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# INHIBITION OF EPOXIDE HYDROLASE FROM HUMAN, MONKEY, BOVINE, RABBIT AND MURINE LIVER BY *TRANS*-3-PHENYLGLYCIDOLS

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**Abstract**—1. *trans*-3-Phenylglycidols were potent inhibitors of cytosolic epoxide hydrolases in all species tested.

2. The order of inhibitor potency varied from species to species but *trans*-3-(4-nitrophenyl)glycidols were always the most potent inhibitors tested for cytosolic epoxide hydrolase.

3. The S,S-enantiomer was a more potent cytosolic epoxide hydrolase inhibitor than the R,R-enantiomer when a free hydroxyl group was present. However, (2R,3R)-1-benzoyloxy-2,3-epoxy-3-(4-nitrophenyl)propane was always a better inhibitor than the (2S,3S)-enantiomer.

4. All microsomal epoxide hydrolases were poorly inhibited by the *trans*-3-phenylglycidols, and related compounds, tested. The best new microsomal epoxide hydrolase inhibitor tested was (1S,2S)-1-phenylpropylene oxide which gave 18–63% inhibition, at 2 mM, depending on the species tested.

## INTRODUCTION

Epoxide hydrolases (EC 3.3.2.3) have been found in all mammals studied (Armstrong, 1987; Meijer and DePierre, 1988; Oesch, 1973; Wixtrom and Hammock, 1985) and are also found in other vertebrates (Gregus *et al.*, 1983; Lauren *et al.*, 1989; Knight and Walker, 1982; Walker *et al.*, 1978), insects (Brooks *et al.*, 1970; Hammock and Quistad, 1981; Ottea and Hammock, 1986; Harshman *et al.*, 1991), fungi (Kolattukudy and Brown, 1975), other plants (Croteau and Kolattukudy, 1975; Kolattukudy *et al.*, 1973), and bacteria (Michaels *et al.*, 1980). Epoxide hydrolases catalyze the addition of water to the electrophilic epoxide ring producing a diol (a dihydrodiol in the case of epoxides derived from aromatic systems). In general, this represents a detoxication step since the diol product is much more polar and may be excreted or further metabolized (Thomas and Oesch, 1988; Wixtrom and Hammock, 1985). Indeed, epoxide hydrolases have been shown to decrease mutagenicity of epoxide-containing compounds in the Ames assay (El-Tantawy and Hammock, 1980; Oesch, 1988). However, some diols, such as benzo(a)pyrene-4,5-dihydrodiol, are metabolized to more toxic compounds (Bentley *et al.*, 1977; Sims *et al.*, 1974).

Cytosolic epoxide hydrolase (CEH) is one of four distinct epoxide hydrolases (Levin *et al.*, 1983; Thomas and Oesch, 1988; Meijer and DePierre, 1988; Wixtrom and Hammock, 1985). The others are microsomal (MEH), cholesterol, and leukotriene $A_4$ epoxide hydrolases. CEH is distinguished by its physical properties (Dietze *et al.*, 1990), location in the cell, substrate specificities, inhibitor specificities, and induction by xenobiotics (Meijer and DePierre, 1988; Wixtrom and Hammock, 1985). It has been studied primarily in the mouse (*Mus musculus*). Both CEH and MEH, characteristic of xenobiotic metabolizing enzymes, accept a wide range of substrates (Wixtrom and Hammock, 1985). MEH is thought to be mainly involved in the hydrolysis of epoxides generated from oxidative metabolism of xenobiotics (Lu and Miwa, 1980; Seidegard and DePierre, 1983). The endogenous role of CEH has not yet been determined. However, given the rapid turnover of lipid epoxides by CEH, it is thought that they may be important in the hydrolysis of lipid epoxides *in vivo* (Halarnkar *et al.*, 1989; Hammock *et al.*, 1985).

Mouse liver is a rich, readily available source of CEH. Affinity purification from mouse liver yields large amounts of CEH rapidly (Prestwich and Hammock, 1985; Wixtrom *et al.*, 1988). However,

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*Abbreviations*—CEH: cytosolic epoxide hydrolase; MEH: microsomal epoxide hydrolase; TSO: *trans*-stilbene oxide; CSO: *cis*-stilbene oxide; PPO: *trans*-1-phenylpropylene oxide; NPGO; *trans*-3-(4-nitrophenyl) glycidol; PGO: *trans*-3-phenylglycidol; EPB: *trans*-3,4- epoxy-4-(4-nitrophenyl)-2-butanol; BEP: *trans*-1-benzoyloxy-2,3-epoxy-3-(4-nitrophenyl)propane; EPP: *trans*-2,3- epoxy-3-(4-nitro-phenyl)-1-phenylpropanol; 4-FCO:4-fluorochalcone oxide; BSA: bovine serum albumin; DFP: diisopropyl fluorophosphate.

despite many physical and structural similarities (Dietze *et al.*, 1990; Silva and Hammock, 1987), it is unclear that the mouse is an ideal model system for extrapolation to man (*Homo sapiens*). There are substantial differences in substrate specificity between murine and human CEH (Halarnkar *et al.*, 1989; Meijer and DePierre, 1988; Silva and Hammock, 1987) and a second soluble epoxide hydrolase, resembling MEH, has been characterized in human liver (Schaldt *et al.*, 1988). Only a single form of murine CEH has been described to date. In this study a new class of CEH inhibitors (Dietze *et al.*, 1991), *trans*-3-phenylglycidols (Table 1), have been used to characterize CEH, and MEH, from human, rhesus monkey (*Macaca mulatta*), bovine (*Bos taurus*), rabbit (*Oryctolagus cuniculus*), and murine liver. Studies with these inhibitors will allow further assessment of the similarity of CEH across species boundaries and provide additional information on the suitability of the mouse as a model animal for the study of human CEH.

## MATERIALS AND METHODS

### *Chemicals*

Chiral *trans*-3-phenylglycidols, cyclohexene oxide, *trans*-stilbene oxide (TSO), and all synthetic precursors were purchased from Aldrich Chemicals (Milwaukee, WI). Bovine serum albumin, fraction V (BSA), disodium EDTA, and diisopropyl fluorophosphate (DFP) were purchased from Sigma (St Louis, MO). Clofibrate was a gift from Ayerst (New York, NY). All water was deionized and glass-distilled. Buffer salts were obtained from Fisher (Pittsburgh, PA). BCA reagent was from Pierce Chemical (Rockford, IL) and Bradford reagent from Bio-Rad (Richmond, CA). All other reagents were of the best quality commercially available.

*cis*-Stilbene oxide (CSO) and 4-fluorochalcone oxide (4-FCO) were synthesized by *meta*-chloroperbenzoic acid or hydrogen peroxide oxidation of *cis*-stilbene and 4-fluorochalcone respectively (Schwartz and Blumbergs, 1964; Mullin and Hammock, 1982). [$^3$H]-TSO and [$^3$H]-CSO were synthesized according to Hammock *et al.* (1984). *trans*-2,3-Epoxy-3-(4-nitrophenyl)-1-phenylpropanol (EPP) and the (2S,3S)- and (2R,3R)-enantiomers of *trans*-1-benzoyloxy-2,3-epoxy-3-(4-nitrophenyl)propane (S-BEP and R-BEP) were synthesized as described previously (Dietze *et al.*, 1991).

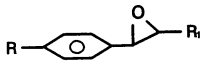
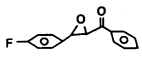
*trans*-3,4-Epoxy-4-(4-nitrophenyl)-2-butanol (EPB) was synthesized by the same method as EPP starting from *trans*-3,4-epoxy-4-(4-nitrophenyl)butan-2-one. The purified product gave one u.v. dense spot by thin layer chromatography with two different solvent systems. The molecular weight of EPB, $C_{10}H_{11}NO_4$, was calculated to be 209.0688 and gave an M + 1 peak with an *m/e* of 210.0749 by high resolution mass spectrometry. The mass spectrum was obtained using a VG-TRIO-2 mass spectrometer coupled to a VG11-250 data system (VG Instruments, Altrincham, England). Chemical ionization with *iso*-butane was employed. The [$^1$H]-NMR spectrum was: $d = 1.36$ (3H, *d*, $J = 6$ Hz), 2.1 (1H, *d*, $J = 21$ Hz, $D_2O$ exchangeable), 3.04 (1H, s), 4.03 (2H, dd, $J_a = 18$ Hz, $J_b = 39$ Hz), 7.44 (2H, *d*, $J = 3$ Hz), 8.21 (2H, *d*, $J = 9$ Hz). The NMR spectrum was collected with a Varian EM-290 spectrometer (Varian Assoc., Palo Alto, CA) and the sample was dissolved in $CDCl_3$ containing tetramethylsilane as an internal standard.

### *Tissue samples*

Human liver was obtained from the SRI organ bank (Menlo Park, CA) and was stored at −80°. The liver used in this study was from a 30-year-old female motorcycle accident victim. The liver was removed, perfused with urocolins, and, not being suitable for transplant, transported to SRI on ice and frozen in liquid nitrogen. The victim tested negative for alcohol, drugs, HIV and hepatitis. Other information on the donor is unknown.

Liver samples from monkey, cow, and rabbit were taken from animals which were not sacrificed specifically for epoxide hydrolase isolation and testing. The monkey liver was from a male rhesus monkey, No. MMU 7457, which died accidentally. It was obtained from the Northern California Regional Primate Research Center (Davis, CA).

Table 1. Structure of inhibitors used for testing epoxide hydrolase

| Inhibitor | Structure | | Enantiomers Tested |
|---|---|---|---|
| |  | | |
| | R | $R_1$ | |
| PPO | H | $CH_3$ | R,R/S,S |
| PGO | H | $CH_2OH$ | R,R/S,S |
| NPGO | $NO_2$ | $CH_2OH$ | R,R/S,S |
| BEP | $NO_2$ | $CH_2OC(O)C_6H_5$ | R,R/S,S |
| EPP | $NO_2$ | $CH(OH)C_6H_5$ | Racemic |
| EPB | $NO_2$ | $CH(OH)CH_3$ | Racemic |
| 4-FCO |  | | Racemic |
| Cyclohexene oxide |  | | Racemic |

The bovine liver was obtained fresh from a cow which had been routinely sacrificed by the Department of Animal Science at UC Davis, CA. It was kept on ice, cut into approximately 50 g sections, and frozen. Rabbit liver was taken from male New Zealand white rabbits which had been sacrificed for antibody production. Rabbits were purchased from Herbert's Rabbitry (Plymouth, CA) and were housed at 23°C in a stainless steel cage with a 12 hr light/dark cycle. The rabbits were fed Purina Rabbit Chow (Purina Mills, St Louis, MO) and given water *ad libitum*. The rabbits were sacrificed by lethal injection and their livers were removed, sectioned and frozen immediately. Male Swiss Webster mice, 20–25 g, were obtained from Charles River (Cambridge, MA) and housed in the same manner as the rabbits. The mice were kept on a diet of Purina Rodent Chow for 1 week after receipt and then switched to a diet of Ground Rodent Chow, corn oil and clofibrate (94.5/5/0.5, wt/wt/wt) for 2 weeks. [Clofibrate has been shown to induce liver CEH in the mouse. The induced and uninduced enzymes are indistinguishable (Dietze *et al.*, 1990; Meijer and DePierre, 1988).] The animals were then sacrificed by cervical dislocation. The livers were harvested, perfused with ice-cold 1.15% KCl, and immediately processed. All liver samples not immediately processed were stored at −80°C.

*Enzyme preparation*

Purified CEH, from all sources, was prepared from cytosol essentially as described (Wixtrom *et al.*, 1988). In addition, DFP was added to the homogenization buffer (0.1 mM final concentration) to help block proteolysis of CEH during preparation. In brief the purification procedure is outlined below. Liver was homogenized in ice-cold pH 7.4 phosphate buffer (76 mM, 0.1 mM EDTA, 0.1 mM DFP). A 20% wt/vol (liver/buffer) homogenate was made using either fresh or frozen liver. The homogenate was centrifuged at 10,000 *g* for 20 min at 4°C and the resulting supernatant centrifuged at 100,000 *g* for 60 min at 4°C. The supernatant from the 100,000 *g* spin, the cytosolic fraction, was removed, frozen in 3 ml aliquots, and stored at −80°C until needed. The pellet from the 100,000 *g* spin, the microsomal fraction, was washed once with 100 mM pH 9.0 Tris buffer as follows. The microsomes were resuspended in a volume of Tris buffer equal to the volume of phosphate buffer used for homogenization, repelleted at 100,000 *g*, and, finally, resuspended in a volume of Tris equal to the weight of liver homogenized. Three-milliliter aliquots were frozen and stored at −80°C until needed. No loss of activity was seen in either the cytosol or the microsomes during storage (data not shown).

The activities of the purified CEH and the MEH from the washed microsomes were assayed with 50 μM TSO and 80 μM CSO, respectively (Wixtrom and Hammock, 1985). Unless otherwise indicated, 76 mM phosphate buffer (pH 7.4, 0.1 mM EDTA, 0.1 mg/ml BSA) was used for all CEH assays and inhibition studies and 100 mM Tris buffer (pH 9.0) was used for all MEH assays and inhibition studies. No attempt was made to optimize assay conditions for each species. However, the linear range of the assays was determined for both CEH and MEH in all species (data not shown). In the case of purified CEH, protein concentrations were determined using the BCA assay modified for use in a 96-well microtiter plate (Dietze *et al.*, 1990). In all other cases, the Bradford assay (Bradford, 1976) was used. This assay was also modified for use in a 96-well microtiter plate by using 0.30 ml of working strength Bradford reagent in each well and adding 50 μl of the standard or unknown. The plate was shaken, allowed to incubate at room temperature for 15 min and then read. BSA was used as the standard protein in all cases. The protein content and specific activities of all preparations are reported in Table 2. The specific activities of monkey, bovine, rabbit and murine MEH (Silva and Hammock, 1987; Wisniewski *et al.*, 1987) as well as purified human and murine CEH (Dietze *et al.*, 1990) were consistent with prior results.

*Inhibition of CEH and MEH*

Stock solutions (100×) of inhibitor were prepared fresh in either absolute ethanol or acetone. With the exception of murine samples, inhibitions were carried out at least two times in quadruplicate on two different preparations of CEH and MEH. Inhibition of murine CEH was carried out four times in triplicate on samples from one purification. The $I_{50}$ values observed were consistent with those seen in an earlier study (Dietze *et al.*, 1991). 4-FCO and cyclohexene oxide inhibitions were done three times in quadruplicate for all samples tested and were included in order

Table 2. Concentration and specific activity of epoxide hydrolase preparations used for inhibition studies*

| Source | Microsomal EH | Cytosolic EH |
|---|---|---|
| Human | | |
| Concentration | 4.3 | 0.090 |
| Specific activity | 29 (1.8) | 395 (24) |
| Dilution used | 1/200 | 1/100 |
| Monkey | | |
| Concentration | 5.8 | 0.025 |
| Specific activity | 46 (5.1) | 520 (53) |
| Dilution used | 1/350 | 1/100 |
| Bovine | | |
| Concentration | 4.2 | 0.10 |
| Specific activity | 60 (7.8) | 750 (34) |
| Dilution used | 1/250 | 1/250 |
| Rabbit | | |
| Concentration | 2.5 | 0.20 |
| Specific activity | 68 (6.7) | 411 (39) |
| Dilution used | 1/250 | 1/50 |
| Murine | | |
| Concentration | 3.9 | 0.91 |
| Specific activity | 5.4 (0.12) | 1600 (140) |
| Dilution used | 1/60 | 1/1000 |

*Protein concentrations determined by the Bradford assay (MEH) or BCA assay (CEH). Specific activities determined at 37°C and at pH 9.0 with 80 μM CSO (MEH) or pH 7.4 with 50 μM TSO (CEH). Value for specific activity is the mean (standard deviation) with $N = 6$. Concentration (mg/ml); specific activity (nmol/min/mg).

to facilitate comparison with prior studies. For CEH inhibition, *trans*-1-phenylpropylene oxide (S- and R-PPO), *trans*-3-phenylglycidol (S- and R-PGO), and (2R,3R)-3-(4-nitrophenyl)glycidol (R-NPGO) were tested between 5000 and 10 $\mu$M final concentration. S- and R-BEP were tested between 1000 $\mu$M (the approximate solubility limit) and 0.1 $\mu$M. (2S,3S)-3-(4-Nitrophenyl)glycidol (S-NPGO), EPP, and EPB were tested between 500 and 0.1 $\mu$M. 4-FCO was tested between 24 and 0.10 $\mu$M. For MEH, the S,S- and R,R-enantiomers of PPO, PGO, NPGO, EPP and EPB were tested at 2000, 500 and 125 $\mu$M. The (2S,3S)- and (2R,3R)-enantiomers of BEP were tested at 125 and 62.5 $\mu$M. Finally, cyclohexene oxide was tested at 10 $\mu$M.

In all cases, an aliquot of 0.5 ml of CEH or crude MEH, diluted (Table 2) to give activity in the linear range of the assay, was placed in an ice cold test tube, 5 $\mu$l of 100× stock inhibitor was added, and the tube was mixed and allowed to incubate on ice for 5 min. Four 100 $\mu$l aliquots were removed, placed in tubes which had been prewarmed to 37°C, and allowed to incubate for 5 min in a shaking, 37°C water bath. Substrate was then added and the assay allowed to proceed for 15 min. $I_{50}$s were calculated for each inhibition assay and the mean and standard deviations calculated from the resulting data.

## RESULTS AND DISCUSSIONS

MEH was poorly inhibited by all of the *trans*-3-phenylglycidol inhibitors tested (Table 3). The best inhibitor tested against MEH was cyclohexene oxide which is known to be a potent MEH inhibitor (Magdalou and Hammock, 1988; Oesch, 1973; Wixtrom and Hammock, 1985). At 10 $\mu$M, cyclohexene oxide gave 58% inhibition, for murine MEH, to 92% inhibition, for rabbit MEH. The next most potent MEH inhibitor tested was S-PPO which gave, at 2 mM, from 20% inhibition, for human MEH, to 63% inhibition, for rabbit MEH.

S-PPO was a more potent inhibitor of bovine, rabbit and murine MEH than R-PPO. Human and monkey MEH were inhibited to approximately the same extent by both R- and S-PPO. The R,R-enantiomer of the two *trans*-3-phenylglycidols tested, PGO and NPGO, was as potent or more potent than the corresponding S,S-enantiomer as an inhibitor of MEH in all five species. However, there was no clear pattern in the degree of inhibition for the benzoate ester of NPGO (BEP) at 125 $\mu$M. S-BEP showed more inhibition than R-BEP for rabbit MEH while inhibition of human and murine MEH by the two enantiomers was similar. Monkey and bovine MEH were slightly stimulated by BEP. Monkey MEH was more stimulated by S-BEP and bovine MEH was

Table 3. Inhibition of microsomal epoxide hydrolase

| MEH | Inhibitor | Percent inhibition R,R | Percent inhibition S,S |
|---|---|---|---|
| Human | PPO | 22 (4.1) | 20 (2.6) |
| | PGO | 16 (4.6) | 8.6 (4.0) |
| | NPGO | 7.4 (4.8) | 2.7 (4.1) |
| | BEP | 5.1 (2.7) | 5.9 (2.3) |
| | Cyclohexene oxide | 75 (1.7) | |
| Monkey | PPO | 22 (2.1) | 25 (3.9) |
| | PGO | 14 (3.8) | 12 (1.4) |
| | NPGO | 12 (5.5) | 10 (3.7) |
| | BEP | +0.27 (2.1) | +4.3 (1.2) |
| | Cyclohexene oxide | 68 (2.8) | |
| Bovine | PPO | 22 (2.9) | 30 (6.5) |
| | PGO | 13 (6.5) | 10 (1.5) |
| | NPGO | 7.5 (4.4) | 7.3 (3.7) |
| | BEP | +2.4 (2.0) | +0.15 (0.50) |
| | Cyclohexene oxide | 63 (5.5) | |
| Rabbit | PPO | 26 (2.5) | 63 (9.0) |
| | PGO | 28 (4.9) | 14 (6.9) |
| | NPGO | 29 (2.6) | 28 (2.4) |
| | BEP | 0.60 (3.3) | 7.0 (5.3) |
| | Cyclohexene oxide | 92 (2.3) | |
| Murine | PPO | 18 (8.2) | 52 (3.8) |
| | PGO | 14 (4.4) | 11 (4.7) |
| | NPGO | 26 (2.3) | 23 (4.4) |
| | BEP | 3.0 (3.7) | 2.0 (5.7) |
| | Cyclohexene oxide | 58 (4.1) | |

*Inhibitor preincubated with MEH for 5 min on ice, 5 min at 37°, and assayed for 15 min with 80 $\mu$M CSO at pH 9.0. All inhibitions were carried out at [I] = 2 mM except BEP (125 $\mu$M) and racemic cyclohexene oxide (10 $\mu$M). Inhibitors were not checked for enantiomeric purity. Standard deviation ( ), +indicates stimulation.

Table 4. Inhibition of cytosolic epoxide hydrolase by *trans*-3-phenylglycidols and related epoxides*

| CEH | Inhibitor | $I_{50}$ ($\mu$M) R,R | $I_{50}$ ($\mu$M) S,S | $I_{50}$ ($\mu$M) Racemic |
|---|---|---|---|---|
| Human | PPO | >5000 | 4965 | — |
| | PGO | >5000 | >5000 | — |
| | NPGO | >5000 | 9.3 (1.4) | — |
| | BEP | 125 (23) | >1000 | — |
| | EPP | — | — | 25 (1.9) |
| | EPB | — | — | 85 (0.97) |
| | 4-FCO | — | — | 3.2 (0.15) |
| Monkey | PPO | >5000 | >5000 | — |
| | PGO | 3100 (240) | 1000 (188) | — |
| | NPGO | >5000 | 8.2 (0.76) | — |
| | BEP | 52 (1.9) | 780 (9.5) | — |
| | EPP | — | — | 23 (0.99) |
| | EPB | — | — | 8.1 (0.53) |
| | 4-FCO | — | — | 2.0 (0.27) |
| Bovine | PPO | 1600 (267) | >5000 | — |
| | PGO | >5000 | 770 (61) | — |
| | NPGO | >5000 | 360 (78) | — |
| | BEP | 14 (1.2) | >1000 | — |
| | EPP | — | — | 6.4 (0.48) |
| | EPB | — | — | 24 (1.8) |
| | 4-FCO | — | — | 1.5 (0.068) |
| Rabbit | PPO | >5000 | >5000 | — |
| | PGO | 3800 (990) | 4600 | — |
| | NPGO | >5000 | 5.1 (0.65) | — |
| | BEP | 47 (1.3) | >1000 | — |
| | EPP | — | — | 2.3 (0.23) |
| | EPB | — | — | 8.0 (0.43) |
| | 4-FCO | — | — | 2.3 (0.27) |
| Murine | PPO | 1500 (82) | 2300 (83) | — |
| | PGO | 680 (30) | 500 (23) | — |
| | NPGO | 690 (36) | 1.1 (0.040) | — |
| | BEP | 10 (1.4) | 20 (1.6) | — |
| | EPP | — | — | 2.7 (0.44) |
| | EPB | — | — | 3.6 (0.17) |
| | 4-FCO | — | — | 1.5 (0.15) |

*Inhibitor preincubated with CEH for 5 min on ice, 5 min at 37°, and assayed for 15 min with 50 $\mu$M TSO. Inhibitors not checked for enantiomeric purity. Human CEH gave 55–60% inhibition with 5 mM S,S-PPO and rabbit CEH gave a similar level of inhibition with 5 mM S,S-PGO. Standard deviation ( ), — not tested.

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