ABSTRACT

We characterized the inhibitory activity of several acetylenic and olefinic compounds on cytochrome P450 (CYP)-derived arachidonic acid \( \omega \)-hydroxylation and epoxidation using rat renal cortical microsomes and recombinant CYP proteins. Among the acetylenic compounds, 6-(2-propargyloxyphenyl)hexanoic acid (PPOH) and N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide were found to be potent and selective inhibitors of microsomal \( \omega \)-hydroxylation and epoxidation of arachidonic acid with IC\(_{50}\) values of 2 \( \mu \)M, whereas the IC\(_{50}\) values for epoxidation were 60 and 51 \( \mu \)M for DDMS and DBDD, respectively. Studies using recombinant rat CYP4A isoforms showed that PPOH caused a concentration-dependent inhibition of \( \omega \)-hydroxylation and 11,12-epoxidation by CYP4A1 and 4A2 but had no effect on CYP4A1-catalyzed \( \omega \)-hydroxylation activity. On the other hand, DDMS inhibited both CYP4A1- and CYP4A3- or CYP4A2-catalyzed arachidonic acid oxidations. Inhibition of microsomal activity by PPOH, but not DDMS, was time- and NADPH-dependent, a result characteristic of a mechanism-based irreversible inhibitor. These studies provide information useful for evaluating the role of the CYP-derived arachidonic acid metabolites in the regulation of renal function and blood pressure.

The CYP monooxygenases constitute a major metabolic pathway for arachidonic acid in the rat kidney. The CYP proteins are primarily localized to the cortex with high concentrations in the proximal tubules, vasculature and thick ascending limb of Henle's loop (TALH), where they metabolize arachidonic acid mainly to 20-HETE as well as to 19-HETE and EETs (Hardwick, 1991; Omata et al., 1992; Dees et al., 1982; Ma et al., 1993). Hydroxylation of arachidonic acid at the \( \omega \)-carbon to form 20-HETE is primarily catalyzed by isoforms of the CYP4A family (Kimura et al., 1989a; Kimura et al., 1989b; Stromstedt et al., 1990; Laniado Schwartzman et al., 1996). On the other hand, arachidonic acid epoxidation is much less specific and can be carried out by numerous CYP isoforms from different CYP gene families, including 1A1, 1A2, 2B1, 2B4, 2C2, 2C9, 2C11, 2C23, 2E1 and 2G1 (Laethem et al., 1994; Laethem and Koop, 1993; Laethem et al., 1993; Rifkind et al., 1995). In addition to having a broad spectrum of substrate specificity, CYP isoforms also demonstrate a lack of product specificity; i.e., one protein can catalyze the oxidation of arachidonic acid at multiple sites. For example, we have shown that CYP4A4 functions not only as an arachidonate \( \omega \)-hydroxylase but also as an arachidonate 11,12-epoxygenase (Wang et al., 1996).

The potential role of the CYP-derived arachidonic acid metabolites in the regulation of renal function and in hypertension has been documented in numerous studies. 20-HETE, in vitro, is a potent vasconstrictor of renal arterioles (Ma et al., 1993; Imig et al., 1996; Zou et al., 1996c) and an inhibitor of renal tubular transport and K+ channel activity (Escalante et al., 1994; Wang and Lu, 1995). In vivo, 20-HETE affects renal vascular resistance, autoregulation of renal blood flow and tubuloglomerular feedback (Gebremedhin et al., 1993; Kauser et al., 1991; Zou et al., 1994a; Zou et al., 1996a; Zou et al., 1994b). In the kidney, EETs exhibit a broad and contrasting spectrum of biological activities, in-
cluding vasodilation and vasoconstriction (Carroll et al., 1992; Zou et al., 1996b; Katoh et al., 1991) as well as inhibition and stimulation of ion transport mechanisms (Harris et al., 1990; Takahashi et al., 1990; Sakairi et al., 1995; Hirt et al., 1989). Studies using different animal models of hypertension and CYP inhibitors have implicated both epoxidation and hydroxylation products of arachidonic acid in the renal responses to changes in dietary salt levels and in hypertension (Sacerdoti et al., 1989; Makita et al., 1994; Imig et al., 1993; Zou et al., 1994c; Capdevila et al., 1992; Brand-Schieber et al., 1996).

In order to elucidate the physiological and pathophysiological roles of these arachidonic acid metabolites, it is important to inhibit one CYP-catalyzed reaction selectively without affecting the others. The fact that the CYP monoxygenases exist in many isoforms with relatively high homology and broad substrate specificity complicates this task; enzyme inhibitors are frequently nonspecific, and multiple isoforms often demonstrate similar catalytic activities (Gonzalez, 1988). A relatively new approach to inhibiting CYP activities selectively involves the use of “suicide substrates” that were designed to resemble the substrate and at the same time to inactivate the enzyme. Inactivation is irreversible, and activity is restored on de novo synthesis of the enzyme. Acetylenic derivatives, such as 17-ODYA, have been shown to inactivate the enzyme. Inactivation is irreversible. It is important to inhibit one CYP-catalyzed reaction selectively without affecting the others. The fact that the CYP monoxygenases exist in many isoforms with relatively high homology and broad substrate specificity complicates this task; enzyme inhibitors are frequently nonspecific, and multiple isoforms often demonstrate similar catalytic activities.

In the present study, we characterize the effect of several acetylenic and olefinic fatty acid analogs on ω-hydroxylation and epoxidation of arachidonic acid in rat renal microsomes and on recombinant rat CYP4A isoforms. This study provides information useful for evaluating the role of the CYP-arachidonic acid metabolites in the regulation of renal function and blood pressure.

**Materials and Methods**

**Materials.** The following drugs and chemicals were used in this study: (1-{14C})-arachidonic acid (56 mCi/mmol) (DuPont-New England Nuclear, Boston, MA), purified recombinant rat NADPH-CYP (c) oxidoreductase (OR) (specific activity, 58 µmol/min/mg) (Oxford Biomedical Research Inc., Oxford, MI), 17-ODYA (Cayman Chemical Co., Ann Arbor, MI), emulgen E811 (KAO Atlas, Tokyo, Japan), tissue culture and molecular biology reagents (Gibco-BRL, Gaithersburg, MD), S9 insect cells and the liposome DNA transfection kit (Invitrogen, San Diego, CA) and purified rat liver cytochrome b (5) (Panvera Corp., Madison, WI). All solvents were HPLC grade.

**Synthesis of CYP inhibitors.** The structures of all compounds are shown in figure 1. The compounds PPOH, MS-PPOH, DDMS, DBDD and DPMS were synthesized and their structures confirmed by spectral analysis as described by Falck et al., in press.

**Tissue and microscopic preparations.** Male Sprague-Dawley rats (8 weeks old, Charles River, Wilmington, PA) were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Kidneys were removed and the cortex was dissected and homogenized in buffer containing 100 mM Tris-Cl and 1.15% KCl, pH 7.4. Homogenates were centrifuged at 10,000 × g for 30 min. Microsomes were obtained by centrifugation of the supernatant at 100,000 × g for 90 min and resuspended in 0.25 M sucrose buffer and stored at −80°C.

**Preparation of recombinant CYP4A cell membranes.** CYP4A proteins were expressed in the baculovirus-S9 insect cell expression system as described previously (Wang et al., 1996a).

Briefly, CYP4A cDNAs were ligated into the Sma I site of the baculovirus expression vector, pVL1393. Transfection of the expression vector was performed individually by an AcNPV linear transfection kit (Invitrogen). AcNPV DNA (1 µg) was mixed with 3 µg of CYP4A-PVL1393 construct in a 5-ml polystyrene tube containing 1.5 ml of serum-free Grace medium. A mixture of 30 µg of cationic liposomes and 1.5 ml of serum-free medium was added to the DNA, mixed and added into cultured S9 cells dropwise. Transfected cells were incubated at 27°C overnight, after which the medium was removed, fresh medium was added, and the cells were incubated for 4 to 6 days. Identification of recombinant viruses was done by visualization of Occ-negative plaques, immunoblot and P450 spectral analysis. Recombinant viruses were amplified in S9 cells. The titer for recombinant viruses was determined by plaque assay. In order to prepare CYP4A recombinant cell membranes, S9 cells were infected with CYP4A recombinant viruses and cultured in the presence of hemin (4 µg/ml). After 72 h, the cells were harvested, washed with 0.14 M NaCl in 50 mM potassium phosphate (pH 7.2) and resuspended in sucrose buffer (50 mM potassium phosphate, pH 7.4, and 0.5 M sucrose). Cells lysates were prepared by brief sonication (4–5 bursts of 4-s duration) and subjected to high-speed centrifugation (100,000 × g) for 60 min. The membrane pellet was then resuspended in sucrose buffer and stored at −80°C. Protein concentration was measured according to the method of Bradford (BioRad; Melville, NY). CYP content was calculated from the reduced CO-difference spectrum using an extinction coefficient of 91 mM⁻¹ cm⁻¹ (Omura and Sato, 1964).

**Incubation conditions, metabolite extraction and HPLC separation.** Compounds (stock solution in ethanol) were diluted 10 to 20 fold with 100 mM potassium phosphate buffer, pH 7.5. The
final concentration of ethanol in the incubations was 0.5% (v/v). At this concentration of ethanol, there was no effect on the activities of ω-hydroxylation and epoxidation of arachidonic acid. The diluted solution was then added into the incubation mixture consisting of microsomes (150 μg of protein), 1 mM NADPH and buffer (100 mM potassium phosphate and 10 mM MgCl₂, pH 7.5). For the recombinant CYP4A system, recombinant cell membranes containing the expressed CYP4A isoform were mixed on ice with purified OR and b₅, at a molar ratio of 1:14:4, and the diluted inhibitor solution at the indicated concentration, 1 mM NADPH and buffer (150 μl final volume) were added. Mixtures containing either microsomes or recombinant proteins were preincubated at 37°C for 10 min. [1-14C]-arachidonic acid (0.4 μCi, 7 nmol) was then added and incubated at 37°C for 30 min. To determine whether inhibition of arachidonic acid oxidation is reversible or irreversible, a two-stage incubation protocol for time-dependent inhibition was carried out. In the first-stage incubation, microsomes (1.5 mg) were incubated at 37°C with 0.1 mM inhibitors either with or without 1 mM NADPH (300 μl final volume). At given time intervals, an aliquot was diluted 10-fold by the addition of a second-stage assay mixture consisting of the same buffer, 0.9 mM NADPH and [1-14C]-arachidonic acid (0.4 μCi, 7 nmol) in a total volume of 0.4 ml. The reactions were continued for 20 min. The reaction was terminated by acidification to pH 3.5 to 4.0 with 2 M formic acid, and the metabolites were extracted with ethyl acetate. The organic extract was evaporated under nitrogen, resuspended in methanol and injected onto the HPLC column. Reverse-phase HPLC was performed on a 5-μm ODS-Hypersil column, 4.6 × 200 mm (Hewlett-Packard, Palo Alto, CA) using a linear gradient ranging from acetonitrile/water/acetic acid (50:50:0.1) to acetonitrile/acetic acid (100:0) at a flow rate of 1 ml/min for 30 min. The elution profile of the radioactive products was monitored by a flow detector (Inus System Inc., Tampa, FL). The identity of each metabolite was confirmed by its comigration with an authentic standard. Specific activity in nmol/mg/min was calculated from the added arachidonic acid, and results were generally expressed as the mean ± S.E.M. of the percent of the control activity. IC₅₀ was determined by quantal dose-response analysis.

COX assay. The effect of the various inhibitors on COX activity was measured using the purified ram seminal vesicles PGH₁ synthase (Cayman Chemical Co., Ann Arbor, MI). The purified enzyme (10 units) was incubated with 1 nmol of arachidonic acid in 0.5 ml of incubation buffer (0.1 M Tris-HCl, pH 8, 1 mM EDTA, 2 mM phenol and 1 μM hematin) with or without PPOH (10 and 50 μM), MS-PPOH (15 and 50 μM), DBDD (2 and 20 μM), DDMS (2 and 20 μM), 17-ODYA (5 and 50 μM) and indomethacin (5 μM). Reaction mixtures were incubated at 37°C shaking-water bath for 10 min before the addition of arachidonic acid and for 15 min thereafter. All samples were run in duplicate. Amounts of PGE₂ were determined using a PGE2-EIA Kit (Cayman Chemical Co., Ann Arbor, MI) after 1000-fold dilution with Tris buffer.

Results

Because of the presence of epoxide hydrolase in microsomal preparations (Oliw et al., 1982), EETs are readily converted to their corresponding metabolites, dihydroxyicosatrienoic acids (DHETs). We therefore considered epoxigenase activity as the sum of EET and DHET formation. Both PPOH and MS-PPOH were designed to target arachidonic acid epoxidation specifically. Addition of PPOH (1–50 μM) decreased microsomal arachidonate epoxigenase activity in a concentration-dependent manner with an IC₅₀ of 9 μM but had almost no effect on ω-hydroxylation (fig. 2). Similar results were obtained with MS-PPOH; no effect on microsomal arachidonic acid ω-hydroxylation (20-HETE formation) and a concentration-dependent inhibition of EETs and DHETs formation with an IC₅₀ of 13 μM (data not shown). These results indicate that PPOH and MS-PPOH specifically and potently inhibit renal microsomal-derived epoxidation of arachidonic acid without affecting ω-hydroxylation.

In order to examine the structure-function relationship of these acetylenic compounds, we tested another aliphatic acetylenic fatty acid, 17-ODYA. The effect of 17-ODYA on renal microsomal ω-hydroxylation and epoxidation of arachidonic acid is shown in figure 3. 17-ODYA was a very potent inhibitor of both ω-hydroxylation and epoxidation of arachidonic acid with similar IC₅₀ values of 7 and 5 μM, respectively.

In addition to these acetylenic compounds, we also examined some dibromo-olefinic fatty acids (DBDD, DPMS and DDMS). Addition of DDMS to the incubation mixture of renal microsomes and 1 mM NADPH for 10 min before the addition of [1-14C]-arachidonic acid (0.4 μCi). Reactions were carried out at 37°C for 30 min. Arachidonic acid metabolites were extracted and separated by HPLC as described in "Materials and Methods." Results are expressed as percent of control and are the mean ± S.E.M., n = 3. The control activities for arachidonic acid ω-hydroxylation (formation of 20-HETE) and epoxidation (formation of EET + DHET) were 231 ± 96 and 54 ± 9 pmol/mg/min, respectively.
microsomes caused a concentration-dependent inhibition of \(\omega\)-hydroxylase activity with an \(IC_{50}\) of 2 \(\mu M\), but it caused only a weak inhibition of epoxygenase activity with an \(IC_{50}\) of 60 \(\mu M\) (fig. 4). These results indicate that DDMS is a specific and potent inhibitor of microsomal arachidonic acid \(\omega\)-hydroxylation. Both DDMS and DBDD demonstrated a pattern of inhibition similar to that of DMS with different potencies. The \(IC_{50}\) values for all of inhibitors are summarized in Table 1.

The specificity of these inhibitors was further examined by using recombinant CYP4A isoforms. We have recently demonstrated that the baculovirus-Sf9 cell-expressed CYP4A2 membranes possess dual catalytic activity: \(\omega\)-hydroxylation and 11,12-epoxidation (Wang et al., 1996). Preliminary results indicated that CYP4A3 exhibits similar catalytic activities (Nguyen et al., 1995). In contrast, the baculovirus-Sf9 cell-expressed CYP4A1 membranes (Nguyen et al., 1997), as well as other forms of recombinant CYP4A1 (Alterman et al., 1995; Imaoka et al., 1989), exhibit only arachidonic acid \(\omega\)-hydroxylation activity. Given these characteristics, we examined the effect of PPOH as an epoxygenase inhibitor and that of DDMS as an \(\omega\)-hydroxylation inhibitor on CYP4A3- and CYP4A1-catalyzed arachidonic acid oxidations. The results are shown in figures 5 and 6. PPOH at 30 \(\mu M\) inhibited both CYP4A3-mediated \(\omega\)-hydroxylation and 11,12-epoxidation of arachidonic acid by 90%. In contrast, PPOH at the same concentration had little effect on CYP4A1-catalyzed \(\omega\)-hydroxylation of arachidonic acid (8% inhibition). On the other hand, DDMS inhibited both CYP4A1- and CYP4A3-catalyzed arachidonic acid oxidations without differentiating between the two reactions (data not shown).

In order to characterize further the mechanisms of the inhibitory action of these inhibitors, we carried out a two-stage incubation. In the first stage, microsomes were preincubated with either acetylenic (PPOH) or olefinic (DDMS) compounds in the presence and absence of NADPH. Aliquots were then taken at different time-points and added to the incubation reaction, which contained \(^{14}\text{C}\)-arachidonic acid.

Fig. 4. Effect of DDMS on arachidonic acid \(\omega\)-hydroxylase and epoxygenase activities in rat cortical microsomes. DDMS was dissolved in ethanol (0.5% final concentration) and preincubated with 150 \(\mu g\) of microsomes and 1 mM NADPH for 10 min before the addition of [\(^1\text{H}\)CO]-arachidonic acid (0.4 \(\mu g\)). Reactions were carried out at 37°C for 30 min. Arachidonic acid metabolites were extracted and separated by HPLC as described in “Materials and Methods.” Results are expressed as percent of control and are the mean \pm S.E.M., \(n = 3\). The control activities for arachidonic acid \(\omega\)-hydroxylation (formation of 20-HETE) and epoxidation (formation of EET + DHET) were 231 \pm 96 and 54 \pm 9 pmol/ng/min, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>(\omega)-Hydroxylation (IC_{50}) ((\mu M))</th>
<th>Epoxidation (IC_{50}) ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPOH</td>
<td>&gt;200</td>
<td>13</td>
</tr>
<tr>
<td>MS-PPOH</td>
<td>&gt;200</td>
<td>9</td>
</tr>
<tr>
<td>DDMS</td>
<td>31</td>
<td>&gt;100</td>
</tr>
<tr>
<td>DBDD</td>
<td>2</td>
<td>51</td>
</tr>
<tr>
<td>17-ODYA</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Over a 15-min period in which PPOH was preincubated in the absence of NADPH, there was no time-dependent inhibition of DHET and EET formation (fig. 7A). However, when PPOH was preincubated in the presence of NADPH, there was a rapid, time-dependent decrease in the formation of DHETs and EETs. After a 15-min preincubation, the activity was significantly decreased by 60% (fig. 7A). The same approach was used to test the inhibitory mechanism of DDMS. As shown in figure 7B, neither preincubation time nor the presence of NADPH affected the degree of inhibition by DDMS.

We further examined the effect of these compounds on COX activity by measuring PGH\(_1\), synthase-catalyzed conversion of arachidonic acid to PGE\(_2\). The results indicated that whereas this activity was readily inhibited by indomethacin (95% inhibition at 5 \(\mu M\)), it was unaffected by the various compounds tested in this study. DDMS, DBDD, DPMS and 17-ODYA at concentrations up to 20 \(\mu M\) had no effect on COX activity; however, PPOH and MS-PPOH at 50 \(\mu M\) inhibited COX activity by 20 and 40%, respectively. Nevertheless, these concentrations are much above the \(IC_{50}\) for epoxygenase activity, and thus they provide a window of selectivity in differentiating between CYP and COX activities.

### Discussion

It is well recognized that the CYP-derived eicosanoids constitute a new member of the arachidonic acid cascade with important implications in the regulation of physiological and pathophysiological processes. These metabolites are formed endogenously in various tissues and exert potent biological effects on cellular functions. Studies of their role in normal and diseased cells and tissues are impeded by the difficulty in selectively targeting their synthesis or their effects, because these metabolites are generated from multiple closely related proteins of the CYP superfamily. Consequently, the development of enzyme inhibitors that target specific isoforms/reactions should aid in the study of their pathophysiological roles.

We have synthesized a series of fatty acid/arachidonic acid analogs and tested their potency and selectivity in inhibiting arachidonic acid epoxidation and \(\omega\)-hydroxylation reactions in rat renal microsomes. Our study confirms that the widely used, terminal acetylenic 17-carbon aliphatic compound 17-ODYA is a potent but nonselective inhibitor of both arachidonic acid epoxidation and \(\omega\)-hydroxylation in rat renal microsomes. In contrast to 17-ODYA, the other terminal acetylenic compounds, PPOH and MS-PPOH, selectively inhibited microsomal arachidonic acid epoxidation with \(IC_{50}\) values of 9 and 13 \(\mu M\), respectively, while having no effect on \(\omega\)-hydroxylation at concentrations up to 50 \(\mu M\). The major
structural difference among these compounds is the presence of a benzene ring moiety in the PPOH derivatives, which suggests that the benzene ring confers selectivity toward the epoxidation reaction. To examine this possibility, Mancy et al. (1996) have proposed a model for the active site of CYP2C9, an arachidonate epoxygenase (Rifkind et al., 1995), in which hydrophobic and/or π-π interactions may be important for binding between the benzene ring of substrates and aromatic amino acid residues of the protein. Alternatively, the ω-hydroxylases may have sterically hindered binding sites that do not accommodate aryl moieties. In a result similar to that observed with 17-ODYA, PPOH inhibitory activity in renal microsomes increased with time and required NADPH, which suggests the formation of an NADPH-dependent intermediate that accounts for inactivation of the enzyme. Ortiz de Montellano and Reich (1984) and Helvig et al. (1997) showed that terminal acetylenes are CYP suicide-substrate inhibitors; they can be further oxidized to a ketene moiety, which then results in alkylation and inactivation of the CYP proteins. PPOH may fit in as a suicide-substrate inhibitor. Furthermore, the time dependence and NADPH dependence of PPOH inhibitory activity are consistent with a mechanism-based irreversible inhibitor (Ortiz de Montellano and Reich, 1984; Muerhoff et al., 1989).

The acyclic compounds, i.e., DDMS, DBDD and DPMS, were selective inhibitors of ω-hydroxylation. The rank order of inhibitory potency was DBDD = DDMS > DPMS, which suggests that a carboxylic acid or methyl sulfimide with 12 carbons (DBDD and DDMS) may be the best fit for the active site of the ω-hydroxylase CYP4A isoforms. Indeed, we and others have demonstrated that recombinant CYP4A proteins have a much higher lauric acid ω-hydroxylase activity than

Fig. 5. Representative reverse-phase HPLC elution profiles showing the effect of PPOH on CYP4A1-catalyzed arachidonic acid metabolism. Sf9 cell membranes containing 5 pmol of expressed CYP4A1 were preincubated with 20 pmol of purified b5 and 70 pmol of purified OR without (panel A) or with (panel B) 30 μM PPOH. [1-14C]-arachidonic acid (0.4 μCi; 7 nmol) was added, and the reaction mixture was incubated for an additional 30 min. 20-HETE coeluted with authentic 20-HETE standard at 12 min.
an arachidonic acid $\omega$-hydroxylase activity (Wang et al., 1996; Alterman et al., 1995; Imaoka et al., 1989). Modification of the carboxyl group in DBDD to a methyl sulfonate in DDMS did not change the potency or selectivity of the inhibitory activity. This modification may be important for in vivo studies, where blocking the carboxyl group renders the compound resistant to $\beta$-oxidation and makes the compound a more effective inhibitor. Alonso-galicia et al. (1997) administered DDMS locally into an isolated perfused renal arteriolar preparation and systemically into anesthetized rats and demonstrated a high selectivity for DDMS in inhibiting 20-HETE formation. However, unlike 17-ODYA and PPOH, these acyclic dibromide derivatives did not exhibit time- and NADPH-dependent inhibitory activity, a result that emphasizes the importance of a terminal acetylenic bond in providing the mechanisms for the enzyme’s inactivation. These experiments also imply that inhibition by DDMS is reversible. Indeed, when microsomes were incubated with DDMS or DBDD, inhibition of 20-HETE formation could be washed out (data not shown).

The dual catalytic activity of the recombinant CYP4A2/CYP4A3 proteins as $\omega$-hydroxylases and epoxygenases provides a means for examining whether the inhibitors distinguish between these two reactions. The results showed that PPOH caused a significant inhibition of CYP4A3-catalyzed $\omega$-hydroxylation and 11,12-epoxidation of arachidonic acid, but had no effect on CYP4A1-dependent $\omega$-hydroxylase activity; this suggests that PPOH inhibitory activity is isoform specific. In contrast to PPOH, DDMS potently inhibited both CYP4A1- and CYP4A3-catalyzed arachidonate $\omega$-hydroxylation as well as CYP4A3-catalyzed 11,12-epoxidation. Taken together, these results suggest that CYP4A1 and CYP4A2/4A3 may have similar size or topology of the active site, which allows the binding of common substrates such as lauric and arachidonic acids. Conversely, there may be more space or freedom for the orientation of substrates or inhibitors in the active sites of CYP4A2/4A3 compared with those of CYP4A1. Furthermore, the amino acid residues involved in the binding of substrates and inhibitors may be quite different. In other words, it is possible that the active site of CYP4A1 is more rigid than that of CYP4A2/4A3. Indeed, Bambal et al. have proposed that the active site of CYP4A1 is sterically hindered and rigid around the heme iron or ferryl moiety, which may explain the prominent regioselectivity of CYP4A1-catalyzed $\omega$-hydroxylase activity (Bambal and Hanzlík, 1996) and accounts for the differences between CYP4A1 and CYP4A2/4A3 that we observed in catalytic activity and inhibitor sensitivity.
Previous studies by Capdevila et al. (1988) examined the selectivity and potency of various arachidonic acid analogs in inhibiting CYP-dependent arachidonic acid oxygenases. The results demonstrated that these analogs were quite potent at low μM concentrations in inhibiting rat liver microsomal CYP-dependent oxygenase reactions without affecting ram seminal vesicle COX or soybean lipoxygenase activities at concentrations of up to 100 μM. However, these analogs did not significantly distinguish between the different oxygenase reactions; i.e., they inhibited both epoxidation and ω-hydroxylation of arachidonic acid to the same extent. In the same study, the authors documented the potency and selectivity of two imidazole derivatives, clotrimazole and ketoconazole, in preferentially inhibiting arachidonic acid epoxygenases (Capdevila et al., 1988). These imidazole derivatives are being used extensively in studies to evaluate the physiological role of arachidonic acid epoxides, especially as they relate to the control of vascular tone and renal function (Makita et al., 1994; Alkayed et al., 1996; Alonso-galicia et al., 1997; Fulton et al., 1995). However, they do affect many CYP-dependent reactions as well as cellular processes unrelated to CYP (Testa and Jenner, 1981; Alvarez et al., 1992).

Our study describes specific inhibitors that, at least in vitro, can distinguish between CYP-catalyzed arachidonate epoxidation and ω-hydroxylation reactions as well as between CYP4A isoform-catalyzed reactions. Additional studies to examine their potency and specificity in vivo, as well as thorough examination of their selectivity with regard to other CYP-catalyzed reactions, should accompany studies designed to evaluate the role of the CYP-arachidonic acid metabolites in the regulation of renal function and blood pressure.

Acknowledgments

The authors thank Dr. Richard J. Roman (Department of Physiology, Medical College of Wisconsin, Milwaukee, WI) for providing the CYP4A1, 4A2, 4A3 and 4A8 cDNAs.

References


(1991) Glomerular stereospecific synthesis and hemodynamic actions of 8,9-
Kauffman KM, Chinetti GS, Ortiz de Montellano PR, Mu Y-H, Harder DR and
Roman RJ (1991) Inhibitors of cytochrome P450 attenuate the myogenic response
Kimura S, Hanika N, Matsunaga E and Gonzalez FJ (1989a) The rat clorafibrate-
inducible hepatic microsomal CYP2C subfamily. I. Complete intron and exon
sequence of the CYP2C1 and CYP2C2 genes, unique exon organization, and
Kimura S, Hardwick JP, Kozak CA and Gonzalez FJ (1989b) The rat clorafibrate-
inducible CYP4A gene subfamily. II. cDNA sequence of the Cyp4a locus to mouse chromosome 4, and coordinate and tissue-specific regulation
of the CYP4A genes. DNA 8:517–525.
19(S), 19(R), and 18(R)-hydroxyeicosatetraenoic acids by alcohol-inducible cyto-
Laethem RM, Halpert JR and Koop DR (1994) Epoxidation of arachidonic acid as an
active-site probe of cytochrome P-450 2B isoforms. Biochim Biophys Acta 1209:
42–48.
Ma Y-H, Gebremedhin D, Schwartzman ML, Falck JR, Clark JE, Masters BS, Harder DR and Roman RJ (1995) 20-Hydroxyeicosatetraenoic acid is an endoge-
Experimental and/or genetically controlled alterations of the renal microsomal
clorochrome P450 epoxygenase induced hypertension in rats fed a high salt diet.
Mancy A, Dijols S, Poli S, Guengerich FP and Mansuy D (1996) Interaction of
sulfanilamidine derivatives with human liver clorochrome P450 2C. Molecular
origin of the specific inhibitory effects of sulfanilamide on CYP 2C9 and conse-
16212.
Mukherjee RS, Williams DE, Reich NO, CaJacob CA, Ortiz de Montellano PR and
Masters BSS (1989) Prostaglandin and fatty acid ω- and ω-1-oxidation in rabbit
lung. Acetylenic fatty acid mechanism-based inactivators as specific inhibitors.
cytochrome P450 4A3 expressed in baculovirus-insect cell system. FASEB J 11:
A805.
Olivo PH, Guengerich FP and Oates JA (1982) Oxidation of arachidonic acid by
hepatic monooxygenase: Isolation and metabolism of four exoform intermediates.
Omura K, Abraham NG and Laniado-Schwartzman M (1992) Arachidonic acid ω-ω-
hydroxylase along the nephron of the spontaneously hypertensive rat. Am J Physiol
262:F592–599.
Omura T and Sato R (1964) The carbon monoxide binding pigment of liver micro-
Ortiz de Montellano PR and Reich NO (1984) Specific inactivation of hepatic fatty
by human cytochrome P450s 2C9, 2C7 and 1A2: Regioselective oxygenation and
evidence for a role for CYP2C enzymes in arachidonic acid epoxygenation in
Sacerdotti D, Escalante B, Abraham NG, McGiff JC, Levere RD and Schwartzman
ML (1989) Treatment with tin prevents the development of hypertension in sponta-
Sakairi Y, Jacobson HR, Noland TD, Capdevila JH, Falck JR and Breyer MD (1995)
5,6-EET inhibits ion transport in collecting ducts by stimulating endogenous
Sakairi Y, Hatashi S, Zaphiropoulos PG and Gustafsson JA (1990) Cloning and
characterization of a novel member of the cytochrome P450 subfamily IVA in
Cytochrome P450 arachidonate metabolites in rat kidney: Characterization and
Testa B and Jenner P (1981) Inhibitors of cytochrome P450s and their mechanism of
Schwartzman M (1996) Cloning, sequencing and cDNA-directed expression of the
rat renal CYP4A2: Arachidonic acid ω-hydroxylation and 11,12-epoxidation by
Wang W and Lu M (1995) Effect of arachidonic acid on activity of the apical K-
channel in the thick ascending limb of the rat kidney. J Gen Physiol 106:727–745.
Zou A-P, Imig JD, Kaldunski M, Ortiz de Montellano PR, Sui Z and Roman RJ
(1994a) Inhibition of renal vascular 20-HETE production impairs autoregulation
Zou A-P, Drummond HA and Roman RJ (1996a) Role of 20-HETE in elevating loop
Zou A-P, Fleming JT, Falck JR, Jacobs ER, Gebremedhin D, Harder DR and Roman
RJ (1996b) Stereospecific effects of epoxyeicosatrienoic acids on renal vascular
Zou A-P, Fleming JT, Falck JR, Jacobs ER, Gebremedhin D, Harder DR and Roman
RJ (1996c) 20-HETE is an endogenous inhibitor of the large-conductance Ca 2
Zou A-P, Imig JD, Ortiz de Montellano PR, Sui Z, Falck JR and Roman RJ (1994)
Effect of P450 ω-hydroxylase metabolites of arachidonic acid on tubuloglomerular
feedback. Am J Physiol (Renal Fluid Electrolyte Physiol) 266:F924–F941.
Zou A-P, Ma Y-H, Sui Z-H, Ortiz de Montellano PR, Clark JE, Masters BS and
Roman RJ (1994c) Effect of 17-octadecynoic acid, a suicide-substrate inhibitor of
cytochrome P450 fatty acid ω-hydroxylase, on renal function. J Pharmacol Exp
Ther 266:414–481.
Send reprint requests to: Dr. Michal Laniado Schwartzman, Department of
Pharmacology, New York Medical College, Valhalla, NY 10595.