Effects of Acetylenic Epoxygenase Inhibitors on Recombinant Cytochrome P450s

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Received November 24, 2010; accepted April 1, 2011

ABSTRACT:

Arachidonate epoxidation, which mediates important biological functions in several tissues, is catalyzed by specific cytochrome P450 (P450) enzymes. Two fatty acid derivatives [2-(2-propynyloxy)-benzenehexanoic acid (PPOH) and *N*-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH)] are used as general, mechanism-based P450 epoxigenase inactivators, but the effects of these drugs on nearly all P450 isoforms are unknown. Here, the activity of these compounds on nine human and three rat recombinant P450s was studied. As expected, PPOH inhibited five known epoxigenases [CYP2B1, 2B6, 2C6, 2C9, and 2C11 (IC_{50} = 23–161 μ M)] but had little or no activity on P450s typically not considered to be epoxigenases (CYP1A1, 1A2, 1B1, 2A6, 2D6, and 2E1). PPOH was only a very weak inhibitor (IC_{50} = ~300 μ M) of CYP2C19, an important human epoxy-

genase. An unexpected finding was that MS-PPOH (a metabolically stable congener of PPOH) potentially inhibited only two P450 epoxigenases (2C9 and 2C11, IC_{50} = 11–16 μ M) and showed considerably lower activity (IC_{50} = >90 μ M) on all other P450s tested, including three epoxigenases (CYP2B1, 2B6, and 2C19). In addition, PPOH and MS-PPOH displayed time- and NADPH-dependent inhibition of CYP2C9 and other epoxigenases. These results support the putative mechanism of action of PPOH and MS-PPOH on recombinant P450s and (with one exception) confirm a general epoxigenase inhibitory profile for PPOH. However, the heterogeneity of inhibitory potencies for MS-PPOH on these enzymes suggests caution in the use of this drug as a general epoxigenase inhibitor. These results will facilitate the judicious use of PPOH and MS-PPOH for epoxigenase research.

Introduction

Cytochrome P450s (P450s) belong to a superfamily of heme-containing mixed-function oxidases, which catalyze the production of hydroxylated, epoxidated, or dealkylated metabolites (Correia and Ortiz de Montellano, 2005). In addition to their well established roles in xenobiotic metabolism, P450s function in diverse endogenous pathways, including steroid biosynthesis and fatty acid metabolism (Capdevila and Falck, 2002). In particular, P450 epoxigenases (Capdevila and Falck, 2002; Spector, 2009), which catalyze the conversion of arachidonate to epoxyeicosatrienoic acids, are receiving increased attention because of their pharmacological activities (McGiff and Quilley, 2001; Roman, 2002; Spector and Norris, 2007; Spector, 2009; Iliff et al., 2010).

PPOH and MS-PPOH are fatty acid derivatives (Fig. 1), which are used as experimental epoxigenase inhibitors (Wang et al., 1998; Brand-Schieber et al., 2000). Both compounds selectively inhibit arachidonate epoxigenase (versus arachidonate hydroxylase) activity in renal cortical microsomes (Wang et al., 1998; Brand-Schieber et al., 2000). Studies of the P450 isoforms thought to be important for kidney epoxigenase activity showed that PPOH inhibits recombinant

rat CYP4A2 and CYP4A3 (Nguyen et al., 1999). A related study showed that MS-PPOH blocks human CYP2C8 epoxigenase activity (Brand-Schieber et al., 2000). However, PPOH and MS-PPOH have been used in vitro as selective P450 epoxigenase inhibitors not only in the kidney (Wang et al., 1998; Imig et al., 1999; Brand-Schieber et al., 2000) but also in astrocytes (Rzagalinski et al., 1999) and in pulmonary arteries (Zhu et al., 2000). MS-PPOH, a metabolically stable congener of PPOH (Schaaf and Hess, 1979; Wang et al., 1998), has been used both in vivo and in vitro to block renal (Brand-Schieber et al., 2000) and brain (Bhardwaj et al., 2000; Peng et al., 2002; Conroy et al., 2010) epoxigenases. Thus, PPOH and MS-PPOH have been used to target P450 epoxigenases throughout the body, but the P450 isoforms responsible for the extrarenal actions of these drugs are unknown. Because arachidonic acid epoxidation can be catalyzed by numerous P450s [primarily of the CYP1 and CYP2 families (Choudhary et al., 2004; Imaoka et al., 2005; Fer et al., 2008; Lucas et al., 2010)], it is important to assess the activity of these drugs on CYP1 and CYP2 isoforms.

Terminal acetylenic fatty acids function as “suicide substrates,” which undergo catalytic oxidation, followed by inactivation of the enzyme (Ortiz de Montellano and Reich, 1984; Zou et al., 1994). These suicide substrates, also known as mechanism-based inactivators, require an active enzyme and therefore demonstrate time- and NADPH-dependent activity. PPOH and MS-PPOH both possess terminal acetylene groups (Fig. 1) and are thought to act as mechanism-

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants DA03816, DA07307, DA027835].

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.110.037424.

ABBREVIATIONS: P450, cytochrome P450; PPOH, 2-(2-propynyloxy)-benzenehexanoic acid; MS-PPOH, *N*-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide; EFC, 7-ethoxy-4-trifluoromethylcoumarin; MFC, 7-methoxy-4-trifluoromethylcoumarin; HFC, 7-hydroxy-4-trifluoromethylcoumarin; DBF, dibenzylfluorescein.

FIG. 1. Chemical structures of MS-PPOH, PPOH, and miconazole.

based inactivators, but this theory is not well established. Although PPOH showed time- and NADPH-dependent inhibition of renal microsomal epoxigenase activity (Wang et al., 1998), MS-PPOH actions have never been studied by such criteria. In vivo, MS-PPOH showed time-dependent (i.e., irreversible) enzyme inhibition, but NADPH-dependence was not assessed (Brand-Schieber et al., 2000). Neither time-dependent nor NADPH-dependent inhibitory actions by these drugs on any recombinant P450 have been documented. Here, the selectivity and mode of action of PPOH and MS-PPOH have been investigated in several human and rat recombinant P450s.

Materials and Methods

Materials. 7-Ethoxy-4-trifluoromethylcoumarin (EFC) was purchased from Invitrogen (Carlsbad, CA). Resorufin benzyl ether was purchased from AnaSpec, Inc. (San Jose, CA). 7-Methoxy-4-trifluoromethylcoumarin (MFC), 7-hydroxy-4-trifluoromethylcoumarin (HFC), dibenzylfluorescein (DBF), 3-cyano-7-ethoxycoumarin, 3-[2-(*N,N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin, coumarin, and recombinant P450 microsomes from baculovirus-infected insect cells (Supersomes) were purchased from BD Biosciences (Woburn, MA). Acetonitrile (high-performance liquid chromatography grade) and magnesium chloride hexahydrate were purchased from Thermo Fisher Scientific (Waltham, MA). Fluorescein, potassium phosphate monobasic, potassium phosphate dibasic, glucose 6-phosphate, NADP⁺, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). PPOH and MS-PPOH were purchased from Cayman Chemical (Ann Arbor, MI), and miconazole was purchased from MP Bioscience (Buxton, UK). Fluorometric assays were conducted in black Costar 96-well plates (Corning Incorporated, Corning, NY).

Recombinant Human P450 Enzyme Assays. Human P450 assays were performed with the following enzyme amounts and substrates: CYP2C9 (1 pmol, 75 μ M MFC), CYP2C19 (1 pmol, 0.5 μ M DBF), CYP2B6 (1 pmol, 2.5 μ M EFC), CYP1A1 (1 pmol, 12.5 μ M resorufin benzyl ether), CYP1A2 (0.5 pmol, 5 μ M 3-cyano-7-ethoxycoumarin), CYP1B1 (1 pmol, 12.5 μ M resorufin benzyl ether), CYP2A6 (1 pmol, 3 μ M coumarin), CYP2D6 (1 pmol, 1.5 μ M 3-[2-(*N,N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin), and CYP2E1 (1 pmol, 70 μ M MFC). All incubations were performed at 37°C. Except as noted above, all other reagents and conditions were as described by BD Biosciences for high-throughput P450 screening kits and by Crespi et al. (1997). IC₅₀ values were determined and are reported for three human P450s.

Recombinant Rat P450 Enzyme Assays (CYP2B1, 2C6, and 2C11). Fluorometric high-throughput screens have been developed for human P450s, but applications of these methods for rat P450s have been limited. Assays for rat P450s were optimized with respect to potential substrate, a NADPH-regenerating system, incubation time, buffer conditions, and enzyme concentration. Unless otherwise stated, the incubations were performed in a total volume of 200 μ l of 50 mM potassium phosphate buffer (pH 7.4) with 1% acetonitrile.

For CYP2B1 or CYP2C6 assays, EFC in 100% acetonitrile was added into the reaction mixtures containing the "low" NADPH-regenerating system (final concentrations of 8.1 μ M NADP⁺, 0.4 mM glucose 6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase, and 0.4 mM magnesium chloride) in 0.1 ml of buffer, followed by preincubation at 37°C for 15 min. Unless specified otherwise, final EFC concentrations were 37.5 μ M (CYP2B1) and 5 μ M (CYP2C6). The reactions were initiated by the addition of 0.1 ml of enzyme (1.0 pmol of CYP2B1 or 0.5 pmol of CYP2C6) in buffer and then incubated at 37°C for 30 min. Reactions were terminated by the addition of 75 μ l of ice-cold 80% acetonitrile-20% 0.5 M Tris base ("STOP" solution). Sample

blanks were prepared by preincubation of the NADPH-regenerating system (37°C for 15 min) and addition of STOP solution followed by addition of enzyme/substrate and incubation as stated above. Fluorescent signals from the blanks were subtracted from all other samples. Formation of HFC, the EFC O-dealkylated product, was monitored by measuring the fluorescent signal using a Victor3 140 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) with an excitation filter at 405 nm (bandwidth 10 nm) and emission filter at 535 nm (bandwidth 25 nm). HFC standard curves were created by spiking HFC into the blank incubation mixture.

To measure CYP2C11 (1 pmol), the conditions discussed above were used except that EFC was replaced by DBF (0.5 μ M unless specified otherwise), a "high" NADPH-regenerating system (final concentrations of 1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride) replaced the low NADPH-regenerating system, and the reactions were terminated by the addition of 75 μ l of 2 N NaOH followed by a 2-h postincubation at 37°C. Product formation was determined from a fluorescein standard curve, prepared by spiking fluorescein into the blank incubation mixture. An excitation filter 485 nm (bandwidth 14 nm) and emission filter at 535 nm (bandwidth 25 nm) were used to monitor fluorescein formation.

IC₅₀ Determinations for Rat P450s. The incubation system contained 50 mM potassium phosphate buffer (pH 7.4), an appropriate amount of NADPH-regenerating system (from the preceding section), recombinant P450, substrate, and inhibitor in a final volume of 200 μ l. After a 10-min preincubation at 37°C of the NADPH-regenerating system, the reaction was initiated by adding enzyme and substrate, followed by a 30-min incubation, and stopped as described above. Enzyme amounts and substrate concentrations were 1 pmol of CYP2B1 (37.5 μ M EFC), 0.5 pmol of CYP2C6 (5 μ M EFC), or 1 pmol of CYP2C11 (0.5 μ M DBF). The control incubations contained the NADPH-regenerating system, substrate, and enzyme without inhibitors in buffer.

Calculations. Rat P450 substrate-velocity curves were fitted to the Michaelis-Menten equation for a single enzyme model and kinetic parameters calculated by nonlinear regression using Prism 5.0 (GraphPad Software Inc., San Diego, CA). IC₅₀ values were estimated from concentration-response curves by nonlinear regression (Prism 5.0) using the sigmoidal dose-response with variable slope function. Data were normalized to percentage control responses, such that tops of all curves were constrained to 100%. In cases in which inhibition was not complete at the highest drug concentrations tested, the bottoms of the curves were constrained to zero. Converged fits yielded IC₅₀ values along with 95% confidence intervals.

Results

Recombinant Rat P450 Activities. Preliminary studies using the substrates at the specified concentrations showed that product formation from the rat enzymes was linear with time for at least 30 min (Fig. 2). After 30-min incubations, product formation was linear with amounts of enzyme added (0.5–1.5, 0.25–1.5, and 0.25–4.0 pmol of

FIG. 2. Linearity of rat CYP2B1 and CYP2C6 enzyme activities with time. Enzymes (1 and 0.5 pmol, respectively) were incubated with EFC (37.5 and 5 μ M, respectively) as described. Product formation [ordinate, in relative fluorescence units (RFU), mean $\pm$ S.E.M. for four to eight replicates from a typical experiment] are plotted versus incubation time (abscissa, minutes).

enzyme for CYP2B1, CYP2C6, and CYP2C11, respectively; data not shown). Varying the ionic strength of the buffer had no effect on CYP2C6 and CYP2C11 from 25 to 100 mM potassium phosphate, pH 7.4. However, activity for CYP2B1 was improved at lower ionic strengths (25 or 50 versus 100 mM; data not shown). Substrate-velocity curves for all three rat enzymes gave good fits to Michaelis-Menten kinetics (Table 1). Lineweaver-Burk analysis of the same data gave good agreement (data not shown).

IC₅₀ Determinations. PPOH and MS-PPOH were screened on nine human and three rat P450s (Table 2). PPOH and MS-PPOH showed little to no inhibitory activity on human CYP1A1, 1A2, 1B1, 2A6, 2D6, or 2E1 (<20% inhibition at 80 μ M). Inhibitory concentration-response curves for PPOH and MS-PPOH were constructed on the remaining P450 isoforms (Fig. 3). On the human enzymes, PPOH was a potent inhibitor of 2C9 and 2B6 (IC₅₀ = 23 and 63 μ M, respectively) (Fig. 3B) but showed considerably weaker activity toward 2C19 (IC₅₀ = ~300 μ M) (Fig. 3B). On the rat P450 isoforms, PPOH had potent inhibitory activity on 2B1 and 2C11 (IC₅₀ = 23 and 73 μ M, respectively), with weaker activity (IC₅₀ = 161 μ M) on 2C6 (Fig. 3D).

We were surprised to find that the spectrum of action of MS-PPOH did not always mirror that of PPOH on rat or human P450 isoforms. Similar to the effects of PPOH, MS-PPOH was a potent inhibitor of human 2C9 and rat 2C11 (IC₅₀ = 11 and 16 μ M, respectively) (Fig. 3, A and C) and showed weak activity on human 2C19 (Fig. 3A). Unlike PPOH (which was a moderate to potent inhibitor of the 2B isoforms), MS-PPOH was very weakly active as an inhibitor of rat CYP2B1 and human CYP2B6 (Fig. 3).

Time and NADPH Dependence Studies. On CYP2C9, MS-PPOH (5 μ M) inhibited activity by 49 and 83%, respectively, in the absence and presence of a 30-min preincubation with the NADPH-regenerating system (Fig. 4A). When this preincubation was performed without an NADPH-regenerating system, the inhibition was 50%, similar to the results obtained in the absence of preincubation (Fig. 4A). PPOH (5 μ M) inhibited CYP2C9 activity by 63 and 90%, respectively, in the absence and presence of the 30-min NADPH preincubation and 63% when the 30-min preincubation omitted NADPH (Fig. 4B). Miconazole, an imidazole-based inhibitor presumed to act as a competitive, reversible inhibitor, showed neither time nor NADPH dependence on human CYP2C9 when tested between 0.05 and 1 μ M (Fig. 4C). Preincubation of CYP2B1 with PPOH also demonstrated time-dependent enzyme inactivation, which was reversed by omission of the NADPH-regenerating system (Fig. 4D). Experiments similar to those of Fig. 4 found time- and NADPH-dependent inactivation of rat CYP2C6 (Fig. 5B) and CYP2C11 (Fig. 5D) by PPOH; MS-PPOH had similar actions on these enzymes (Fig. 5, A and C).

TABLE 1

K_m and V_{max} values for rat CYP2B1, 2C6, and 2C11

Recombinant enzymes (1 pmol of CYP2B1 and CYP2C11 or 0.5 pmol of CYP2C6) were incubated with various amounts of substrate (EFC for CYP2B1 and CYP2C6 or DBF for CYP2C11) and regenerating system in potassium phosphate buffer, pH 7.4, at 37°C for 30 min. Product formation in picomoles of product per picomoles of enzyme per minute was plotted at each concentration of substrate. Data were fitted to the Michaelis-Menten equation for a single enzyme model, and kinetic parameters were estimated by nonlinear regression. Values are means $\pm$ S.E.M. for a total of four measurements.

Enzyme	Substrate	<i>K_m</i> μ M	<i>V_{max}</i> min^{-1}
2B1	EFC	66.7 $\pm$ 7.6	7.1 $\pm$ 0.3
2C6	EFC	6.0 $\pm$ 0.9	20.2 $\pm$ 0.7
2C11	DBF	1.3 $\pm$ 0.1	0.52 $\pm$ 0.02

TABLE 2

Activity of epoxygenase inhibitors on select human and rat P450 isoforms

IC₅₀ values were taken from Fig. 3 or literature cited. Potencies are classified according to the IC₅₀ values as either high (bold type, <25 μ M), intermediate (italic type, 26–99 μ M), or low (regular type, >100 μ M). Literature citations in support of the epoxygenase activity of each P450 isoform are shown as footnotes in the right column.

Isoform	IC ₅₀ ^a		Epoxygenase?
	MS-PPOH	PPOH	
	μ M or % inhibition		
1A1 h	N.A.	N.A.	Some ^b
1A2 h	N.A.	N.A.	Minor ^c
1B1 h	N.A.	N.A.	No
2A6 h	N.A.	N.A.	No
2B1 r	23% at 80 μ M	23 (13–40)	Yes ^{d,e,f}
2B6 h	~300 (166–526)	63 (39–103)	Yes ^{d,e}
2C6 r	99 (70–140)	161 (137–189)	Yes ^g
2C8 h	15^h	N.T.	Yes ^{c,f}
2C9 h	11 (6–21)	23 (19–28)	Yes ^{d,e,f}
2C11 r	16 (11–25)	73 (61–87)	Yes ^{c,f}
2C19 h	23% at 80 μ M	~300 (213–444)	Yes ^{d,e}
2D6 h	N.A.	N.A.	No
2E1 h	N.A.	N.A.	Minor ^c
4A2 r	N.T.	22ⁱ	Yes ⁱ
4A3 r	N.T.	6.5ⁱ	Yes ⁱ

h, human; r, rat; N.A., not active (<20% inhibition at 80 μ M, duplicate measurements); N.T., not tested.

^a Values are fitted IC₅₀ values with 95% confidence intervals in parenthesis or percentage inhibition at the specified concentration of inhibitor.

^b See Choudhary et al. (2004).

^c Epoxygenase products from these enzyme most often are identified as minor or variable in amounts (Rifkind et al., 1995; Capdevila and Falck, 2002; Choudhary et al., 2004; Fer et al., 2008; however, see also Lucas et al., 2010).

^d See Imaoka et al. (2005).

^e See Roman (2002).

^f See Capdevila et al. (2000).

^g See Iliff et al. (2010).

^h See Brand-Schieber et al. (2000).

ⁱ See Nguyen et al. (1999).

Discussion

Extrahepatic P450s are known to regulate many aspects of cellular homeostasis, including steroid hormone biosynthesis, vitamin bioactivation, and cholesterol and fatty acid metabolism (Correia and Ortiz de Montellano, 2005). In addition, many organ-specific specialized roles for these enzymes have been revealed. For example, recent studies have shown that brain P450 activity is important for neural-glial-vascular coupling (Iliff et al., 2010) and in mediation of opioid analgesic drug action (Conroy et al., 2010). Such studies emphasize the need for rapid, efficient, and cost-effective assays for P450s from laboratory animals. The microplate-based fluorescence assays presently optimized for rat P450s have many advantages over traditional enzymatic assays. At present, these methods permitted measurements of the potency of PPOH and MS-PPOH on these enzymes (Table 2). Because the effects of some P450 inhibitors have been found to be substrate-dependent (Schwarz and Roots, 2003), complementary studies with other substrates would also be of interest.

Very little is known about the molecular targets for PPOH and MS-PPOH. In the initial report (Wang et al., 1998), both compounds potentially inhibited arachidonic acid epoxygenase activity in rat renal cortical microsomes (IC₅₀ = 9 and 13 μ M, respectively). A 10- to 20-fold selectivity for inhibition of arachidonate epoxygenase versus hydroxylase activity by these drugs was demonstrated (Wang et al., 1998). Subsequently, PPOH was shown to block the kidney-related CYP4A2 and CYP4A3 epoxygenase activity (IC₅₀ = 22 and 6.5 μ M, respectively) but not CYP4A1 ω -hydroxylase activity (Nguyen et al., 1999). In another study related to kidney epoxygenases, MS-PPOH was reported to potentially inhibit human CYP2C8 epoxygenase activity

FIG. 3. Inhibition of human (A and B) and rat (C and D) P450s by MS-PPOH (A and C) and PPOH (B and D). Recombinant enzymes were incubated with substrate and the regenerating system in the presence of varying concentrations of inhibitors at 37°C. Percentage control activity (ordinate) is shown as the log of each concentration of inhibitor (abscissa). Each data point represents the mean ($\pm$ S.E.M.) of a total of four determinations. IC_{50} values, estimated by non-linear regression, are given in parentheses.

(Brand-Schieber et al., 2000). Thus, despite the use of these drugs as general epoxidegenase inhibitors in the literature (Imig et al., 1999; Rzigalinski et al., 1999; Bhardwaj et al., 2000; Brand-Schieber et al.,

2000), only three recombinant epoxidegenases were previously known to be blocked by PPOH and MS-PPOH (Table 2). As discussed below, the present results have 1) identified several new P450 epoxidegenase

FIG. 4. Time- and NADPH-dependent inhibition of CYP2C9 (A-C) and CYP2B1 (D). Enzymes were preincubated at 37°C with (+N) or without (-N) the NADPH-regenerating system, along with the designated concentrations of inhibitor [MS-PPOH (A), PPOH (B and D), and miconazole (C)] for the times shown (abscissa), followed by incubation with substrates and NADPH-regenerating systems as in Fig. 3. The natural log (Ln) of the percentage remaining activity (ordinate) is shown for each inhibitor concentration at the specified preincubation time (abscissa). Data are mean ($\pm$ S.E.M.) of a total of four determinations. Control (100%) activity was measured without inhibitor present. Dashed lines (A-C) show no loss of activity when preincubations were performed in the absence of the regenerating system (-N groups, solid square). A single time point for the (-N) group is shown in D (solid square, compare with open square).

FIG. 5. Time- and NADPH-dependent inhibition of CYP2C6 (A and B) and CYP2C11 (C and D) by MS-PPOH (A and C) and PPOH (B and D). Enzymes were preincubated at 37°C for the times shown (abscissa) with (+N, open symbols) or without (-N, solid symbols) the NADPH-regenerating system, along with the designated concentrations of inhibitors, followed by incubation with substrate and the regenerating system as in Fig. 4. The ordinate shows the natural log (Ln) of the percentage remaining activity (mean $\pm$ S.E.M. of at least four determinations) for each inhibitor concentration. In these experiments, regenerating systems were omitted only during the 30-min preincubation (-N groups, shown as solid symbols, compare with respective open symbols).

isoforms as targets for these drugs, 2) found large potency differences among P450 epoxygenases in their sensitivity to these compounds, 3) discovered important differences between the inhibitory actions of PPOH and MS-PPOH, and 4) confirmed that both compounds act as mechanism-based inactivators on the newly documented epoxygenase targets.

Although PPOH and MS-PPOH selectively block kidney epoxygenase activity (Wang et al., 1998), these drugs cannot be assumed a priori to block all P450 epoxygenase isoforms. On the contrary, the present results show that, of the six newly identified PPOH epoxygenase targets, the drug is a potent inhibitor of CYP2B1 and CYP2C9, a moderate inhibitor of CYP2B6 and CYP2C11, but only a weak inhibitor of CYP2C6 and CYP2C19 (Table 2). As discussed further below, the inhibitory profile of MS-PPOH was similar to that of PPOH, except for the CYP2B isoforms (Table 2). In addition, the findings that both drugs are inactive on the nonepoxygenase P450 isoforms (human CYP1A1, 1A2, 1B1, 2A6, 2D6, or 2E1) are consistent with the putative epoxygenase-inhibitory profile of these compounds. These results confirm that an inhibitory action of PPOH or MS-PPOH in a biological system may indeed imply a mechanistic role for epoxygenase activity. However, because there is considerable heterogeneity in potencies of these drugs across the epoxygenases tested (Table 2), the absence of an effect of one of these drugs does not exclude the importance of an epoxygenase-based mechanism. Additional studies are needed to assess the significance in vivo of the presently described potency differences for MS-PPOH, but it can be estimated (Brand-Schieber et al., 2000) that low systemic doses of this drug achieve tissue levels in the low micromolar range commensurate with the potent activities reported (Table 2).

The presently observed potencies of MS-PPOH on rat CYP2C11 and human CYP2C9 (IC_{50} = 11–16 μ M) (Table 2) are comparable to those reported previously for this drug on human CYP2C8 (15 μ M) (Brand-Schieber et al., 2000), rat CYP4A2 (22 μ M), and rat CYP4A3 (6.5 μ M) (Nguyen et al., 1999). This inhibition of specific 2C epoxygenases (Table 2) by MS-PPOH validates some of the prior uses of this drug. For example, inhibition of neural-glial-vascular coupling by in vivo administration of MS-PPOH was speculated to occur via blockade of the brain epoxygenase CYP2C11 (Peng et al., 2002; Iliff et al., 2010). The present findings, which are the first to directly

measure activity of the drug on CYP2C11, show potent inhibition of this enzyme, supporting this hypothesis. By similar reasoning, Table 2 suggests that in vivo administration of MS-PPOH may or may not block CYP2C6 (another brain epoxygenase) (Iliff et al., 2010), because the drug is 6-fold less potent on CYP2C6 versus CYP2C11. Given the very high homology between human CYP2C19, rat CYP2C11, and rat CYP2C6 (demonstrated readily by BLAST searches), the notable heterogeneity in the inhibitory actions of PPOH and MS-PPOH on these 2C epoxygenases (Table 2) requires further study. These findings will also aid in the search for the identity of the brain epoxygenases relevant to the relief of pain. For example, brain-administered MS-PPOH was recently shown to block morphine analgesia (Conroy et al., 2010). It should be noted that the rat brain contains several epoxygenases in addition to those found in Table 2, which have not yet been studied for sensitivity to MS-PPOH. These include members of the 2J, 4X, and 2D families (Iliff et al., 2010).

MS-PPOH was designed to be a systemically acting congener of PPOH. Conversion of the free carboxylic acid function of PPOH to the methylsulfonyl amide derivative (Fig. 1) was expected to confer resistance to β -oxidation (Schaaf and Hess, 1979; Wang et al., 1998), but resulting alterations in pharmacodynamic properties were not anticipated and have never been reported. The present studies show both strong similarities and notable differences between the effects of MS-PPOH and its parent drug. As discussed, the drugs exerted similar, but heterogeneous, potencies across the human and rat 2C isoforms. However, the findings that the carboxylate-modified MS-PPOH is a 5- to 10-fold weaker inhibitor of the rat and human 2B enzymes compared with PPOH shows the latter drug to be generally a more versatile inhibitor of the CYP2B and CYP2C epoxygenases. Although the mechanisms accounting for the low potency of MS-PPOH on the CYP2B epoxygenases are unknown, the present studies suggest caution when interchanging these compounds as epoxygenase inhibitors. In addition, although PPOH is of limited value in vivo, the pharmacological differences identified here between PPOH and MS-PPOH may assist in the in vitro characterization of the enzymatic basis for epoxygenase activity in specific tissues.

The terminal acetylenic drugs PPOH and MS-PPOH were designed to be mechanism-based epoxygenase inactivators (Wang et al., 1998; Brand-Schieber et al., 2000), but such a mode of action has never been

established on any recombinant P450 epoxigenase. The present results, which confirm both time and NADPH dependence of PPOH and MS-PPOH on human CYP2C9 and rat CYP2B1, CYP2C6, and CYP2C11, are consistent with the proposed mechanisms of action of these drugs. The present identification and characterization of specific P450 epoxigenases as targets for PPOH and MS-PPOH will facilitate the appropriate use of these important pharmacological tools.

Acknowledgments

We thank Dr. Jun Yang for excellent assistance and Julia Nalwalk for discussions and proofreading.

Authorship Contributions

Participated in research design: VanAlstine and Hough.

Conducted experiments: VanAlstine.

Performed data analysis: VanAlstine and Hough.

Wrote or contributed to the writing of the manuscript: VanAlstine and Hough.

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