} > 120$ min). In contrast, pretreatment of human liver microsomes with quinidine (P4502D6 inhibitor), sulfaphenazole (P4502C9 inhibitor), ticlopidine (P4502C19 inhibitor), and ketoconazole (P4503A4 inhibitor) was not as effective in inhibiting leflunomide metabolism (data not shown). Thus, in human liver microsomes, P4501A2 appears to be the major isozyme responsible for leflunomide metabolism to A771726.

To further establish the role of P450 enzymes in the metabolism of leflunomide to A771726, leflunomide (2 μ M) was incubated with several recombinant human P450 enzymes (P4501A2, -2B6, -2C9, -2C19, -2D6, -3A4, and -3A5) in the presence of NADPH cofactor at 37°C. Although relatively stable in P4502B6, -2C9, -2D6, and -3A5, leflunomide was metabolized to A771726 in an NADPH-dependent fashion in the presence of P4501A2, -2C19, and -3A4 isozymes with $t_{1/2}$ values of 8, 25, and 29 min, respectively (Fig. 8). Thus, in addition to P4501A2, P4502C19 and P4503A4 isozymes are also capable of catalyzing the isoxazole ring opening in leflunomide.

Liver Microsomal Metabolite Profile of 3-Methylleflunomide. The effect of 3-methyl substitution on the isoxazole ring opening in leflunomide was also investigated by incubating 3-methylleflunomide in NADPH-supplemented rat and human liver microsomes. 3-Methylleflunomide was synthesized via the nucleophilic substitution of 3,5-dimethylisoxazole-4-carbonyl chloride with 4-trifluoromethylaniline. LC/MS-MS analysis in the negative ion mode indicated the NADPH-dependent formation of two polar metabolites **M1** and **M2** (the R_f s of 3-methylleflunomide, **M1**, and **M2** were 16.5, 16.2, and 15.8 min, respectively) (Fig. 9). The LC/MS-MS spectra of **M1** and **M2** displayed deprotonated molecular ions at 299 (i.e., 16 mass units higher than those observed for 3-methylleflunomide) consistent with two monohydroxylated 3-methylleflunomide metabolites. No other

3-methylleflunomide metabolites were detected in these microsomal incubation mixtures.

The CID spectrum of the molecular ion at 299 from **M1** (Fig. 10B) indicated fragment ions at m/z 269 (15%), 160 (25%), and 82 (100%) and that from **M2** (Fig. 10C) displayed an intense fragment ion at 160 (100%). The fragment ion at m/z 160 in **M1**, **M2**, and 3-methylleflunomide corresponded to the trifluoromethylaniline moiety suggesting that the 3,5-dimethylisoxazole ring was the site of hydroxylation in both **M1** and **M2**. The mass spectrum of **M1** was remarkably similar to that of leflunomide and A771726. In the case of leflunomide and A771726, the fragment ion m/z 269 corresponds to their respective molecular ions, whereas the fragment ion m/z 82 corresponds to the 5-methylisoxazolyl moiety in leflunomide and the cyanomethyl methyl ketone group in A771726, respectively. Therefore, a proposed structure for **M1** that accounts for the fragment ions m/z 269 and 82 is 3-hydroxymethylleflunomide, since elimination of formaldehyde from this structure resulting in A771726 is the only conceivable pathway that affords fragment ions 269 and 82 (Fig. 10B). On this basis, we propose 5-hydroxymethylleflunomide to be the structure of **M2** (Fig. 10C).

Studies on the Mechanism of the P450-Catalyzed Isoxazole Ring Opening. The lack of isoxazole ring opening in 3-methylleflunomide strongly suggested that the hydrogen at C-3 in leflunomide is essential for ring opening. In an attempt to provide further evidence for the P450-mediated deprotonation of the C-3 hydrogen in leflunomide, we examined the possibility of C-3 hydrogen/deuterium exchange in leflunomide/recombinant P4501A2 incubations in deuterated phosphate buffer. LC/MS-MS analysis of the incubation mixtures did not reveal the presence of C-3-deuterio-leflunomide.

To assess whether the P450-mediated isoxazole ring opening in leflunomide is also catalyzed by the Fe(II) form of the enzyme, leflunomide-human liver microsomal or recombinant P4501A2 incubations were conducted in the presence of NADPH under anaerobic conditions and also in the presence of carbon monoxide. As previously determined, the presence of NADPH was essential for efficient isoxazole ring opening, but the presence of molecular oxygen in the incubation mixtures greatly inhibited the formation of A771726. For

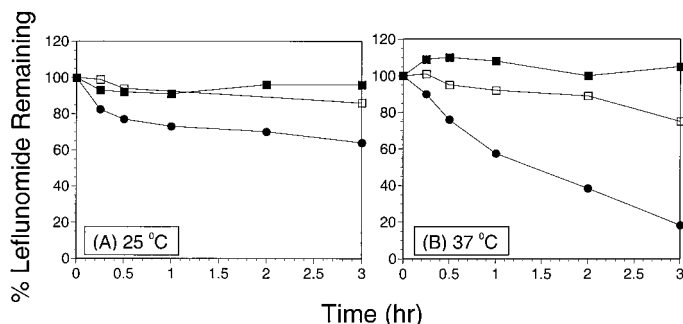


FIG. 4. pH and temperature stability of the isoxazole ring in leflunomide.

Leflunomide (2 μ M) in methanol was incubated with pH 4.0 (■), pH 7.4 (□), and pH 10.0 (●) buffer. Incubations were conducted at 25°C (A) and at 37°C (B) for 3 h in a shaking water bath. Periodically (0–3 h), aliquots (75 μ l) of the reaction mixture were added to acetonitrile (200 μ l) containing internal standard, and the samples were analyzed for leflunomide disappearance by LC/MS-MS. Incubations were conducted in duplicate.

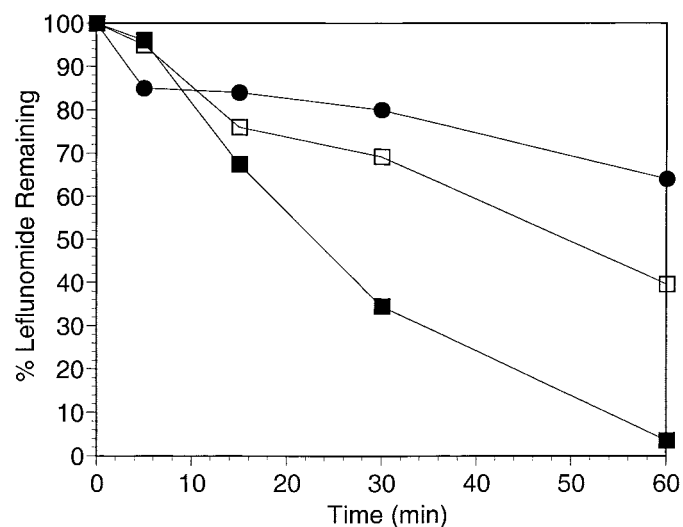


FIG. 5. Leflunomide stability in human plasma, whole blood, and albumin.

Leflunomide (2 μ M) in methanol was incubated with plasma (■), whole blood (□), and albumin (●) at 37°C for 60 min in a shaking water bath. Periodically (0–60 min), aliquots (75 μ l) of the reaction mixture were added to acetonitrile (200 μ l) containing internal standard, and the samples were analyzed for leflunomide disappearance by LC/MS-MS. Incubations were conducted in duplicate.

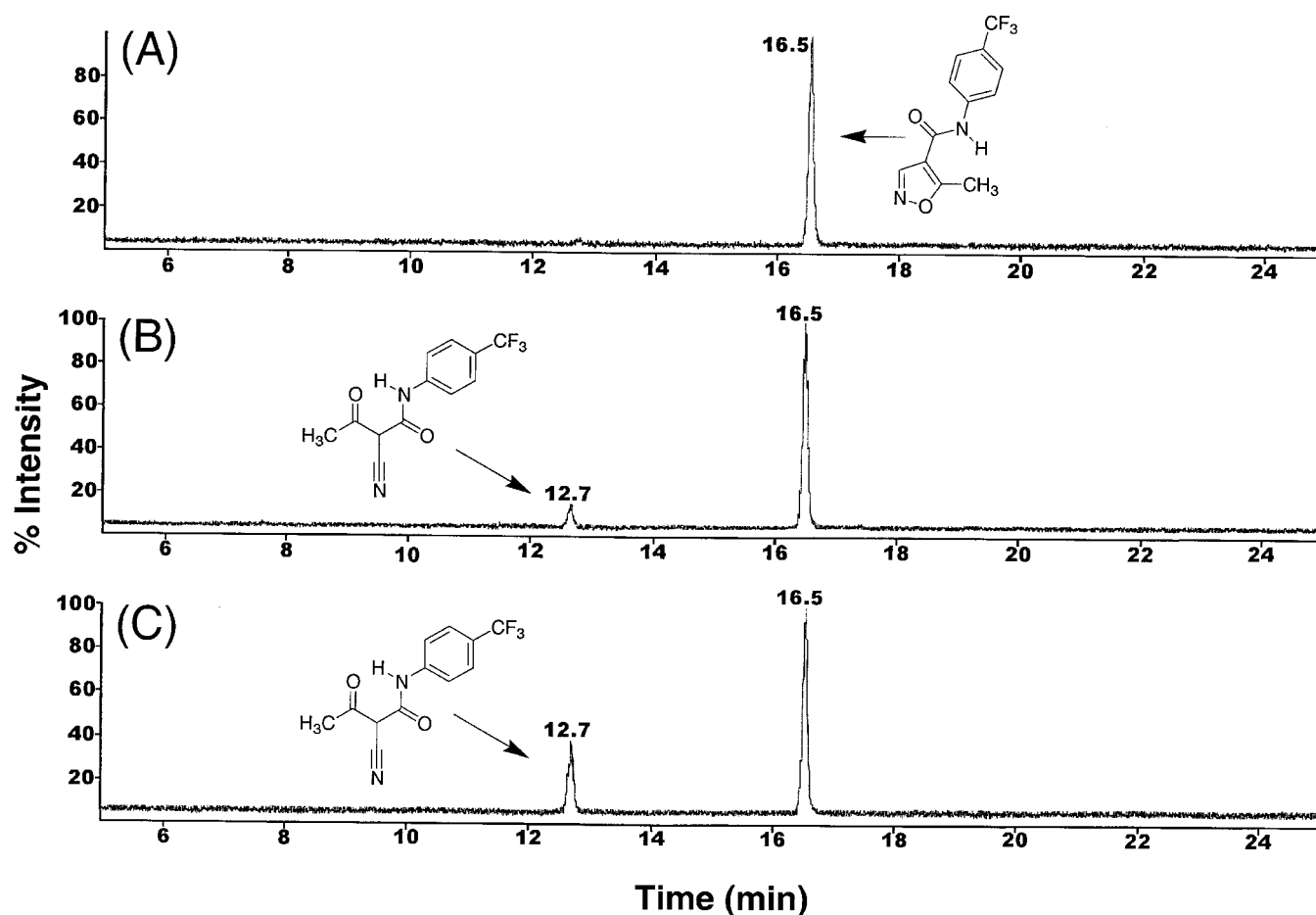


FIG. 6. LC/MS-MS chromatogram (precursor ion scan of m/z 269 $\rightarrow$ m/z 82) in the negative ionization mode of a human liver microsomal incubation mixture containing leflunomide (50 μ M) at time 0 (A) and at 30 min in the absence (B) or the presence (C) of NADPH cofactor.

instance, in aerobic human liver microsomal and recombinant P4501A2 incubations, the A771726/leflunomide peak height ratio was reduced by approximately 90 and 80%, respectively, when compared with that observed in anaerobic incubations. Furthermore, addition of carbon monoxide strongly decreased A771726 formation by >90% in anaerobic human liver microsomal and recombinant P4501A2 incubations.

Discussion

Leflunomide is the first orally active, disease-modifying, anti-inflammatory drug to be approved in the past decade. Approximately 95% of orally administered leflunomide is converted to its active metabolite A771726. A771726 displays linear pharmacokinetics at the dosing regimen of leflunomide used in clinical practice. It has a long elimination $t_{1/2}$ (approximately 2 weeks), reaching a steady state after approximately 20 weeks (Rozman, 2002). The present findings constitute the first report on the in vitro characterization of the chemical and the biochemical isoxazole ring opening in leflunomide to its active α -cyanoenol metabolite A771726.

Consistent with the known base instability of 3-unsubstituted isoxazoles, the isoxazole ring in leflunomide was efficiently cleaved as a function of increased pH and temperature. Under physiological conditions (pH 7.4 at 37°C), the isoxazole ring in leflunomide also underwent facile ring opening to A771726 in the presence of rat and human plasma and blood. Human plasma and whole blood catalyzed the ring opening at a slightly faster rate than rat plasma and whole blood. Pretreatment of human plasma with the nonspecific hydrolase

inhibitor sodium fluoride did not prevent the isoxazole ring opening, suggesting that esterases/amidases are not responsible for leflunomide decomposition to A771726. In contrast, albumin, the most abundant

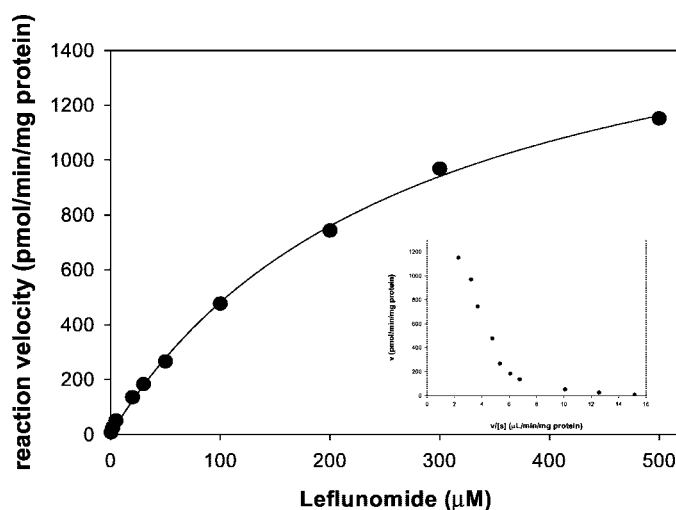


FIG. 7. Leflunomide (0–500 μ M) was incubated with human liver microsomes pooled from 56 individual donors (P450 concentration, 0.25 μ M) in the presence of NADPH.

A771726 concentrations were determined as described in the experimental procedures. Each point represents the average of duplicate determinations. Inset, Eadie-Hofstee plot.

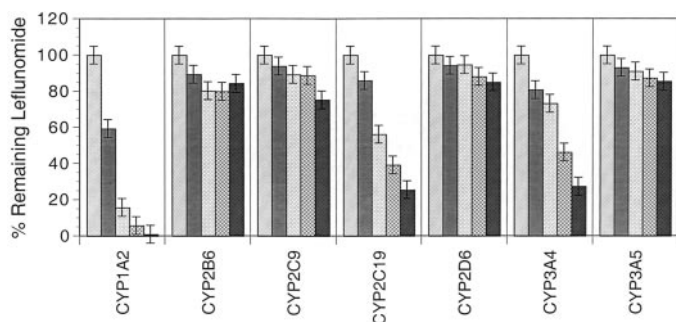


FIG. 8. Leflunomide stability in human recombinant P450 isozymes.

Leflunomide (2 μ M) was incubated with several recombinant human P450 enzymes (P4501A2, -2B6, -2C9, -2C19, -2D6, -3A4, and -3A5) in the presence of NADPH cofactor at 37°C, and periodically (0–60 min), aliquots of the reaction mixture were analyzed for leflunomide disappearance. All incubations were conducted in triplicate.

protein in blood serum (Peters, 1996), catalyzed the leflunomide ring opening, albeit at a slower rate than that observed in human whole blood or plasma. Thus, it is likely that components of human serum other than albumin may also catalyze the isoxazole ring opening in leflunomide. Overall, the involvement of albumin in leflunomide metabolism is not surprising considering its well established role in the metabolism of xenobiotics, particularly its esterase activity with drugs such as aspirin (Walker, 1976) and its ability to catalyze β -elimination reactions on drugs such as 4-hydroxycyclophosphamide (Kwon et al., 1987), felbamate (Roller et al., 2002), and certain prostaglandin analogs (Fitzpatrick et al., 1984; Kozak et al., 2001).

Although leflunomide was stable in cytosol, the drug was extensively metabolized to A771726 in rat and human liver microsomes in an NADPH-dependent fashion. No other metabolites, including the aldoxime intermediate **1** depicted in Fig. 1, pathway A, were discernible in the microsomal or cytosolic reaction mixtures. Examination of the Eadie-Hofstee plot of leflunomide metabolism in human liver microsomes indicated the presence of at least two kinetically distinguishable activities. Chemical inhibition studies in human liver microsomes suggested P4501A2 as the principal P450 isozyme responsible for leflunomide metabolism. In addition to P4501A2, subsequent studies with human recombinant P450 isozymes also indicated a role for P4502C19 and -3A4 in leflunomide metabolism. Our *in vitro* finding on the involvement of P4501A2 as the principal P450 isozyme responsible for leflunomide metabolism is consistent with the *in vivo* observation on the increased plasma clearance of leflunomide in smokers, presumably via the induction of P4501A2 (Rozman, 2002). *In vivo*, the individual contribution of these biological systems (gut wall and hepatic P450 and blood) toward overall leflunomide plasma clearance remains to be characterized.

The results of our present investigation also shed light on the mechanism of isoxazole ring scission in leflunomide. The lack of ring opening with 3-methylleflunomide indicated that the C3–H in leflunomide is essential for ring opening. This observation strongly suggests that the isoxazole ring opening in leflunomide must proceed through the C3–H deprotonation and elimination process depicted in Fig. 1, pathway B, rather than the reductive ring opening pathway shown in Fig. 1, pathway A. This outcome is not surprising, considering that chemical reaction of 3-unsubstituted isoxazoles with bases, leading to

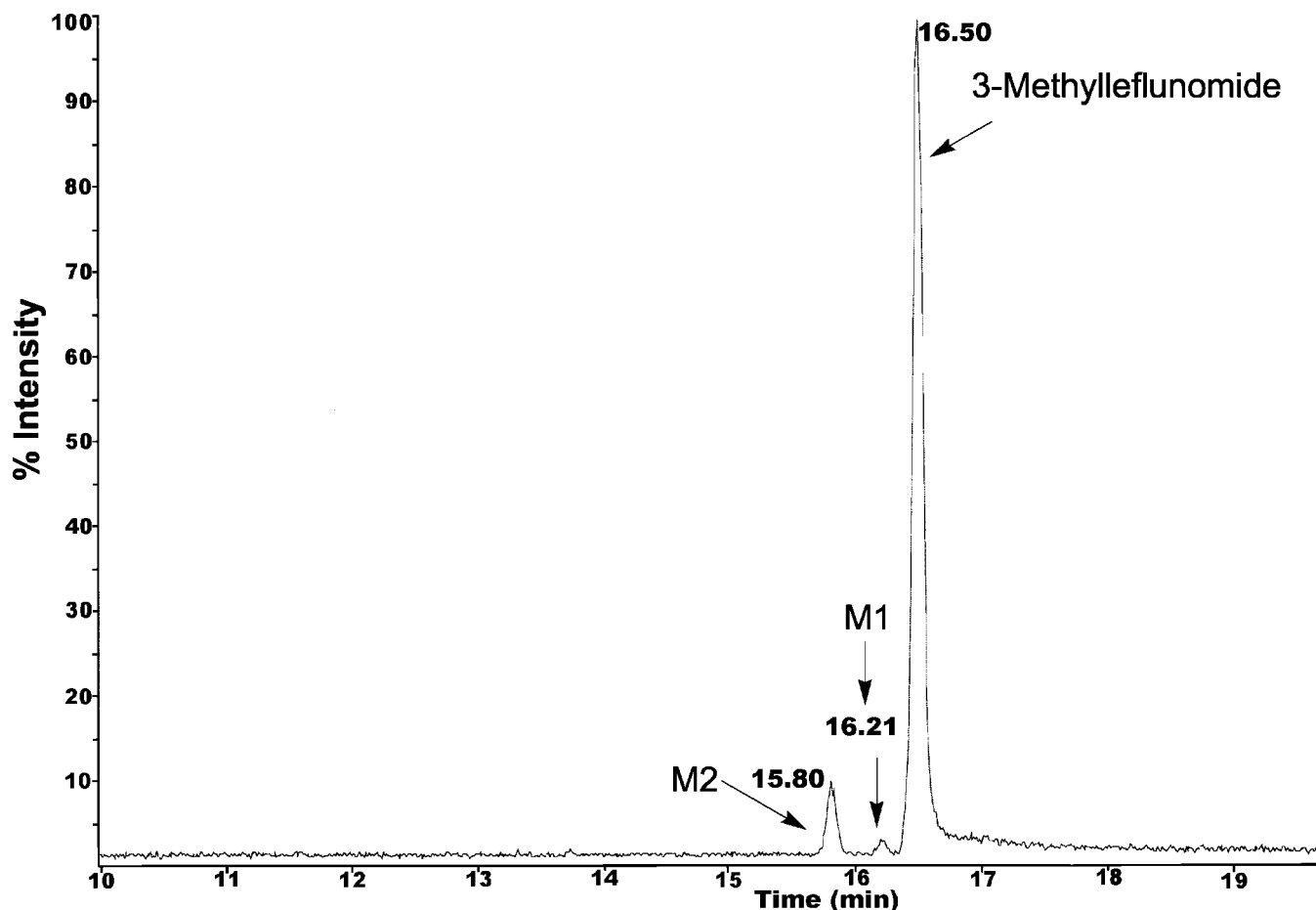


FIG. 9. Representative LC/MS-MS chromatogram of an incubation of 3-methylleflunomide (50 μ M) in NADPH-supplemented human liver microsomes.

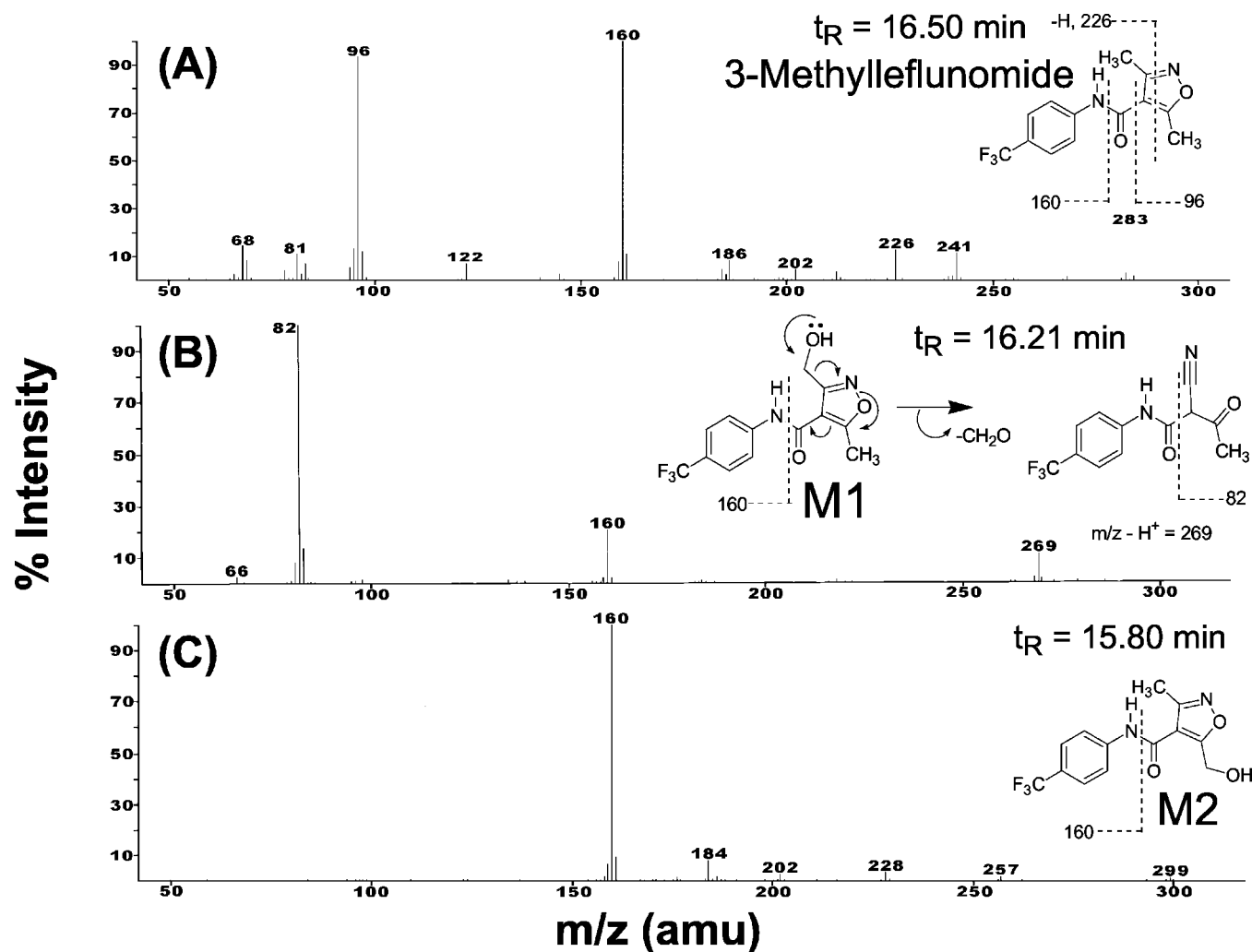


FIG. 10. Product ion spectra obtained by CID of $M-H^+$ ions m/z 283 (3-methylleflunomide, A), m/z 299 (M1, B), and m/z 299 (M2, B).

The origins of characteristic ions are as indicated.

α -cyanoenol-like intermediates via ring scission, has been established over a century ago (for a review on isoxazole chemistry, see Wakefield and Wright, 1979), and this property has been conveniently exploited as a building block in synthetic methodology (McGregor et al., 1969; Wakefield and Wright, 1979; Cillar et al., 1985; Perez et al., 1996).

Base (hydroxide ion)-catalyzed deprotonation of the C3-H in 3-unsubstituted isoxazoles has been studied in detail with 4- and 5-arylisoxazole derivatives (De Munno et al., 1977). Studies with 3-deuterio-5-phenylisoxazole reveal a primary deuterium isotope effect ($k_H/k_D = 3.1$), suggesting that deprotonation of the C3-H by hydroxide is rate determining for ring opening, and UV spectra of the reaction mixtures indicate sharp isosbestic points, suggesting an "intermediate-free" conversion of isoxazole to α -cyanoenol, consistent with a concerted E2 elimination process. This E2 elimination hypothesis is also consistent with the recent work of Zipse et al. (1995), indicating that the anion formed by deprotonation of isoxazole is unstable, opening spontaneously to cyanoenolate. Given the available chemical evidence on the instability of the isoxazole C-3 anion, failure to observe hydrogen/deuterium exchange in leflunomide/P4501A2 incubations in deuteriated phosphate buffer in the present investigation is not altogether surprising. Based on the lack of H/D exchange in the enzymatic reaction, it is tempting to speculate that the P450-catalyzed

reaction also proceeds through a concerted C-3 deprotonation/isoxazole ring-opening pathway. Kinetic isotope effect measurements on the P450-mediated ring opening of C-3-deuterio-leflunomide should help resolve some of these issues.

The results from the studies by De Munno et al. (1977) also reveal that the rate of isoxazole ring scission is faster in 4-phenylisoxazole than in 5-phenylisoxazole and that electron-withdrawing groups (e.g., Br and NO_2) in the *para* position of 4-phenylisoxazole dramatically accelerate the rate of ring opening. Thus, in the case of leflunomide, it is conceivable that under chemical and biochemical conditions, the electron-withdrawing *N*-(4-trifluoromethylphenyl)amino-carbonyl moiety at the C4 position is responsible for the resonance stabilization of the negatively charged enolate ion in A771726 after ring scission (see Fig. 1, pathway B).

In retrospect, the enzymatic/nonenzymatic C3-H deprotonation and elimination reaction sequence in 3-unsubstituted isoxazoles such as leflunomide appears to be analogous to the P450-catalyzed dehydrations of alkylaldoximes ($R-CH=N-OH$) to the corresponding cyano ($R-CN$) metabolites (Boucher et al., 1994; Mathews et al., 1998), since the isoxazole ring can be considered a cyclic *O*-substituted aldoxime derivative. The proposed mechanism for the cytochrome P450-mediated N-O bond cleavage in aldoximes presumably involves initial coordination of the aldoxime nitrogen to the reduced form of

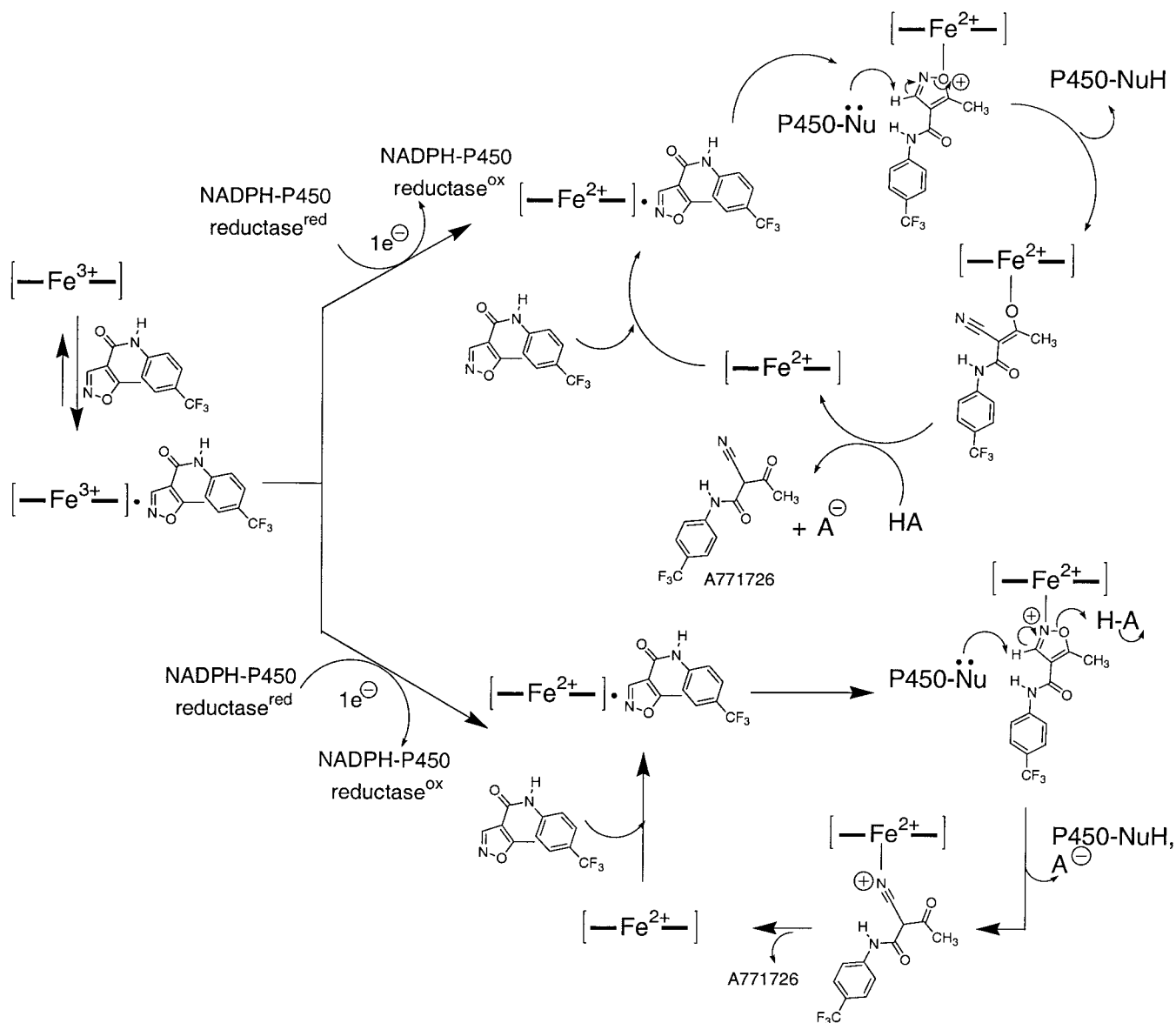


FIG. 11. Proposed mechanisms of the P450-mediated isoxazole ring scission of leflunomide under anaerobic conditions.

the heme [P450Fe(II)] followed by charge transfer from P450Fe(II) to the C=N bond, leading to cleavage of the N–O bond (Boucher et al., 1994). In the case of aldoximes, this pathway is supported by the key observations that NADPH but not molecular oxygen is essential for catalysis and that the dehydration is inhibited by carbon monoxide. Similar mechanistic observations were also noted in the P450-catalyzed isoxazole ring opening in human liver microsomes and P4501A2. Therefore, in the case of isoxazoles, we speculate that either isoxazolyl oxygen or nitrogen atom could coordinate to P450Fe(II) (Fig. 11). In either case, the ferrous form of the enzyme P450Fe(II) acts as a Lewis acid lowering the pK_a of the C3–H and weakening the N–O bond. This would allow a weakly basic active-site amino acid or water molecule to deprotonate the C3 proton. Spectral measurements that will address the formation of P450Fe(II)-leflunomide complexes and kinetic studies on the comparison of the rates of NADPH-dependent leflunomide ring opening in liver microsomes and recombinant enzymes under aerobic and anaerobic conditions are currently underway.

Finally, it is worth commenting on the differences in the P450-

mediated metabolism of the isoxazole ring in 3-unsubstituted isoxazoles and 3-substituted-isoxazoles and -1,2-benzisoxazoles. It is obvious that the presence of the C3-substituent in 3-substituted isoxazoles (e.g., 3-methylleflunomide) and -1,2-benzisoxazoles (e.g., zonisamide, risperidone, and iloperidone) is mainly responsible for hydroxylation or reductive ring-opening reactions. Absence of the C3-substituent in the case of leflunomide results in metabolic switching from reductive isoxazole ring opening to deprotonation and elimination. In this aspect, it would be interesting to characterize the biotransformation of a zonisamide analog that lacks the 3-methylsulfonyl substituent.

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