INDUCTION OF CYP1A AND CYP2-MEDIATED ARACHIDONIC ACID EPOXYGENATION AND SUPPRESSION OF 20-HYDROXYEICOSATETRAENOIC ACID BY IMIDAZOLE DERIVATIVES INCLUDING THE AROMATASE INHIBITOR VOROZOLE

Silvia Diani-Moore, Fotini Papachristou, Erin Labitzke, and Arleen B. Rifkind
Department of Pharmacology, Weill Medical College of Cornell University, New York, New York

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ABSTRACT:
Cytochrome P450 (P450) enzymes metabolize the membrane lipid arachidonic acid to stable biologically active epoxides [eicosatetraenoic acids (EETs)] and 20-hydroxyeicosatetraenoic acid (20-HETE). These products have cardiovascular activity, primarily acting as vasodilators and vasoconstrictors, respectively. EET formation can be increased by the prototype CYP1A or CYP2 inducers, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or phenobarbital (PB), respectively. We report here that imidazole derivative drugs: the anthelmintics, albendazole and thiabendazole; the proton pump inhibitor, omeprazole; the thromboxane synthase inhibitor, benzylimidazole; and the aromatase (CYP19) inhibitor vorozole (R76713, racemate; and R83842, (+) enantiomer) increased hepatic microsomal EET formation in a chick embryo model. Albendazole increased EETs by transcriptional induction of CYP1A5 and the others by combined induction of CYP1A5 and CYP2H, the avian orthologs of mammalian CYP1A2 and CYP2B, respectively. All inducers increased formation of the four EET regioisomers, but TCDD and albendazole had preference for 5,6-EET and PB and omeprazole for 14,15-EET. Vorozole, benzylimidazole, and TCDD also suppressed 20-HETE formation. Vorozole was a remarkably effective and potent inducer of multiple hepatic P450s at a dose range which overlapped its inhibition of ovarian aromatase. Increased CYP1A activity in mouse Hepa 1-6 and human HepG2 cells by vorozole and other imidazole derivatives demonstrated applicability of the findings to mammalian cells. The findings suggest that changes in P450-dependent arachidonic acid metabolism may be a new source of side effects for drugs that induce CYP1A or CYP2. They demonstrate further that in vivo induction of multiple hepatic P450s produces additive increases in arachidonic acid epoxygenase activity and can occur concurrently with inhibition of ovarian aromatase activity.

Cytochrome P450 (P450)-dependent arachidonic acid (AA) epoxides (EETs) and 20-HETE ω-OH AA) are biologically active AA metabolites that participate in the physiologic regulation of vascular tone. EETs primarily cause vasodilatation and 20-HETE, vasoconstriction (see reviews of Capdevila et al., 2000; Roman, 2002). Hepatic EET production is mediated by P450s in the CYP1A and CYP2 families (Rifkind et al., 1995; Capdevila et al., 2000). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and β-naphthoflavone (β-NF), inducers of hepatic CYP1A enzymes, and phenobarbital (PB), a CYP2 enzyme inducer, increase EET production in mammalian and avian liver (Nakai et al., 1992; Rifkind et al., 1994; Capdevila et al., 2000).

In prior work using the chick embryo model, we found that TCDD and β-NF induce CYP1A-dependent AA epoxygenation via the transcriptional enhancement of CYP1A5, the chick ortholog of mammalian CYP1A2 (Gilday et al., 1996) and PB by induction of CYP2H, the chick ortholog of mammalian CYP2B (Hansen et al., 1989; Nakai et al., 1992). CYP1A4, the chick ortholog of mammalian CYP1A1, is virtually inactive in AA epoxygenation (Nakai et al., 1992; Rifkind et al., 1994).

To determine whether therapeutically used drugs that induce P450 enzymes might alter AA metabolism, we examined the effects of treatment with a group of imidazole derivatives (Fig. 1) on hepatic P450-dependent AA metabolism in the chick embryo model. The compounds studied included several reported to induce CYP1A alone or together with CYP2 enzymes in mammalian liver or cultured cells: albendazole, benzylimidazole, omeprazole, and thiabendazole (Papac and Franklin, 1988; Southall-el Amri et al., 1988; Aix et al., 1994; Curi-Pedrosa et al., 1994; Rey-Grobellet et al., 1996; Price et al., 2004). In addition, we examined the effect of a third-generation aromatase inhibitor, the triazole derivative vorozole (De Coster et al., 1990; Goss, 1998) for which reports of P450-inducing activities were not found.

We discovered that vorozole was a remarkably effective and potent inducer of multiple hepatic P450 enzymes including CYP1A5 and CYP2H, notwithstanding its inhibition of ovarian CYP19 aromatase.

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activity and that the other imidazole derivatives induced AA epoxigenation via CYP1A alone or both CYP1A and CYP2H, to varying degrees. Immunoinhibition studies showed that the two P450s contributed independently to the induced epoxigenase activity, producing additive enhancement of EET formation reflecting the degree of CYP1A and/or CYP2H induction by the drugs.

In addition, we present evidence that some imidazole derivatives, in particular, vorozole and benzimidazole, like TCDD (Lee et al., 1998), decreased the formation by hepatic microsomes of 20-HETE, a CYP4 product whose cardiovascular effects are often opposed to those of EETs (Roman, 2002; Kroetz and Xu, 2005).

**Materials and Methods**

**Reagents.** Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. High pressure liquid chromatography (HPLC)-grade reagents were used for HPLC. Fertilized chicken eggs, White Leghorn strain, were obtained from Burr Farms (Hampton, CT); TCDD, from the NCI Chemical Carcinogen Repository (Kansas City, MO), benzimidazole, from BIOMOL (Plymouth Meeting, PA); vorozole [R76713, the racemate (±, d,l), and R83842, the (+, d) enantiomer], from Janssen Pharmaceutical N.V. (Beerse, Belgium); and omeprazole, from Astra Hassle AB (Molndal, Sweden). Rabbit antisera to chick CYP2H1/2H2 was a gift from Dr. Brian K. May (Adelaide, Australia).

**Treatment of Chick Embryos and Preparation of Liver Microsomes.** Fertilized chicken eggs were incubated at 37°C at high humidity. Embryos were treated at 15 days of gestation (hatching occurs at 21 days), unless otherwise indicated, by injecting the drugs through a small hole in the shell into the fluids surrounding the embryo. Treatment with the imidazole derivatives (see Fig. 1) was at the doses indicated in the figures or figure legends in 0.05 ml of dioxane. PB at 20 mg and TCDD at 1 nmol per egg are maximal P450-inducing doses (Nakai et al., 1992; Rifkind et al., 1994). Embryos that were untreated or treated with the solvents alone were included as negative controls. Livers were removed after 48 h, weighed, and homogenized in 3 volumes of 0.1 M KPO4, pH 7.4, and used to prepare microsomes as previously reported (Nakai et al., 1992). Microsomes were stored at −80°C.

**Hepa 1-6 (Mouse) and HepG2 Cells (Human).** The mammalian cell lines were obtained from The American Type Culture Collection (Manassas, VA) and grown according to the supplier’s directions in α-minimal essential medium for Hepa 1-6 and Dulbecco’s minimal essential medium for HepG2 cells. The cells were plated in 24-well plates (0.5 × 10^6/well) and treated at semiconfluence, using three wells for each treatment. β-NF, 10 μM, and the solvent DMSO were used as positive and negative controls, respectively.

**AA Metabolism.** Reaction mixtures for AA metabolism (Capdevila et al., 1990a; Rifkind et al., 1994) were in 0.25-ml total volume, and contained 75 μg of microsomal protein unless otherwise indicated and 30 μM [1-14C]AA (48 mCi/mmol) (Perkin Elmer, Torrance, CA). After preincubation for 2 min at 37°C, reactions were started with 1 mM NADPH, 10 mM isotonic acid, 0.2 α unit of isocitric dehydrogenase/ml, and 10 mM MgCl2 and incubated for 10 min at 37°C. Addition of 0.1 ml of acetic acid was followed by two extractions, each with 3 ml of ethyl acetate containing 0.005% butylated hydroxytoluene. The organic phases were pooled, dried under N₂, and resuspended in 0.05 ml of isocitric acid containing 0.1% acetic acid. Products in 0.05 ml were resolved by reverse phase HPLC using a Vydac C18 column (Vydac, Hesperia, CA) (90 Å, 5-μm particle size, 4.6 × 250 mm) on a linear gradient from 50 to 100% acetonitrile in water containing 0.1% acetic acid, at 1 ml per min for 40 min. Radioactivity was measured using a Flo-One Beta Model S radioactivity flow monitor (Packard Instrument Company, Downers Grove, IL). Products were identified by reference to HPLC retention times of pure standards (Rifkind et al., 1994). For immunoinhibition of P450-dependent AA metabolism (Kanetoshi et al., 1992), chick embryo liver microsomes were preincubated for 15 min at room temperature with 5 mg IgG/mmol P450 from preimmune rabbit serum or serum of rabbits immunized with CYP1A5 or CYP2H1/2H2, before adding other constituents of the reaction mixture for AA metabolism as described above.

**Aromatase.** An adaptation of the method of Leppard and Simpson (1991) was used. 1H18O released during the formation of estrone from androst-4-ene-3,17-dione ([1-^18O]N); (specific activity, 25.5 Ci/mmol) (Perkin Elmer) by CYP19 (aromatase) was measured. Ovaries were removed from 17-day-old chick embryos 48 h after treatment with R76713 at 1 or 0.1 mg per egg, or with...
the solvent. Three groups of ovaries (three ovaries per group) were assayed separately for each treatment. Each group of ovaries was homogenized using a hand homogenizer in 0.25 ml of 0.1 M KPO4, pH 7.4. Then, 0.95 ml of the KPO4 were added for a total of 1.2 ml. An aliquot was used to measure protein levels. Reaction mixtures containing protein equivalent to 1/10 of an ovary in 1 mM NADPH, 10 mM isocitric acid, 10 mM MgCl2, 0.2 U/ml isocitrate dehydrogenase, and 100 mM 2-[3H]NADPH (specific radioactivity 1.4 Ci/mM) were added, and the KPO4 buffer in a total volume of 0.2 ml were preincubated at 37°C for 3 min before adding the NADPH regenerating system, and then for 10 min. Controls without ovaries or without the NADPH regenerating system were included. Reactions were stopped with 0.8 ml of 12.5% (v/v) trichloroacetic acid. Three milliliters of chloroform were added and samples were centrifuged at 1700 g for 15 min. Then, 0.8 ml of the supernatant was mixed with 5% dextran-coated charcoal and centrifuged again at 1700 g for 15 min; 0.8 ml of that supernatant was added to 4.5 ml of Ecolume (ICN, Costa Mesa, CA). Radioactivity was measured in a LS 6000IC scintillation counter (Beckman, Fullerton, CA).

7-Ethoxyresorufin Deethylase (EROD) in Hepa 1-6 and HepG2 Cells. Cells were treated for 24 h. Then, the medium was removed, cells were washed with phosphate-buffered saline (Cellgro by Mediatech, Herndon, VA), and an EROD reaction mixture (4 µM 7-ethoxyresorufin and 10 µM dicyanol in 0.5 ml of medium) was added to each well. After 30 min at 37°C, two 0.2-ml aliquots from each well were transferred to separate tubes. Cold acetone, 0.25 ml, was added to each tube followed by centrifugation at 1875 g for 15 min. Resorufin was measured in the supernatants in a Perkin Elmer MFP3 spectrophotofluorimeter at excitation and emission A of 558 nm and 590 nm, respectively, using a quinine sulfate standard previously calibrated against resorufin. Emission spectra were obtained for selected samples to confirm the presence of a resorufin peak at 590 nm.

Reverse Transcriptase-PCR. Total RNA was extracted from chick embryo liver using RNA STAT-60 (Tel-Test “B”, Friendswood, TX). First-strand cDNA was synthesized from the total RNA using Moloney murine leukemia virus reverse transcriptase (Roche, Indianapolis, IN) as directed by the manufacturer: 4 µl of 5X incubation buffer, 2 µg of total RNA, 2 µl of 10X concentration 1st strand mix, 2 µl of 10 mM deoxyribonucleoside-5'-triphosphates, 0.5 µl of 10 U/µl RNAse inhibitor, 2 µl of 20 U/µl reverse transcriptase, and diethyl pyrocarbonate-water, up to 20 µl, were incubated for 2 h at 37°C; all components were obtained from Roche. PCR amplification for CYP1A4, 1A5, 2H1, and 2H2 and GAPDH, as an internal control, was performed. The primers and conditions reported (Diani-Moore et al., 2006) were used for CYP1A5, CYP2H1, and GAPDH. For CYP1A4 and 2H2, the following primers (Invitrogen, Carlsbad, CA) were used: for CYP1A4 (GenBank accession number X94953), 5'-5'-ggagggattgaagaggtcgggtg-3' and 5'-tgccagttctgagctggtg-3'; for CYP2H2 (GenBank accession number M25469), 5'-tggaggttctgatgtgaga-3' and 5'-tgccagttctgagctggtg-3'. Amplification conditions were: for CYP1A4, 94°C x 5 min for 1 cycle, 94°C x 1 min, 59°C x 30 s, 72°C x 40 s for 30 cycles, 72°C x 10 min for 1 cycle; and for CYP2H2, 94°C x 5 min for 1 cycle, 94°C x 1 min, 56°C x 30 s, 72°C x 40 s for 28 cycles, 72°C x 10 min for 1 cycle. PCR products were obtained at cycle numbers below plateau levels. PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide using a UV transilluminator. Expected PCR product sizes were: CYP1A4, 404 bp; CYP1A5s, 219 bp; CYP2H1, 286 bp; CYP2H2, 304 bp; GAPDH, 170 bp. Band intensities were measured by densitometry using AlphaEaseFC software (Alpha Innotech, San Leandro, CA). We verified that amplified PCR products represented the target genes of interest by DNA sequencing. In addition, CYP1A5s and CYP2H primers recognized the targeted chicken P450s using the University of California, Southern California In-Silico PCR application against the chicken genome (assembly date, February 2004) (http://genome.ucsc.edu/).

SDS-Polyacrylamide Gel Electrophoresis (PAGE)/Western Blotting. SDS-PAGE (Laemmli, 1970) was performed on slab gels (14.5 cm x 14.5 cm x 1.0 mm) using 3% and 7.5% acrylamide in the stacking and separating gels, respectively. Samples were prepared in 2X sample buffer (125 mM Tris/HCl, pH 6.8, 4% SDS, 16% glycerol, 10% β-mercaptoethanol, 0.002% bromphenol blue) and kept at 100°C for 2 min. Microsomal protein, 20 µg per lane, was run into the stacking gel for 2 h at 15 mA and through the separating gel at 30 mA for 3 to 4 h. Gels were stained with Coomassie Blue. For Western blotting, proteins were separated on SDS-gels, amounts per lane as indicated in the figure legends, and transferred to nitrocellulose at 90 mA overnight at 4°C. Completeness of transfer was monitored by post-transfer staining with Coomassie Blue. Membranes were washed with 10 mM Tris, pH 7.4, in 0.9% NaCl (Tris/NaCl), blocked for 1 h with 3% BSA in Tris/NaCl, washed with Tris/NaCl, and then incubated with immunopurified antisera to CYP1A4 or CYP1A5 (1:400) or anti-CYP2H IgG (1:1000) in 3% BSA in Tris/NaCl for 1 h. The anti-CYP2H antibody used was raised against the major PB-induced P450s, 2H1 and 2H2 (Kanetoshi et al., 1992). N-terminal amino acid sequencing of the PB-induced bands recognized by the antisemur on Western blots verified that the antibody recognized CYP2H. After washing, blots were incubated with horseradish peroxidase-linked goat anti-rabbit IgG (1:1000) for 1 h at room temperature in 3% BSA in Tris/NaCl. Immunoblots were developed in 30 ml of 0.1% 4-chloro-1-naphthol and 0.03% hydrogen peroxide in 50 mM Tris, pH 7.6 (Rifkind et al., 1994). Band intensities were measured by densitometry as described above.

Other Procedures. Total cytochrome P450 was measured by carbon monoxide-reduced versus reduced difference spectra, using sodium dithionite as a reducing agent and an extinction coefficient of 91 mM⁻¹ cm⁻¹ for the difference in absorbance at 490 nm and 450 nm (Omura and Sato, 1964). Protein concentrations were measured by the method of Lowry et al. (1951). Statistical analysis included unpaired t tests for differences between group means or analyzes of variance (GraphPad Prism 4 software; GraphPad, San Diego, CA).

Other Procedures. Total cytochrome P450 was measured by carbon monoxide-reduced versus reduced difference spectra, using sodium dithionite as a reducing agent and an extinction coefficient of 91 mM⁻¹ cm⁻¹ for the difference in absorbance at 490 nm and 450 nm (Omura and Sato, 1964). Protein concentrations were measured by the method of Lowry et al. (1951). Statistical analysis included unpaired t tests for differences between group means or analyzes of variance (GraphPad Prism 4 software; GraphPad, San Diego, CA).

FIG. 2. Effect of imidazole derivatives on P450-dependent AA metabolism. P450-dependent AA epoxygenase activity was measured as described under Materials and Methods in liver microsomes from 17-day-old chick embryos that had been treated for 48 h with albendazole (ALB), R76713, thiabendazole (TBZ), omeprazole (OP), benzimidazole (BZ), benzylimidazole (BZY), or PB (gray bars; doses indicated below the abscissa) or with TCDD (1 nmol, 0.0003 mg) or 0.1 ml of DMSO (black bar). a, epoxygenase activity: formation of EETs and DHETs; b, 20-HETE formation. Mean values (nmol product/mg protein/min ± S.E.) are for n (number of independent experiments) = 11 for TCDD, 10 for DMSO, 5 for R76713 at 1 mg, and 3 or 4 for the others. In each experiment, each determination was based on two separate replicates. * p < 0.05; ** p < 0.01 versus DMSO.
Results

All of the imidazole derivatives increased AA epoxygenase activity (EETs and DHETs) over the DMSO controls (Fig. 2a). TCDD, PB, and R76713 produced the greatest increases in EET formation: 17.7-fold for TCDD and 18-fold for PB, at maximal P450-inducing doses, and a mean of 12.8-fold for R76713 at 1 to 9 mg per egg. EETs were increased 9.2- and 13.3-fold by albendazole at 1 and 9 mg per egg, 3.5- and 10.4-fold by omeprazole at 3 and 9 mg per egg, respectively, 12.6-fold by benzimidazole at 9 mg per egg (not tested at a lower dose), and 2.7- and 2.4-fold by thiabendazole at 9 and 18 mg per egg, respectively. Benzimidazole produced a 2.4-fold increase only at 18 mg/egg.

20-HETE was not significantly increased by any of the imidazoles (Fig. 2b). However, TCDD significantly suppressed 20-HETE (38.7 ± 4.3%), as did R76713 (mean suppression of 53.1 ± 3.5% at 1–9 mg per egg) and benzimidazole (59.4 ± 3.5%) (p < 0.001 for all). Figure 3 shows representative HPLC chromatograms for P450-dependent AA metabolism by the imidazole derivatives at 9 mg per egg. Increases in the four regioisomeric EETs (5,6-, 8,9-, 11,12-, and 14,15-), their secondary epoxyde hydroxylase products (DHETs), 18- and 19-HETE, and other HETEs (Capdevila et al., 1990a) are evident for all of the compounds except benzimidazole. The chromatograms also show the suppression of the 20-HETE peak by TCDD, R76713, and benzimidazole.

Table 1 shows means ± S.E. for the production and distribution of the four regioisomeric EETs + DHETs after treatment by the test compounds. For TCDD, the 5,6-regiosomer was the major product [mean 33 ± 1.8% of the total EET + DHET production (p < 0.01)], and the other regiosomers were approximately evenly distributed [21 ± 1.5% to 24 ± 1.2% (not significant; N.S.)]. In contrast, for PB, formation of the 14,15-regiosomer was highest [40 ± 0.8% (p < 0.001 versus 5,6-)] and that of the 5,6-, lowest (7 ± 0.6%). For PB, overall regiosomer production was less evenly distributed than for TCDD; each product differed significantly from the others (see Table 1). For the imidazole derivatives, differences in regiosomer distribution were not as pronounced as for TCDD or PB. The distributions for albendazole resembled those of TCDD and the distributions for omeprazole and thiabendazole, those for PB. For R76713 and benzylimidazole, more mixed induction patterns were seen. Analysis of variance showed no significant differences between TCDD, PB, or the other test compounds with respect to relative formation of HETEs, 18-HETE (o-2-OH AA) or 19 HETE (o-1-OH AA) (not shown).

To investigate the basis for the increase in EETs, we examined effects of the imidazole derivatives on P450 levels (Fig. 4a). All of the compounds increased total P450 1.5-fold or more over the DMSO controls [mean for DMSO, 0.22 ± 0.01 (S.E.) nmol/mg protein]. R76713 was the most potent and also the most effective P450 inducer among the imidazole derivatives. It produced the highest P450 levels we have seen for chick embryo liver: mean levels of 1.95 and 3.1 nmol/mg protein at 1 and 9 mg per egg, respectively, over 3- and 5-fold higher than TCDD (p < 0.01) and 1.4- and more than 2-fold higher than PB at 20 mg per egg (p = 0.055). Albendazole was equieffective with TCDD and had a maximal inducing effect at 1 mg per egg. Benzimidazole at 9 mg per egg increased total P450 1.7-fold more than TCDD (p < 0.01). Total P450 was increased by thiabendazole and omeprazole, about 1.5-fold at 3 mg per egg or higher, and 2.4-fold by benzimidazole at 18 mg per egg.

TABLE 1
Regiosomeric production and distribution of arachidonic acid epoxides in response to 48 h of treatment with TCDD, PB, and imidazole derivatives

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EETs + DHETs</th>
<th>14,15</th>
<th>11,12</th>
<th>8,9</th>
<th>5,6</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td></td>
<td>0.188 ± 0.019</td>
<td>0.175 ± 0.014</td>
<td>0.200 ± 0.020</td>
<td>0.283 ± 0.029</td>
<td>0.844 ± 0.067</td>
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<tr>
<td></td>
<td></td>
<td>(22 ± 1.2)***</td>
<td>(21 ± 1.5)***</td>
<td>(24 ± 1.2)***</td>
<td>(33 ± 1.8)***</td>
<td>(32 ± 1.8)***</td>
</tr>
<tr>
<td>PB</td>
<td></td>
<td>0.339 ± 0.014</td>
<td>0.162 ± 0.019</td>
<td>0.294 ± 0.001</td>
<td>0.067 ± 0.003</td>
<td>0.862 ± 0.027</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(40 ± 0.8)<strong>b</strong>*</td>
<td>(19 ± 2)***</td>
<td>(34 ± 0.9)<strong>b</strong>*</td>
<td>(7 ± 0.6)<strong>b</strong>*</td>
<td>(32 ± 0.9)<strong>b</strong>*</td>
</tr>
<tr>
<td>ALB</td>
<td></td>
<td>0.161 ± 0.014</td>
<td>0.123 ± 0.014</td>
<td>0.168 ± 0.009</td>
<td>0.182 ± 0.011</td>
<td>0.635 ± 0.043</td>
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<tr>
<td></td>
<td></td>
<td>(25 ± 0.9)<strong>b</strong>*</td>
<td>(19 ± 1.3)<strong>b</strong>*</td>
<td>(27 ± 1.8)<strong>b</strong>*</td>
<td>(29 ± 0.3)<strong>b</strong>*</td>
<td>(29 ± 0.3)<strong>b</strong>*</td>
</tr>
<tr>
<td>BZY</td>
<td></td>
<td>0.193 ± 0.033</td>
<td>0.145 ± 0.024</td>
<td>0.142 ± 0.028</td>
<td>0.126 ± 0.017</td>
<td>0.605 ± 0.099</td>
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<td></td>
<td></td>
<td>(32 ± 0.7)<strong>p</strong>*</td>
<td>(24 ± 0.6)***</td>
<td>(23 ± 1.2)***</td>
<td>(21 ± 1.6)<strong>p</strong>*</td>
<td>(21 ± 1.6)<strong>p</strong>*</td>
</tr>
<tr>
<td>OP</td>
<td></td>
<td>0.191 ± 0.005</td>
<td>0.108 ± 0.009</td>
<td>0.140 ± 0.008</td>
<td>0.058 ± 0.007</td>
<td>0.497 ± 0.018</td>
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<tr>
<td></td>
<td></td>
<td>(38 ± 1.6)<strong>b</strong>*</td>
<td>(22 ± 1.3)<strong>c</strong>*</td>
<td>(28 ± 1.4)<strong>b</strong>*</td>
<td>(12 ± 1.3)<strong>c</strong>*</td>
<td>(12 ± 1.3)<strong>c</strong>*</td>
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<tr>
<td>R76713</td>
<td></td>
<td>0.178 ± 0.018</td>
<td>0.119 ± 0.008</td>
<td>0.190 ± 0.013</td>
<td>0.130 ± 0.013</td>
<td>0.616 ± 0.040</td>
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<td></td>
<td></td>
<td>(28.5 ± 1.6)<strong>b</strong>*</td>
<td>(19.5 ± 0.7)<strong>b</strong>*</td>
<td>(31 ± 1.2)<strong>p</strong>*</td>
<td>(21 ± 1.7)<strong>p</strong>*</td>
<td>(21 ± 1.7)<strong>p</strong>*</td>
</tr>
<tr>
<td>TBZ</td>
<td></td>
<td>0.045 ± 0.008</td>
<td>0.024 ± 0.006</td>
<td>0.038 ± 0.008</td>
<td>0.021 ± 0.005</td>
<td>0.128 ± 0.013</td>
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<tr>
<td></td>
<td></td>
<td>(35 ± 4.3)<strong>b</strong>*</td>
<td>(20 ± 4.5)***</td>
<td>(29 ± 4.4)***</td>
<td>(16 ± 3.4)<strong>p</strong>*</td>
<td>(16 ± 3.4)<strong>p</strong>*</td>
</tr>
</tbody>
</table>

*Significantly different from all other regiosomers for the same treatment (exceptions: ALB, 11,12 did not differ from 14,15; R76713, 11,12 did not differ from 5,6 nor 14,15; 8,9). **Relative regiosomer production significantly different from TCDD. ***Relative regiosomer production significantly different from PB. For a, b, c: * p < 0.05, ** p < 0.01, *** p < 0.001. For a, b TCDD, p < 0.01; PB, p < 0.01, except p < 0.05 for 8,9 vs. 14,15; ALB, p < 0.05; OP, p < 0.001, except p < 0.01 for 11,12 vs. 8,9; R76713, p < 0.01, except p < 0.05 for 14,15. n = number of independent experiments. Abbreviations as in Fig. 2.
increased the CYP1A bands less than TCDD and the CYP2H bands more than PB. [Fig. 7b shows that an even lower dose of R76713 (1 mg per egg) also induced the CYP2H and 52-kDa bands more than a maximal inducing dose of PB (20 mg per egg).] Benzylimidazole also produced strong increases and omeprazole, much weaker increases in multiple P450 bands. Benzimidazole increased the CYP1A bands more than R76713 and the CYP2H bands less. Benzimidazole lacked an effect. Although the loading for microsomes from benzimidazole-treated livers seems low in the gel shown, no effects were also seen in other gels.

Western blotting results for effects on CYP1A4, 1A5, and 2H for the imidazole derivatives at 9 mg per egg (Fig. 4c) were consistent with the SDS-PAGE patterns. As expected (Nakai et al., 1992; Rifkind et al., 1994), TCDD increased CYP1A4 and 1A5 but not CYP2H, whereas PB increased CYP2H but not CYP1A4 or 1A5. Albendazole, like TCDD, increased CYP1A4 and 1A5 and not
CYP2H1, whereas thiabendazole increased only a small increase in CYP1A5. R76713 and benzylimidazole increased both CYP2H and CYP1A4 and 1A5. R76713 increased CYP2H more than benzylimidazole, and CYP1A4 and 1A5, less. Omeprazole produced small increases in CYP2H and CYP1A5 but no detectable increase in CYP1A4. No effects were seen for benzimidazole.

Liver microsomes were also subjected to Western blotting using equal amounts of P450 per lane to reveal changes in P450 distribution and induction effects not apparent at equal protein loading (see Supplemental Data). Under those conditions, small increases for CYP1A4, CYP1A5, and CYP2H were observed for thiabendazole and benzimidazole. However, omeprazole increased only CYP1A5 as seen in Fig. 4c.

Figure 5a shows that TCDD, R76713, albendazole, benzylimidazole, and thiabendazole, to a much smaller extent, increased CYP1A4 I A5 and thiabendazole. However, omeprazole increased only CYP1A5 as seen in Fig. 4c.

To assess the contribution of CYP1A5 and CYP2H to the enhanced epoxygenase activity by each of the imidazole derivatives, we examined the ability of CYP1A5 or CYP2H antisera to immunoinhibit EET formation at equal concentrations of total P450 (Fig. 6). Consistent with prior evidence (Kanetoshi et al., 1992) TCDD-induced EET formation was entirely suppressed by anti-CYP1A5 IgG and unaffected by anti-CYP2H IgG, whereas PB-induced EET formation was entirely suppressed by anti-CYP2H IgG and unaffected by anti-CYP1A5 IgG. Consistent with the selective induction of CYP1A5 by
albendazole shown in Figs. 4 and 5, albendazole-enhanced EET formation was exclusively and entirely inhibited by anti-CYP1A5 IgG. Anti-CYP1A5 and anti-CYP2H IgG each partially suppressed EET formation by R76713, benzylimidazole, and omeprazole, indicating that both P450s contributed to the total EET formation by those agents, consistent with their mixed pattern of P450 induction.

The sum of the residual induced EET formation after immunoinhibition with the two antisera was close to the activity in the presence of preimmune serum, indicating that CYP1A5 and CYP2H made additive contributions to the total EET formation. The results showed further that CYP1A5 and CYP2H could account for virtually all of the epoxygenase induction without the need to invoke other P450s.

We investigated further whether R76713 suppressed ovarian aromatase (CYP19) activity in the chick model used here and did so at doses at which it induced hepatic P450s. At 1 mg per egg, R76713 inhibited ovarian aromatase activity 94% and at 0.1 mg per egg, submaximally (73%) (Fig. 7a).

To learn whether the capacity to induce hepatic P450s resided in one or both vorozole enantiomers, we compared P450 induction by the vorozole racemate, R76713, and the (+) enantiomer, R83842. Both compounds increased the same bands on SDS-PAGE (Fig. 7b) and did so to about the same extent at equal doses as confirmed by densitometry (not shown). Both also increased the CYP2H bands at 1 and 0.1 mg per egg but not below, and the CYP1A bands only at the higher dose. At 1 mg per egg, both increased the CYP2H bands approximately 20% more than a maximal inducing dose of PB. On more sensitive Western blots using chemiluminescence detection (not shown), R76713 and R83842 increased CYP1A4 and 1A5 at 0.1 mg per egg and CYP2H even at 0.01 mg per egg. Thus, the vorozole (+) enantiomer, like the racemate, can induce P450 enzymes with high potency. Because R76713 and R84832 produced similar induction responses at the same concentrations and the racemate by definition contains less of the (+) enantiomer, it can be inferred that the (-) enantiomer also has hepatic P450-inducing capacity.

R83842 and R76713 increased total P450 13.4- and 11.7-fold, respectively, at 1 mg per egg and 2.5- and 4-fold at 0.1 mg per egg.
increased EROD the same or more than increasing EROD. At the highest doses tested, benzylimidazole and vorozole, as well as TCDD, suppress 20-HETE formation. 3) The triazole aromatase inhibitor, vorozole, is an unusually potent and effective inducer of multiple hepatic P450 enzymes at doses inhibiting aromatase activity.

Both R83842 and R76713 also suppressed 20-HETE: 58% and 44%, respectively, at 1 mg per egg, 17% and 12% at 0.1 mg per egg, and 42% and 54% at 0.01 mg per egg. The anomalous dose-response relationships were seen in two independent experiments for R76713 and in two separate groups of livers for R83842.

To learn whether R76713 increased P450 activity in mammalian cells, the effects of R76713, albendazole, and benzylimidazole on CYP1A1-mediated EROD were examined in mouse Hepa 1-6 and human HepG2 cells (Fig. 8). In the Hepa 1-6 cells, all three increased EROD significantly compared with the solvent controls. Albendazole was equipotent and equipotent with β-NF, the positive control, in increasing EROD. At the highest doses tested, benzylimidazole and R76713 increased EROD the same or more than β-NF, respectively, indicating that both are highly effective CYP1A1 inducers in the mouse cells, as in chick liver. As expected, PB, the negative control, failed to increase EROD.

R76713 also increased EROD in the HepG2 cells, but less than albendazole. EROD was significantly increased over the control levels, even at 10 μM R76713, the lowest concentration tested.

Discussion

This paper reports the following new findings. 1) Imidazole drugs can increase P450-dependent AA EET formation and do so by induction of CYP1A or CYP2 enzymes, or both. 2) Two of the imidazole derivatives, benzylimidazole and vorozole, as well as TCDD, suppress 20-HETE formation. 3) The triazole aromatase inhibitor, vorozole, is an unusually potent and effective inducer of multiple hepatic P450 enzymes at doses inhibiting aromatase activity.

The first two findings are significant because they identify several therapeutic agents as potential modifiers of endogenous AA metabolism. Furthermore, the results here show that CYP1A, which has been regarded principally as a metabolizer and activator of toxic or carcinogenic xenobiotics (Neberdt et al., 2004), increases the metabolism of endogenous AA to biologically active products. It was previously demonstrated that prototype xenobiotic inducers of either CYP1A or CYP2 enzymes could increase EET production (Rifkind et al., 1994; Capdevila et al., 2000). We show here that the coinduction of CYP1A and CYP2 may produce additive effects on EET formation and that the induction by each P450 does not interfere with the EET-inducing capacity of the other.

The suppression of 20-HETE, as shown here for vorozole and benzylimidazole, as well as TCDD, is another new and potentially significant drug effect. The mechanism for the suppression of 20-HETE will require investigation: 20-HETE is generated by CYP4 family enzymes (Kroetz and Xu, 2005), which are not known to be affected by the drugs tested here or by PB or TCDD. Moreover, the suppression of 20-HETE cannot be a direct effect of the induced CYP1A or 2H per se, inasmuch as albendazole and phenobarbital, selective inducers of CYP1A and 2H, respectively, failed to suppress 20-HETE.

EETs and 20-HETE are major physiologic regulators of vascular reactivity (Roman, 2002; Kroetz and Xu, 2005). EETs mainly cause vasodilation and 20-HETE, vasoconstriction, although they can have reverse effects in the pulmonary vasculature. EETs and 20-HETE often have opposite effects but also some common actions. Thus, both are angiogenic (Michaels et al., 2003; Chen et al., 2005; Wang et al., 2005) and both increase production of reactive oxygen species (Roman, 2002). EETs also increase intracellular calcium and cell proliferation, and suppress hormone secretion (reviewed in Capdevila et al., 2000). It has been suggested that EETs are cardioprotective in ischemic injury (Seubert et al., 2004) and that suppression of 20-HETE may be antihypertensive, cardioprotective in ischemic injury, and antiangiogenic (Nithipatikom et al., 2004; Chen et al., 2005). Moreover, there is new evidence that EETs are required for the cardioprotective effect of inhibition of 20-HETE (Nithipatikom et al., 2006). In that light, agents such as those shown here, which both induce EETs and suppress 20-HETE, may have an enhanced positive effect in ischemic-reperfusion injury. Conversely, there is also evidence that EETs may have adverse effects in ischemic heart disease (Granville et al., 2004), and that 20-HETE may be antihypertensive (Gainer et al., 2005). Accordingly, it cannot yet be predicted whether alterations in production of EETs or 20-HETE would be beneficial or harmful.

EETs and 20-HETE are stable products and can be detected in the circulation (Nithipatikom et al., 2001). Therefore, EETs or 20-HETE generated in liver or in extrahepatic organs such as the gastrointestinal tract or kidney, where TCDD-inducible CYP1A2 and PB-inducible
CYP2 enzymes are present (Ding and Kaminsky, 2003; Yu et al., 2006), could have systemic as well as local effects. The evidence that CYP1A5 and CYP2P2, which are selectively induced by TCDD and PB, respectively, produced relatively more of the 5,6- and 14,15-AA regioisomers, respectively, indicates that these P450s have distinct regioselective specificities, even though both generated all four regioisomers. These findings are consistent with evidence for preferential formation of 5,6-EET by human CYP1A2 (Choudhary et al., 2004) and of 14,15-EET by CYP2B (Capdevila et al., 1990b; Laethem et al., 1994) and human CYP2C8 and 2C9 (Daikh et al., 1994; Zeldin et al., 1996).

Preferential production of 5,6- or 14,15-EET could potentially lead to different effects. For example, 5,6-EET but not 14,15-EET enhanced angiogenesis (Pozzi et al., 2005), and 14,15-EET but not 5,6-EET suppressed endothelial cell apoptosis (Dhanasekaran et al., 2006). In vivo, however, such effects are likely to be subjected to modification by 1) the presence of other EET regioisomers, inasmuch as all four EETs are usually generated even when one is preferentially produced, 2) endogenous P450-dependent AA metabolites, which vary among organs and species and over which drug-induced EETs will be superimposed, and 3) 20-HETE, which commonly has opposite effects to EETs (Roman, 2002) and which can be suppressed by some EET inducers (as shown here for TCDD, benzylmidazole, and vorozole).

Imidazole drugs induce CYP1A by AhR-mediated transcriptional signaling, although they do not bind to the AhR (Aix et al., 1994; Curi-Pedrosa et al., 1994). Our evidence that increases in CYP1A mRNAs are associated with the increases in CYP1A proteins is consistent with a transcriptional mechanism for CYP1A induction by these compounds, also, in the chick embryo model. AhR involvement is also supported by Western blotting evidence that AhR protein levels in chick embryo liver cytosol were diminished 7 h after treatment with benzylmidazole or alendronate (not shown).

The following considerations support the relevance of these findings in the chick embryo to mammalian species. 1) The P450 induction responses in the chick model were qualitatively and quantitatively similar to those reported in mammalian species. Thus, a strong mixed CYP1A and CYP2 induction for benzylmidazole and a weaker mixed induction for omeprazole and thabendazole, selective induction of CYP1A by alendronate, and scanty effects for benzimidazole were also reported in mammalian liver (Papac and Franklin, 1988; Souhaili-el Amri et al., 1988; Curi-Pedrosa et al., 1994; Rey-Grobellet et al., 1996; Masubuchi et al., 1997; Price et al., 2004). Although in the chick model, omeprazole did not produce the high CYP1A induction seen in human liver cells, omeprazole, in particular, has shown specificity differences in CYP1A induction (Kikuchi et al., 1996). 2) Human CYP1A2 and CYP2 enzymes, like chick CYP1A5 and CYP2H, are highly active AA epoxygenases (Rifkind et al., 1994, 1995; Capdevila et al., 2000), and as noted above, the chick and human orthologs also have similar regioselective specificities. 3) CYP1A-mediated EROD was induced by R76713 in mammalian cells as well as chick embryo liver (see Fig. 8). 4) The chick embryo is the accepted model for prediction of drug exacerbations in human acute intermittent porphyria, notwithstanding that much higher doses (mg/kg) are required in the chick to induce hepatic δ-aminolevulinic acid synthase, the rate-limiting enzyme in synthesis of heme, an essential component for P450 enzyme function (www.porphryafoundation.com).

Our third new finding, that the aromatase inhibitor vorozole can induce hepatic P450 enzymes, demonstrates that inhibition of ovarian aromatase can occur concurrently with hepatic P450 induction. Vorozole was further distinguished by its remarkably high potency and efficacy for hepatic P450 induction, producing P450 induction equivalent to that of PB but at about 1/100 the dose of PB. Moreover, vorozole induced multiple P450s, including P450s not yet identified, as well as CYP2H and CYP1A (see Fig. 4b). Vorozole may also have post-transcriptional effects, as suggested by greater expression in CYP1A mRNAs (Fig. 5), than might have been expected from the relatively low increases in CYP1A proteins (Fig. 4, b and c). Although the (+) enantiomer of vorozole is substantially more potent than the (−) enantiomer in aromatase inhibition (De Coster et al., 1990; Goss, 1998), the results reported here show that the (−) enantiomer may be more potent with respect to P450 induction.

Aromatase inhibitors are an increasingly important part of the breast cancer armamentarium (Swain, 2005). Clinical drug interactions associated with P450 induction have not been reported for aromatase inhibitors, but these studies should draw new attention to possible P450 induction effects. Inasmuch as newer aromatase inhibitors in current use, i.e., anastrozole and letrozole, are triazole derivatives structurally similar to vorozole, they may also have P450-inducing effects. Accordingly, these findings warrant further study of aromatase inhibitor effects on hepatic P450 enzyme levels and on P450-dependent AA metabolism.

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References


Address correspondence to: Arleen B. Rifkind, Department of Pharmacology, Weill Medical College of Cornell University, 1300 York Avenue, Room LC401, New York, NY 10021. E-mail: arifkind@med.cornell.edu
**Western blots comparing induction of CYP1A4, 1A5 and 2H1/2H2 by PB, TCDD and imidazole derivatives at equal amounts of total P450.** Chick embryo liver microsomes immunoblotted at equal microsomal protein concentrations in Fig. 4c, were immunoblotted at equal concentrations of total P450 (7 pmol per lane) to compare the relative amounts of CYPs 1A4, 1A5 and 2H present in a given amount of P450 after treatment with the different inducing agents. Western blotting was with monospecific immunopurified antisera to CYP1A4 (top panel), CYP1A5 (middle panel) or antiserum to CYP2H1/2H2 (lower panel) as in Fig. 4c, as described in “Material and Methods”. Microsomal protein (µg per lane) was as follows: Lane 1, 16.1; lane 2, 11.4; lane 3, 11; lane 4, 11.6; lane 5, 2.2; lane 6, 15.6; lane 7, 14.2; lane 8, 21.5; lane 9, 7.4. Lanes 10 and 11, 1 pmol purified CYP1A4 or 1A5, respectively, in each blot. MW, molecular weights in kDa.