MECHANISM OF CYTOCHROME P4503A4- AND 2D6-CATALYZED DEHYDROGENATION OF EZLOPITANT AS PROBED WITH ISOTOPE EFFECTS USING FIVE DEUTERATED ANALOGS

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ABSTRACT:

Ezlopitant is metabolized by cytochrome P450 primarily to two metabolites: a benzyl alcohol and a corresponding alkene. The alkene arises as a direct product of metabolism of ezlopitant rather than through dehydration of the benzyl alcohol. The mechanism of this cytochrome P450 (P450)-catalyzed dehydrogenation reaction was probed with five different deuterium-labeled analogs of ezlopitant. At saturating ezlopitant concentrations, deuterium substitution resulted in small differences in reaction velocity. When deuterium was incorporated into the benzyl position ([\textit{d}1]ezlopitant and [\textit{d}2]ezlopitant), low isotope effects on the formation of both the benzyl alcohol and alkene were observed (1.25–1.55 for CYP3A4 and 1.48–2.61 for CYP2D6), suggesting that abstraction of the benzyl hydrogen is obligatory in the formation of both metabolites. A small amount of metabolic switching occurred because isotope effects were slightly higher for alkene and alcohol formation than for ezlopitant consumption. Intramolecular deuterium isotope effects of the dehydrogenation reaction for tri- and tetra-deuterated analogs were very low (1.13–1.15) for both CYP3A4 and CYP2D6, whereas intramolecular isotope effects for the chemical dehydration of correspondingly deuterated ezlopitant benzyl alcohol (CJ-12,764) were 3.8 to 5.9. Thus, dehydrogenation does not appear to occur via enzyme-mediated general acid catalysis of the benzyl alcohol. A mechanism for the dehydrogenation of ezlopitant is proposed in consideration of the data presented.

Ezlopitant, (2S,3S,4S)-2-diphenylmethyl-3-(5-isopropyl-2-methoxybenzylamino)-1-azabicyclo[2.2.2]octane (Fig. 1), is a novel nonpeptide antagonist of the substance P receptor. It represents a potential therapeutic agent for pathologies that involve the substance P receptor, such as inflammation, pain, and emesis. In the course of the characterization of the metabolism of this compound, it was discovered that a major metabolite in human liver microsomes (Obach, 2000) and the systemic circulation of human study subjects was a dehydrogenated analog (CJ-12,458; Fig. 1). The other major metabolite of ezlopitant observed in vitro and in vivo was CJ-12,764, a benzyl alcohol analog (Fig. 1). In previous work, evidence was obtained that suggested that CJ-12,458 did not arise by a chemical- or enzyme-catalyzed dehydration of the benzyl alcohol (Obach, 2000). Thus, CJ-12,458 appears to arise via the direct action of cytochrome P450 (P450), and as such, ezlopitant represents another example substrate that undergoes the unusual reaction of P450-catalyzed dehydrogenation.

Examples of other P450-catalyzed dehydrogenation reactions (Ortiz de Montellano, 1995) include the formation of alkene metabolites of valproic acid (Rettie et al., 1987, 1988; Fisher et al., 1998), the dehydrogenation of the 6 and 7 positions of testosterone (Nagata et al., 1986), dehydrogenation of lauric acid (Guan et al., 1998), 2-ethylhexanoic acid (Pennanen et al., 1996), warfarin (Fasco et al., 1978), and the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors lovastatin (Vyas et al., 1990) and simvastatin (Vickers and Duncan, 1991). The metabolism of valproic acid to the Δ(4,5) alkene metabolite represents a dehydrogenation reaction of an sp3-hybridized center, which is not adjacent to any sp2-hybridized carbon center. Experiments by Rettie et al. (1988) used deuterated analogs of valproic acid to obtain evidence that the P450-catalyzed dehydrogenation reaction proceeded via initial hydrogen atom abstraction of the 4 position. The alkene arises by either abstraction of a second hydrogen atom by the Fe(IV)-OH intermediate and subsequent collapse of the diradical or via abstraction of an electron and deprotonation of the cationic intermediate to yield the alkene (Ortiz de Montellano, 1995). In contrast to valproic acid, the formation of the Δ(6,7) alkene testosterone metabolite represents a P450-catalyzed dehydrogenation occurring at a position adjacent to an sp2-hybridized center, ultimately leading to a diene conjugated to a carbonyl group (Nagata et al., 1986). Deuterium isotope effects were also used in this case to gain mechanistic insight for this reaction (Korzekwa et al., 1990). The data supported a mechanism of sequential hydrogen atom abstraction reactions from the 6 and 7 positions.

Ezlopitant represents another type of substrate that undergoes dehydrogenation: a benzyl compound. Due to the fact that the dehydrogenation reaction represents a substantial component of ezlopitant metabolism by CYP3A4, CYP3A5, and CYP2D6 enzymes (Obach, 2000) and that formation of the corresponding benzyl alcohol represents the remaining amount of metabolism of ezlopitant by these enzymes, ezlopitant represented an appealing probe through which the
mechanism of dehydrogenation of a benzylic position could be explored with deuterium isotope effects. Furthermore, the fact that the substituent that undergoes dehydrogenation in ezlopitant is symmetrical (isopropyl group) opened the possibility of the exploration of intramolecular isotope effects for this reaction. Since the rate-limiting step in the P450-catalytic process occurs before the C-H bond-breaking step, intramolecular isotope effects can provide mechanistic information in the event that isotope effects are masked by a high “commitment to catalysis” of the enzyme.

Thus, the objective of the experiments described here was to gather evidence to determine the mechanism of P450-catalyzed dehydrogenation of benzylic positions. Five deuterated analogs of ezlopitant, each targeted to address a specific mechanistic aspect, were synthesized to accomplish this objective. Furthermore, two human P450 enzymes of different evolutionary families (CYP3A4 and CYP2D6) that both catalyze the dehydrogenation of ezlopitant were compared with regard to deuterium isotope effects to determine whether the dehydrogenation mechanism was consistent among P450 enzymes or whether differences were possible among the enzymes.

**Experimental Procedures**

**Materials.** Toluene sulfonyl chloride, hexadeuterated acetone, trideuterated methylmagnesium iodide, zinc iodide, LiAlD₄, LiAlH₄, NaBH₃CN, NaB⁻-

To 6.4 ml of 2-deuteroisopropanol in 80 ml of dry pyridine was added analytical procedures was subjected to gas-phase silylation using hexameth-

Department (Pfizer). The preparations contained coexpressed cytochrome prepara-

tions containing recombinant human CYP3A4 and CYP2D6 heterolo-

sized to accomplish this objective. Furthermore, two human P450 enzymes of different evolutionary families (CYP3A4 and CYP2D6) that both catalyze the dehydrogenation of ezlopitant were compared with regard to deuterium isotope effects to determine whether the dehydrogenation mechanism was consistent among P450 enzymes or whether differences were possible among the enzymes.

**Synthesis of Deuterated Ezlopitant Analogs.** Monodeuterated 4-isopropyl anisole.

To 0.93 g of Mg turnings (previously acid-washed) in 5 ml of ether, under N₂, was added 4.7 ml of 4-bromoanisole in 20 ml of ether. The mixture was gently heated to effect synthesis of the Grignard intermediate over 1 h. The monodeuterated isopropyl toluene sulfonate (8.2 g) in 30 ml of ether was added to the reaction in a dropwise manner, and a precipitate was observed. The reaction was stirred for 1 h, after which it was poured onto 300 ml of ice water. NH₄Cl (975 ml, 26% solution) was added; the mixture was extracted with ether (200 ml); the ether was washed with 0.1 M NaOH (2 × 100 ml), dried (MgSO₄), filtered, and evaporated under N₂ to yield a pale yellow oil. The oil was subjected to chromatography on Silica (40 g) using hexane, and the fractions containing the monodeuterated isopropyl anisole product were pooled and evaporated under N₂ to yield 920 mg of clear oil. A minor contaminant was observed on TLC and NMR, but this material was subsequently used as such in the preparation of 2-methoxy-5-(2-(2-deuteropropyl))benzaldehyde. All subsequent products using this material as a starting material were devoid of impurities.

**Trideuterated 2-(4-methoxyphenyl)-2-propanol.** To a three-neck flask fitted with nitrogen line, condenser, and delivery funnel was added 50 ml of a 1 M solution of CD₃MgI in ether. The flask was cooled in an ice bath, and 7.5 g of 4’-methoxyacetophenone in 40 ml of ether was slowly added from the delivery funnel. The reaction was allowed to come to ambient temperature. After stirring for 4 h, it was cooled on ice, and 50 ml of saturated NH₄Cl solution was slowly added. The mixture was extracted with ether (2 × 50 ml); the organic fraction was dried (MgSO₄), filtered, and evaporated under N₂ to yield 7.1 g of yellow oil. This material was purified on Silica (100 g) by first washing with hexane, followed by hexane/ethyl acetate (25:1), and the product eluted with hexane/ethyl acetate (9:1). The fractions were pooled and the solvent removed in vacuo to yield 2.7 g of oil product that gave one band on TLC.

**Trideuterated 4-isopropyl anisole.** To 2.7 g of trideuterated 2-(4-methoxy-

phenyl)-2-propanol in 80 ml dichloroethane was added 7.6 g of ZnI₂, followed by 7.4 g of NaBH₃CN. The reaction mixture was stirred for 4 h, after which it was filtered through celite. The solvent was removed under a stream of N₂ to yield a clear oil. The oil was applied to a Silica column (18 g), and the trideuterated 4-isopropyl anisole was eluted with hexane. The solvent was evaporated under N₂ to yield 0.85 g of product that gave one band on TLC.

**1,1,3,3-Tetradeuterato-2-(4-methoxyphenyl)-propane-1,3-diol.** A solution of 2-(4-methoxyphenyl)diethylmalonate (30 g) in ether (120 ml) was slowly added to a suspension of LiAlD₄ (5.0 g) in ether under N₂. Ten hours later, an additional 1.5 g of LiAlD₄ was added followed by an additional 0.5 g 6 h later. Four hours after the final addition of reagent, 100 ml of 10% HCl was added dropwise, while stirring in an ice bath under N₂. The precipitate was dissolved in an additional 300 ml of 10% HCl, and the entire mixture was extracted with ethyl acetate. The organic fraction was washed with 5% NaHCO₃, dried (MgSO₄), filtered, and the solvent was removed under N₂. The resulting oil (8 g) was subjected to Silica chromatography (14 g). The column was washed with hexane/ethyl acetate (1:1), and the product eluted with ethyl acetate. The fractions containing the product were pooled, and the solvent was evaporated under N₂ to yield 3 g of white solid, pure by TLC.

**tetradeuterated 4-isopropyl anisole.** A solution of 1,1,3,3-tetradeto-2-(4-methoxyphenyl)-propane-1,3-diol (3.0 g) in dry pyridine (15 ml) was added...
to a solution of toluenesulfonyl chloride (6.8 g) in dry pyridine (15 ml), and
the reaction was stirred overnight. The reaction mixture was poured into 300 ml of
ice water and extracted with ethyl acetate (300 ml). The ethyl acetate was
washed with 2% HCl until the washes were acidic. The ethyl acetate fraction was
dried (MgSO₄), filtered, and the solvent was evaporated under a stream of
nitrogen to yield 6.3 g of oil that was pure by TLC. The ditoluenesulfonate was
dissolved in tetrahydrofuran (60 ml) and added to a 1 M solution of LiAIH₄ in
tetrahydrofuran under N₂. The reaction was refluxed for 1 h, cooled, and
slowly added to water (300 ml). The mixture was vacuum filtered, and the
filtered solids were stirred with ethyl acetate overnight. The mixture was
vacuum filtered, the filtrate was dried (MgSO₄), filtered, and the solvent was
removed under N₂ to yield 870 mg of tetradeterated 4-isopropyl anisole as an
oil, pure by TLC.

Hexadeterated 2-(4-methoxyphenyl)-2-propanol. To 1.22 g of Mg turnings in
was slowly added 6.24 ml of 4-bromoisouline in 25 ml of ether with gentle
heating to generate the Grignard intermediate. The reaction was cooled on ice,
after which 3.7 ml of hexadedeterated acetone in 5 ml of ether was added,
during which the reaction was observed to generate heat. After stirring for 30
min, 26% NH₄Cl was added, the mixture was filtered, and the solids were
washed with ether. The filtrate was dried down under N₂ to generate a yellow
oil. This procedure was repeated in 2-fold scale, and the materials were
combined. The combined material was subjected to vacuum distillation (5 mm
Hg), and the fraction between 125–130°C was collected to yield 5 g of
determinated 2-(4-methoxyphenyl)-2-propanol as a colorless oil. The product
was pure by TLC and [1H]NMR.

Hexadeterated 4-Isopropyl anisole. To 3.4 g of hexadeterated 2-(4-
methoxyphenyl)-2-propanol in 100 ml of 1.1-dichloroethane was added 99.6 g of
Zn and 9.4 g of NaBH₄CN. The reaction mixture was stirred for 2 h, after
which it was filtered through celite. The filtrate was evaporated under N₂ to
obtain 2.8 g of oil. The oil was subjected to silica chromatography using
hexane. The fractions containing the product were evaporated under N₂ to
yield 1.0 g of pure hexadeterated 4-isopropyl anisole as a colorless oil.

Heptadeterated 4-isopropyl anisole. To 12.8 ml of octadeuterated isopro-
palin in 80 ml of pyridine was added 31.6 g of toluenesulfonyl chloride,
portion-wise, while stirring. The reaction mixture was stirred overnight, after
which it was poured into 500 ml of ice water. The mixture was stirred 30 min,
followed by extraction with ether (200 ml). The ether was washed with 5%
aqueous acetic acid until the washes were acidic (4 × 125 ml). The ether was
then dried (MgSO₄), filtered, and evaporated under N₂ until the acetic acid
odor was gone to yield 25.8 g of heptadeterated isopropyl toluenesulfonate as
a yellow oil. This was used in the subsequent reaction below.

To 1.86 g of Mg turnings in 5 ml of ether under N₂ was added 9.4 ml of
metabolite eluted just after the void volume. To the CJ-12,764 fractions was
added 0.01 ml of NaOH (1 M), which was extracted with methyl-
butyl ether (30 ml), the organic fraction was evaporated under N₂ and the residue was
reconstituted in 0.1 ml of H₂O/CH₃CN (1:1). The deuterated CJ-12,764 was purified by reverse phase
HPLC containing a Waters Symmetry C₁₈ column (3.9 × 150 mm; Milford, MA) using an injection volume of 85 μl. The initial mobile phase composition
was 36.5% CH₃CN in 20 ml HHOAc, pH 4 (with NaOH), at a flow rate of
0.8 ml/min. This composition was held for 5 min, followed by a linear gradient
to 63.5% CH₃CN at 10 min, and a second gradient to 95% CH₃CN at 20 min.
Fractions (1 min) were collected into vials containing 0.05 ml of 1 M NaHCO₃
and analyzed for product. CJ-12,764 eluted in the sixth fraction (5–6 min)
while CJ-12,458 and ezlopitant eluted at 10 and 11 to 14 min. A dichroxy
metabolite eluted just after the void volume. To the CJ-12,764 fractions was
added 0.01 ml of NaOH (1 M), which was extracted with methyl-
butyl ether (4 ml). The organic fraction was evaporated under N₂ and reconstituted in
CH₃CN for use in chemical dehydration experiments.

Deuterium Isotope Effect Experiments. Before conducting isotope effect
experiments, incubation conditions were established to ensure initial rate
linearity. Initially, enzyme kinetics for each of the deuterated analogs were
examined in triplicate to determine whether isotope effects were on Vₘₐₓ or on
V₉₉.5K₉₅. For measurement of isotope effects, unlabeled ezlopitant and the five
deoxygenated analogs of ezlopitant were incubated with SF9 cell microsomes
containing heterologously expressed human CYP3A4 (0.052 nmol of P450
of protein; 0.5 mg of protein/ml incubation) or CYP2D6 (0.012 nmol/mg of
protein; 0.4 mg of protein/ml incubation), in a total volume of 0.2 ml of 25 mM
KH₂PO₄, pH 7.5, containing 3.3 mM MgCl₂ and 1.3 mM NADPH (n = 6).
The substrate concentrations were 50 μM for CYP3A4 and 10 μM for CYP2D6.
After a 2-min preincubation period at 37°C in which all components were
mixed except for NADPH, the reactions were commenced by the addition of 0.1 ml of NaOH
(10 M). The reaction was stirred for 60 min, followed by the addition of 0.1 ml of NaOH
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(10 M).
Deuterium Isotope Effects on the Chemical Dehydration of CJ-12,764.

Solutions of \([d_3]\)CJ-12,764 or \([d_4]\)CJ-12,764 (1.25 \(\mu\)g/ml) were prepared in CH\(_3\)CN containing FeCl\(_3\) (1 mM) or trifluoroacetic acid (0.01%, v/v). Initial experiments using nonlabeled CJ-12,764 demonstrated that the rate of dehydration to CJ-12,458 was linear for greater than 16 h. The incubations containing \([d_3]\)CJ-12,764 or \([d_4]\)CJ-12,764 (n = 3) were allowed to react for 16 h (in the dark), followed by direct injection (10 \(\mu\)l) onto the analytical HPLC system described below.

Analysis of Ezlopitant, CJ-12,458, and CJ-12,764. Measurement of concentrations of ezlopitant, CJ-12,458 and CJ-12,764, and their deuterated counterparts was accomplished by liquid extraction followed by HPLC-MS analysis. To terminate incubation mixtures (already containing 3 ml of methyl tertiary butyl ether) was added 100 ng of internal standard CJ-11,957 in 0.1 ml of water (CJ-11,957 is identical to ezlopitant with the exception that the isopropyl group in the latter is replaced with an ethyl group). The mixtures were vortex mixed for 1 min, followed by separation of the layers by spinning at 3000 rpm at ambient temperature in a Jouan model CT422 swinging bucket tabletop centrifuge (Jouan, Inc., Winchester, VA). The samples were placed in a dry-ice acetone bath to effect freezing of the aqueous layer, and the organic layer was decanted into a fresh, silylated glass test tube. The solvent was removed under N\(_2\) at 30°C in a Zymark TurboVap (Zymark Corporation, Hopkinton, MA), and the residue reconstituted in 0.1 ml of HPLC mobile phase.

The HPLC-MS system consisted of a Hewlett-Packard 1100 HPLC system (Palo Alto, CA) coupled to a PE Sciex API 100 single quadrupole mass spectrometer (Toronto, ON, Canada) containing an APCI interface. The column was a Waters Symmetry C\(_18\) (3.9 \(\times\) 150 mm; 5-\(\mu\)m particle size packing), and the initial mobile phase consisted of 45.5% CH\(_3\)CN in 20 mM acetic acid, adjusted to pH 4 with NH\(_4\)OH at a flow rate of 0.8 ml/min. Samples (75 \(\mu\)l) were injected, and the initial mobile phase composition was maintained for 2 min, after which a linear gradient was applied resulting in 95% CH\(_3\)CN at 6 min.
Synthesis of Deuterated Ezlopitant Analogs. A general scheme of preparing variously deuterated 4-isopropyl anisoles (Fig. 2) followed by formylation and reductive amination (Fig. 3) was successful in generating deuterated analogs of ezlopitant. The use of deuterium-labeled precursors of high isotopic purity resulted in high isotopic purity of the 4-isopropyl anisoles, as assessed via [1H]NMR spectra (Table 1). By [1H]NMR, contamination with undesired hydrogen atoms was not detectable. Deuterated ezlopitant analogs were also of high isotopic purity, as assessed through mass spectrometry. Again, contamination with hydrogen was not detectable. [1H]NMR spectra for ezlopitant are very complicated; therefore, the extent of deuterium incorporation assessed by NMR was better accomplished by examination of the spectra of the 4-isopropyl anisole and 5-isopropyl-2-methoxybenzaldehyde precursors.

Deuterium Isotope Effects on the Formation of CJ-12,458 and CJ-12,764 Catalyzed by CYP3A4 and CYP2D6. In initial experiments, the enzyme kinetics were determined for both CYP3A4 and CYP2D6 for ezlopitant and all five deuterated analogs. For each enzyme, the $K_M$ values were nearly identical regardless of the position and extent of deuteration, but reaction velocities differed for the different isotopically labeled analogs. $K_M$ values ranged between 0.8 and 1.4 $\mu$M and 8.2 and 11 $\mu$M for CYP2D6 and CYP3A4, respectively. Thus, deuterium isotope effects were equivalent for $V_{\text{max}}$ and $V_{\text{max}}/K_M$, and subsequent isotope effect measurements were conducted at saturating substrate concentrations. Mean deuterium isotope effects for all five deuterated ezlopitant analogs are listed in Table 2. For CYP3A4, deuterium isotope effects of 1.25 to 1.55 were observed for the formation of both CJ-12,458 and CJ-12,764 when the benzylic hydrogen was replaced with deuterium (i.e., $[d_1]$ezlopitant and $[d_2]$ezlopitant). When the benzylic position possessed a hydrogen, but the other positions of the isopropyl substituent were variously substituted with deuterium, isotope effects were barely detected for formation of the alkene (1.06–1.12), and small inverse isotope effects were observed for the formation of the benzylic alcohol (0.84–0.87; Table 2).

For CYP2D6, a similar pattern of isotope effects was observed, but the magnitude differed. For analogs possessing deuterium at the benzylic position, isotope effects of 1.48 to 2.61 were observed. Barely detectable effects were observed for alkene formation (1.07–1.12) when the other isopropyl positions were deuterated (Table 2). As with CYP3A4, small inverse isotope effects were observed for benzylic hydroxylation (0.78–0.90).

The ratios of benzylic alcohol to alkene formed by CYP3A4 and CYP2D6 for ezlopitant and all five deuterated analogs are listed in Table 3. For CYP3A4, the ratio was 1.08 (±0.04) for nondeuterated ezlopitant. When the benzylic position was deuterated but the other isopropyl carbons possessed only hydrogen atoms ($[d_1]$ezlopitant), the alcohol to alkene ratio was fairly similar at 0.94 ± 0.01. When the 1° carbons had 3, 4, or 6 hydrogens replaced with deuterium, the alcohol became somewhat favored over the alkene with ratios ranging from 1.32 to 1.43. Increased deuteration incorporation (from three to seven deuterium atoms) did not yield greater ratios. The corresponding ratio of alcohol to alkene for unlabeled ezlopitant was 1.27 (±0.03) for CYP2D6. Ratios for $[d_1]$ezlopitant, $[d_2]$ezlopitant, $[d_3]$ezlopitant, and $[d_6]$ezlopitant were similar and ranged between 1.58 and 1.75, again with no trend toward higher ratios with increasing deuteration incorporation. For CYP2D6, the ratio of alcohol to alkene formation was 2.12 (±0.09) for heptadeuterated ezlopitant.

Deuterium Isotope Effects on the Overall Consumption of Ezlopitant Catalyzed by CYP3A4 and CYP2D6. In P450-catalyzed reactions, incorporation of deuterium can result in the switching of the position of metabolism to a distant site. To measure this, the rate of consumption of ezlopitant and its deuterated analogs at $[S] < K_M$ was determined and compared (it should be noted that since no effect of deuteration on $K_M$ had been observed, measurement of reaction velocity at $[S] < K_M$ provides a meaningful estimate of isotope effect). The overall rates of metabolism of ezlopitant and deuterated analogs were measured at a substrate concentration well below $K_M$.

**TABLE 1**

Summary of [1H]NMR spectral data for deuterated 4 isopropyl anisoles

<table>
<thead>
<tr>
<th>Compound</th>
<th>$H_1$ (Aromatic)</th>
<th>$H_6$ (Methoxy)</th>
<th>$H_8$ (Benzyl)</th>
<th>$H_4$ (Isopropyl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monodeuterated</td>
<td>7.2 and 6.8 ppm;</td>
<td>3.8 ppm;</td>
<td>s; 3H</td>
<td>absent; 1.2 ppm; s; 6H</td>
</tr>
<tr>
<td>Trideuterated</td>
<td>7.2 and 6.8 ppm;</td>
<td>3.8 ppm;</td>
<td>s; 3H</td>
<td>2.9 ppm; q; 1H</td>
</tr>
<tr>
<td>Tetraduterated</td>
<td>7.2 and 6.8 ppm;</td>
<td>3.8 ppm;</td>
<td>s; 3H</td>
<td>2.9 ppm; 1.2 ppm 2s; t; 1H</td>
</tr>
<tr>
<td>Hexaduterated</td>
<td>7.2 and 6.8 ppm;</td>
<td>3.8 ppm;</td>
<td>s; 3H</td>
<td>2.8 ppm; Absent</td>
</tr>
<tr>
<td>Heptaduterated</td>
<td>7.2 and 6.8 ppm;</td>
<td>3.8 ppm;</td>
<td>s; 3H</td>
<td>Absent</td>
</tr>
</tbody>
</table>

All [1H]NMR spectra were taken in CDCl$_3$ on a Bruker 300 MHz instrument (Newark, DE).
TABLE 2
Deuterium isotope effects for the metabolism of ezlopitant to hydroxylated (CJ-12,764) and dehydrogenated (CJ-12,458) metabolites by CYP3A4 and CYP2D6

<table>
<thead>
<tr>
<th>Deuterated Analog</th>
<th>CYP3A4</th>
<th>CYP2D6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CJ-12,458 Formation</td>
<td>CJ-12,764 Formation</td>
</tr>
<tr>
<td>[d1]Ezlopitant</td>
<td>1.34 ± 0.01</td>
<td>2.12 ± 0.09</td>
</tr>
<tr>
<td>[d2]Ezlopitant</td>
<td>1.43 ± 0.05</td>
<td>1.58 ± 0.05</td>
</tr>
<tr>
<td>[d4]Ezlopitant</td>
<td>1.32 ± 0.03</td>
<td>1.62 ± 0.10</td>
</tr>
<tr>
<td>[d6]Ezlopitant</td>
<td>1.44 ± 0.05</td>
<td>1.56 ± 0.05</td>
</tr>
<tr>
<td>[d8]Ezlopitant</td>
<td>1.36 ± 0.03</td>
<td>1.75 ± 0.04</td>
</tr>
<tr>
<td>[d10]Ezlopitant</td>
<td>1.52 ± 0.05</td>
<td>1.82 ± 0.04</td>
</tr>
</tbody>
</table>

TABLE 3
Effect of deuterium substitution on the ratio of benzyl alcohol (CJ-12,764) and alkene (CJ-12,458) metabolites formed by CYP3A4- and CYP2D6-catalyzed metabolism of ezlopitant

<table>
<thead>
<tr>
<th>Deuterated Analog</th>
<th>CYP3A4</th>
<th>CYP2D6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio of Benzyl Alcohol to Alkene</td>
<td></td>
</tr>
<tr>
<td>[d1]Ezlopitant</td>
<td>1.08 ± 0.04</td>
<td>1.27 ± 0.03</td>
</tr>
<tr>
<td>[d2]Ezlopitant</td>
<td>0.94 ± 0.01</td>
<td>1.62 ± 0.10</td>
</tr>
<tr>
<td>[d4]Ezlopitant</td>
<td>1.32 ± 0.03</td>
<td>1.62 ± 0.11</td>
</tr>
<tr>
<td>[d6]Ezlopitant</td>
<td>1.43 ± 0.05</td>
<td>1.58 ± 0.05</td>
</tr>
<tr>
<td>[d8]Ezlopitant</td>
<td>1.34 ± 0.01</td>
<td>2.12 ± 0.09</td>
</tr>
</tbody>
</table>

Intramolecular Isotope Effects of CJ-12,458 Formation. Intramolecular isotope effects were measured for dehydrogenation by examining the ratio of [d1]CJ-12,458 versus [d2]CJ-12,458 formed from [d1]Ezlopitant and the ratio of [d4]CJ-12,458 versus [d2]CJ-12,458 formed from [d4]Ezlopitant. Isotope effects were very low at 1.13 to 1.15 for each deuterated substrate for both P450 isoforms (Table 5).

TABLE 4
Deuterium isotope effects for the overall consumption of ezlopitant by CYP3A4 and CYP2D6

<table>
<thead>
<tr>
<th>Deuterated Ezlopitant</th>
<th>CYP3A4</th>
<th>CYP2D6</th>
</tr>
</thead>
<tbody>
<tr>
<td>[d1]Ezlopitant</td>
<td>1.07 ± 0.04</td>
<td>1.39 ± 0.07</td>
</tr>
<tr>
<td>[d2]Ezlopitant</td>
<td>0.95 ± 0.05</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>[d4]Ezlopitant</td>
<td>0.97 ± 0.05</td>
<td>0.94 ± 0.05</td>
</tr>
<tr>
<td>[d6]Ezlopitant</td>
<td>0.93 ± 0.04</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>[d8]Ezlopitant</td>
<td>1.05 ± 0.07</td>
<td>1.53 ± 0.08</td>
</tr>
</tbody>
</table>

Intramolecular isotope effect of alkene formation for CYP3A4 and CYP2D6 using [d1]ezlopitant and [d6]ezlopitant

For CYP3A4, isotope effects on overall consumption of ezlopitant were not observed (Table 4). However for CYP2D6, overall isotope effects of 1.39 and 1.53 were observed for [d1]ezlopitant and [d6]ezlopitant, respectively, representing both compounds that possess deuterium substitution at the benzylic position. When the benzylic position possessed a hydrogen ([d2]ezlopitant, [d4]ezlopitant, and [d8]ezlopitant), no isotope effects on overall consumption of ezlopitant were observed (Table 4).

Intramolecular Isotope Effects of CJ-12,458 Formation. Intramolecular isotope effects were measured for dehydrogenation by examining the ratio of [d1]CJ-12,458 versus [d2]CJ-12,458 formed from [d1]Ezlopitant and the ratio of [d4]CJ-12,458 versus [d2]CJ-12,458 formed from [d4]Ezlopitant. Isotope effects were very low at 1.13 to 1.15 for each deuterated substrate for both P450 isoforms (Table 5).

Isotope Effects on Chemical Dehydration of CJ-12,764 (Benzyl Alcohol) to CJ-12,458 (Alkene). In the chemical dehydration experiments, no detectable CJ-12,458 was observed when CJ-12,764 was incubated in CH3CN (control). In incubations containing acetonitrile solutions of FeCl3 and TFA, dehydration of the alcohol to the alkene was observed. For [d1]CJ-12,764, intrinsic isotope effects of 5.90 and 4.47 were observed for the FeCl3- and TFA-catalyzed dehydration reactions, respectively (Table 6). For [d4]CJ-12,764, the isotope effects were 4.51 and 3.79, respectively.

Discussion
The use of deuterium-containing analogs of substrates in the measurement of deuterium isotope effects on reaction rates/kinetics is a standard approach in the examination of mechanisms of reactions in organic and bio-organic chemistry. Such an approach has been extensively and successfully applied to the P450 enzymes to understand the mechanisms of aliphatic hydroxylation and heteroatom dealkylation reactions (Ortiz de Montellano, 1995). An interesting aspect of the application of deuterium isotope effects to P450-catalyzed reactions resides in the fact that the enzyme exhibits a high “commitment to catalysis”. That is, in the multistep reaction cycle for many of these enzymes, a step or steps before the isotope-sensitively sensitive step is essentially rate limiting and irreversible. This can result in the masking of isotope effects; little change is observed in the overall consumption of substrate when hydrogen is replaced with deuterium despite there being a substantial slowing down of the C-H bond-breaking step. Many examinations of deuterium isotope effects of P450-catalyzed reactions have relied upon the observation of intramolecular isotope effects. In this approach, identical, chemically equivalent positions simultaneously possess both deuterium and hydrogen atoms, and the product of possessing deuterium versus that containing hydrogen is measured. This results in an “unmasking” of the isotope effect and generates useful data for mechanistic interpretation. A second possibility in the application of isotope effects to study P450-catalyzed reactions is the observation of metabolic switching. In this case, replacement of hydrogen atoms with deuterium atoms results in a diminished rate of metabolism at the site where the deuterium atoms are present and a concomitant acceleration of the rate of metabolism at chemically unequivalent sites in which hydrogen atoms were maintained with little or no overall change in the rate of substrate consumption.

In addition to its use in understanding the mechanisms of the common P450-catalyzed hydroxylation and heteroatom dealkylation reactions, the approach of measuring deuterium isotope effects has also been applied to the study of P450-catalyzed dehydrogenation reactions. Two noteworthy deuterium isotope effect studies have been conducted on the P450-catalyzed dehydrogenation of valproic acid (Rettie et al., 1988) and testosterone (Korzekwa et al., 1990). In the former case, the dehydrogenation reaction occurs at an sp3-hybridized center of an alkane carbon and is catalyzed by liver microsomes of phenobarbital-induced rabbits. The intramolecular isotope effect ob-
The P450-catalyzed dehydrogenation of ezlopitant exhibits almost no intramolecular isotope effect when chemically equivalent positions are partially deuterium substituted (i.e., \([d_1]\)ezlopitant and \([d_2]\)ezlopitant; Table 5). In Fig. 4, possible mechanisms for ezlopitant dehydrogenation are depicted in consideration of these data. All require the initial abstraction of the benzylic hydrogen to yield a common benzylic radical intermediate. In pathway A, the Fe(IV)-OH intermediate abstracts a second hydrogen atom from the terminal position, water is released, and the diradical collapses to yield the alkene. In pathway B, the benzylic alcohol is formed but undergoes dehydration to the alkene (possibly via a carbocation intermediate), which must occur before release of the alcohol (since benzyl alcohol + P450 does not yield alkene). The dehydration could be catalyzed by the heme Fe(III) or by an amino acid in the active site acting as a general acid catalyst. These possibilities were tested by measuring the intrinsic isotope effect of dehydration of \([d_1]\)CJ-12,764 and \([d_2]\)CJ-12,764 catalyzed by both Lewis (FeCl3) and protic acids (TFA) acids. These isotope effects were substantially higher than those observed for P450-catalyzed CJ-12,458 formation (Tables 5 and 6), which is evidence against a general acid-catalyzed dehydration of CJ-12,764. Furthermore, incubation of ezlopitant with CYP3A4 and CYP2D6 in \([^{18}\text{O}]\text{H}_2\text{O} \) did not result in any incorporation of oxygen-18 into CJ-12,764, indicating that a benzylic carbocation is not formed as an intermediate in the metabolism of ezlopitant (data not shown).

The mechanism in pathway A is that which has been proposed for valproic acid (Rettie et al., 1988). The data obtained with \([d_1]\)ezlopitant, a very small isotope effect of 1.15, is similar to that observed for the CYP2B1-catalyzed dehydrogenation of \([5,5,5-^{2}\text{H}_3]\)valproic acid (Rettie et al., 1995). However, the distinction of \([d_1]\)ezlopitant versus \([5,5,5-^{2}\text{H}_3]\)valproic acid is that the competition between hydrogen versus deuterium for the second H-atom abstraction would occur between exactly equivalent positions that are directly bonded to the carbon from which the initial hydrogen was abstracted. The very low isotope effect for \([d_1]\)ezlopitant is consistent with pathways A or C, and with the previous observations for valproic acid.

A mechanism is proposed in pathway C, which is consistent with but not unequivocally proven by the data. In this proposal, the benzylic radical is partially stabilized by the p-methoxy substituent leading to a delocalization of the electron density throughout the aromatic ring and isopropyl group, resulting in restricted rotation around the benzylic bond. The P450 Fe(IV)-OH intermediate could then abstract a hydrogen atom from the terminal position but due to the restricted rotation could not selectively abstract a hydrogen atom over a deuterium atom in \([d_2]\)ezlopitant. Thus, intrinsic isotope effects would be masked for the trideuterated analog. However, no intramolecular isotope effects were observed for the \([d_1]\)ezlopitant analog either. In pathway C, abstraction of the benzylic hydrogen. Thus, the benzylic radical is an intermediate that partitions between formation of the benzylic alcohol and alkene. This is also what has been proposed for the alkene formation reactions for valproic acid and testosterone (Rettie et al., 1988; Korzekwa et al., 1990) and is consistent with the accepted hypothesis of P450 mechanism.

### Table 6

<table>
<thead>
<tr>
<th>Acid Catalyst</th>
<th>([d_1])CJ-12,764 Ratio</th>
<th>([d_1])CJ-12,764 Corrected Ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl3</td>
<td>5.90 ± 0.13</td>
<td>4.51 ± 0.12</td>
</tr>
<tr>
<td>TFA</td>
<td>4.47 ± 0.21</td>
<td>3.79 ± 0.12</td>
</tr>
</tbody>
</table>

\(a\) Ratio corrected for statistics of two deuterium atoms per one hydrogen atom present on each \(1^\text{st}\) position of \([d_1]\)ezlopitant.

The authors used this as evidence that the two products arise from a common radical intermediate. The enzyme-bound radical intermediate can partition between two subsequent reactions: rebound of the hydroxyl radical to yield the alcohol or abstraction of a second hydrogen atom followed by collapse of the diradical to form the alkene. For testosterone, the CYP2A1-catalyzed dehydrogenation occurs at a position adjacent to an sp\(^2\)-hybridized center. Measurement of intrinsic isotope effects supported a mechanism of formation of a common radical intermediate that partitions between formation of the alcohol and the alkene (Korzekwa et al., 1990).

Unlike valproic acid and testosterone, the dehydrogenation of ezlopitant occurs at a benzylic position. The possibility that the alcohol metabolite gives rise to the alkene via either a spontaneous dehydrogenation or dehydration catalyzed by an unknown component in the incubation mixtures was considered and explored. Several lines of direct and indirect evidence had been previously obtained that support the notion that CJ-12,458 does not arise via a simple dehydrogenation of CJ-12,764: 1) no CJ-12,458 was observed when radiolabeled CJ-12,764 was incubated under conditions in which ezlopitant gave rise to CJ-12,458 (Obach, 2000); 2) no CJ-12,458 was observed when CJ-12,764 was incubated under conditions in which ezlopitant gave rise to CJ-12,458 using a sensitive HPLC-MS assay; 3) in other metabolic systems, including liver microsomes from several preclinical species and heterologously expressed recombinant CYP3A5, the ratio of CJ-12,764 to CJ-12,458 was markedly different from metabolite ratios observed for CYP3A4, CYP2D6, and human liver microsomes; and 4) incubation of CJ-12,764 and CJ-12,458 with human liver microsomes yields distinctly different product profiles. Thus, the conversion of ezlopitant to CJ-12,458 appears to be another example of direct P450-catalyzed dehydrogenation of a saturated alkane.

For ezlopitant, isotope effects were observed on \(V_{\text{max}}\), which is common for P450 reactions since the rate-limiting step in the catalytic cycle for many of these enzymes occurs before the first C-H bond-breaking step. The low isotope effects for the metabolism of ezlopitant to the alkene suggest that there is a very high commitment to catalysis for this reaction. Only small isotope effects were observed on the overall consumption of ezlopitant, which is typical for P450 enzymes (Table 4). However, there was also little effect on the formation of the two metabolites (Table 2) indicating that metabolic switching did not occur to a great extent. When the benzylic position possessed a deuterium atom (\([d_1]\)ezlopitant and \([d_2]\)ezlopitant), isotope effects were observed for formation of both the alcohol and alkene. However, when the benzylic position possessed a hydrogen atom while the adjacent positions possessed three or more deuterium atoms, isotope effects were not observed for alkene formation, with small inverse isotope effects observed for benzyl alcohol formation. These data support the notion that the formation of the alkene requires initial abstraction of the benzylic hydrogen. Thus, the benzylic radical is an intermediate that partitions between formation of the benzylic alcohol and alkene. This is also what has been proposed for the alkene formation reactions for valproic acid and testosterone (Rettie et al., 1988; Korzekwa et al., 1990) and is consistent with the accepted hypothesis of P450 mechanism.
due to a high drive to re-aromatize the ring, which would be effected by abstraction of the second hydrogen atom. A second possibility is that the restricted rotation proposed for the benzylic bond due to resonance stabilization from the \( p \)-methoxy group could extend to the adjacent C-C bond, thereby reducing selectivity for removal of a hydrogen atom versus a deuterium atom (as in pathway C of Fig. 4). This proposed mechanism requires an important role for the \( p \)-methoxy substituent, and further testing of this hypothesis requires the
examination of the P450-catalyzed metabolism of close-in analogs that possess electronically different substituents at this position (i.e., measurement of the propensity of other analogs to undergo dehydrogenation). However, if the low intramolecular isotope effect observed for \([\text{d}_3]\) and \([\text{d}_4]\) is merely due to a rapid rate/high commitment to catalysis for the second hydrogen atom abstraction, then the data do not distinguish between pathways A and C.

In conclusion, the P450-catalyzed dehydrogenation of ezlopitant represents a mechanistically interesting reaction. The isotope effect data obtained appear to distinguish this reaction from other P450-catalyzed dehydrogenation reactions. Whether, this distinction is due to the fact that the dehydrogenation of ezlopitant occurs at a benzylic position or whether this is due to some unique characteristic of this particular substrate remains to be determined.

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References