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# Induction of CYP1A and CYP2 mediated arachidonic acid epoxygenation and suppression of 20-HETE 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, NY, NY, 10021

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Running title: Imidazole derivatives and CYP arachidonic acid metabolism

Corresponding author: Arleen B. Rifkind, Department of Pharmacology, Weill Medical College of Cornell University, 1300 York Avenue, Room LC401, New York, NY 10021. Tel. 212-746-6236; FAX 212-746-8835; email: arifkind@med.cornell.edu

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Abbreviations: AhR, aryl hydrocarbon receptor; BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; EETs, eicosatetraenoic acids; DHETs, EET-diols, epoxide hydrolase metabolites of EETs; HETE, hydroxyeicosatrienoic acids; HPLC, high pressure liquid chromatography; MEM, minimal essential medium; R76713, (+/-, *d,l*) vorozole; R83842, (+, *d*) vorozole; SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ω-OH AA, omega hydroxy arachidonic acid, 20-HETE, 20-hydroxyeicosatetraenoic acid.

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ABSTRACT

Cytochrome P450 enzymes metabolize the membrane lipid arachidonic acid to stable biologically active epoxides (EETs) and 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, TCDD or phenobarbital, respectively. We report here that imidazole derivative drugs: the antihelminthics, 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 CYPs 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 CYP-dependent arachidonic acid metabolism may be a new source of side for drugs that induce CYP1A or CYP2. They demonstrate further that *in vivo* induction of multiple hepatic CYPs produces additive increases in AA epoxygenase activity and can occur concurrently with inhibition of ovarian aromatase activity.

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## INTRODUCTION

Cytochrome P450 (CYP) dependent arachidonic acid (AA) epoxides (EETs) and 20-HETE 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 CYPs in the CYP1A and CYP2 families (Capdevila et al., 2000; Rifkind et al., 1995). 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 (Capdevila et al., 2000; Nakai et al., 1992; Rifkind et al., 1994).

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 (Nakai et al., 1992; Hansen et al., 1989). 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 CYP enzymes might alter AA metabolism, we examined the effects of treatment with a group of imidazole derivatives (Fig. 1) on hepatic CYP 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 (Souhaili-el Amri et al., 1988; Aix et al., 1994; Rey-Grobellet et al., 1996; Curi-Pedrosa et al., 1994; Papac and Franklin, 1988; Price et al., 2004). In addition, we examined the effect of a

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third generation aromatase inhibitor, the triazole derivative vorozole (De Coster et al., 1990; Goss, 1998) for which reports of CYP-inducing activities were not found.

We discovered that vorozole was a remarkably effective and potent inducer of multiple hepatic CYP enzymes including CYP1A5 and CYP2H, notwithstanding its inhibition of ovarian CYP19 aromatase activity and that the other imidazole derivatives induced AA epoxygenation via CYP1A alone or both CYP1A and CYP2H, to varying degrees. Immunoinhibition studies showed that the two CYPs contributed independently to the induced epoxygenase activity, producing additive enhancement of EET formation reflecting the degree of CYP1A and/or CYP2H induction by the drugs.

Additionally, we present evidence that some imidazole derivatives, in particular, vorozole and benzylimidazole, 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 effects of EETs (Roman, 2002; Kroetz and Xu, 2005).

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## MATERIALS AND METHODS

*Reagents* - Chemicals were from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. HPLC (high pressure liquid chromatography) 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), benzylimidazole from Biomol (Plymouth, PA); vorozole (R76713, the racemate (+/-, *d,l*) and R83842, the (+,*d*) enantiomer) from Janssen Pharmaceutical N.V. (Beerse, Belgium); 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° 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 0.05-0.1 ml DMSO (dimethylsulfoxide), with PB at 9 or 20 mg per egg in 0.1 ml of 0.9% sodium chloride, and with TCDD at 1 nmol ($3 \times 10^{-4}$ mg) per egg in 0.005 ml dioxane. PB at 20 mg and TCDD at 1 nmol per egg are maximal CYP 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 hr, weighed and homogenized in three volumes of 0.1 M $KPO_4$, pH 7.4, and used to prepare microsomes as previously reported (Nakai et al., 1992). Microsomes were stored at -80°.

*Hepa 1-6 (mouse) and HepG2 cells (human)*- The mammalian cell lines were obtained from ATCC (Manassas, VA), and grown according to the supplier's directions in

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αMEM for Hepa 1-6 and DMEM for HepG2 cells. The cells were plated in 24 well plates ($0.5 x 10^6$/well) and treated at semiconfluence, using three wells for each treatment. β-NF, 10 μM, or the solvent DMSO were used as positive and negative controls, respectively.

*Arachidonic Acid (AA) metabolism* – Reaction mixtures for AA metabolism (Capdevila et al., 1990a; Rifkind et al., 1994) were in 0.25 ml total vol and contained 75 μg of microsomal protein unless otherwise indicated and 30 μM [1-$^{14}$C] AA (48 mCi/mmol) (Perkin Elmer, Torrance, CA). After preincubation for 2 min at 37°, reactions were started with 1 mM NADPH, 10 mM isocitric acid, 0.2 U isocitric dehydrogenase/ml and 10 mM $MgCl_2$ and incubated for 10 min at 37°. 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 $N_2$ and resuspended in 0.11 ml of 50% acetonitrile in water and 0.1% acetic acid. Products in 0.05 ml were resolved by reverse phase HPLC using a Vydac $C_{18}$ column (Vydac, Hesperia, CA) (90 Å, 5 μm particle size, 4.6 x 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 CYP-dependent AA metabolism (Kanetoshi et al., 1992) chick embryo liver microsomes were preincubated for 15 min at room temperature with 5 mg IgG/nmol 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 Lephart and Simpson (1991) was used. $^3H_2O$ released during the formation of estrone from androst-4-ene-3,17-dione, [1β-$^3$H (N)]- (specific

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activity 25.5 Ci/mmol), (Perkin Elmer, Torrance, CA) by CYP19 (aromatase) was measured. Ovaries were removed from 17-day old chick embryos 48 hr after treatment with R76713 at 1 or 0.1 mg per egg, or 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 $KPO_4$ pH 7.4. Then 0.95 ml of the $KPO_4$ were added for a total of 1.2 ml. An aliquot was used to measure protein levels. Reaction mixtures containing protein equivalent to 1/5 of an ovary in 1 mM NADPH, 10 mM isocitric acid, 10 mM $MgCl_2.6H_2O$, 0.2 U/ml of isocitrate dehydrogenase and 100 nM of $^3H$ androstenedione and the $KPO_4$ buffer in a total volume of 0.2 ml were preincubated at 37$^o$ 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% (w/v) trichloroacetic acid. Three ml of chloroform were added and samples were centrifuged at 1,700 x *g* for 15 min. 0.8 ml of the supernatant was mixed with 5% dextran coated charcoal and centrifuged again at 1,700 x *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 hr. Then the medium was removed, cells were washed with phosphate buffered saline (PBS) (Cellgro by Mediatech) and an EROD reaction mixture (4 µM 7-ER and 10 µM dicumarol in 0.5 ml of medium) was added to each well. After 30 min at 37º, 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 1,875 x g for 15 min. Resorufin was measured in the supernatants in a Perkin Elmer MPF 3 spectrophotofluorimeter at excitation (Ex) and emission (Em) λ of 558 nm and

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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 (RT)-PCR-* Total RNA was extracted from chick embryo livers 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 concentrated hexamers, 1 µl of 10 mM dNTPs, 0.5 µl of 40 U/µl RNase inhibitor, 1 µl of 20 U/µl of the reverse transcriptase, and DEPC-water up to 20 µl, were incubated for 2 hr at 37°; all components from Roche. PCR amplification for *CYPs 1A4, 1A5, 2H1, 2H2* and *GAPDH*, as an internal control, was performed. The primers and conditions reported (Diani-Moore et al., 2006) were used for *CYPs 1A5, 2H1,* and *GAPDH.* For *CYPs 1A4* and *2H2,* the following primers (Invitrogen, Carlsbad, CA) were used: for *CYP1A4* (Gene Bank Accession No. X99453): 5'-GGACGGAGGCTGACAAGGTG-3' and 5'-GTGCAAACGCTGAGGGATTC-3'; for *CYP2H2* (Gen Bank Accession No. M25469): 5'-GTGGGTGACTGCATAAGGAAG-3' and 5'-TCTGACGGAGTGAAGTTGTT-3'. Amplification conditions were for *CYP1A4*, 94° x 5 min for 1 cycle, 94° x 1 min, 59° x 30 s, 72° x 40 s for 30 cycles, 72° x 10 min for 1 cycle; and for *CYP2H2*, 94° x 5 min for 1 cycle, 94° x 1 min, 56° x 30 s, 72° x 40 s for 28 cycles, 72° 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; *CYP1A5*, 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

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that amplified PCR products represented the target genes of interest by DNA sequencing. Additionally, CYP1A5 and CYP2H primers recognized the targeted chicken CYPs using the UCSC In-Silico PCR application against the chicken genome (assembly date, February 2004) (http://genome.ucsc.edu/).

*SDS-polyacrylamide gel electrophoresis (SDS-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% bromophenol blue) and kept at 100$^o$ for 2 min. Microsomal protein, 20 μg per lane, was run into the stacking gel for 2hr at 15 mA and through the separating gel at 30 mA for 3-4 hr. 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$^o$. 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 hr with 3% BSA in Tris/NaCl, washed with the Tris/NaCl and then incubated with immunopurified antisera to CYP1A4 or CYP1A5 (1:400) or antiCYP2H IgG (1:1000) in 3% BSA in Tris/NaCl for 1 hr. The antiCYP2H antibody used was raised against the major PB-induced chicken CYPs, 2H1 and 2H2 (Kanetoshi et al., 1992). N-terminal amino acid sequencing of the PB-induced bands recognized by the antiserum 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 hr 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%

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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 *vs.* 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 analyses of variance (ANOVA) (Graph Pad Prism 4 software, San Diego CA). Newman-Keuls multiple comparison tests were used for post-test analysis; p values <0.05 were accepted as statistically significant.

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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 CYP 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 benzylimidazole 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% $\pm$ 4.3 ), as did R76713 (mean suppression of 53.1% $\pm$ 3.5 at 1 to 9 mg per egg) and benzylimidazole (59.4% $\pm$ 3.5) ($p < 0.001$ for all).

Fig. 3 shows representative HPLC chromatograms for CYP-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 epoxide hydrolase 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 benzylimidazole.

Table 1 shows means $\pm$ SE for the production and distribution of the four regioisomeric EETs +DHETs after treatment by the test compounds. For TCDD, the 5,6 regioisomer was the major product (mean 33% $\pm$ 1.8 of the total EET + DHET production ($p < 0.01$)), and the other regioisomers were approximately evenly distributed (21% $\pm$ 1.5 to 24% $\pm$ 1.2 (NS (not significant)). In contrast, for PB, formation of the 14,15 regioisomer was highest (40% $\pm$ 0.8 (p<



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0.001 *vs.*5,6)) and of the 5,6, lowest (7% $\pm$ 0.6). For PB overall regioisomer production was less evenly distributed than for TCDD; each product differed significantly from the others (see Table). For the imidazole derivatives, differences in regioisomer distribution were not as pronounced as for TCDD or PB. The distributions for albendazole resembled those of TCDD and for omeprazole and thiabendazole, those for PB. For R76713 and benzylimidazole the percentages of the 5,6 and 14,15 regioisomers did not differ significantly.

Analysis of variance showed no significant differences between TCDD, PB or the other test compounds with respect to relative formation of HETEs, 18-HETE (ω-2-OH AA) or 19 HETE (ω-1-OH AA) (not shown).

To investigate the basis for the increase in EETs we examined effects of the imidazole derivatives on CYP levels (Fig. 4a). All of the compounds increased total P450 1.5-fold or more over the DMSO controls (mean for DMSO: 0.22 nmol per mg protein $\pm$ 0.01 (SE)). 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 over 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. Benzylimidazole 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.

SDS-PAGE for liver microsomes from chick embryos treated with TCDD at 1 nmol per egg and the other compounds at 9 mg per egg is shown in Fig. 4b. TCDD increased staining of a broad band at 55-55.5 kDa corresponding to CYPs 1A4 and 1A5 (Gilday et al., 1996) while PB

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increased bands corresponding to CYPs 2H1 and 2H2 at 48 and 49 kDa, respectively (Nakai et al., 1992). Albendazole and thiabendazole, to a much smaller extent, like TCDD, selectively increased the CYP1A bands.

R76713 increased at least five bands in the P450 region, the CYP2H1 and 2H2 bands most, lesser increases in the CYP1A4/1A5 band, a band at 52 kDa previously shown to be increased by PB at 20 mg per egg (Nakai et al., 1992), and another unidentified band at 53 kDa, which was not increased by any of the other agents. R76713 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 CYP bands. Benzylimidazole 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 appears low in the gel shown, no effects were also seen in other gels.

Western blotting results for effects on CYPs 1A4, 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 CYPs 1A4 and 1A5 but not CYP2H while PB increased CYP2H but not CYPs 1A4 or 1A5. Albendazole, like TCDD, increased CYPs 1A4 and 1A5 and not CYP2H while thiabendazole produced only a small increase in CYP1A5. R76713 and benzylimidazole increased both CYP2H and CYPs 1A4 and 1A5. R76713 increased CYP2H more than benzylimidazole and CYPs 1A4 and 1A5 less. Omeprazole produced small increases in CYP2H and CYP1A5 but no detectable increase in CYP1A4. No effects were seen for benzimidazole.

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Liver microsomes were also subjected to Western blotting using equal amounts of P450 per lane to reveal changes in CYP distribution and induction effects not apparent at equal protein loading (see Supplemental Data). Under those conditions small increases for CYP1A4, 1A5 and CYP2H were observed for thiabendazole and benzimidazole. However, omeprazole increased only CYP1A5 as seen in Fig. 4c.

Fig. 5a shows that TCDD, R76713, albendazole, benzylimidazole and thiabendazole to a much smaller extent, increased *CYP1A4* and *1A5* mRNAs consistent with the Western blotting results, confirming a small CYP1A4-inducing effect for thiabendazole. Omeprazole, however, (Fig. 5b) increased *CYP1A5* mRNA and not *CYP1A4*, supporting its selective increase of CYP1A5 protein in chick embryo liver.

R76713 produced the greatest increase in *CYP2H* mRNA among the imidazole derivatives followed by benzylimidazole and omeprazole. Thiabendazole increased *CYP2H1* mRNA slightly, consistent with its effects on Western blots at equal P450 loading noted above (see Supplemental Data). Albendazole and TCDD failed to increase *CYP2H1* mRNA. In other experiments essentially identical results to those for *CYP2H1* were found for *CYP2H2*, which has 92% homology to *CYP2H1* (Hansen et al., 1989) (data not shown).

Summarizing the results in Fig. 4 and 5: Albendazole selectively increased CYP1A4 and 1A5 in the chick model with comparable efficacy to TCDD. The other agents produced mixed induction of both CYP1A and CYP2H. R76713, by far the most effective and potent inducer, increased CYP2H primarily and also CYP1A4 and 1A5 and some unidentified CYPs. Benzylimidazole followed R76713 in efficacy for mixed CYP1A and 2H induction, and omeprazole, thiabendazole and benzimidazole were successively less effective. CYP1A induction by omeprazole appeared to involve only CYP1A5.

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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 antiCYP1A5 IgG and unaffected by antiCYP2H IgG, while PB-induced EET formation was entirely suppressed by antiCYP2H IgG and unaffected by antiCYP1A5 IgG. Consistent with the selective induction of CYP1A by albendazole shown in Fig. 4 and 5, albendazole-enhanced EET formation was exclusively and entirely inhibited by antiCYP1A5 IgG. AntiCYP1A5 and antiCYP2H IgG each partially suppressed EET formation by R76713, benzylimidazole and omeprazole indicating that both CYPs contributed to the total EET formation by those agents, consistent with their mixed pattern of CYP 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 CYPs.

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 CYPs. 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 CYPs resided in one or both vorozole enantiomers we compared CYP induction by the vorozole racemate, R76713, and the (+) enantiomer, R83842. Both compounds increased the same bands on SDS-PAGE (Fig. 7b) and

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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 about 20% more than a maximal inducing dose of PB. On more sensitive Western blots using chemiluminescence detection (not shown) R76713 and R83842 increased CYPs 1A4 and 1A5 at 0.1 mg per egg and CYP2H even at 0.01 mg egg. Thus, the vorozole (+) enantiomer, like the racemate, can induce CYP enzymes with high potency. As 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 CYP-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 (Fig. 7c). They increased AA epoxygenation, 13.6- and 17.8-fold, respectively, at 1 mg per egg and 5.5- and 14.7-fold at 0.1 mg per egg. Thus the racemate and the (+) enantiomer were approximately equipotent and equieffective in increasing total hepatic P450, but the racemate was more effective for AA epoxygenation, indicating that the enantiomers may differ in their relative capacities to induce particular CYPs.

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 CYP 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 to the solvent controls. Albendazole was equieffective and

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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.

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# DISCUSSION

This paper reports the following new findings: (1) Imidazole drugs can increase CYP-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 CYP enzymes at doses inhibiting aromatase activity.
The first two findings are significant because they identify several therapeutic agents as potential modifiers of endogenous AA metabolite production. Further, the results here show that CYP1A, which has been regarded principally as a metabolizer and activator of toxic or carcinogenic xenobiotics (Nebert 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 (Capdevila et al., 2000; Rifkind et al., 1994). We show here that the coinduction of CYP1A and CYP2 can produce additive effects on EET formation and that the induction by each CYP 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*, as albendazole and phenobarbital, selective inducers of CYP1A and 2H respectively, failed to suppress 20-HETE.

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EETs and 20-HETE are major physiologic regulators of vascular reactivity (Roman, 2002; Kroetz and Xu, 2005). EETs mainly cause vasodilatation 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 (Chen et al., 2005; Michaelis et al., 2003;Wang et al., 2005) and 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. On the other hand, 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 GI 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 CYP2H which are selectively induced by TCDD and PB, respectively, produced relatively more of the 5,6 and 14,15 AA regioisomers, respectively,

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indicates that these CYPs 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 (a) the presence of other EET regioisomers, as all four EETs are usually generated even when one is preferentially produced, (b) endogenous CYP-dependent AA metabolites, which vary among organs and species and over which drug-induced EETs will be superimposed and (c) 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, benzylimidazole and vorozole).

Imidazole drugs induce CYP1A by AhR mediated transcriptional signaling, although they do not bind to the AhR (Curi-Pedrosa et al., 1994; Aix 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 hr after treatment with benzylimidazole or albendazole (not shown).

The following considerations support the relevance of these findings in the chick embryo to mammalian species: (1) The CYP induction responses in the chick model were qualitatively

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and quantitatively similar to those reported in mammalian species. Thus, a strong mixed CYP1A and CYP2 induction for benzylimidazole and weaker mixed induction for omeprazole and thiabendazole, selective induction of CYP1A by albendazole, and scanty effects for benzimidazole were also reported in mammalian liver (Papac and Franklin, 1988; Price et al., 2004; Masubuchi et al., 1997; Curi-Pedrosa et al., 1994; Souhaili-el Amri et al., 1988; Rey-Grobellet et al., 1996). Although in the chick model, omeprazole did not produce the high CYP1A induction seen in human liver cells, omeprazole, in particular, has shown species 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; Rifkind et al., 1995; Capdevila et al., 2000) and as noted above, the chick and human orthologs also have similar regioisomeric selectivities. (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 CYP enzyme function (see www.porphyriafoundation.com).

Our third new finding, that the aromatase inhibitor vorozole can induce hepatic CYP enzymes demonstrates that inhibition of ovarian aromatase can occur concurrently with hepatic CYP induction. Vorozole was further distinguished by its remarkably high potency and efficacy for hepatic CYP induction producing CYP induction equivalent to that of PB but at about 1/100 the dose of PB. Moreover, vorozole induced multiple CYPs, including CYPs not yet identified, as well as CYP2H and CYP1A (see Fig. 4b). Vorozole may also have posttranscriptional effects as suggested by greater increases in CYP1A mRNAs (Fig. 5) than might have been expected

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from the relatively low increases in CYP1A proteins (Fig. 4b and 4c). 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 CYP induction.

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

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REFERENCES

Aix L, Rey-Grobellet X, Larrieu G, Lesca P and Galtier P (1994) Thiabendazole is an inducer of cytochrome P4501A1 in cultured rabbit hepatocytes. *Biochem Biophys Res Commun* **202**: 1483-1489.

Capdevila J H, Falck J R, Dishman E and Karara A (1990a) Cytochrome P-450 arachidonate oxygenase. *Methods Enzymol* **187**: 385-394.

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

Capdevila J H, Karara A, Waxman D J, Martin M V, Falck J R and Guenguerich F P (1990b) Cytochrome P-450 enzyme-specific control of the regio- and enantiofacial selectivity of the microsomal arachidonic acid epoxygenase. *J Biol Chem* **265**: 10865-10871.

Chen P, Guo M, Wygle D, Edwards P A, Falck J R, Roman R J and Scicli A G (2005) Inhibitors of cytochrome P450 4A suppress angiogenic responses. *Am J Pathol* **166**: 615-624.

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

Curi-Pedrosa R, Daujat M, Pichard L, Ourlin J C, Clair P, Gervot L, Lesca P, Domergue J, Joyeux H, Fourtanier G and et al. (1994) Omeprazole and lansoprazole are mixed inducers of CYP1A and CYP3A in human hepatocytes in primary culture. *J Pharmacol Exp Ther* **269**: 384-392.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 19, 2024

DMD Fast Forward. Published on May 19, 2006 as DOI: 10.1124/dmd.106.009498
This article has not been copyedited and formatted. The final version may differ from this version.


Daikh B E, Lasker J M, Raucy J L and Koop D R (1994) Regio- and stereoselective epoxidation of arachidonic acid by human cytochromes P450 2C8 and 2C9. *J Pharmacol Exp Ther* **271**: 1427-1433.

De Coster R, Wouters W, Bowden C R, Vanden Bossche H, Bruynseels J, Tuman R W, Van Ginckel R, Snoeck E, Van Peer A and Janssen P A (1990) New non-steroidal aromatase inhibitors: focus on R76713. *J Steroid Biochem Mol Biol* **37**: 335-341.

Dhanasekaran A, Al-Saghir R, Lopez B, Zhu D, Gutterman D D, Jacobs E R and Medhora M M (2006) Protective Effects of Epoxyeicosatrienoic Acids (EETs) on Human Endothelial Cells from the Pulmonary and Coronary Vasculature. *Am J Physiol Heart Circ Physiol.*

Diani-Moore S, Labitzke E, Brown R, Garvin A, Wong L and Rifkind A B (2006) Sunlight generates multiple tryptophan photoproducts eliciting high efficacy CYP1A induction in chick hepatocytes and in vivo. *Toxicol Sci* **90**: 96-110.

Ding X and Kaminsky L S (2003) Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol* **43**: 149-173.

Gainer J V, Bellamine A, Dawson E P, Womble K E, Grant S W, Wang Y, Cupples L A, Guo C Y, Demissie S, O'Donnell C J, Brown N J, Waterman M R and Capdevila J H (2005) Functional variant of CYP4A11 20-hydroxyeicosatetraenoic acid synthase is associated with essential hypertension. *Circulation* **111**: 63-69.

Gilday D, Gannon M, Yutzey K, Bader D and Rifkind A B (1996) Molecular cloning and expression of two novel avian cytochrome P450 1A enzymes induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Biol Chem* **271**: 33054-33059.

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DMD Fast Forward. Published on May 19, 2006 as DOI: 10.1124/dmd.106.009498
This article has not been copyedited and formatted. The final version may differ from this version.


Goss P E (1998) Pre-clinical and clinical review of vorozole, a new third generation aromatase inhibitor. *Breast Cancer Res Treat* **49 Suppl 1**: S59-65; discussion S73-57.

Granville D J, Tashakkor B, Takeuchi C, Gustafsson A B, Huang C, Sayen M R, Wentworth P, Jr., Yeager M and Gottlieb R A (2004) Reduction of ischemia and reperfusion-induced myocardial damage by cytochrome P450 inhibitors. *Proc Natl Acad Sci U S A* **101**: 1321-1326.

Hansen A J, May B K and Elferink L A (1989) Sequence of a chicken phenobarbital-inducible cytochrome P450 cDNA: regulation of two P450 mRNAs transcribed from different genes. *DNA* **8**: 179-191.

Kanetoshi A, Ward A M, May B K and Rifkind A B (1992) Immunochemical identity of the 2,3,7,8-tetrachlorodibenzo-p-dioxin- and beta-naphthoflavone-induced cytochrome P-450 arachidonic acid epoxygenases in chick embryo liver: distinction from the omega-hydroxylase and the phenobarbital-induced epoxygenase. *Mol Pharmacol* **42**: 1020-1026.

Kikuchi H, Kato H, Mizuno M, Hossain A, Ikawa S, Miyazaki J and Watanabe M (1996) Differences in inducibility of CYP1A1-mRNA by benzimidazole compounds between human and mouse cells: evidences of a human-specific signal transduction pathway for CYP1A1 induction. *Arch Biochem Biophys* **334**: 235-240.

Kroetz D L and Xu F (2005) Regulation and inhibition of arachidonic acid omega-hydroxylases and 20-HETE formation. *Annu Rev Pharmacol Toxicol* **45**: 413-438.

Laemmli U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Laethem R M, Halpert J R and Koop D R (1994) Epoxidation of arachidonic acid as an active-site probe of cytochrome P-450 2B isoforms. *Biochim Biophys Acta* **1206**: 42-48.

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DMD Fast Forward. Published on May 19, 2006 as DOI: 10.1124/dmd.106.009498
This article has not been copyedited and formatted. The final version may differ from this version.


Lee C A, Lawrence B P, Kerkvliet N I and Rifkind A B (1998) 2,3,7,8-Tetrachlorodibenzo-p-dioxin induction of cytochrome P450-dependent arachidonic acid metabolism in mouse liver microsomes: evidence for species-specific differences in responses. *Toxicol Appl Pharmacol* **153**: 1-11.

Lephart E D and Simpson E R (1991) Assay of aromatase activity. *Methods Enzymol* **206**: 477-483.

Lowry O H, Rosebrough N J, Farr A L and Randall R J (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275.

Masubuchi N, Hakusui H and Okazaki O (1997) Effects of pantoprazole on xenobiotic metabolizing enzymes in rat liver microsomes: a comparison with other proton pump inhibitors. *Drug Metab Dispos* **25**: 584-589.

Michaelis U R, Fisslthaler B, Medhora M, Harder D, Fleming I and Busse R (2003) Cytochrome P450 2C9-derived epoxyeicosatrienoic acids induce angiogenesis via cross-talk with the epidermal growth factor receptor (EGFR). *Faseb J* **17**: 770-772.

Nakai K, Ward A M, Gannon M and Rifkind A B (1992) Beta-naphthoflavone induction of a cytochrome P-450 arachidonic acid epoxygenase in chick embryo liver distinct from the aryl hydrocarbon hydroxylase and from phenobarbital-induced arachidonate epoxygenase. *J Biol Chem* **267**: 19503-19512.

Nebert D W, Dalton T P, Okey A B and Gonzalez F J (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* **279**: 23847-23850.

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DMD Fast Forward. Published on May 19, 2006 as DOI: 10.1124/dmd.106.009498
This article has not been copyedited and formatted. The final version may differ from this version.


Nithipatikom K, DiCamelli R F, Kohler S, Gumina R J, Falck J R, Campbell W B and Gross G J (2001) Determination of cytochrome P450 metabolites of arachidonic acid in coronary venous plasma during ischemia and reperfusion in dogs. *Anal Biochem* **292**: 115-124.

Nithipatikom K, Gross E R, Endsley M P, Moore J M, Isbell M A, Falck J R, Campbell W B and Gross G J (2004) Inhibition of cytochrome P450omega-hydroxylase: a novel endogenous cardioprotective pathway. *Circ Res* **95**: e65-71.

Nithipatikom K, Moore J M, Isbell M A, Falck J R and Gross G J (2006) Epoxyeicosatrienoic acids (EETs) in cardioprotection: Ischemic versus reperfusion injury. *Am J Physiol Heart Circ Physiol*.

Omura T and Sato R (1964) The Carbon Monoxide-Binding Pigment of Liver Microsomes. Ii. Solubilization, Purification, and Properties. *J Biol Chem* **239**: 2379-2385.

Papac D I and Franklin M R (1988) N-benzylimidazole, a high magnitude inducer of rat hepatic cytochrome P-450 exhibiting both polycyclic aromatic hydrocarbon- and phenobarbital-type induction of phase I and phase II drug-metabolizing enzymes. *Drug Metab Dispos* **16**: 259-264.

Pozzi A, Macias-Perez I, Abair T, Wei S, Su Y, Zent R, Falck J R and Capdevila J H (2005) Characterization of 5,6- and 8,9-epoxyeicosatrienoic acids (5,6- and 8,9-EET) as potent in vivo angiogenic lipids. *J Biol Chem* **280**: 27138-27146.

Price R J, Scott M P, Walters D G, Stierum R H, Groten J P, Meredith C and Lake B G (2004) Effect of thiabendazole on some rat hepatic xenobiotic metabolising enzymes. *Food Chem Toxicol* **42**: 899-908.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 19, 2024

Rey-Grobellet X, Ferre N, Eeckhoutte C, Larrieu G, Pineau T and Galtier P (1996) Structural requirements for the induction of cytochromes P450 by benzimidazole anthelmintic derivatives in cultured rabbit hepatocytes. *Biochem Biophys Res Commun* **220**: 789-794.

Rifkind A B, Kanetoshi A, Orlinick J, Capdevila J H and Lee C (1994) Purification and biochemical characterization of two major cytochrome P-450 isoforms induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in chick embryo liver. *J Biol Chem* **269**: 3387-3396.

Rifkind A B, Lee C, Chang T K and Waxman D J (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 R J (2002) P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* **82**: 131-185.

Seubert J, Yang B, Bradbury J A, Graves J, Degraff L M, Gabel S, Gooch R, Foley J, Newman J, Mao L, Rockman H A, Hammock B D, Murphy E and Zeldin D C (2004) Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K+ channels and p42/p44 MAPK pathway. *Circ Res* **95**: 506-514.

Souhaili-el Amri H, Fargetton X, Benoit E, Totis M and Batt A M (1988) Inducing effect of albendazole on rat liver drug-metabolizing enzymes and metabolite pharmacokinetics. *Toxicol Appl Pharmacol* **92**: 141-149.

Swain S M (2005) Aromatase inhibitors--a triumph of translational oncology. *N Engl J Med* **353**: 2807-2809.

Wang Y, Wei X, Xiao X, Hui R, Card J W, Carey M A, Wang D W and Zeldin D C (2005) Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and

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DMD Fast Forward. Published on May 19, 2006 as DOI: 10.1124/dmd.106.009498
This article has not been copyedited and formatted. The final version may differ from this version.


angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways. *J Pharmacol Exp Ther* **314**: 522-532.

Yu Z, Ng V Y, Su P, Engler M M, Engler M B, Huang Y, Lin E T and Kroetz D L (2006) Induction of Renal Cytochrome P450 Arachidonic Acid Epoxygenase Activity by {gamma}-Linolenic Acid. *J Pharmacol Exp Ther*.

Zeldin D C, Moomaw C R, Jesse N, Tomer K B, Beetham J, Hammock B D and Wu S (1996) Biochemical characterization of the human liver cytochrome P450 arachidonic acid epoxygenase pathway. *Arch Biochem Biophys* **330**: 87-96.

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## FOOTNOTES

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# FIGURE LEGENDS

**Figure 1. Chemical structures.** Structures of the following imidazole derivatives are shown: albendazole (methyl 5-(propylthio)-2-benzimidazolecarbamate); vorozole ((6-[(4-chlorophenyl)(1H-1,2,4-triazol-1-yl)methyl]-1-methyl-1H- benzotriazole); thiabendazole (2-(4-thiazolyl)benzimidazole); omeprazole (5-Methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]-1H-benzimidazole); benzimidazole (1,3-benzodiazole); benzylimidazole (1-(phenylmethyl)-1H-imidazole). Structures of phenobarbital (5-ethyl-5-phenylbarbituric acid) and TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), used here as positive controls, are also shown.

**Figure 2. Effect of imidazole derivatives on CYP-dependent arachidonic acid (AA) metabolism.** CYP-dependent AA epoxygenase activity was measured as described in "Materials and Methods" in liver microsomes from 17 day-old chick embryos that had been treated for 48 hr with albendazole (ALB), R76713, thiabendazole (TBZ), omeprazole (OP), benzimidazole (BZ), benzylimidazole (BZY), or phenobarbital (PB) (grey 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 $\pm$ SE) 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 2 separate replicates. $*p<0.05$; $**p<0.01$ *vs.* DMSO.

**Figure 3. HPLC chromatograms for CYP-dependent AA metabolism in chick embryo liver microsomes 48 hr after treatment with the imidazole derivates**. Representative reverse-phase

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HPLC chromatograms for AA metabolism by liver microsomes from 17 day-old chick embryos 48 hr after treatment with PB (20 mg/egg), TCDD (1 nmol/egg), imidazole derivatives (9 mg/egg) or the DMSO control. Assay and chromatography procedures were as described in "Materials and Methods". Abbreviations as in Fig. 2. The chromatograms shown reflect AA metabolites generated by 50% of reaction mixtures at similar concentrations of microsomal protein per reaction mixture and total cpm. Peak identities are shown as an example in the PB chromatogram. Elution times were: 14,15-DHET, 12-12.5 min; 11,12-DHET; 13-13.2 min; 8,9-DHET, 13.5-14.5 min; ω-1 OH AA (19-HETE), 15-15.5 min; ω-OH AA, (20-HETE), 15.6-16.1 min; ω-2 OH AA (18-HETE), 16.5-17 min; other HETEs, 17.1-20.1 min; 5,6-EET lactone (δ-lactone), 20.-.5-22 min; 14,15-EET, 21.6-23min; 11,12- EET, 23.5-24 min; 8,9-EET, 24.4-24.8, 5,6-EET, 25–25.5 min; AA, 32.1-33 min. Non-CYP mediated polar products and non-enzymatic breakdown products eluting between 0 and 10 min are not shown.

**Figure 4. Effects of the imidazole derivatives on cytochrome P450. a. Total P450**. Liver microsomal CYP levels were measured by difference spectroscopy as described in "Material and Methods". Liver microsomes were from 17 day-old chick embryos 48 hr after treatment with imidazole derivatives, or with PB or TCDD, or with 0.1 ml of DMSO. Doses for the drugs (mg/egg) are shown below the x axis. TCDD was administered at 1 nmol (0.0003 mg)/egg. Mean values $\pm$ SE are for n=3 or more independent determinations . **p<0.01 *vs*. DMSO. **b. SDS-polyacrylamide gel electrophoresis** (SDS-PAGE). Liver microsomes from 17 day-old chick embryos treated for 48 hr as described above or with the imidazole derivatives at 9 mg per egg. 20 µg of microsomal protein per lane. Arrows show bands increased compared to the DMSO control: 55, 53, 52, 49, 48 kDa (top to bottom); MW, molecular weight in kDa. **c. Western**

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**Blotting.** Liver microsomes from chick embryos treated as for Fig. 4b were subjected to Western blotting as described in "Materials and Methods" using monospecific immunopurified antisera to CYP1A4 (top panel) or CYP1A5 (middle panel) or antiserum to CYP2H1/2H2 (lower panel). The Western blots for CYP1A4 and 1A5 (two top panels) contained purified CYP1A4 and 1A5 on the same blots. The dashed lines show where lanes containing duplicative control treatments were excised in the preparation of the image. Lanes 1-9, liver microsomes (10 μg/lane); lane 10, 1 pmol of purified CYP1A4; lane 11, 1 pmol of purified CYP1A5.

**Figure 5. *CYP1A4*, *CYP1A5*, and *CYP2H1* expression by imidazole derivatives.** RT-PCR was performed as indicated in "Materials and Methods" using gene specific primers and total RNA from livers of chick embryos treated for 48 hr with 9 mg of the imidazole derivatives, and 9 mg PB or 1 nmol of TCDD or the indicated vehicle controls. The results shown are representative of two to three independent experiments. Specific PCR products for *CYP1A4*, *CYP1A5*, *CYP2H1* (a and b) are 404, 219, and 286 bp, respectively. *GAPDH* (170 bp) was used as an internal control. Abbreviations for imidazole derivatives as in Fig. 2. NaCl, sodium chloride; bp, base pair marker (top to bottom): 653, 517, 453, 394, 298, 234/220, 154 bp.

**Figure 6. Induction of AA epoxygenase activity by imidazole derivatives is mediated by CYP1A5, CYP2H or both.** Epoxygenase activity (EET and DHET formation) by liver microsomes from 17 day-old chick embryos 48 hr after treatment with TCDD, PB, DMSO, or the different imidazole derivatives. AA metabolites were measured as described in "Material and Methods". For each treatment group, reaction mixtures containing microsomes at the same amounts of total P450 (0.05 nmol per reaction mixture) were incubated with the other ingredients

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of the AA metabolism reaction mixture, see "Materials and Methods" with antiCYP1A5 (grey bars) or antiCYP2H IgG (clear bars) or pre-immune IgG (black bars) at 5 mg per nmol of P450 as previously shown to produce maximal inhibition of the target CYP activity. The preimmune IgG was not inhibitory. Mean data for duplicate determinations are shown.

**Figure 7. Suppression of aromatase activity by R76713 and comparison of CYP inducing effects of R76713 and R83842 at low doses. a. Aromatase inhibition by R76713.** Ovarian aromatase activity was measured as described in "Materials and Methods". Mean values are shown. Dots indicate values for separate groups of eggs at each dose. **b. Effects of R76713 and R83842 on SDS-PAGE**. SDS-PAGE on liver microsomes from 17 day old chick embryos treated for 48 hr with R76713 or its (+) enantiomer, R83842, at 0.01, 0.1 or 1 mg per egg, or with PB at 9 or 20 mg per egg, TCDD at 1 nmol per egg, or DMSO (20 µg microsomal protein per lane). Arrows point to increased bands for CYP1A, CYP2H and a broad unidentified band at 53/52 kDa. **c. Effects of R76713 and R83842 on total P450 and CYP dependent AA metabolism.** Total P450 and CYP-dependent AA metabolism were measured as described in "Material and Methods" in liver microsomes from 17 day-old chick embryos treated for 48 hr with R76713 (squares, solid lines) or R83842 (triangles, dotted lines). The DMSO control value for 20-HETE is indicated by a filled circle on the y axis. Means $\pm$ SE are shown for three experiments for R76713 and two for R83842 (dots for individual means). Error bars or dots not shown are hidden within the symbols. Mean values for PB, 20 mg per egg, and TCDD, 1 nmol per egg, in the experiments shown were: for total P450, 1.43 and 0.34 nmol/mg protein, respectively; for EETs and DHETs, 0.84 and 0.81 nmol/mg protein/min and for 20-HETE, 0.36 and 0.15 nmol/mg protein per min, respectively.

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**Figure 8. Effect of imidazole derivatives on EROD activity in mouse Hepa 1-6 and human HepG2 cells.** Mouse Hepa 1-6 (left panel) or human HepG2 cells (right panel) were plated (0.5 x $10^6$ cells/well) in 24-well plates. At semiconfluence cells were treated (triplicate wells for each treatment) with the imidazole derivates shown in the figure, β-NF, PB or with the solvent (DMSO). After 24 hr EROD was measured as described in "Materials and Methods". Mean values ± SE are shown. UT, untreated. *$p<0.05$; **$p<0.01$ *vs.* DMSO.

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TABLE 1

*Regioisomeric production and distribution of arachidonic acid epoxides in response to 48 hr treatment with TCDD, PB, and imidazole derivatives.*

Each regioisomer reflects the sum of the EETs + DHETs; 5,6-EET includes the 5,6-δ-lactone. The values in parentheses represent the regioisomers as a percent ± SE of total EETs + DHETs (100%). TCDD, 1 nmol/egg; PB, 20 mg/egg; R76713 for 1, 3 and 9 mg/egg combined (all produced maximal induction and did not differ significantly); all others, 9 mg/egg. Total EETs + DHETs for the DMSO control were (n = 10) 0.048±0.013 (nmol/mg microsomal protein/min).

| | | EETs + DHETs | | | | |
|---|---|---|---|---|---|---|
| Treatment | n | 14,15 | 11,12 | 8,9 | 5,6 | Total |
| | | *nmol/mg microsomal protein/min* | | | | |
| TCDD | 11 | 0.188±0.019<br>(22±1.2)[c***] | 0.175±0.014<br>(21±1.5) | 0.200±0.020<br>(24±1.2)[c***] | 0.283±0.029<br>(33±1.8)[a, c***] | 0.844±0.067 |
| PB | 3 | 0.339±0.014<br>(40±0.8)[a, b***] | 0.162±0.019<br>(19±2)[a] | 0.294±0.001<br>(34±0.9)[a, b**] | 0.067±0.003<br>(7±0.6)[a, b***] | 0.862±0.027 |
| ALB | 4 | 0.161±0.014<br>(25±0.9)[c**] | 0.123±0.014<br>(19±1.1)[a] | 0.168±0.009<br>(27±1.8) | 0.182±0.011<br>(29±0.3)[c***] | 0.635±0.043 |
| BZY | 4 | 0.193±0.033<br>(32±0.7)[b***, c**] | 0.145±0.024<br>(24±0.6) | 0.142±0.028<br>(23±1.2)[c*] | 0.126±0.017<br>(21±1.6)[b**, c*] | 0.605±0.099 |
| OP | 4 | 0.191±0.005<br>(38±1.6)[a, b***] | 0.108±0.009<br>(22±1.3)[a] | 0.140±0.008<br>(28±1.4)[a] | 0.058±0.007<br>(12±1.3)[a, b***] | 0.497±0.018 |
| R76713 | 13 | 0.178±0.018<br>(28.5±1.6)[b**, c**] | 0.119±0.008<br>(19.5±0.7)[a] | 0.190±0.013<br>(31±1.2)[b**] | 0.130±0.013<br>(21±1.7)[a, b***, c**] | 0.616±0.040 |
| TBZ | 4 | 0.045±0.008<br>(35±4.3)[b***] | 0.024±0.006<br>(20±4.5) | 0.038±0.008<br>(29±4.4) | 0.021±0.005<br>(16±3.4)[b***] | 0.128±0.013 |

[a] Significantly different from all other regioisomers for the same treatment (except ALB for which 11,12 differed only from 5,6 and 8,9). [b] Relative regioisomer production significantly different from TCDD. [c] Relative regioisomer production significantly different from PB. For [b] and [c]: *p < 0.05, **p < 0.01, ***p < 0.001. For [a]: TCDD, p<0.01; PB, p<0.01, except p<0.05 for



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8,9 *vs.* 14,15; ALB, $p<0.05$; OP, $p<0.001$, except $p<0.01$ for 11,12 *vs.* 8,9; R76713, $p<0.001$, except $p<0.05$ for 11,12 *vs.* 5,6 and 14,15 *vs.* 8,9. n= number of independent experiments.

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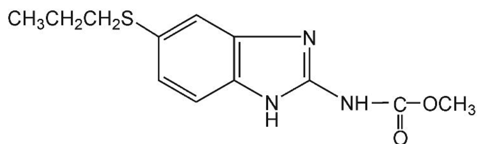
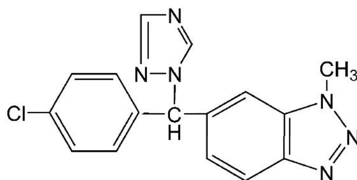
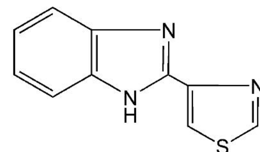
Figure 1



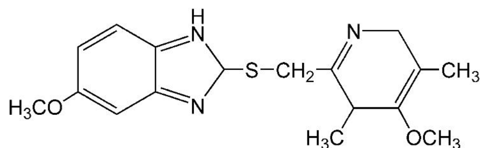
Albendazole



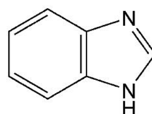
Vorozole



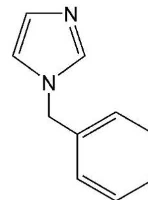
Thiabendazole



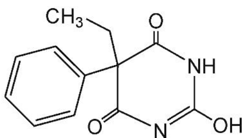
Omeprazole



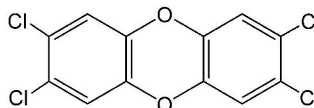
Benzimidazole



Benzylimidazole

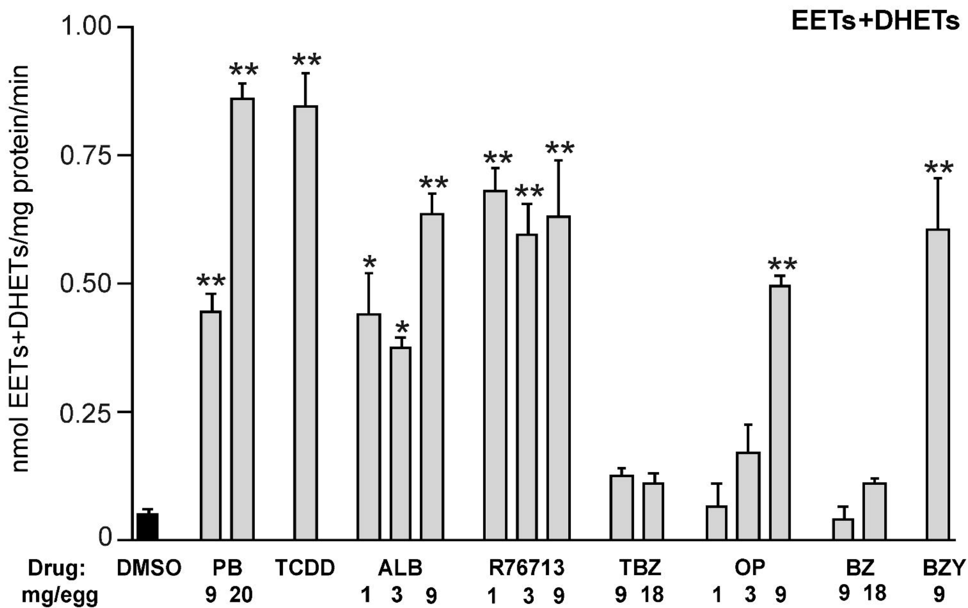


Phenobarbital



TCDD

Figure 2




b

20-HETE

nmol 20-HETE/mg protein/min

0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0

** ** ** ** **

Drug: DMSO PB TCDD ALB R76713 TBZ OP BZ BZY

mg/egg 9 20 1 3 9 1 3 9 9 18 1 3 9 9 18 9

Figure 3

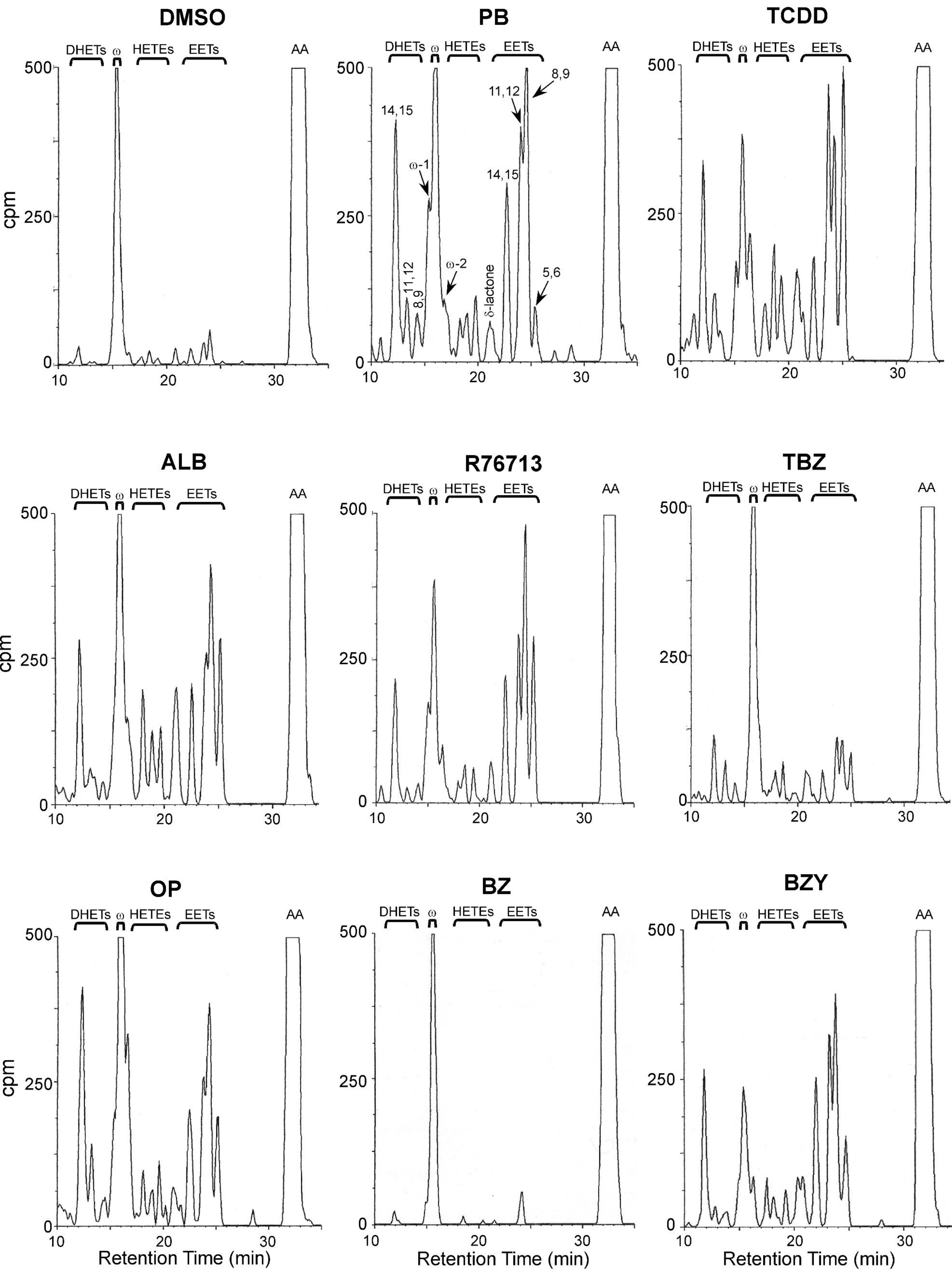
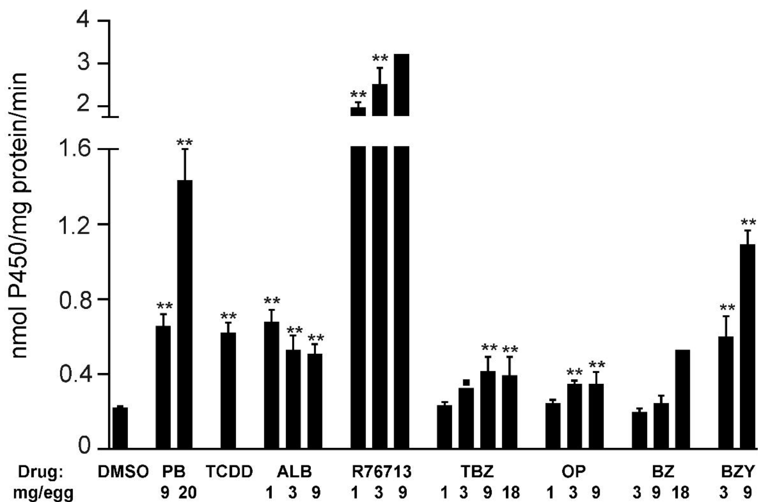
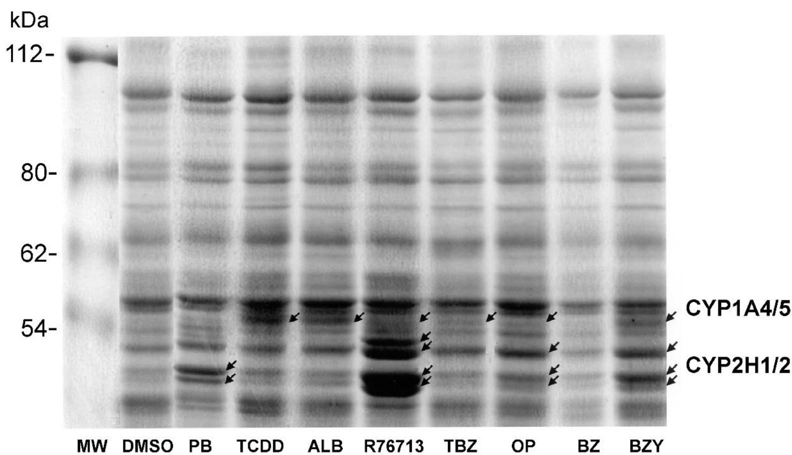


Figure 4



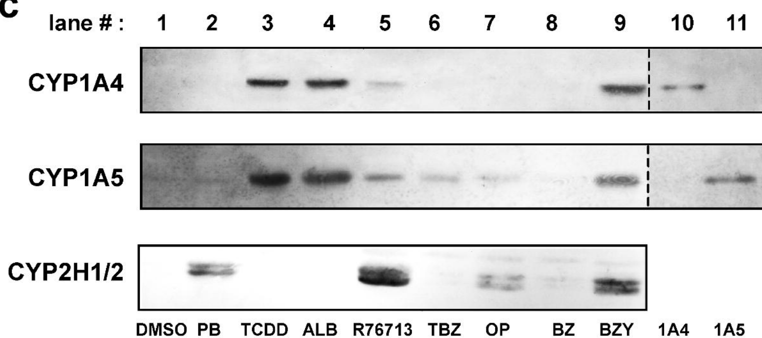



Figure 5

a

ALB BZY R76713 TBZ NaCl PB Dioxane TCDD bp

CYP1A4 →
GAPDH →

CYP1A5 →
GAPDH

CYP2H1 →
GAPDH →

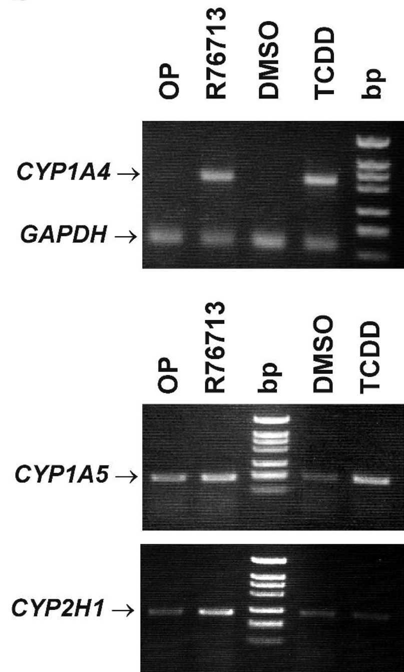


Figure 6

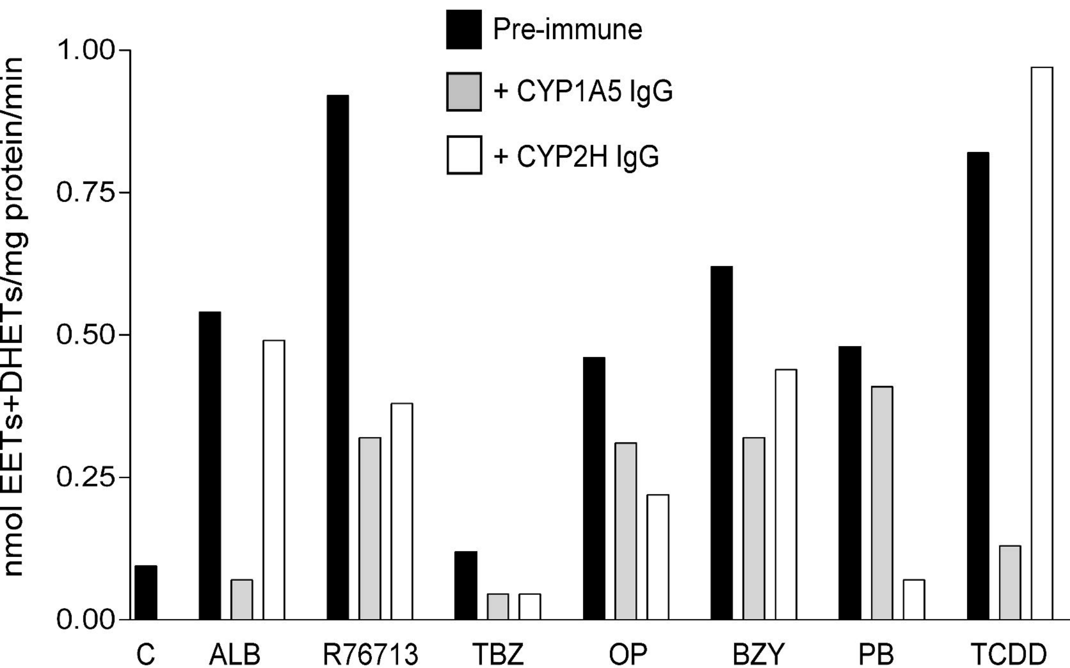
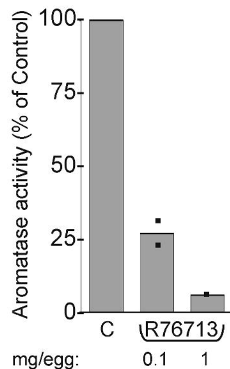
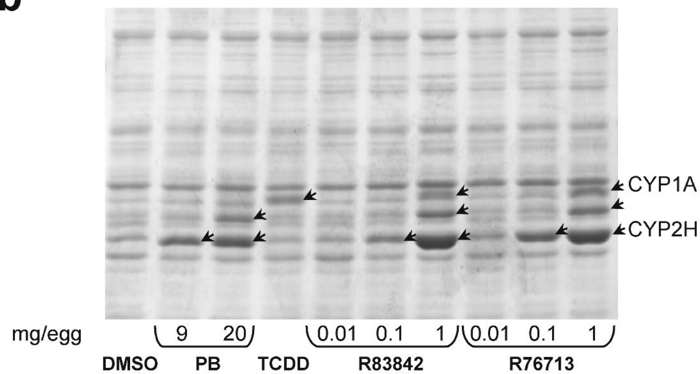


Figure 7

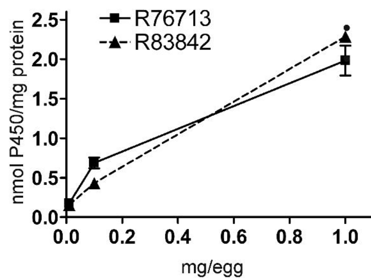
**a**

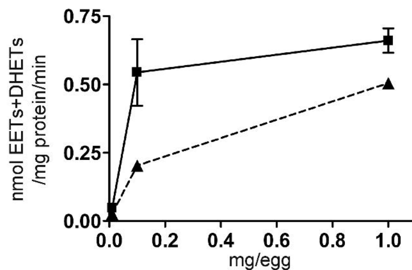



**b**




**c**



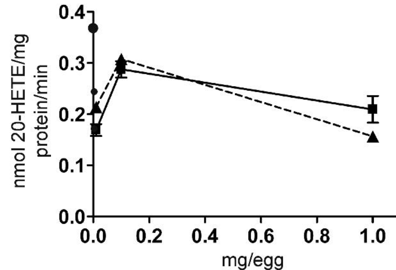



Figure 8