

Effects of Acetylenic Epoxygenase Inhibitors on Recombinant Cytochrome P450s

Melissa A. VanAlstine and Lindsay B. Hough

Chemistry Department, Adelphi University, Garden City, NY, USA (MAV)

Center for Neuropharmacology and Neuroscience, Albany Medical College, Albany, NY, USA
(LBH)

Corresponding Author: Lindsay B. Hough, Ph.D., Center for Neuropharmacology and
Neuroscience, Albany Medical College MC-136, 47 New Scotland Ave., Albany, NY 12208
USA Phone: 518-262-5786 Fax: 518-262-5799 Email: houghl@mail.amc.edu

Running Title: Epoxygenase Inhibitors

Corresponding Author: Lindsay B. Hough, Ph.D., Center for Neuropharmacology and Neuroscience, Albany Medical College MC-136, 47 New Scotland Ave., Albany, NY 12208
USA Phone: 518-262-5786 Fax: 518-262-5799 Email: houghl@mail.amc.edu

Number of text pages: 24

Number of tables: 2

Number of figures: 5

Number of references: 27

Words in Abstract: 246

Words in Introduction: 490

Words in Discussion: 1,155

Abbreviations: CYP: cytochrome P450; DBF: dibenzylfluorescein; EFC: 7-Ethoxy-4-trifluoromethylcoumarin; HFC: 7-hydroxy-4-trifluoromethylcoumarin; MFC: 7-methoxy-4-trifluoromethylcoumarin; MS-PPOH: *N*-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide; NADP⁺: nicotinamide adenine dinucleotide phosphate; NADPH: β -nicotinamide adenine dinucleotide 2'-phosphate reduced; PPOH 2-(2-propynyloxy)-benzenehexanoic acid.

Abstract

Arachidonate epoxidation, which mediates important biological functions in several tissues, is catalyzed by specific cytochrome P450 (CYP) 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 CYP epoxygenase inactivators, but the effects of these drugs on nearly all CYP isoforms are unknown. Presently, the activity of these compounds on 9 human and 3 rat recombinant CYPs were studied. As expected, PPOH inhibited 5 known epoxygenases (CYPs 2B1, 2B6, 2C6, 2C9, and 2C11 [IC_{50} = 23 – 161 μ M]), but had little or no activity on CYPs typically not considered to be epoxygenases (CYPs 1A1, 1A2, 1B1, 2A6, 2D6 and 2E1). PPOH was only a very weak inhibitor (IC_{50} ~ 300 μ M) of CYP2C19, an important human epoxygenase. Unexpectedly, MS-PPOH (a metabolically stable congener of PPOH) potently inhibited only two CYP epoxygenases (2C9 and 2C11, IC_{50} = 11-16 μ M), and showed considerably lower activity (IC_{50} > 90 μ M) on all other CYPs tested, including 3 epoxygenases (CYPs 2B1, 2B6, and 2C19). In addition, PPOH and MS-PPOH displayed time- and NADPH-dependent inhibition of CYP2C9 and other epoxygenases. These results support the putative mechanism of action of PPOH and MS-PPOH on recombinant CYPs, and (with one exception) confirm a general epoxygenase 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 epoxygenase inhibitor. These results will facilitate the judicious use of PPOH and MS-PPOH for epoxygenase research.

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

PPOH and MS-PPOH are fatty acid derivatives (see Fig. 1) which are used as experimental epoxygenase inhibitors (Brand-Schieber, et al., 2000; Wang, et al., 1998). Both compounds selectively inhibit arachidonate epoxygenase (vs. arachidonate hydroxylase) activity in renal cortical microsomes (Brand-Schieber, et al., 2000; Wang, et al., 1998). Studies of the CYP isoforms thought to be important for kidney epoxygenase activity found that PPOH inhibits recombinant rat CYP4A2 and CYP4A3 (Nguyen, et al., 1999). A related study showed that MS-PPOH blocks human CYP2C8 epoxygenase activity (Brand-Schieber, et al., 2000). However, PPOH and MS-PPOH have been used in vitro as selective CYP epoxygenase inhibitors not only in the kidney (Brand-Schieber, et al., 2000; Wang, et al., 1998; Imig, et al., 1999), but also in astrocytes (Rzagalinski, et al., 1999) and in pulmonary arteries (Zhu, et al., 2000). MS-PPOH, a metabolically stable congener of PPOH (Wang, et al., 1998; Schaaf and Hess, 1979), has been used both in vivo and in vitro to block renal (Brand-Schieber, et al., 2000) and brain (Peng, et al., 2002; Bhardwaj, et al., 2000; Conroy, et al., 2010) epoxygenases. Thus, PPOH and MS-PPOH have been used to target CYP epoxygenases throughout the body, but the CYP isoforms

responsible for the extra-renal actions of these drugs are unknown. Since arachidonic acid epoxidation can be catalyzed by numerous CYPs (primarily of the CYP1 and CYP2 gene families [Imaoka, et al., 2005; Fer, et al., 2008; Choudhary, et al., 2004; 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 (Zou, et al., 1994; Ortiz de Montellano and Reich, 1984). 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-based inactivators, but this is not well-established. Although PPOH showed time- and NADPH-dependent inhibition of renal microsomal epoxygenase 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 has been documented by these drugs on any recombinant CYP. Presently, the selectivity and mode of action of PPOH and MS-PPOH have been investigated on several human and rat recombinant CYPs.

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 CYP

microsomes from baculovirus-infected insect cells (Supersomes) were purchased from BD Bioscience (Woburn, MA). Acetonitrile (HPLC grade) and magnesium chloride hexahydrate were purchased from Fisher Scientific (Pittsburg, PA). Fluorescein, potassium phosphate monobasic, potassium phosphate dibasic, glucose-6 phosphate, nicotinamide adenine dinucleotide phosphate (NADP⁺), and glucose-6 phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). PPOH and MS-PPOH were purchased from Cayman Chemicals (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 CYP enzyme assays. Human CYP 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 for 37°. Except as noted above, all other reagents and conditions were performed as described by BD Bioscience high-throughput P450 screening kits and by Crespi et al. (1997). IC₅₀ values were determined and reported for 3 human CYPs.

Recombinant rat CYP enzyme assays (CYP2B1, 2C6, and 2C11). Fluorometric high-throughput screens have been developed for human CYPs, but applications of these methods for rat CYPs have been limited. Assays for rat CYPs were optimized with respect to potential substrate, β -nicotinamide adenine dinucleotide 2'-phosphate reduced (NADPH)-regenerating system, incubation time, buffer conditions, and enzyme concentration. Unless otherwise stated,

the incubations were carried out in a total volume of 200 μL 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 μM 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 buffer, followed by pre-incubation at 37° C for 15 min. Unless specified otherwise, final EFC concentrations were 37.5 μM (CYP2B1) and 5 μM (CYP2C6). The reactions were initiated by the addition of 0.1 mL 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 μL of ice-cold 80% acetonitrile/20% 0.5 M Tris base (“STOP” solution). Sample blanks were prepared by preincubation of NADPH-regenerating system (37° C for 15 minutes), 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 (Perkin Elmer) with 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 μM 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 μL of 2N

NaOH followed by a 2 h post incubation at 37°C. Product formation was determined from a fluorescein standard curve, prepared by spiking fluorescein into the blank incubation mixture. 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 CYPs. The incubation system contained 50 mM potassium phosphate buffer (pH 7.4), appropriate amount of NADPH-regenerating system (from above section), recombinant CYP, substrate, and inhibitor in a final volume of 200 μL. 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 CYP2B1 (37.5 μM EFC), 0.5 pmol CYP2C6 (5 μM EFC), or 1 pmol CYP2C11 (0.5 μM DBF). The control incubations contained NADPH-regenerating system, substrate and enzyme without inhibitors in buffer.

Calculations. Rat CYP 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 percent of control responses, such that tops of all curves were constrained to 100%. In cases where 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 CYP activities. Preliminary studies using the substrates at the specified concentrations found that product formation from the rat enzymes was linear with time

for at least 30 min (e.g. see Fig. 2). Following 30 min incubations, product formation was linear with amounts of enzyme added (0.5-1.5 pmol, 0.25-1.5 pmol, and 0.25-4.0 pmol of enzyme for CYP2B1, CYP2C6 and CYP2C11, respectively, not shown). Varying the ionic strength of the buffer had no effect on CYP2C6 and CYP2C11 from 25 mM to 100 mM potassium phosphate, pH 7.4. However, activity for CYP2B1 was improved at lower ionic strengths (25 or 50 mM vs. 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 9 human and 3 rat CYPs (Table 2). PPOH and MS-PPOH showed little to no inhibitory activity on human CYPs 1A1, 1A2, 1B1, 2A6, 2D6, or 2E1 (<20 % inhibition at 80 μM). Inhibitory concentration-response curves for PPOH and MS-PPOH were constructed on the remaining CYP isoforms (Fig. 3). On the human enzymes, PPOH was a potent inhibitor of 2C9 and 2B6 (IC₅₀ values = 23 and 63 μM, respectively, Fig. 3B), but showed considerably weaker activity toward 2C19 (IC₅₀ ~ 300 μM, Fig. 3B). On the rat CYP isoforms, PPOH had potent inhibitory activity on 2B1 and 2C11 (IC₅₀ values = 23 and 73 μM, respectively), with weaker activity (IC₅₀ = 161 μM) on 2C6 (Fig. 3D).

Surprisingly, the spectrum of action of MS-PPOH did not always mirror that of PPOH on rat or human CYP isoforms. Similar to the effects of PPOH, MS-PPOH was a potent inhibitor of human 2C9 and rat 2C11 (IC₅₀ values = 11 and 16 μM, respectively, Figs 3A, 3C), 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 NADPH-regenerating system (Fig 4A). When this preincubation was performed without 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- 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 (Figs. 5A, 5C).

Discussion

Extrahepatic CYPs 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 CYP activity is important for neuro-glial-vascular coupling (Iliff, et al., 2010), and in mediating opioid analgesic drug action (Conroy, et al., 2010). Such studies emphasize the need for rapid, efficient and cost- effective assays for CYPs from laboratory animals. The microplate-based, fluorescence assays presently optimized for rat CYPs have many advantages over traditional enzymatic assays. Currently, these methods permitted potency measurements of PPOH and

MS-PPOH on these enzymes (Table 2). Since 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 potently inhibited arachidonic acid epoxygenase activity in rat renal cortical microsomes (IC_{50} values were 9 and 13 μ M, respectively). A 10-20 fold selectivity for inhibition of arachidonate epoxygenase vs. 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 ($IC_{50} = 22$ 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 potently inhibit human CYP2C8 epoxygenase activity (Brand-Schieber, et al., 2000). Thus, despite the use of these drugs as general epoxygenase inhibitors in the literature (Bhardwaj, et al., 2000; Imig, et al., 1999; Rzigalinski, et al., 1999; Brand-Schieber, et al., 2000), only 3 recombinant epoxygenases were previously known to be blocked by PPOH and MS-PPOH (Table 2). As discussed below, the present results have 1) identified several new CYP epoxygenase isoforms as targets for these drugs, 2) found large potency differences among CYP 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 CYP epoxygenase isoforms. On the contrary, the present results show that, of the 6 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 the 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 non-epoxygenase CYP 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, since 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} s = 11 – 16 μ M, Table 2) are comparable to those previously reported 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 glial-neural-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), as the drug is six-fold less potent on CYP2C6 vs. 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 PPOH's free carboxylic acid function to the methylsulfonyl amide derivative (Fig. 1) was expected to confer resistance to β -oxidation (Wang, et al., 1998; Schaaf and Hess, 1979), 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 5-10 fold weaker inhibitor of the rat and human 2B enzymes when compared with PPOH shows the latter drug to be generally a more versatile inhibitor of the CYP2B and 2C 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 presently 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 (Brand-Schieber, et al., 2000; Wang, et al., 1998), but such a mode of action has never been established on any recombinant CYP epoxygenase. 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 CYP epoxygenases as targets for PPOH and MS-PPOH will facilitate the appropriate use of these important pharmacological tools.

Acknowledgements

We thank Dr. Jun Yang for excellent assistance and Julia Nalwalk for discussions and proof-reading.

Authorship Contributions

Participated in research design: Hough and VanAlstine.

Conducted experiments: VanAlstine.

Performed data analysis: VanAlstine and Hough.

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

References

Bhardwaj A, Northington FJ, Carhuapoma JR, Falck JR, Harder DR, Traystman RJ and Koehler RC (2000) P-450 epoxygenase and NO synthase inhibitors reduce cerebral blood flow response to N-methyl-D-aspartate. *Am J Physiol Heart Circ Physiol* **279**:H1616-H1624.

Brand-Schieber E, Falck JF and Schwartzman M (2000) Selective inhibition of arachidonic acid epoxidation in vivo. *J Physiol Pharmacol* **51**:655-672.

Capdevila JH and Falck JR (2002) Biochemical and molecular properties of the cytochrome P450 arachidonic acid monooxygenases. *Prostaglandins Other Lipid Mediat* **68-69**:325-344.

Capdevila JH, Falck JR and Harris RC (2000) Cytochrome P450 and arachidonic acid bioactivation. Molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res* **41**:163-181.

Choudhary D, Jansson I, Stoilov I, Sarfarazi M and Schenkman JB (2004) Metabolism of retinoids and arachidonic acid by human and mouse cytochrome P450 1b1. *Drug Metab Dispos* **32**:840-847.

Conroy JL, Fang C, Gu J, Zeitlin SO, Yang W, VanAlstine MA, Nalwalk JW, Albrecht PJ, Mazurkiewicz JE, Snyder-Keller A, Shan Z, Zhang S, Wentland MP, Behr M, Knapp BI, Bidlack JM, Zuiderveld OP, Leurs R, Ding X and Hough LB (2010) Opioids activate brain analgesic circuits through cytochrome P450/epoxygenase signaling. *Nature Neuroscience* **13**:284-286.

Correia, MM and Ortiz de Montellano PR (2005) Inhibition of Cytochrome P450 Enzymes, In: *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ed. P.R. Ortiz de Montellano) Kluwer Academic/Plenum Publishers, New York.

Crespi CL, Miller VP and Penman BW (1997) Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Anal Biochem* **248**:188-190.

Fer M, Dreano Y, Lucas D, Corcos L, Salaun JP, Berthou F and Amet Y (2008) Metabolism of eicosapentaenoic and docosahexaenoic acids by recombinant human cytochromes P450. *Arch Biochem Biophys* **471**:116-125.

Illiff JJ, Jia J, Nelson J, Goyagi T, Klaus J and Alkayed NJ (2010) Epoxyeicosanoid signaling in CNS function and disease. *Prostaglandins Other Lipid Mediat.* **91**: 68-84.

Imaoka S, Hashizume T and Funae Y (2005) Localization of rat cytochrome P450 in various tissues and comparison of arachidonic acid metabolism by rat P450 with that by human P450 orthologs. *Drug Metab Pharmacokinet* **20**:478-484.

Imig JD, Falck JR and Inscho EW (1999) Contribution of cytochrome P450 epoxygenase and hydroxylase pathways to afferent arteriolar autoregulatory responsiveness. *Br J Pharmacol* **127**:1399-1405.

Lucas D, Goulitquer S, Marienhagen J, Fer M, Dreano Y, Schwaneberg U, Amet Y and Corcos L (2010) Stereoselective epoxidation of the last double bond of polyunsaturated fatty acids by human cytochromes P450. *J Lipid Res* **51**:1125-1133.

McGiff JC and Quilley J (2001) 20-hydroxyeicosatetraenoic acid and epoxyeicosatrienoic acids and blood pressure. *Curr Opin Nephrol Hypertens* **10**:231-237.

Nguyen X, Wang MH, Reddy KM, Falck JR and Schwartzman ML (1999) Kinetic profile of the rat CYP4A isoforms: arachidonic acid metabolism and isoform-specific inhibitors. *Am J Physiol* **276**:R1691-R1700.

Ortiz de Montellano PR and Reich NO (1984) Specific inactivation of hepatic fatty acid hydroxylases by acetylenic fatty acids. *J Biol Chem* **259**:4136-4141.

Peng X, Carhuapoma JR, Bhardwaj A, Alkayed NJ, Falck JR, Harder DR, Traystman RJ and Koehler RC (2002) Suppression of cortical functional hyperemia to vibrissal stimulation in the rat by epoxygenase inhibitors. *Am J Physiol Heart Circ Physiol* **283**:H2029-H2037.

Rifkind AB, Lee C, Chang TK and Waxman DJ (1995) Arachidonic acid metabolism by human cytochrome P450s 2C8, 2C9, 2E1, and 1A2: regioselective oxygenation and evidence for a role for CYP2C enzymes in arachidonic acid epoxygenation in human liver microsomes. *Arch Biochem Biophys* **320**:380-389.

Roman RJ (2002) P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* **82**:131-185.

Rzagalinski BA, Willoughby KA, Hoffman SW, Falck JR and Ellis EF (1999) Calcium influx factor, further evidence it is 5, 6-epoxyeicosatrienoic acid. *J Biol Chem* **274**:175-182.

Schaaf TK and Hess HJ (1979) Synthesis and biological activity of carboxyl-terminus modified prostaglandin analogues. *J Med Chem* **22**:1340-1346.

Schwarz D and Roots I (2003) In vitro assessment of inhibition by natural polyphenols of metabolic activation of procarcinogens by human CYP1A1. *Biochem Biophys Res Commun* **303**:902-907.

Spector AA (2009) Arachidonic acid cytochrome P450 epoxygenase pathway. *J Lipid Res* **50 Suppl**:S52-S56.

Spector AA and Norris AW (2007) Action of epoxyeicosatrienoic acids on cellular function. *Am J Physiol Cell Physiol* **292**:C996-1012.

Wang MH, Brand-Schieber E, Zand BA, Nguyen X, Falck JR, Balu N and Schwartzman ML (1998) Cytochrome P450-derived arachidonic acid metabolism in the rat kidney: characterization of selective inhibitors. *J Pharmacol Exp Ther* **284**:966-973.

Zhu D, Bousamra M, Zeldin DC, Falck JR, Townsley M, Harder DR, Roman RJ and Jacobs ER (2000) Epoxyeicosatrienoic acids constrict isolated pressurized rabbit pulmonary arteries. *Am J Physiol Lung Cell Mol Physiol* **278**:L335-L343.

Zou AP, Ma YH, Sui ZH, Ortiz de Montellano PR, Clark JE, Masters BS and Roman RJ (1994) Effects of 17-octadecynoic acid, a suicide-substrate inhibitor of cytochrome P450 fatty acid omega-hydroxylase, on renal function in rats. *J Pharmacol Exp Ther* **268**:474-481.

Footnote to title page

This work was supported by grants from the National Institute on Drug Abuse [DA-03816, DA-07307, DA-027835].

Legends for Figures:

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

Fig. 2. Linearity of rat CYP2B1 and CYP2C6 enzyme activities with time. Enzymes (1 pmol and 0.5 pmol, respectively) were incubated with EFC (37.5 μ M and 5 μ M, respectively) as described. Product formation (ordinate, in relative fluorescence units, mean \pm SEM for 4-8 replicates from a typical experiment) are plotted vs. incubation time (abscissa, min).

Fig. 3. Inhibition of human (A,B) and rat (C,D) CYPs by MS-PPOH (A,C) and PPOH (B,D). Recombinant enzymes were incubated with substrate and regenerating system in the presence of varying concentrations of inhibitors at 37°C. Percent control activity (ordinate) is shown at the log of each concentration of inhibitor (abscissa). Each data point represents the mean (\pm S.E.M.) of a total of 4 determinations. IC₅₀ values, estimated by non-linear regression, are given in parentheses.

Fig. 4. Time- and NADPH-dependent inhibition of CYP2C9 (A-C) and CYP2B1 (D). Enzymes were pre-incubated at 37° C with (+N) or without (-N) NADPH regenerating system, along with the designated concentrations of inhibitor (MS-PPOH [A], PPOH [B,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 percent 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 4 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 regenerating system (-N groups, solid symbols). 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, B) and CYP2C11 (C, D) by MS-PPOH (A,C) and PPOH (B, D). Enzymes were pre-incubated at 37° C for the times shown (abscissa) with (+N, open symbols) or without (-N, solid symbols) NADPH regenerating system, along with the designated concentrations of inhibitors, followed by incubation with substrate and regenerating system as in Fig. 4. Ordinate shows the natural log (ln) of the percent remaining activity (mean \pm S.E.M. of at least 4 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 open respective open symbols).

TABLE 1

K_m and V_{max} values for rat CYPs 2B1, 2C6 and 2C11

Enzyme	Substrate	K_m (μM)	V_{max} (min⁻¹)
2B1	EFC	66.7 ± 7.6	7.1 ± 0.3
2C6	EFC	6.0 ± 0.9	20.2 ± 0.7
2C11	DBF	1.3 ± 0.1	0.52 ± 0.02

Recombinant enzymes (1 pmol CYP2B1 and CYP2C11 or 0.5 pmol CYP2C6) were incubated with varying 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 minutes. Product formation in pmol product/pmol enzyme/min 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 ± S.E.M. for a total of 4 measurements.

TABLE 2

Activity of epoxygenase inhibitors on select human (h) and rat (r) P450 isoforms

Isoform	IC ₅₀ (μM) or % inhibition ^a		Epoxygenase?
	MS-PPOH	PPOH	
1A1h	NA	NA	some ^b
1A2h	NA	NA	minor ^c
1B1h	NA	NA	no
2A6h	NA	NA	no
2B1r	23% @ 80 μM	23 (13-40)	yes ^{d,e,f}
2B6h	~300 (166-526)	63 (39-103)	yes ^{d,e}
2C6r	99 (70-140)	161 (137-189)	yes ^g
2C8h	15^h	NT	yes ^{e,f}
2C9h	11 (6-21)	23 (19-28)	yes ^{d,e,f}
2C11r	16 (11-25)	73 (61-87)	yes ^{e,f}
2C19h	23% @ 80 μM	~300 (213-444)	yes ^{d,e}
2D6h	NA	NA	no
2E1h	NA	NA	minor ^c
4A2r	NT	22ⁱ	yes ⁱ
4A3r	NT	6.5ⁱ	yes ⁱ

IC₅₀ values were taken from Fig. 3 or literature cited. NA, not active (< 20% inhibition at 80 μM, duplicate measurements); NT, not tested. Potencies are classified according to the IC₅₀ values as either high (bold shaded, < 25 μM), intermediate (bold italics, 26 – 99 μM), or low (regular typeface, > 100 μM). Literature in support of the epoxygenase activity of each CYP isoform is shown at right.

^aValues are fitted IC₅₀ values with 95% confidence intervals in parenthesis or percent inhibition at specified concentration of inhibitor.

^b See Choudhary, et al., 2004.

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

^d See Imaoka, et al., 2005.

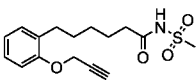
^e See Roman, 2002.

^f See Capdevila, et al., 2000.

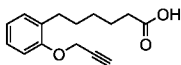
^g See Iliff, et al., 2010.

^h See Brand-Schieber, et al., 2000.

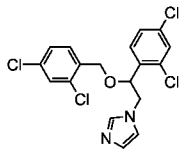
ⁱ See Nguyen, et al., 1999.



MSPPOH



PPOH



Miconazole

Figure 1

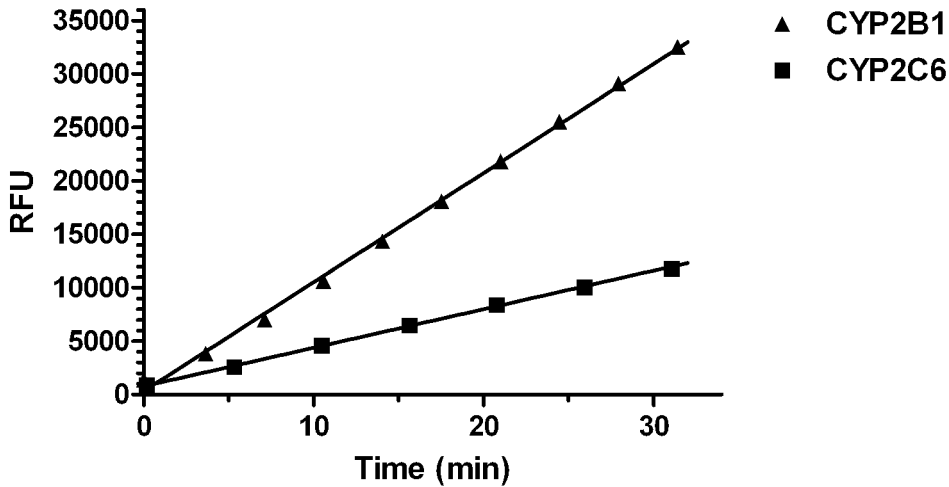


Figure 2

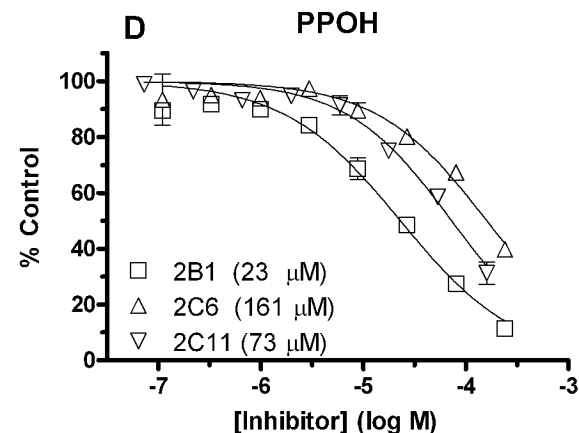
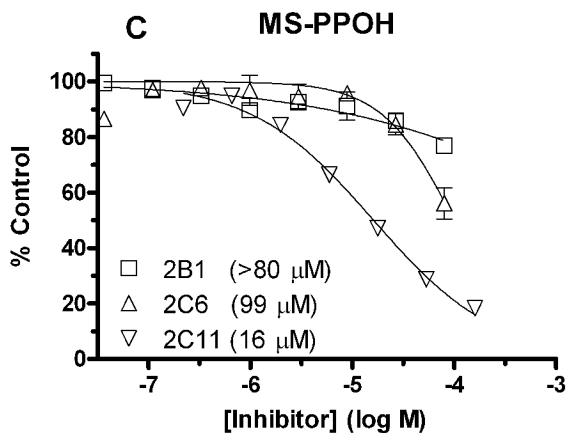
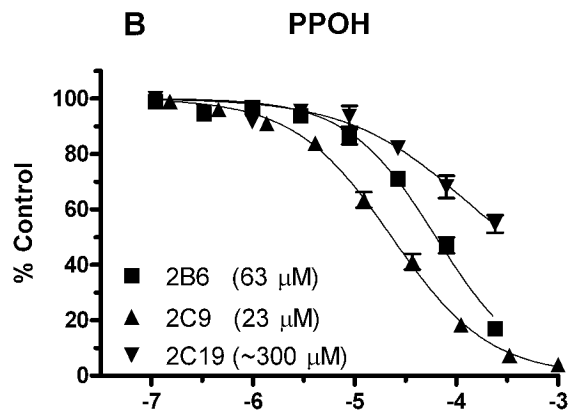
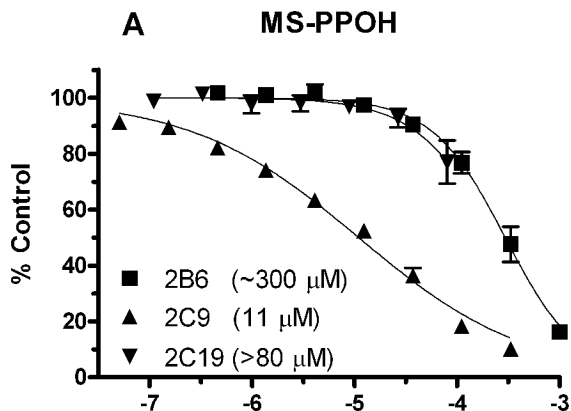


Figure 3

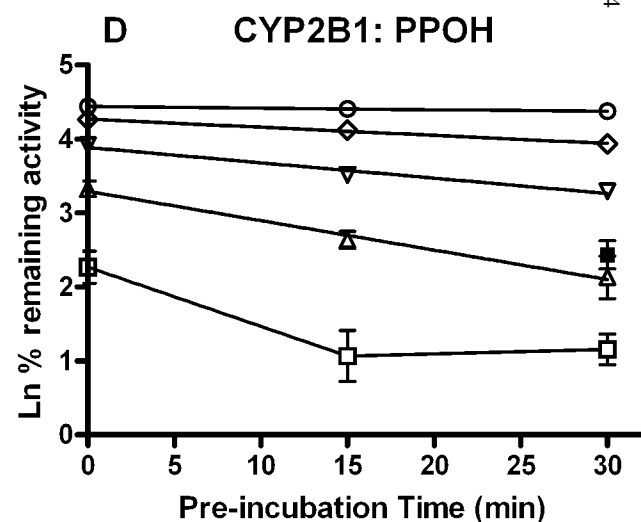
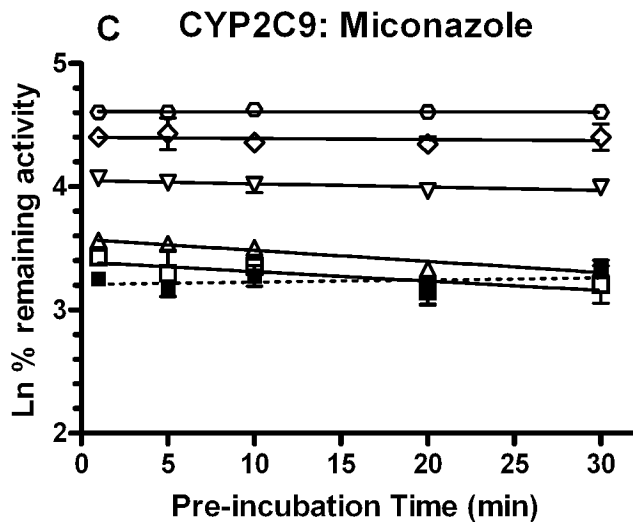
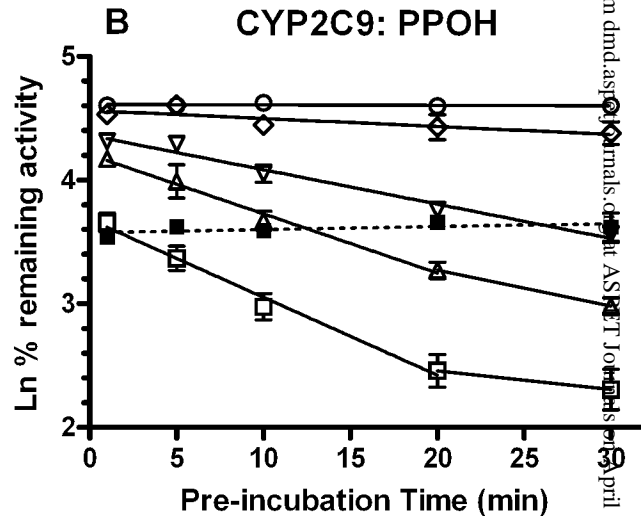
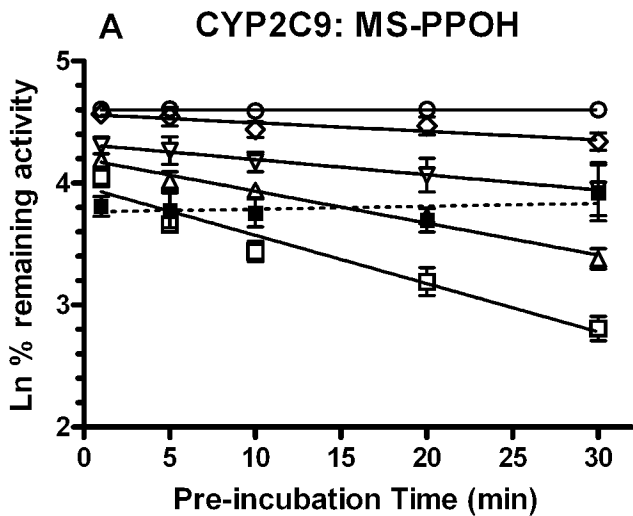


Figure 4

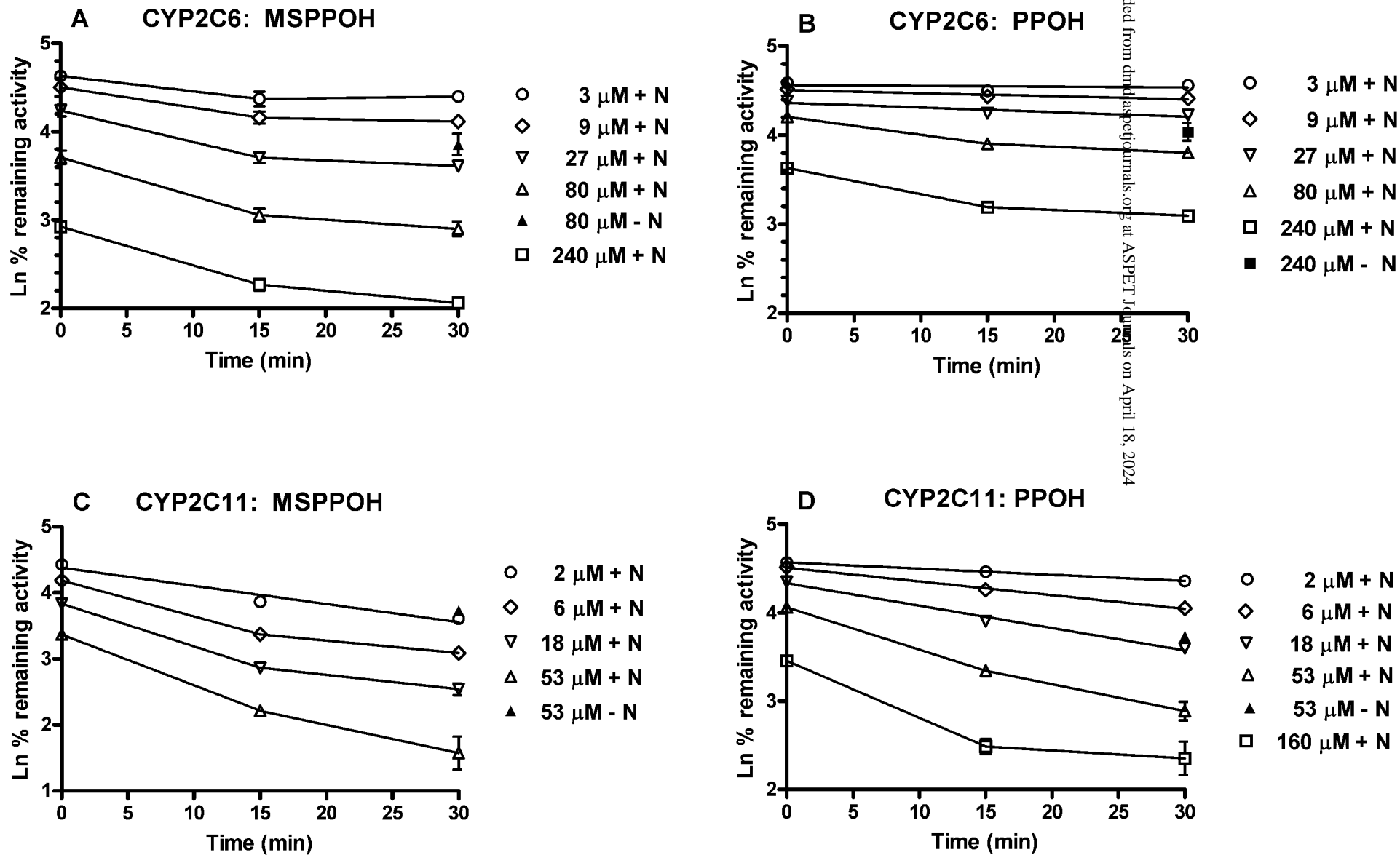


Figure 5