

Novel Cytochrome P450-Mediated Ring Opening of the 1,3,4-Oxadiazole in Setileuton, a 5-Lipoxygenase Inhibitor^S

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ABSTRACT:

Setileuton [4-(4-fluorophenyl)-7-[(5-[(1S)-1-hydroxy-1-(trifluoromethyl)propyl]-1,3,4-oxadiazol-2-yl)amino)methyl]-2H-1-benzopyran-2-one] is a selective inhibitor of the 5-lipoxygenase enzyme, which is under investigation for the treatment of asthma and atherosclerosis. During the development of setileuton, a metabolite (M5) was identified in incubations with rat, dog, and human liver microsomes that represented the addition of 18 Da to the 1,3,4-oxadiazole portion of the molecule. Based on mass spectral data, a ring opened structure was proposed and confirmed through comparison with a synthetic standard. The metabolic ring opening was examined *in vitro* in rat liver microsomes and was determined

to be mediated by cytochrome P450s (P450s). Upon examination of the specific P450s involved using cDNA-expressed rat P450s, it was shown that CYP1A2 likely was the major isoform contributing to the formation of M5. Studies using stable labeled molecular oxygen and water demonstrated that the oxygen was incorporated from molecular oxygen, rather than water, and confirmed that the metabolic formation was oxidative. An alternative, comparatively slow pathway of chemical hydrolysis also was identified and described. Three potential mechanisms for the two-step metabolic ring opening of the 1,3,4-oxadiazole are proposed.

Introduction

Many oxadiazole-containing compounds have been developed as drug candidates since the 1970s (Allen et al., 1971; Saunders et al., 1990; Allan et al., 2006; Bateman et al., 2006; Hall et al., 2007). These compounds have been used for various indications including postsurgical dermal scarring, asthma, pain, Alzheimer's disease, and anticonvulsants. Incorporation of an oxadiazole ring may be used in place of an ester moiety to offer enhanced resistance to hydrolytic cleavage mediated by esterases (Dalvie et al., 2002). Although many oxadiazole-containing drug candidates have been reported, information regarding the biotransformation of the oxadiazole ring is scarce. In fact, the only oxadiazole isomer with reported biotransformation is the 1,2,4-oxadiazole, which can undergo a ring opening through reductive cleavage of the N-O bond (Lan et al., 1973; Dalvie et al., 2002; Allan et al., 2006; Bateman et al., 2006). Setileuton [4-(4-fluorophenyl)-7-[(5-[(1S)-1-hydroxy-1-(trifluoromethyl)propyl]-1,3,4-oxadiazol-2-yl)amino)methyl]-2H-1-benzopyran-2-one] contains a 1,3,4-oxadiazole ring and is under development as an inhibitor of the 5-lipoxygenase (5-LO) enzyme.

The 5-lipoxygenase enzyme catalyzes the initial steps in the conversion of arachidonic acid to leukotrienes (Funk, 2005; Radmark and Samuelsson, 2005). Leukotrienes are potentially important mediators of inflammation and have been implicated in the pathology of several diseases including asthma, psoriasis, rheumatoid arthritis, and atherosclerosis (Ford-Hutchinson, 1985; Israel et al., 1990; Huang et al., 2004a; Funk, 2005). It is thought that inhibition of the 5-LO enzyme could be a means of treating such diseases. Setileuton, a selective inhibitor of 5-LO, is under investigation for the treatment of asthma and atherosclerosis.

During the development of setileuton, a 1,3,4-oxadiazole ring-opened metabolite (M5) was identified in rat, dog, and human liver microsomes and hepatocyte incubations. In a rat disposition study, M5 was identified in the urine, bile, and plasma. The mechanism of the metabolic ring opening, which was examined in rat liver microsomes and recombinantly expressed enzymes, was shown to be cytochrome P450 (P450) mediated. The evidence supporting this conclusion is described in this report, and three potential mechanisms for this two-step biotransformation are proposed. An alternative, comparatively slow pathway of chemical hydrolysis also was identified.

Materials and Methods

Chemicals. Acetonitrile, premixed 0.1% aqueous formic acid, NADPH, 1 M potassium phosphate buffer, and water-¹⁸O (95 atom % excess, Isotec) were purchased from Sigma-Aldrich (St. Louis, MO). Argon was obtained from Airgas (Radnor, PA). Oxygen-18 gas (97.8 atom % excess) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Meperidine hy-

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ABBREVIATIONS: 5-LO, 5-lipoxygenase; RLM, rat liver microsomes; P450, cytochrome P450; LC-MS, high-pressure liquid chromatography coupled with mass spectrometric analysis; 1D, one-dimensional.

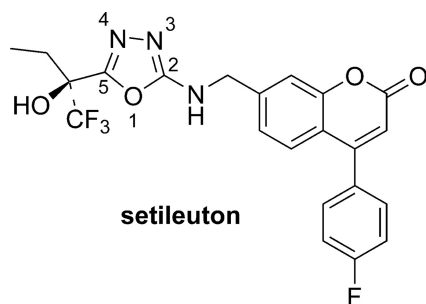


FIG. 1. Structure of setileuton.

drochloride was obtained from Spectrum Chemical and Laboratory Products (Gardena, CA). All other reagents were of analytical grade or higher.

Setileuton (Fig. 1) was synthesized at Merck Frosst (Kirkland, QC, Canada) as described previously (Ducharme et al., 2010). Synthetic M5 (Fig. 2) also was synthesized at Merck Frosst. Analytical amounts of M5 were prepared in a three-step synthesis starting from compound A (see supplemental material in the study by Ducharme et al., 2010). In the presence of sodium hydride and di-*tert*-butyl iminodicarboxylate, compound A was converted to B in a 74% isolated yield. A mixture of trifluoroacetic acid and B was stirred for 16 h at room temperature affording the primary amine C in quantitative yield. A solution of C and 5-((*S*)-1-hydroxy-1-(trifluoromethyl)propyl)-3H-[1,3,4]oxadiazol-2-one (F) (see supplemental data) was stirred at 65°C overnight to provide M5 as a synthetic standard.

Pooled Liver Microsomes and Recombinant P450. Pooled male rat liver microsomes (RLMs) were obtained from XenoTech, LLC (Lenexa, KS). Microsomes prepared from baculovirus-infected insect cells expressing rat CYP1A2, 2A1, 2B1, 2C11, 2C13, 2D1, 2J3, 2J4, 3A1, and 3A2 were obtained in-house from the Department of Drug Metabolism and Pharmacokinetics, Merck Research Labs (West Point, PA; preparation similar to that of Shou et al., 2003). Microsomes prepared from baculovirus-infected insect cells expressing rat CYP1A1, 2A2, 2C6, 2C12, and 2D2 were obtained from BD Gentest (Woburn, MA). The control baculosomes were obtained from PanVera Corp. (Madison, WI).

Incubations with Rat Liver Microsomes. Each incubation mixture contained 0.1 M potassium phosphate buffer (pH 7.4), male RLMs (0.75–1 mg/ml), and setileuton (10 μ M) in the presence or absence of 1 mM NADPH. Samples were incubated for 0 to 30 min at 37°C in a shaking water bath. At the end of the incubation, two volumes of acetonitrile were added to the mixtures to terminate the reaction. A structurally similar internal standard [compound (R)-12 in the study by Ducharme et al., 2010] was added to samples when quantitation was required. Samples were centrifuged, and the supernatants were transferred into glass culture tubes and evaporated to dryness under a nitrogen stream at room temperature. The sample residues were reconstituted in 10% acetonitrile in aqueous 0.1% formic acid or 20% acetonitrile in aqueous 0.1% formic acid before injection.

Metabolite Isolation. Metabolite M5 was isolated from rat urine using a three-step high-pressure liquid chromatography method. In brief, urine was treated with an equal volume of acetonitrile. Samples were centrifuged, and supernatants were transferred into glass culture tubes and evaporated to dryness under a nitrogen stream. For the first step, samples were reconstituted in

25% acetonitrile in aqueous 25 mM ammonium formate at pH 3 and injected onto a Synergi Hydro-RP column (4.6 \times 250 mm, 4 μ m) (Phenomenex, Torrance, CA). The mobile phase consisted of a 25 mM ammonium formate (pH 3.0, solvent A) and acetonitrile (solvent B) gradient operated at 1 ml/min. The initial condition (25% B) was held for 5 min and increased linearly to 65% B over 15 min. The gradient then was increased to 85% B over 0.5 min, held at 85% B for 1 min, and re-equilibrated under the initial conditions for 7 min. Fractions were collected at 30-s intervals using a Foxy 200 fraction collector (ISCO, Lincoln, NE). The fractions determined by UV and retention time to contain M5 were combined, and the solvent was removed under a nitrogen stream. The concentrated samples were reconstituted in 30% acetonitrile in aqueous 25 mM ammonium formate at pH 3. For the second step, the column, mobile phase composition, and flow rate were the same as step 1. The initial condition (15% B) was held for 5 min and increased to 70% B over 15 min. The gradient then was increased to 85% B for 0.5 min, held at 85% B for 1 min, and re-equilibrated under the initial conditions for 7 min. Fractions containing M5 were collected, and the solvent was removed under a nitrogen stream. The concentrated samples were reconstituted as done previously and injected for step 3 onto a Prodigy ODS3 column (4.6 \times 250 mm, 5 μ m) (Phenomenex) with the same mobile phase and gradient conditions as the first step. Fractions were collected again, those containing M5 were combined, and the solvent was removed under a nitrogen stream. The concentrated sample was used for structural elucidation by ^1H - and ^{13}C -NMR analyses.

^1H - and ^{13}C -NMR Analysis. The isolated metabolic M5 and the synthetic M5 samples both were dissolved in deuterated methanol supplied in ampules by Cambridge Isotope Laboratories, Inc. The dissolved metabolic M5 was placed into a 1.7-mm capillary NMR tube, and flame sealed. The dissolved synthetic M5 sample was placed into a 3-mm NMR tube. ^1H - and ^{13}C -NMR experiments were conducted by using a Varian Inova 500- or 600-MHz instrument (Varian, Inc., Palo Alto, CA) equipped with inverse detection proton-carbon-nitrogen triple resonance cold probes. All experiments were acquired at 25°C.

Incubation with Recombinant P450 Isoforms Derived from Rat. Incubation mixtures consisted of 0.1 M potassium phosphate buffer (pH 7.4), recombinant P450 (300 pmol/ml), and setileuton (10 μ M). Mixtures were preincubated at 37°C for 3 min before initiating the reaction by the addition of 1 mM NADPH. After 30 min, the reaction was terminated with two volumes of acetonitrile, and a structurally similar internal standard [compound (R)-12 in the study by Ducharme et al., 2010] was added. Samples were centrifuged, and the supernatants were transferred into glass culture tubes and evaporated to dryness under a nitrogen stream. The sample residues were reconstituted in 20% acetonitrile in aqueous 0.1% formic acid for mass spectrometric analysis.

For kinetic studies using RLMs, CYP3A1/2, and CYP1A1/2, incubation conditions were identified under which the formation of M5 was linear with respect to time and protein concentration (data not shown). Incubation mixtures contained 0.1 M phosphate buffer, protein (0.75 mg/ml RLMs, 100 or 300 pmol/ml recombinant P450), and setileuton (0.2–100 μ M). Samples were preincubated for 3 min at 37°C before initiating reaction by the addition of 1 mM NADPH. Incubations were conducted for 10 min, the reactions were terminated, and samples were processed for mass spectrometric analysis as described above.

Incubation in Oxygen-18 Water. Each incubation mixture contained 0.1 M potassium phosphate buffer (pH 7.4) prepared in either deionized water or

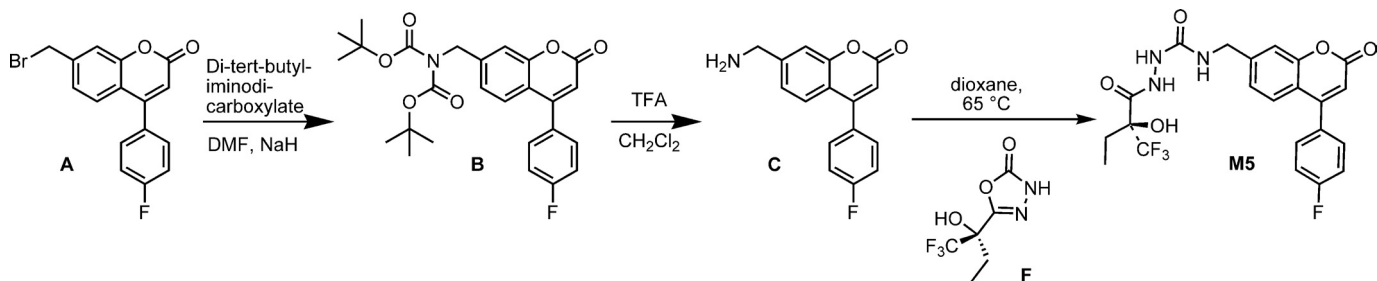


FIG. 2. Synthesis of M5. DMF, dimethylformamide; TFA, trifluoroacetic acid. A, 7-(bromomethyl)-4-(4-fluorophenyl)-2H-chromen-2-one; B, di-*tert*-butyl [4-(4-fluorophenyl)-2-oxo-2H-chromen-7-yl]methyl]imidodicarbonate; C, 7-(aminomethyl)-4-(4-fluorophenyl)-2H-chromen-2-one; F, 5-((*S*)-1-hydroxy-1-(trifluoromethyl)propyl)-3H-[1,3,4]oxadiazol-2-one.

water- ^{18}O , male RLMs (2 mg/ml), setileuton (100 μM), and 1 mM NADPH. Samples were incubated for 30 min at 37°C in a shaking water bath. At the end of the incubation, two volumes of acetonitrile were added to the mixtures to terminate the reaction. Samples were centrifuged, and the supernatants were transferred into glass culture tubes and evaporated to dryness under a nitrogen stream. The sample residues were reconstituted in 20% acetonitrile in aqueous 0.1% formic acid before analysis by high-pressure liquid chromatography coupled with mass spectrometry (LC-MS).

Incubations containing 0.1 M potassium phosphate buffer (pH 7.4) prepared in either deionized water or water- ^{18}O ; male RLMs (1 mg/ml); meperidine (50 μM), a known esterase substrate; and 1 mM NADPH were prepared as a positive control for esterase activity. These samples were incubated for 40 min at 37°C in a shaking water bath. At the end of the incubation, two volumes of acetonitrile were added to the mixtures to terminate the reaction. Samples were centrifuged, and the supernatants were transferred into glass culture tubes and evaporated to dryness under a nitrogen stream. The sample residues were reconstituted in 5% acetonitrile in aqueous 0.1% formic acid before LC-MS analysis.

Incubation under Oxygen-18 Gas Atmosphere. Incubation conditions were modified from Stearns et al. (1995) as follows. Degassed 0.1 M potassium phosphate buffer was prepared through five cycles of freezing under an argon atmosphere followed by thawing under a vacuum. The degassed buffer was stored under a positive argon atmosphere. Incubation mixtures were prepared in a 15-ml three-neck round-bottom flask that was evacuated at room temperature and refilled with argon two times. The flask then was placed on ice, and the incubation mixture was added through a septum using a syringe. The incubation mixture consisted of degassed 0.1 M potassium phosphate buffer (pH 7.4), male RLMs (2 mg/ml), and setileuton (100 μM). The reaction flask then was evacuated and filled with argon three times. The argon atmosphere was replaced by ^{18}O -labeled oxygen. The incubation mixture then was preincubated at 37°C for 3 min with gentle agitation using a magnetic stir bar. The reaction was initiated by the addition of NADPH (1 mM, in degassed 0.1 M potassium phosphate buffer) and incubated at 37°C . After 30 min, an aliquot of the reaction mixture was transferred to a test tube containing two volumes of acetonitrile. Samples were centrifuged, and the supernatants were transferred into glass culture tubes and evaporated to dryness under a nitrogen stream. The sample residues were reconstituted in 20% acetonitrile in aqueous 0.1% formic acid and analyzed by LC-MS.

Chemical Hydrolysis. Approximately 10 mg of setileuton was added to 1 ml of water (deionized or ^{18}O -labeled) in a glass screw-cap tube, capped, and agitated on a vortex mixer forming a saturated aqueous solution of setileuton. The samples were stressed in an oven at 80°C for approximately 6 days. A 200- μl aliquot of each tube was placed in an autosampler vial, and 50 μl of acetonitrile was added before LC-MS analysis.

Sample Analysis. Concentrated samples from each study were reconstituted as described previously. LC-MS was performed by using an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA) coupled to a Finnigan LTQ linear ion trap or Quantum mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

Reconstituted samples for metabolite profiling were injected onto a Prodigy ODS3 column (250 \times 4.6 mm, 5 μm) (Phenomenex). The mobile phase consisted of a 0.1% formic acid (solvent A) and acetonitrile (solvent B) gradient with a flow rate of 1 ml/min. The initial condition was 10% B and was maintained for 5 min. After 5 min, the gradient was increased linearly to 90% B at 40 min and held at 90% B for 5 min. The column was re-equilibrated under the initial conditions for 5 min before the next injection.

Samples for quantitation were injected onto a Phenomenex Luna C18(2) column (50 \times 4.6 mm, 5 μm). The mobile phase composition and flow rate were the same as those described in the previous paragraph. The initial condition was 20% B and was maintained for 1 min. The gradient then was increased linearly to 90% B over 4 min and held at 90% B for 1.5 min. The column was re-equilibrated under the initial conditions for 2.5 min before the next injection. Data were acquired in selected reaction monitoring mode using the following transitions: M5, m/z 482 (MH^+) \rightarrow 296 (collision energy, 18 V; retention time, 4.7 min); and internal standard, m/z 482 (MH^+) \rightarrow 145 (collision energy, 42 V; retention time, 6.2 min). The calibration standards covered a concentration range of 12.5 to 750 nM.

Labeled oxygen experiment samples were injected onto an Allure PFP Propyl column (250 \times 4.6 mm, 5 μm) (Restek, Bellefonte, PA). The mobile phase composition and flow rate were the same as those described above. The

initial condition was 20% B and was maintained for 1 min. The gradient then was increased linearly to 90% B over 39 min and maintained at 90% B for 5 min. The column was re-equilibrated under the initial conditions for 5 min before the next injection.

Meperidine incubation samples were injected onto a Phenomenex Synergi Polar-RP column (250 \times 4.6 mm, 4 μm). The mobile phase composition and flow rate were the same as those described above. The initial condition was 5% B and was held for 5 min. The gradient then was increased linearly to 90% B at 15 min and held at 90% B for 5 min. The column was re-equilibrated under the initial conditions for 5 min before the next injection.

In all cases, column effluent was split such that ~ 150 – 200 $\mu\text{l}/\text{min}$ flowed to the mass spectrometer source and the remainder to waste. All mass spectrometric analyses were performed with electrospray ionization in the positive mode.

Kinetic Analysis. Data from incubations of setileuton with RLMs, CYP3A1/2, and CYP1A1/2 for kinetic studies were used to fit equations describing the classic hyperbolic (eq. 1) or the sigmoidal (eq. 2) kinetic models used previously (Huang et al., 2004b):

$$v = V_{\max}[S]/(K_m + [S]) \quad (1)$$

$$v = V_{\max}[S]^n/(S_{50}^n + [S]^n) \quad (2)$$

where v is the initial velocity, V_{\max} is the maximum velocity, $[S]$ is the substrate concentration, K_m is the Michaelis constant (substrate concentration at which velocity is 50% V_{\max}), n is the Hill coefficient, and S_{50} is the substrate concentration at which velocity is 50% V_{\max} . The kinetic parameters were estimated using SigmaPlot (version 9.01; Systat Software, Inc., Point Richmond, CA).

Results

Metabolism to M5. After incubation of setileuton with NADPH-fortified rat liver microsomes, several oxidative metabolites were formed (data not shown). Mass spectrometric analysis revealed that the metabolite M5 (Fig. 2) had a molecular ion that was 18 Da higher than that of setileuton, and accurate mass analysis supported the conclusion that this was due to the net addition of the elements of water. The product ion spectrum of metabolite M5 (Fig. 3) contained the m/z 253 ion, which was detected in the product ion spectrum of setileuton, resulting from the neutral loss of the oxadiazole portion of the molecule. This suggested that the structural modification occurred to the oxadiazole portion of the molecule. Structural identification was confirmed by matching the retention time and product ion spectrum to the synthetic standard of M5. Metabolite M5 subsequently was isolated from rat urine, and the ^1H -NMR spectrum of the isolated M5 was compared to the synthetically prepared compound (Fig. 4). The two one-dimensional (1D) ^1H -NMR spectra in Fig. 4 have identical chemical shifts and coupling patterns for all proton signals, which indicate that the two samples have the same chemical structure.

To distinguish between oxidation and either chemical or enzymatic hydrolysis, setileuton was incubated with rat liver microsomes in the presence and absence of NADPH, and the formation of M5 was shown to be NADPH dependent (Table 1). This suggested that the formation was oxidative and led to an investigation of the enzymes and mechanism responsible for metabolite formation.

Enzymes in RLMs Responsible for M5 Formation. Of the 15 recombinant rat P450 enzymes studied (CYP1A1, 1A2, 2A1, 2A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2D1, 2D2, 3A1, 3A2, 2J3, and 2J4), only four (CYP1A1, CYP1A2, CYP3A1, and CYP3A2) catalyzed the formation of M5 at appreciable levels (data not shown). Kinetic studies were conducted in male rat liver microsomes as well as recombinant rat CYP1A1, CYP1A2, CYP3A1, and CYP3A2 to determine the K_m and V_{\max} (Fig. 5). Although the metabolic formation of M5 presumably involves two biotransformation steps, it was assumed that one step was rate limiting and the other step was rapid

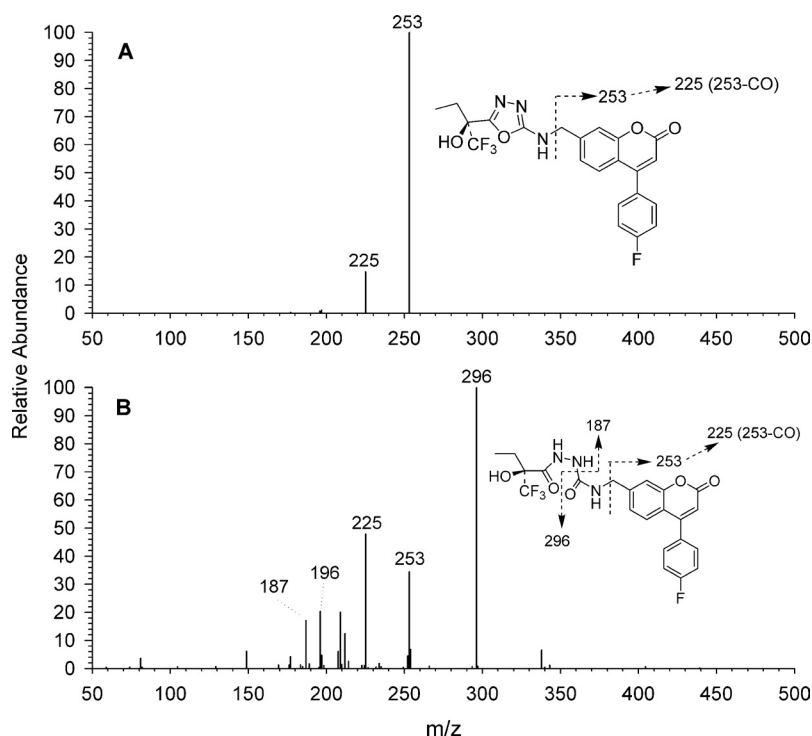


FIG. 3. Product ion spectrum after collision-induced dissociation of setileuton ($[M+H]^+$, m/z 464; A) and M5 ($[M+H]^+$, m/z 482; B).

enough to not impact the kinetics. M5 formation in male rat liver microsomes and in expressed CYP1A2 and CYP3A1 displayed sigmoidal kinetics with comparable Hill coefficients (1.2, 1.2, and 1.3, respectively) and S_{50} values (10.6, 8.2, and 8.2 μ M, respectively) (Table 2). In contrast, catalysis mediated by CYP1A1 and CYP3A2 conformed to Michaelis-Menten kinetics, with K_m values estimated to be 6.0 and 2.2 μ M, respectively.

Synthetic M5. Collision-induced dissociation of the synthetic M5 ($[M+H]^+$, m/z 482) resulted in four characteristic product ions (Fig. 6A). The m/z 187 and m/z 296 product ions result from cleavage of one of the nitrogen-carbon bonds in the opened ring (Fig. 6A). Neutral loss of the hydrazinecarboxamide portion of the molecule gives rise to the product ion of m/z 253, and the further loss of CO from this fragment gives rise to a product ion of m/z 225.

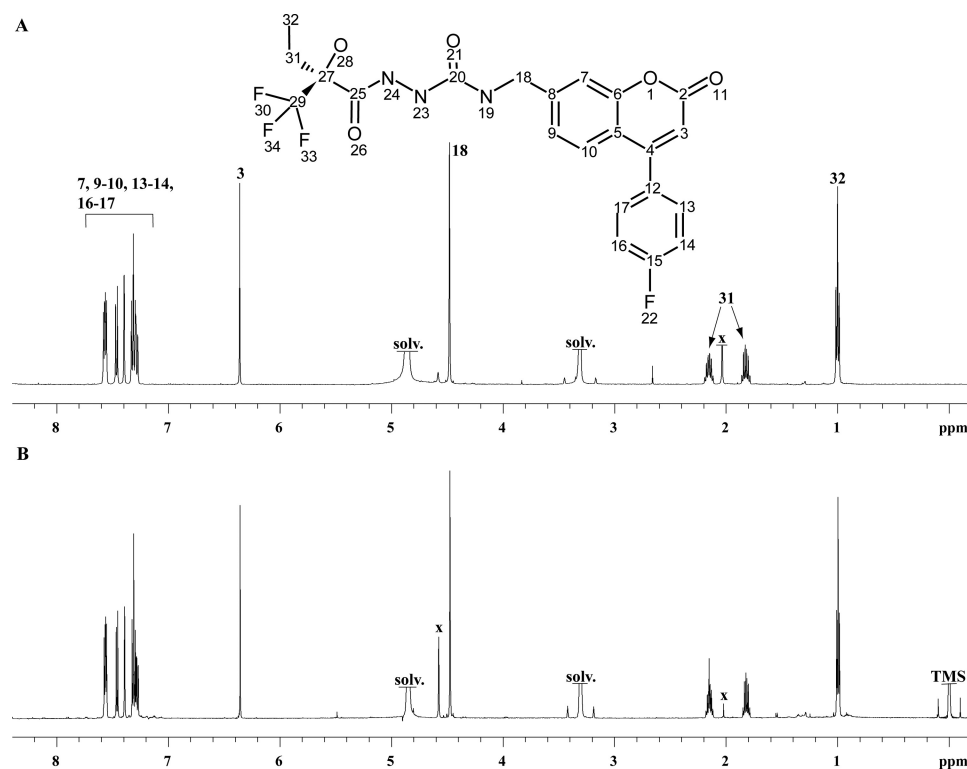


FIG. 4. Full 1D proton NMR spectra of M5 isolated from rat urine (A) and synthetically prepared M5 (B).

TABLE 1

Concentration of M5 (nM) formed after incubation of setileuton with rat liver microsomes in the presence and absence of NADPH

Data are mean \pm S.D.

Time min	In the Presence of NADPH	In the Absence of NADPH
0	8.10 \pm 2.26	1.93 \pm 0.40
2	29.74 \pm 3.17	1.81 \pm 0.06
5	57.93 \pm 12.29	1.66 \pm 0.26
8	74.89 \pm 8.53	1.43 \pm 0.09
10	100.40 \pm 17.19	1.56 \pm 0.15

Analysis of the 1D proton NMR spectra of M5 (Fig. 4) resulted in the observation of six aromatic as well as three aliphatic resonances. The observed aromatic resonances are consistent with expected values and can be identified quite easily based on ^1H - ^1H coupling values. For example, the two resonances at 7.57 and 7.31 ppm are observed as doublets of doublets with 8.4/5.3 and 8.4/8.4 Hz couplings, respectively. These values are consistent with the assignment for the 4-fluorophenyl group. Likewise, the singlet at 6.36 ppm is consistent with the expected resonance adjacent to the lactone moiety. Analysis of the couplings observed for the three remaining aromatic resonances quickly leads to the assignment of the peak at 7.46 ppm (doublet, 8.2 Hz), 7.39 ppm (doublet, 1.6 Hz), and 7.28 ppm (doublet of doublets, 8.2/1.6 Hz). Identification of the aliphatic peaks was also fairly simple based on both the observed chemical shift values as well as the ^1H - ^1H coupling values. As a result, the two protons observed at 4.48 ppm were assigned to the methylene adjacent to the aromatic group, and the protons observed at 2.15/1.82 ppm (methylene) and 1.00 ppm (methyl) were assigned to the ethyl group. Furthermore, a variety of

two-dimensional ^1H - and ^{13}C -NMR experiments allowed the unambiguous assignment of all proton and carbon atoms in the synthetic M5 (supplemental data).

Oxygen-18 Label Experiments. When setileuton was incubated with rat liver microsomes under an atmosphere of oxygen-18 gas, mass spectrometric analysis showed that the molecular ion of the resulting M5 increased by 2 Da relative to M5 formed under normal atmospheric conditions, indicative that the oxygen-18 was incorporated in this metabolite. Further analysis of the labeled metabolite by collision-induced dissociation showed that the m/z 187 product ion had increased by 2 Da to m/z 189, indicating that the labeled oxygen was incorporated on carbon 5 of the oxadiazole ring (Fig. 6B).

When setileuton was incubated with male rat liver microsomes in potassium phosphate buffer enriched with water- ^{18}O in the presence of NADPH, mass spectrometric analysis revealed that the molecular ion $[\text{M}+\text{H}]^+$ of the resulting M5 remained constant, which indicated that there was no incorporation of the oxygen-18 (data not shown). However, in the control incubation with meperidine, the meperidinic acid metabolite formed by esterase hydrolysis increased in mass by 2 Da in the presence of oxygen-18-labeled water. This indicated that the oxygen-18 from water was incorporated in meperidinic acid in accordance with the mechanism of enzyme-mediated hydrolysis and was evidence that the experimental conditions were conducive to esterase activity (Luttrell and Castle, 1988). Overall, the result from the in vitro study suggested that the oxygen incorporated into M5 did not originate from the water.

When a saturated aqueous solution of setileuton was stressed in a capped test tube at 80°C for 6 days, M5 was formed via hydrolysis. When the aqueous solution was prepared in oxygen-18-labeled water, the oxygen-18 was incorporated into the resulting M5, which was shown by a net increase of 2 Da compared to the standard observable by mass spectrometry. Further analysis of the labeled metabolite by collision-induced dissociation suggested that the labeled oxygen was primarily incorporated on carbon 2 of the oxadiazole due to an increase of 2 Da of the m/z 296 ion to 298, whereas the m/z 253 ion remained unchanged (Fig. 6C). This result suggested a second, separate mechanism for hydrolysis.

Discussion

During the development of setileuton, the metabolite M5 was identified, which represented the addition of 18 Da (net addition of the elements of water) to the oxadiazole portion of the parent molecule. Biotransformations for the 1,2,4-oxadiazole are well known in the literature (Lan et al., 1973; Yabuki et al., 1993; Dalvie et al., 2002), but no examples of biotransformations of 1,3,4-oxadiazoles could be identified. The 1,2,4-oxadiazoles undergo metabolic ring opening through reductive cleavage of the *N*-O bond (Lan et al., 1973; Dalvie et al., 2002; Allan et al., 2006; Bateman et al., 2006) or an unknown mechanism (Yabuki et al., 1993). In some instances, there is further hydrolysis of the amide and carboxylic acid resulting from cleavage of the oxadiazole ring (Allan et al., 2006). This led to an investigation into the mechanism of the novel ring opening of the 1,3,4-oxadiazole to determine whether it was oxidative or hydrolytic.

The formation of M5 in male RLM incubations was dependent on the presence of NADPH, which indicated the probable involvement of P450 isoforms. Various recombinant rat P450s were tested, and it was determined that CYP1A1, CYP1A2, CYP3A1, and CYP3A2 were capable of forming the metabolite in significant quantities. Additional kinetic studies revealed that metabolite formation in CYP1A2 and CYP3A1 displayed sigmoidal kinetics similar to that observed in male rat liver microsomes. In contrast, catalysis mediated by CYP1A1 and CYP3A2 conformed to Michaelis-Menten kinetics. These results sug-

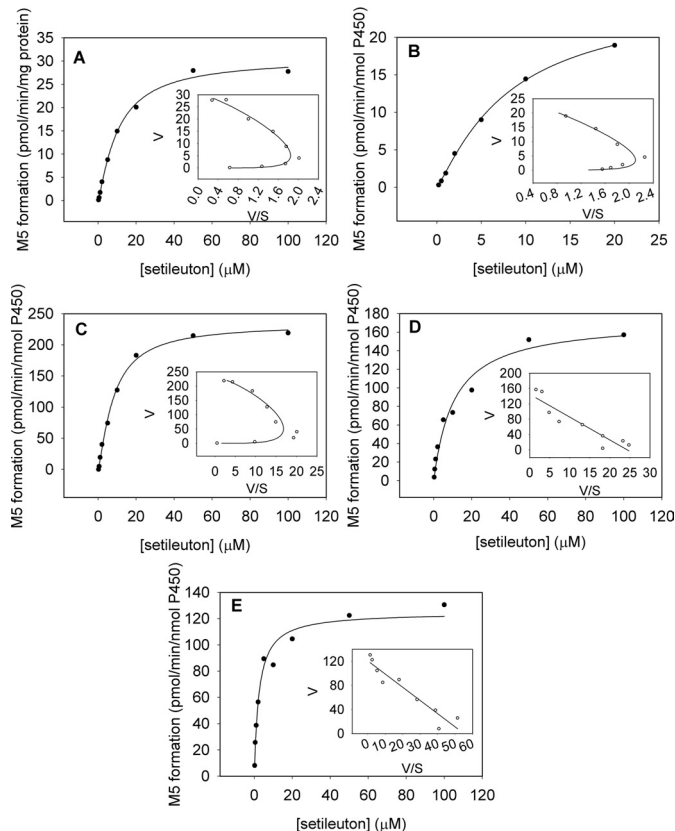


FIG. 5. Kinetics of M5 formation in male rat liver microsomes (A) and in expressed rat CYP1A2 (B), CYP3A1 (C), CYP1A1 (D), and CYP3A2 (E). Eadie-Hofstee plots are shown as insets.

TABLE 2

Kinetic parameters for M5 formation in male rat liver microsomes and expressed P450 enzymes

S.E. is shown in parentheses.

	RLM	1A1	1A2	3A1	3A2
Best model fit	Sigmoidal	Hyperbolic	Sigmoidal	Sigmoidal	Hyperbolic
V_{\max}	30.6 (1.2)	145.1	25.7 (1.4)	233.0 (7.1)	120.8
S_{50} or K_m (μ M)	10.6 (1.1)	6.0	8.2 (0.9)	8.2 (0.7)	2.2
Hill coefficient	1.2 (0.1)		1.2 (0.1)	1.3 (0.1)	

gested that CYP1A2 and CYP3A1 likely were contributing to M5 formation in male rat liver. Since the expression level of CYP3A1 was reported to be very low in adult male rat liver (Ribeiro and Lechner, 1992; Rich and Boobis, 1997; Rodrigues et al., 2003), CYP1A2 was likely the major P450 isoform contributing to M5 formation in male rat liver microsomes. There is little information available on gender differences in the expression of CYP1A2 in rat liver. There is some evidence that the protein level of CYP1A2 is slightly higher in female rat liver than male rat liver (Kato and Yamazoe, 1992; Iba et al., 1999); however, this is not likely to cause a gender difference in the formation of M5.

Additional studies were conducted with stable labeled molecular oxygen and water to investigate the origin of the oxygen incorporated into the metabolite and the mechanism of M5 formation. When

incubations were conducted for 30 min in the presence of NADPH at 37°C under an atmosphere of stable labeled molecular oxygen, the oxygen-18 was incorporated in the M5 metabolite formed. In addition, after collision-induced dissociation, the m/z 187 ion was completely replaced by m/z 189, whereas the m/z 296 ion was unchanged (Fig. 6B, inset). The oxygen-18 gas was 97.8% enriched, thus near total replacement was expected. These results indicated that the incorporation of the oxygen occurred on carbon 5 of the oxadiazole. However, when incubations were conducted in buffer prepared in oxygen-18-labeled water in the presence of NADPH, there was no incorporation of the labeled oxygen in the metabolite after 30 min at 37°C. This supported our hypothesis that metabolite formation was oxidative rather than chemical- or enzyme-mediated hydrolysis.

Chemical hydrolysis of 1,3,4-oxadiazoles is known in acidic environments and leads directly to a ring-opened product (Suyama et al., 2003; Johnson et al., 2008). To determine whether this could occur for setileuton, a saturated aqueous solution of setileuton was stressed for 6 days at 80°C. Under these conditions, it was possible to form M5 through chemical hydrolysis, and when oxygen-18-labeled water was used in this experiment, the labeled oxygen was incorporated. However, the chemical hydrolysis process is slow (Table 1) and therefore not a significant contributor to the M5 metabolite formed in liver microsome incubations. Even so, M5 was detected as a degradation product in the bulk active pharmaceutical material.

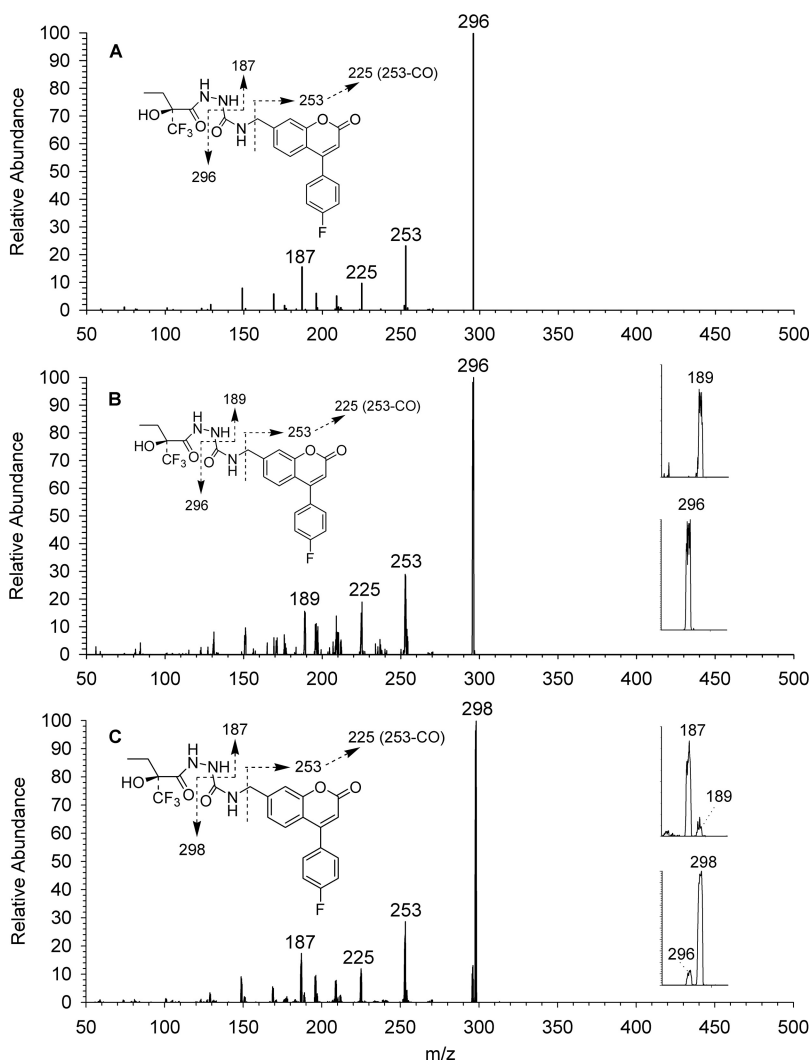


FIG. 6. Product ion spectrum after collision-induced dissociation of M5 standard ($[M+H]^+$, m/z 482; A) and ^{18}O -labeled M5 formed after incubation of setileuton with male rat liver microsomes under an $^{18}\text{O}_2$ atmosphere ($[M+H]^+$, m/z 484; B) or after chemical hydrolysis in water- ^{18}O ($[M+H]^+$, m/z 484; C). Insets highlight the 187/189 and 296/298 fragments.

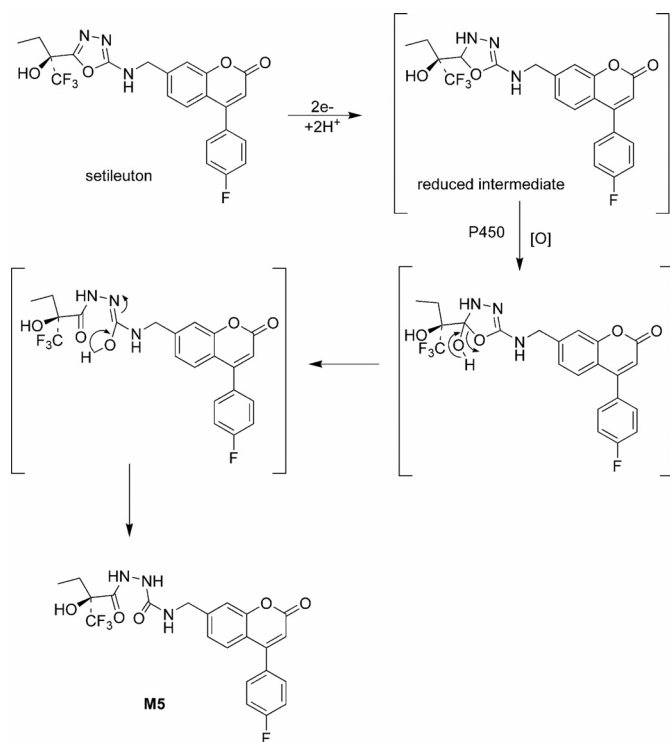


FIG. 7. Proposed mechanism for the formation of M5 from setileuton. Setileuton is reduced, oxidized, and rearranged to form M5.

Mass spectrometric analysis demonstrated that the oxygen was incorporated at a different position on the molecule after chemical hydrolysis than when M5 was formed metabolically. After metabolic formation, the oxygen was incorporated on carbon 5 of the oxadiazole, whereas after chemical hydrolysis, it was primarily incorporated on carbon 2 of the oxadiazole. We propose that two separate mechanisms are responsible for the formation of M5 under hydrolytic and NADPH-dependent conditions.

The chemical formation of M5 involves nucleophilic attack by a hydroxide to directly open the oxadiazole ring. On close examination of the isotope ratios for the fragments of M5 formed by chemical hydrolysis (Fig. 6C, insets), it was found that the m/z 187 fragment accounted for approximately 90% of the total ($187 + 189$), whereas the m/z 189 fragment accounted for approximately 10% with a similar

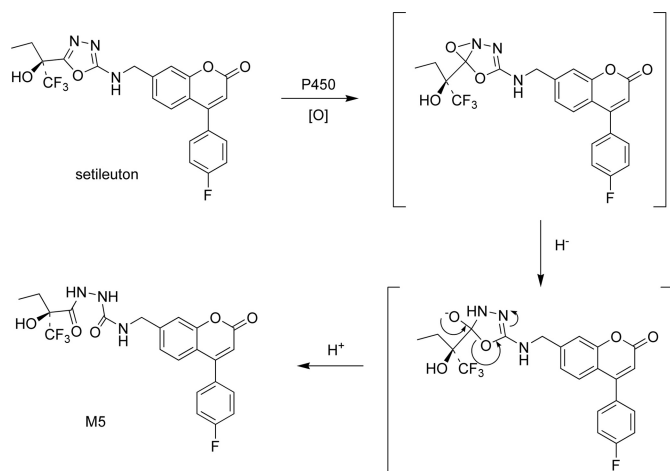


FIG. 8. Proposed mechanism for the formation of M5 from setileuton. Setileuton is oxidized to an epoxide, reduced, and rearranged to form M5.

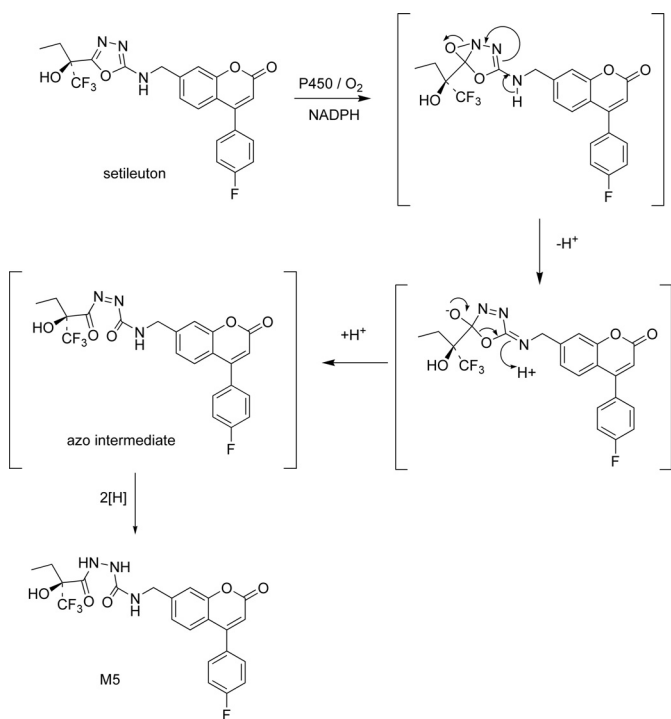


FIG. 9. Proposed mechanism for the formation of M5 from setileuton. Setileuton is oxidized and rearranged to an azo intermediate, which is reduced to produce M5.

ratio for the m/z 296/298 fragment. The water was 95% enriched, so this indicated that a small amount of the M5 formed involved nucleophilic attack of carbon 5 of the oxadiazole, whereas nucleophilic attack of carbon 2 was preferred. Carbon 2 of the oxadiazole ring is more electron deficient because of the substitution pattern. In addition, there is increased steric hindrance on carbon 5 due to the bulky 1-hydroxy-1-(trifluoromethyl)propyl group. These factors make nucleophilic attack on carbon 2 more likely and explain the preference for the labeled oxygen incorporation on carbon 2.

In contrast to the single-step ring opening of the 1,2,4-oxadiazoles, the mechanism for metabolic formation of M5 appears to involve two steps, an oxidation and a reduction, to form the final product. Three possible mechanisms are proposed in this report (Figs. 7–9). The first proposed mechanism (Fig. 7) involves reduction, possibly mediated by P450s, followed by oxidation and rearrangement to form M5. All attempts failed to identify the reduced intermediate (Fig. 7), and although this does not rule out the mechanism, it did lead to consideration of alternative mechanisms. The second proposed mechanism (Fig. 8) involves oxidation by P450 followed by nucleophilic attack by a hydride, likely originating from NADPH (Metzler, 1977), and rearrangement to form M5. The third proposed mechanism (Fig. 9) involves oxidation by P450 and rearrangement to an azo intermediate, followed by reduction of the intermediate. Attempts to identify the azo intermediate (Fig. 9) were unsuccessful, which could be due to instability of the intermediate (Fahr and Lind, 1966). All three mechanisms remain plausible explanations of the two-step process to form M5.

In conclusion, a novel P450-mediated ring opening of a 1,3,4-oxadiazole has been investigated. Metabolite formation displays sigmoidal kinetics in incubations with rat liver microsomes, CYP1A2, and CYP3A1. Expression levels of the enzymes in adult male rat liver indicated that CYP1A2 is most likely responsible for the metabolic formation of M5. Three possible mechanisms for this two-step biotransformation have been proposed, although we have been unable to differentiate between them to this point. The metabolite can be formed

through chemical hydrolysis, and this was detected in the bulk active pharmaceutical material, but the process is slow under physiologic conditions and probably is not a significant contributor to total metabolite formation. The only oxadiazole isomer with reported metabolism is the 1,2,4-oxadiazole, therefore this is the first reported metabolism of the 1,3,4-oxadiazole ring.

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Authorship Contributions

Participated in research design: Maciolek, Ma, Menzel, Laliberte, Bateman, and Gibson.

Conducted experiments: Maciolek, Menzel, Laliberte, Bateman, and Krolkowski.

Contributed new reagents or analytic tools: Laliberte.

Performed data analysis: Maciolek, Ma, Menzel, Laliberte, and Krolkowski.

Wrote or contributed to the writing of the manuscript: Maciolek, Menzel, Laliberte, Krolkowski, and Gibson.

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