THE EFFECT OF CHLORPYRIFOS-OXON AND OTHER XENOBIOTICS ON THE HUMAN CYTOCHROME P450-DEPENDENT METABOLISM OF NAPHTHALENE AND DEET

Taehyeon M. Cho[1], Randy L. Rose[2] and Ernest Hodgson*

Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC, USA
[1]Current address: U. S. Navy Environmental Health Center, Portsmouth, VA, USA

SUMMARY

Chlorpyrifos-oxon (CPO), a metabolite of chlorpyrifos, is a potent inhibitor of acetylcholinesterase and, although the neurotoxicological impact of this organophosphorus compound has been broadly studied both *in vitro* and *in vivo*, there are few studies of metabolic interactions of CPO with other xenobiotics. CPO significantly activated the production of 1-naphthol (5-fold), 2-naphthol (10-fold), trans-1,2-dihydro-1,2-naphthalenediol (1.5-fold), and 1,4-naphthoquinone from naphthalene by human liver microsomes (HLM). It was further demonstrated that the production of naphthalene metabolites by CYP2C8, 2C9*[1], 2C19, 2D6*[1], 3A4, 3A5, and 3A7 was activated by CPO, while the production of naphthalene metabolites by CYP1A1, 1A2, 1B1, and 2B6 was inhibited by CPO. CPO inhibited CYP1A2 production of naphthalene metabolites, while activating their production by CYP3A4. Similarly, CPO inhibited the production of *N,N*-

* Author for correspondence:
Dr. Ernest Hodgson
Department of Environmental and Molecular Toxicology
Campus Box 7633
North Carolina State University
Raleigh, NC 27695-7633, USA
e-mail: ernest_hodgson@ncsu.edu

[2]Deceased. Dr. Rose died in a tragic accident on May 23rd, 2006.

diethyl-*m*-hydroxymethylbenzamide (BALC) from DEET by human liver microsomes, but activated the production of *N*-ethyl-*m*-toluamide (ET) from this substrate. CYP2B6, the most efficient isoform for BALC production, was inhibited by CPO, while CYP3A4, the most efficient isoform for ET production, was activated by CPO. CPO inhibited CYP2B6 production of both BALC and ET from DEET, but activated CYP3A4 production of ET, while inhibiting CYP3A4 BALC production. CPO appears to facilitate the binding of naphthalene to CYP3A4. This metabolic activation is independent of cytochrome b_5, suggesting that activation of CYP3A4 by CPO is associated with a conformational change of the isoform rather than facilitating electron transfer.

KEY WORDS

activation, chlorpyrifos, chlorpyrifos-oxon, CYP, CYP3A4, human CYP isoforms, human hepatocytes, inhibition, naphthalene, DEET

INTRODUCTION

Naphthalene, a component of fossil fuels, as well as products for household and industrial use /1/, is metabolized to 1-naphthol, 2-naphthol, and *trans*-1,2-dihydro-1,2-naphthalenediol by pooled human liver microsomes and individually expressed human CYP isoforms /2/. 1-Naphthol is further metabolized to 1,4-naphthoquinone and 2-naphthol to 2,6- and 1,7-dihydroxynaphthalene, respectively /2/.

N,N-Diethyl-*m*-toluamide (DEET), the active ingredient in most insect repellents, is metabolized to *N,N*-diethyl-*m*-hydroxymethylbenzamide (BALC) and *N*-ethyl-*m*-toluamide (ET) by liver microsomes of human, rat, or mouse, as well as individually expressed human CYP isoforms /3/.

Cytochrome P450 (CYP) monooxygenases are the most important enzymes in the metabolism of xenobiotics, including clinical drugs and environmental chemicals as well as endogenous compounds, such as steroids, bile acids, fatty acids, eicosanoids, and fat-soluble vitamins /4/. Among 57 CYP isoforms known in humans, 15 are involved in the metabolism of xenobiotics, while the function of 13 is

unknown /5/. Metabolism of a xenobiotic by these CYP enzymes can lead to either activation or detoxication of the parent chemical /6,7/.

Although *in vivo* studies of metabolism in animal models, including rodents, produce scientifically important data, xenobiotic metabolism in such surrogate animals frequently deviates from metabolism in humans (reviewed in /8,9/). Metabolism by rodent CYP3A and CYP2D is particularly discrepant from that of human CYP3A4 and CYP2D6, respectively, the latter being collectively involved in the metabolism of more than 70% of clinical drugs /8-10/. *In vitro* metabolic studies have significant advantages for the study of human metabolism of xenobiotics, the determination of kinetic parameters, metabolic efficiency, contributions of individual CYP isoforms and intrinsic clearance rates facilitating *in vitro-in vivo* correlations /11/. For studies of either xenobiotic-xenobiotic metabolic interactions or CYP allosterism, *in vitro* models are more advantageous than *in vivo* models /12/.

Xenobiotic-associated changes in the metabolic activity of human CYP isoforms have frequently been observed. Both ketoconazole and chlorpyrifos inhibit 3-hydroxycarbofuran formation from carbofuran by human liver microsomes /13/ while *in vitro* metabolism of carbaryl by human CYP enzymes is inhibited by chlorpyrifos /14/. Although activation of CYP enzymes by xenobiotics is less frequently encountered and less well understood than inhibition, in some cases the metabolic activity of these enzymes is significantly enhanced by xenobiotics. Enhancement by acetone of the hepatic microsomal *p*-hydroxylation of aniline was first reported in 1968 /15/. Flavone and 7,8-benzoflavone both stimulate benzo[*a*]pyrene metabolism by rabbit CYPs, the extent of the stimulation depending on the type of CYP obtained from rabbit liver microsomes /16/. 6β-Hydroxylation of testosterone by human CYP3A4 is significantly increased by preincubation of the enzyme with pyridostigmine bromide /17/.

The objectives of the current studies were to identify the public health and agriculture-related chemicals most effective in influencing the metabolic activity of human liver microsomes toward naphthalene, to investigate metabolic interactions between naphthalene or DEET and chlorpyrifos-oxon (CPO), to identify the most efficient human CYP isoforms relative to activation or inhibition of naphthalene or DEET metabolism by CPO, to investigate the role of CPO in the activation and inhibition of human oxidative metabolism of

naphthalene, and to investigate the mechanism of activation of CYP3A4-mediated naphthalene metabolism.

MATERIALS AND METHODS

Chemicals

Naphthalene, 1-naphthol, 2-naphthol, 1,4-naphthoquinone and dodecane (DD) were purchased from Sigma-Aldrich (St. Louis, MO). *trans*-1,2-Dihydro-1,2-naphthalenediol was a generous gift from Dr. Alan R. Buckpitt (University of California, Davis, CA). Chlorpyrifos (CPS), chlorpyrifos-oxon (CPO), permethrin (PM), carbaryl (CB), *N,N*-diethyl-*m*-toluamide (DEET), and pyridostigmine bromide (PB) were purchased from ChemService (West Chester, PA). *N,N*-Diethyl-*m*-hydroxymethylbenzamide (BALC) and *N*-ethyl-*m*-toluamide (ET) were generous gifts from Dr. Wesley G. Taylor (Saskatoon Research Center, Saskatoon, Canada). Acetonitrile and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA).

Human liver microsomes and human cytochrome P450 isoforms

Pooled human liver microsomes (pHLM) and human CYP isoforms expressed in baculovirus infected insect (*Autographa californica*) cells (BTI-TN-5B1-4) as follows: CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9*[1](Arg$_{114}$), 2C18, 2C19, 2D6*[1](Val$_{374}$), 2E1, 3A4, 3A5, 3A7, and 4A11, were purchased from BD Gentest (Woburn, MA).

In vitro metabolic interactions of xenobiotics with naphthalene metabolism mediated by pooled human liver microsomes or CYP1A2

Naphthalene metabolism mediated by pHLM or CYP1A2 was tested after pre-incubation of the enzyme with 40 μM of the individual chemicals, such as chlorpyrifos (CPS), chlorpyrifos-oxon (CPO), permethrin (PM), carbaryl (CB), *N,N*-diethyl-*m*-toluamide (DEET), pyridostigmine bromide (PB), or dodecane (DD), followed by the addition of 40 μM naphthalene. These assays were performed with an NADPH-generating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate and 2 U/ml glucose 6-phosphate dehydrogenase) in 100 mM potassium phosphate buffer containing 3.3 mM $MgCl_2$ (pH 7.4).

After each xenobiotic (40 μM) was pre-incubated with pHLM (0.48 mg/ml) or CYP1A2 (40 pmol/ml) at 37°C for 5 minutes, naphthalene metabolism was initiated by the addition of naphthalene (40 μM) followed by incubation at 37°C for 10 minutes. For controls, the same incubation was carried out in the absence of the NADPH-generating system.

Naphthalene metabolism by pHLM, CYP1A2, or CYP3A4 after pre-incubation with chlorpyrifos-oxon (CPO)

After preliminary screening of the metabolic interaction of naphthalene with seven individual chemicals, CPO was chosen for further investigation of its metabolic influence on naphthalene metabolism. After a series of CPO concentrations (0 to 600 μM) were pre-incubated with pHLM (0.48 mg/ml), CYP1A2 (40 pmol/ml), or CYP3A4 (40 pmol/ml) in an NADPH-generating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate and 2 U/ml glucose 6-phosphate dehydrogenase) in 100 mM potassium phosphate buffer containing 3.3 mM $MgCl_2$ (pH 7.4) at 37°C for 5 minutes, naphthalene metabolism was initiated by the addition of the substrate (40 μM) and incubated at 37°C for 10 minutes. Additionally, pHLM was pre-incubated with the same concentrations of CPS or a 1:1 mixture of CPO and CPS (0 to 600 μM) before being incubated with 40 μM naphthalene as described above. For controls, the same incubation was carried out without the NADPH-generating system.

***In vitro* screening for activation or inhibition of human cytochrome P450 isoform-mediated naphthalene metabolism by CPO**

Modulation of the metabolic activity of the 15 human CYP isoforms (40 pmol/ml) listed above for naphthalene (40 μM) by CPO (160 μM) was determined. The enzymatic assays were performed in the same manner as stated above. Generation of each metabolite mediated by individual CYP isoforms was compared. Sf9 insect cell microsomes from wild-type baculovirus infected cells (BD Gentest, Woburn, MA) were used as controls for these assays.

Kinetics of naphthalene metabolism by CYP3A4 in the presence of CPO with or without cytochrome b_5

To investigate the mechanism of activation of naphthalene metabolism by CYP3A4, the kinetics of the reaction was studied in the presence of CPO (0, 5, or 80 μM). CYP3A4, co-expressed with cytochrome b_5, was pre-incubated with CPO at 37°C for 5 minutes, followed by the addition of naphthalene (0 to 500 μM) to initiate the reaction, which was then continued for 10 minutes. The kinetic parameters (V_{max} and K_m) for naphthalene metabolism by CYP3A4 pre-incubated with three different concentrations of CPO were calculated as stated below. The production ratios of the major metabolites, 1-naphthol and 2-napthol, produced by CYP isoforms pre-incubated in the three different concentrations of CPO were calculated to determine whether pre-incubation with CPO can influence the production of those metabolites from naphthalene 1,2-epoxide.

To examine whether electron transfer from cytochrome b_5 was involved in this metabolic activation of naphthalene in CYP3A4 co-expressed with cytochrome b_5, an additional experiment was carried out as follows: CYP3A4, not co-expressed with cytochrome b_5, was pre-incubated with CPO (0 to 600 μM) at 37°C for 5 minutes, followed by the addition of naphthalene (100 μM) to initiate a 10 minute metabolic reaction. The pattern of naphthalene metabolic activation in CYP3A4 without cytochrome b_5 was compared with the metabolism in CYP3A4 co-expressed with cytochrome b_5 as described above.

Effect of pre-incubation with CPO on DEET metabolism by pHLM

After CPO (0 to 600 μM) was pre-incubated with pHLM (0.48 mg/ml) and an NADPH-generating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate and 2 U/ml glucose 6-phosphate dehydrogenase) in 100 mM potassium phosphate buffer containing 3.3 mM $MgCl_2$ (pH 7.4) at 37°C for 5 minutes, DEET metabolism was initiated by the addition of the substrate (100 μM) and incubated at 37°C for 20 minutes. For controls, DEET and pHLM pre-incubated with CPO were incubated in the same buffer system but without the NADPH-generating system.

CPO (40 μM)-associated modulation of the metabolic activity of the human CYP isoforms (40 pmol/ml) toward DEET (40 μM)

metabolism was determined, the CYP isoforms being selected based on a previous study /12/. The enzymatic assays were performed in the same manner as above. Generation of each metabolite mediated by the individual CYP isoforms was compared. Sf9 insect cell microsomes from wild-type baculovirus infected cells (BD Gentest, Woburn, MA) were used as controls for these assays.

Metabolism of DEET (100 μM) by CYP2B6 or CYP3A4 in the presence of CPO was further characterized in order to determine the effect of pre-incubation of each isoform with CPO (0 to 600 μM) at 37°C for 5 minutes. The enzymatic assays of DEET with each CYP isoform (40 pmol/ml) were performed in the same manner as stated above. For controls, DEET and the CYP isoform pre-incubated with CPO were incubated in the same buffer system but without the NADPH-generating system.

All assay reactions were terminated by addition of an equal volume (250 μl) of acetonitrile and vortexing. After 5 minutes centrifugation at 15,000 rpm (21,000 *g*), the supernatant was collected for metabolite characterization using an HPLC system. No metabolites were detected in controls in which the NADPH-generating system was absent.

Analysis of metabolites by HPLC

The generation of metabolites was analyzed using a Waters 2695 HPLC system equipped with a 2996 Photodiode Array (PDA) detector (Milford, MA). This HPLC system was equipped with a degasser and an autoinjector, and data were collected and analyzed using Waters Empower software, version 5.00. The solution for pump A was 3% tetrahydrofuran, 0.2% O-phosphorus acid (85%) and 96.8% water, and for pump B 100% acetonitrile. The gradient in the mobile phase was as follows: 0 to 2 min (20% B), 2 to 22 min (gradient to 80% B), 22 to 25 min (80% B), and 25 to 30 min (gradient to 20% B). The flow rate was 1.0 ml/min. Metabolites were separated by a reversed phase C_{12} column (Synergi 4μ Max-RP, 250 × 4.6 mm, Phenomenex, Torrance, CA) and detected using a PDA detector operated from 190 to 350 nm. Optimal wavelengths for 1-naphthol, 2-naphthol, *trans*-1,2-dihydro-1,2-naphthalenediol, 1,4-naphthoquinone, BALC, and ET were selected as 232.7, 225.6, 262.2, 251.6, 231.5 and 231.5 nm, respectively. Standards of metabolites were prepared in acetonitrile and 50 μl of standard or sample was injected into the HPLC system.

Data analysis and statistics

The apparent V_{max} and K_m were calculated using a nonlinear regression curve fitted to the Michaelis-Menton equation. The coefficients of determination (R^2), a measure of how well a regression model describes the data, were greater than 0.95. Data means were obtained by at least three determinations and the data show the mean and the S.E. The percentages of total normalized rate (%TNR) were determined as described previously /18/. The nominal specific contents of individual CYP proteins in native human livers (10 donors) used for calculating the %TNR were obtained from BD Gentest (2003 product catalog) except for the contents of CYP2C8 and CYP2C18, which were from Rodrigues /18/.

Statistical significance of the data was determined with one-way ANOVA followed by the Tukey's multiple comparisons when three or more data sets needed to be compared with one another, and by Dunnett's comparisons when data sets needed to be compared to their corresponding control. Student's t-test was also applied to compare data with their corresponding controls in the CYP screening assays.

RESULTS

As a preliminary test, 40 μM of the test chemicals (carbaryl, chlorpyrifos, chlorpyrifos-oxon, DEET, dodecane, permethrin and pyridostigmine bromide) were pre-incubated with pHLM followed by the same concentration of naphthalene and incubated under the conditions stated in the Methods section. While most chemicals did not affect 1- or 2-naphthol production from naphthalene by pHLM, their production was significantly enhanced by CPO (Fig. 1). However, CPO did not affect dihydrodiol production under these conditions while CPS or CB significantly reduced the production of dihydrodiol (Fig. 1). In another preliminary test, CYP1A2 was utilized to screen the most effective chemicals for their effects on naphthalene metabolism. In contrast to pHLM-mediated naphthalene metabolism, CPO significantly reduced the metabolic activity of CYP1A2 for the production of these three naphthalene metabolites (Fig. 2). While CPS significantly reduced the activity of CYP1A2 for the production of 2-naphthol and dihydrodiol in naphthalene metabolism, DEET significantly enhanced the production of 1- and 2-naphthol (Fig. 2).

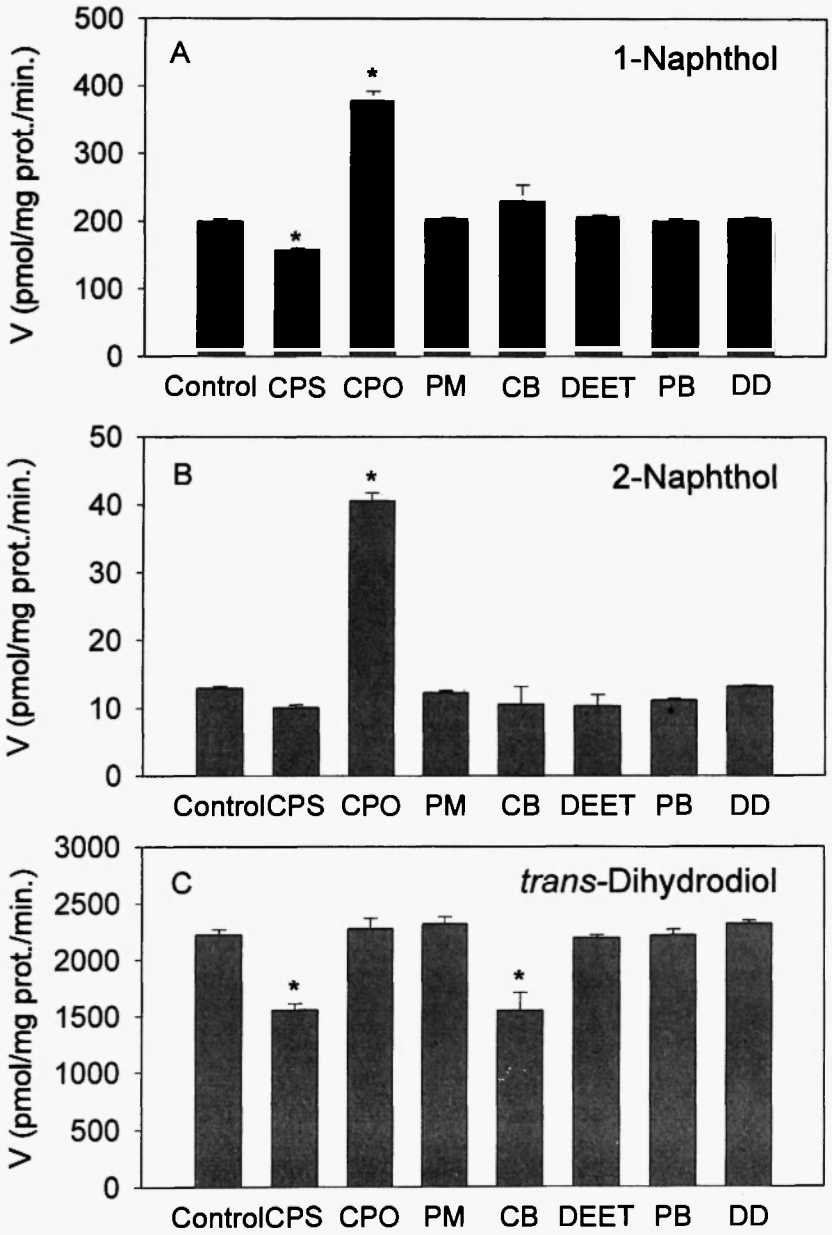

Fig. 1: Naphthalene (40 μM) metabolism by pHLM, which has been pre-incubated with other xenobiotics (40 μM). Each chemical is abbreviated as stated in the Materials and Methods section. Production of 1-naphthol (**A**), 2-naphthol (**B**), or *trans*-dihydrodiol (**C**) is shown. The asterisks indicate a significant difference compared to the corresponding control ($p < 0.05$).

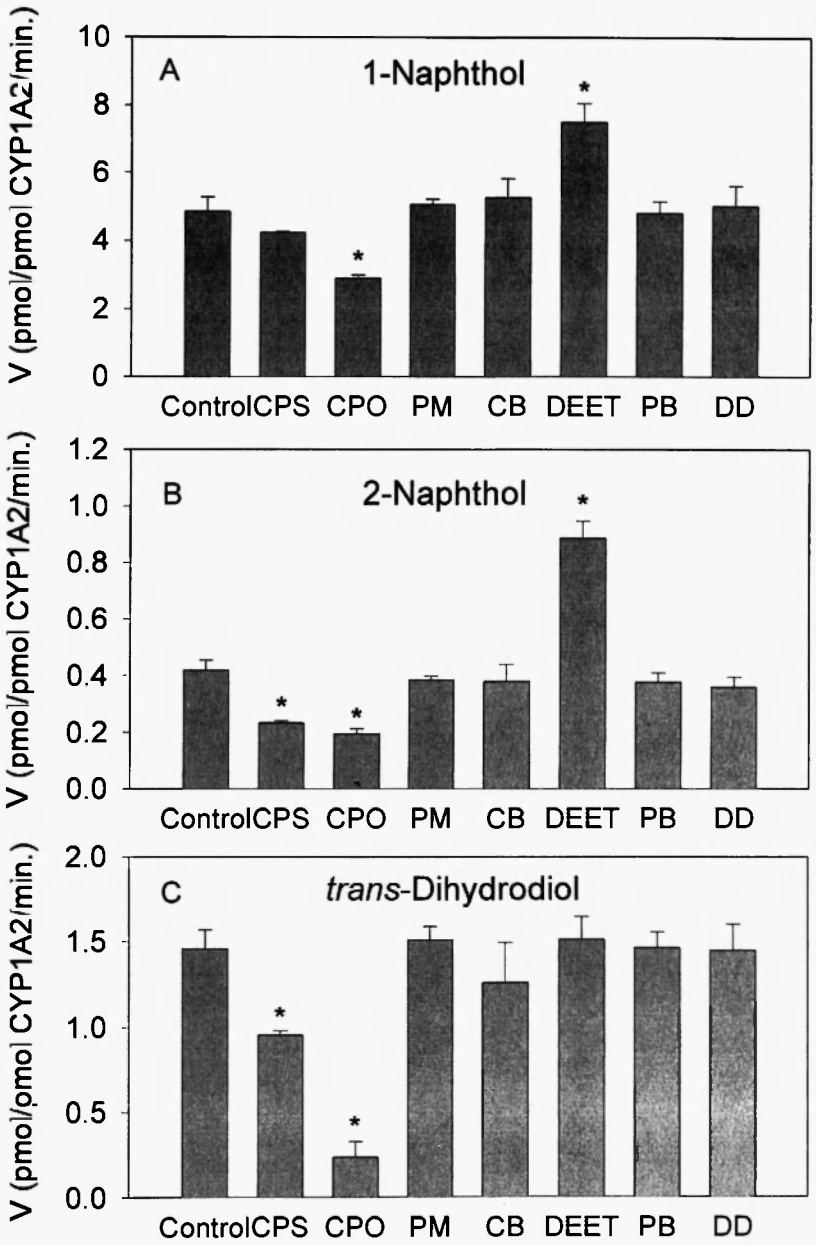

Fig. 2: Naphthalene (40 μM) metabolism by CYP1A2, which has been pre-incubated with other xenobiotics (40 μM). Each chemical is abbreviated as stated in the Materials and Methods section. Production of 1-naphthol (**A**), 2-naphthol (**B**), or *trans*-dihydrodiol (**C**) is shown. The asterisks indicate a significant difference compared to the corresponding control ($p < 0.05$).

Metabolite production in pHLM-associated naphthalene metabolism in the presence of varying concentrations of CPO, CPS, or 1:1 CPO/CPS mixture was examined. The production of 1-naphthol (5.5 fold), 2-naphthol (10 fold), and dihydrodiol (1.6 fold) was significantly increased by pre-incubation with CPO (Fig. 3). CPS did not increase the production of any metabolite at most concentrations; rather it decreased 1-naphthol production at the two lowest concentrations (20 and 40 μM) of CPS and increased it slightly at the highest (600 μM) (Fig. 3A). The production of dihydrodiol was significantly inhibited by CPS (Fig. 3C). The 1:1 mixture of CPO and CPS caused reduced activation for the production of metabolites compared to CPO alone. CPO significantly increased production of naphthalene metabolites in human pHLM at most concentrations of the chemicals (Fig. 3A-C). The production of 1,4-napthoquinone was also significantly enhanced by pre-incubation with CPO (Fig. 3D). The amounts of metabolites produced were dihydrodiol > 1-naphthol > 2-naphthol > 1,4-naphthoquinone.

To investigate which human CYP isoforms are activated or inhibited by CPO for naphthalene metabolism, a screening assay of CYP isoforms for naphthalene metabolism was performed in the presence or absence of CPO. The production of 1-naphthol by CYP1A1, 1A2, 1B1, and 2B6 was inhibited by CPO, while CYP2C8, 2C9*[1], 2C19, 2D6*[1], 3A4, 3A5, and 3A7 were activated for 1-naphthol production by CPO (Fig. 4A). Similar patterns of inhibition and activation were observed for 2-naphthol and dihydrodiol production (Fig. 4B,C).

The percentages of total normalized rate (%TNR) for the production of naphthalene metabolites in individual CYP isoforms pre-incubated without or with 160 μM CPO are shown in Table 1. CYP isoforms including CYP1A2, 2A6, and 2B6 showed decreased %TNR, while others including CYP2C19 and 3A4 demonstrated increased %TNR values for the production of three naphthalene metabolites (Table 1).

To examine how much the metabolic activity of CYP1A2 for naphthalene is inhibited by CPO, a study of the metabolism of 40 μM naphthalene was performed with CYP1A2 pre-incubated with varying concentrations of CPO. CPO significantly reduced the production of three metabolites, 1-naphthol, 2-naphthol, and dihydrodiol, in a dose-dependant manner (Fig. 5). The highest dose of CPO (160 μM)

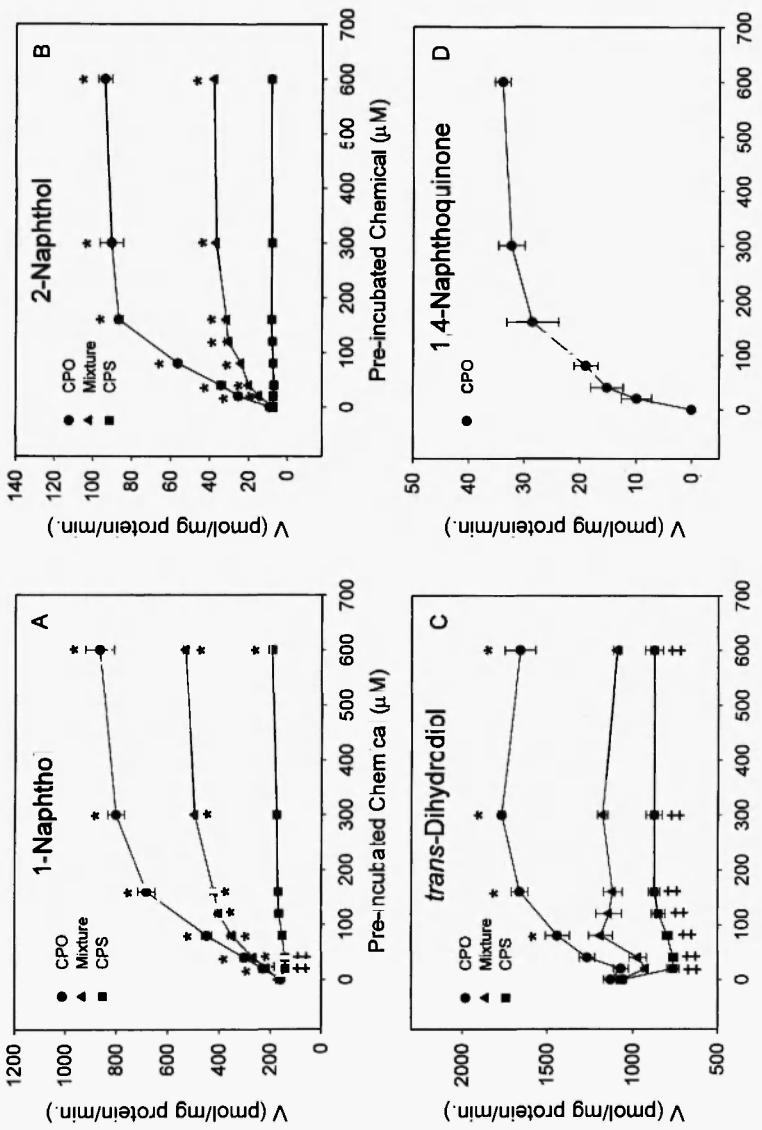

Fig. 3: Naphthalene (40 μM) metabolism by pHLM, which has been pre-incubated with CPO, CPS, or their 1:1 mixture, respectively. Production of 1-naphthol (**A**), 2-naphthol (**B**), trans-dihydrodiol (**C**), or 1,4-naphthoquinone (**D**) is shown. * and ‡ indicate significant increases or decreases in the production of each metabolite compared to the corresponding control ($p < 0.05$).

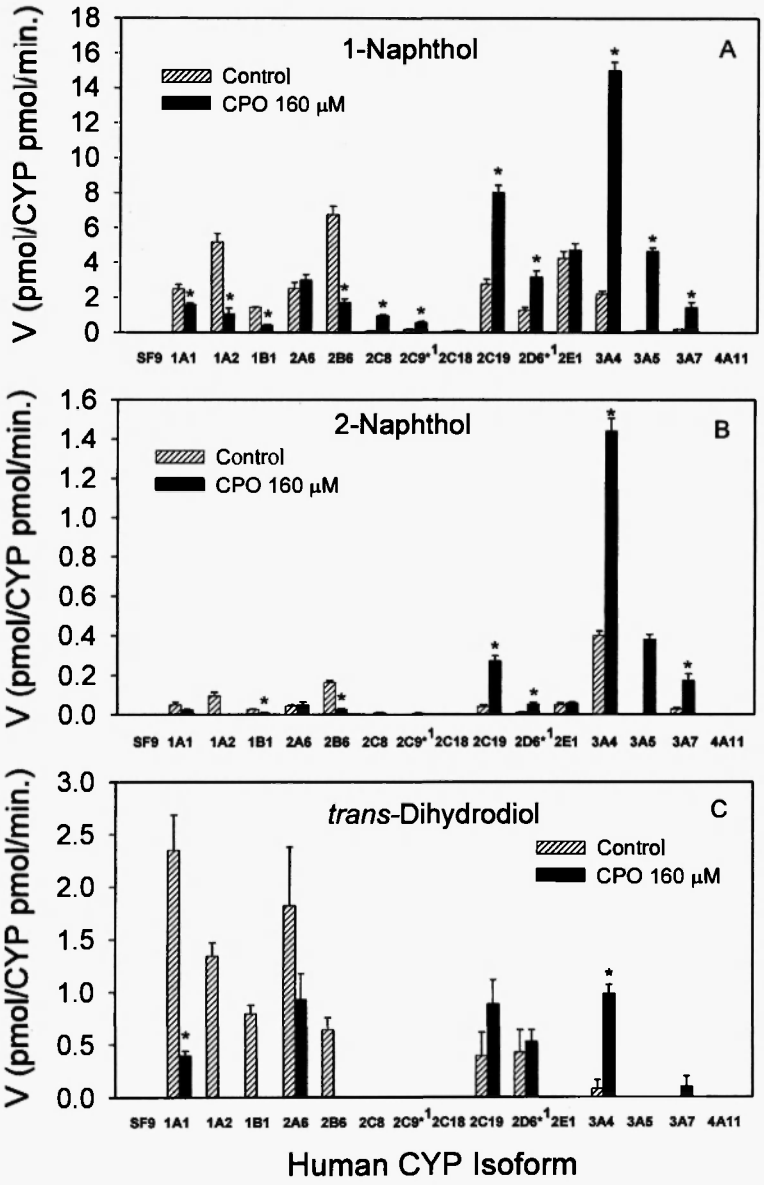

Fig. 4: Activation and inhibition of naphthalene (40 μM) metabolism by various human CYP isoforms, which have been pre-incubated with CPO (160 μM). Production of 1-naphthol (**A**), 2-naphthol (**B**), or trans-dihydrodiol (**C**) is shown. The asterisks indicate significant difference compared to the corresponding control ($p < 0.05$).

TABLE 1

The total percentage normalized rate (%TNR) of naphthalene metabolites of individual CYP isoforms pre-incubated with or without 160 μM CPO, and the specific content of each CYP protein in human liver microsomes

CYP isoform	% TNR						Mean content of CYP[†] (pmol CYP/mg prot.)
	1-Naphthol		2-Naphthol		Dihydrodiol		
	Control	CPO 160 μM	Control	CPO 160 μM	Control	CPO 160 μM	
1A1	ND	ND	ND	ND	ND	ND	NA
1A2	23.4	2.0 (↓ 11.7)	7.5	0.0 (↓)	34.7	0.0 (↓)	55
1B1	ND	ND	ND	ND	ND	0.0	NA
2A6	10.8	5.3 (↓ 2.0)	3.3	1.2 (↓ 2.8)	44.5	22.0 (↓ 2.0)	52
2B6	11.7	1.2 (↓ 9.8)	5.0	0.3 (↓ 16.7)	6.3	0.0 (↓)	21
2C8[‡]	0.5	2.1 (↑ 4.2)	0.0	0.3 (↑)	0.0	0.0	64
2C9*[‡]	1.1	1.5 (↑ 1.4)	0.0	0.1 (↑)	0.0	0.0	76
2C18[‡]	0.01	0.01	0.0	0.0	0.0	0.0	2.5
2C19	8.9	10.6 (↑ 1.2)	2.3	5.1 (↑ 2.2)	7.1	15.7 (↑ 2.2)	39
2D6*[‡]	1.3	1.3	0.1	0.3 (↑ 3.0)	2.4	2.9 (↑ 1.2)	12
2E1	18.2	8.3 (↓ 2.2)	3.8	1.4 (↓ 2.7)	0.0	0.0	52
3A4	24.1	67.4 (↑ 2.8)	77.9	91.1 (↑ 1.2)	5.1	59.5 (↑ 11.7)	133
3A5	0.01	0.2 (↑ 20.0)	0.0	0.2 (↑)	0.0	0.0	1.2
3A7	ND	ND	ND	ND	0.0	ND	NA
4A11	0.0	0.0	0.0	0.0	0.0	0.0	NA

[†] Mean content data were obtained from the BD Gentest (2003). [‡] Mean content data for 2C8 and 2C18 were obtained from Rodrigues (1999).

ND = not determined; NA = not available.

The numbers in parentheses are the decreased (↓) or increased (↑) percentages (×100) compared to their corresponding controls.

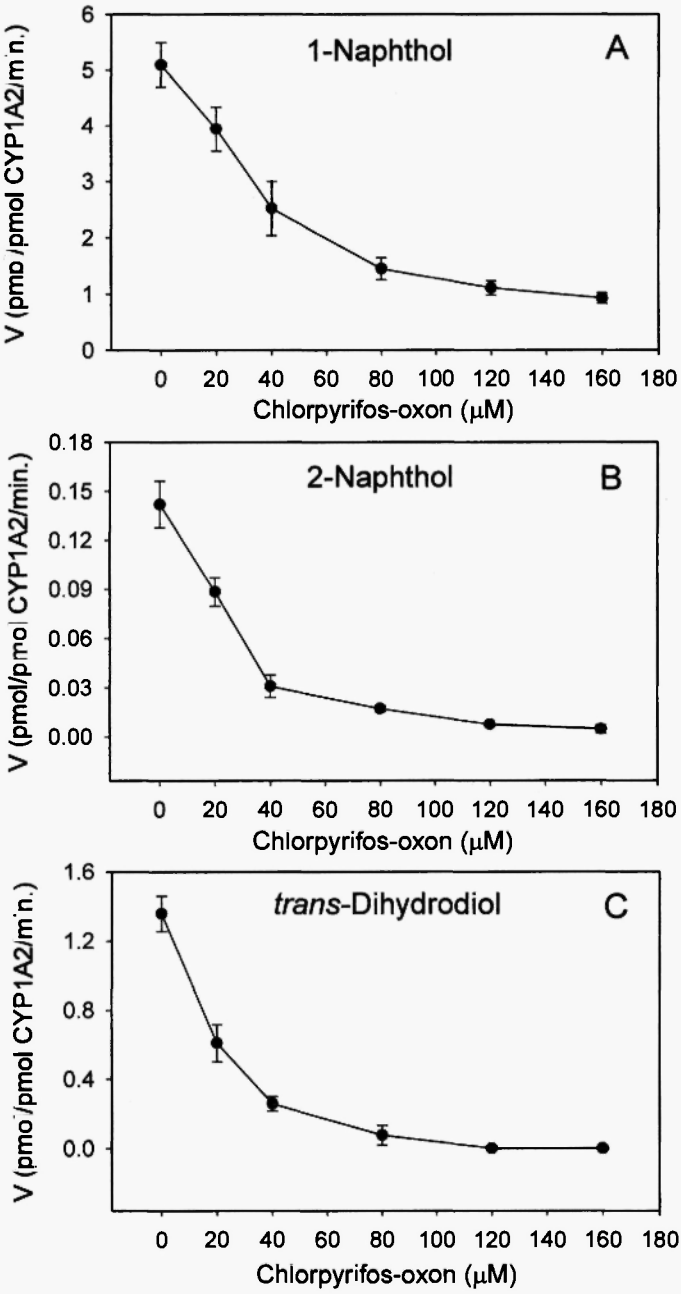

Fig. 5: CPO inhibition of naphthalene (40 μM) metabolism by human CYP1A2. Production of 1-naphthol (**A**), 2-naphthol (**B**), or *trans*-dihydrodiol (C) is shown.

reduced CYP1A2-mediated 1-naphthol and 2-naphthol production by 82% and 97%, respectively (Fig. 5A,B). Doses of 120 μM CPO and higher abolished the production of dihydrodiol (Fig. 5C).

To investigate how much the metabolic activity of CYP3A4 for naphthalene is activated by CPO, a study of the metabolism of 40 μM naphthalene was performed using CYP3A4 pre-incubated with varying concentrations of CPO. Metabolic activity of CYP3A4 for naphthalene was significantly activated by CPO in a dose-dependant manner (Fig. 6). The production of 1-naphthol and 2-naphthol was significantly enhanced by CPO up to 9 fold and 4.6 fold compared to the corresponding controls (Fig. 6A,B). Concentrations of CPO as low as 5 μM significantly enhanced the production of dihydrodiol mediated by CYP3A4 (Fig. 6C). The production of 1,4-naphthoquinone in naphthalene metabolism was also increased by the pre-incubation of CYP3A4 with CPO (Fig. 6D).

To investigate the mechanism of CPO activation of naphthalene metabolism by CYP3A4, naphthalene kinetics studies were performed in the presence and absence of CPO (5 and 80 μM) (Fig. 7). CPO activated the production of the major metabolites, 1-naphthol and 2-naphthol, by CYP3A4 in a dose-dependant manner (Fig 7A,B). The apparent K_m values for both metabolites were significantly reduced by pre-incubation of CYP3A4 with CPO, and V_{max} values were significantly increased by CPO in a dose-dependant manner (Fig. 7C,D). The production of 1-naphthol was more accelerated than that of 2-naphthol as the concentration of CPO was increased. The production of 1-naphthol was 5.15 ± 0.07 (CPO 0 μM), 7.86 ± 0.07 (CPO 5 μM), and 10.88 ± 0.33 (CPO 80 μM) times as much as that of 2-naphthol based on the V_{max} values. The acceleration of 1-naphthol over 2-naphthol production by CPO in a dose dependent manner shows significant differences from one another.

To investigate whether cytochrome b_5 is involved in this activation of naphthalene metabolism by CYP3A4, naphthalene metabolism by CYP3A4 co-expressed with and without cytochrome b_5 was observed in preliminary experiments using varying concentrations of CPO. The control velocity of naphthalene metabolism was lower in CYP3A4 without cytochrome b_5 compared to CYP3A4 with cytochrome b_5, but the degree of activation by CPO in a dose dependant manner is similar to naphthalene metabolism in CYP3A4 co-expressed with cytochrome b_5 (data not shown).

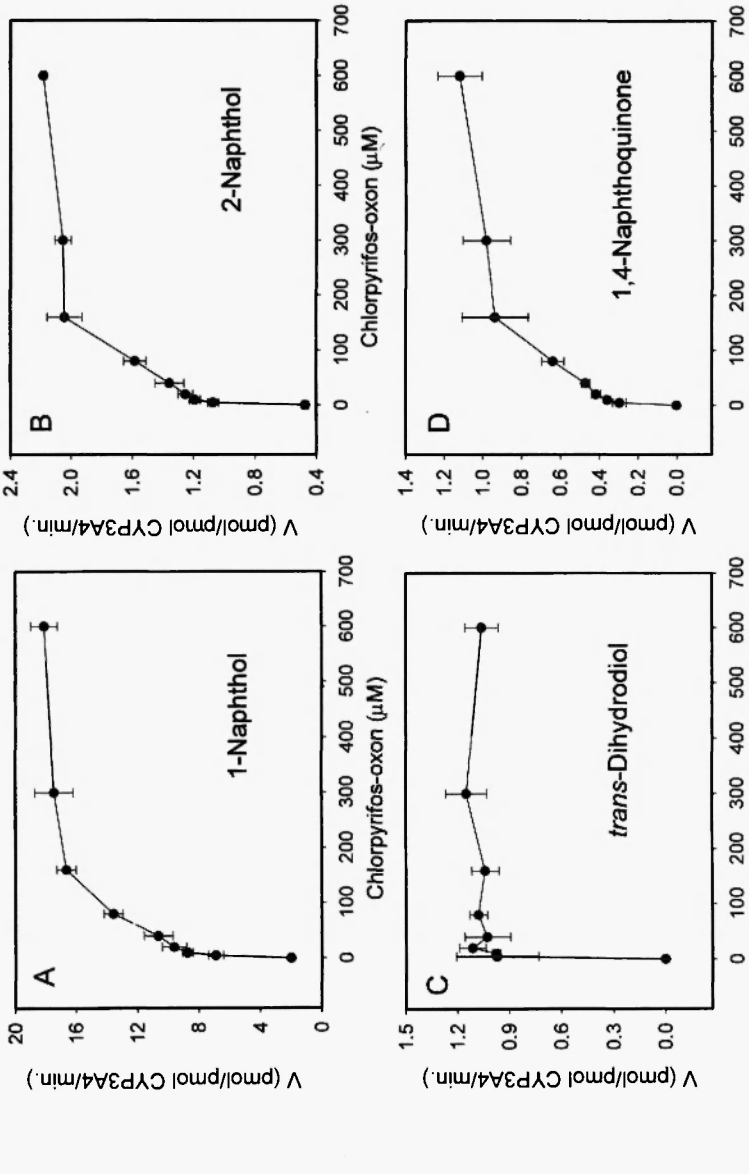

Fig. 6: CPO activation of naphthalene (40 μM) metabolism by human CYP3A4. Production of 1-naphthol (**A**), 2-naphthol (**B**), *trans*-dihydrodiol (**C**), or 1,4-naphthoquinone (**D**) is shown.

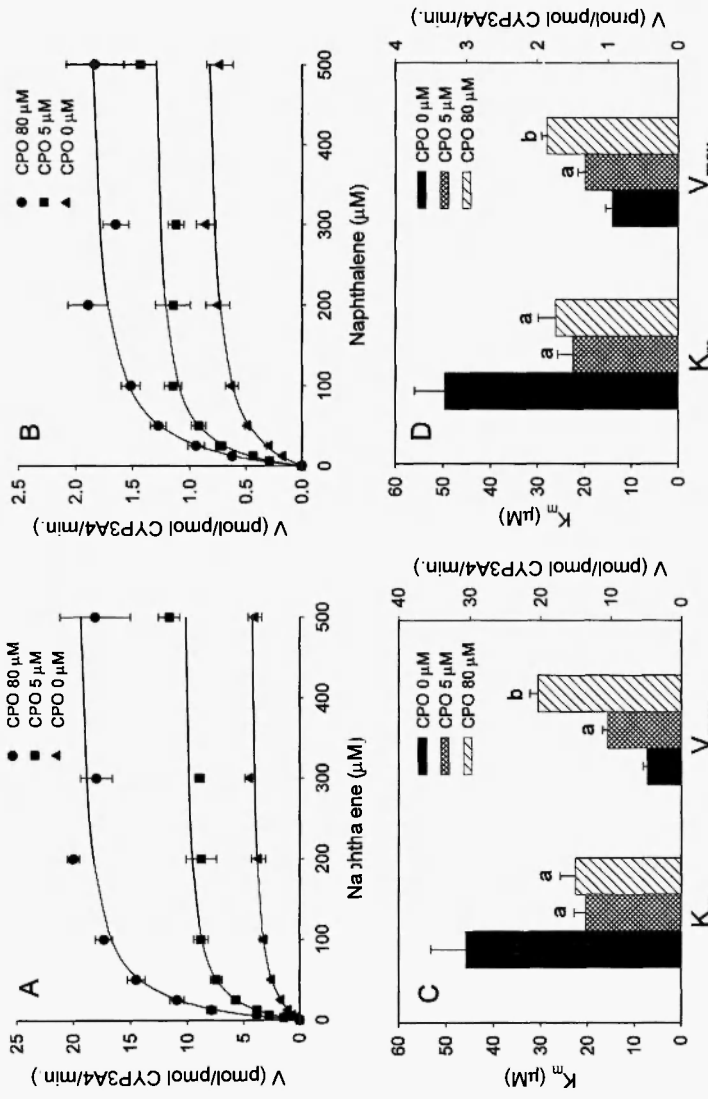

Fig. 7: Kinetics of naphthalene metabolism in the presence of three different concentrations of CPO. **A** and **B** present production of 1-naphthol and 2-naphthol, respectively. Kinetic parameters, K_m and V_{max}, of 1-naphthol (**C**) and 2-naphthol (**D**) production are shown. The letters, a or b, indicate a significant difference compared to their corresponding controls (CPO 0 µM), and different letters indicate a statistical difference in V_{max} between these data (C and D) ($p < 0.05$).

Metabolic activity of pHLM for DEET metabolism in the presence of varying concentrations of CPO was also investigated. While the production of ET was significantly increased 4.7 fold by CPO, the production of BALC was decreased 4 fold by this chemical in a dose-dependant manner (Fig. 8).

To investigate which human CYP isoforms among those chosen are activated or inhibited by CPO for DEET metabolism, a screening assay of CYP isoforms for DEET metabolism was performed in the absence and presence of CPO. CYP1A2, 2B6, and 2C19 were significantly inhibited for the production of BALC by CPO, while metabolic activity of other isoforms for BALC was not significantly affected by this chemical (Fig. 9A). CYP3A4 and 3A5 were significantly activated by CPO for the production of ET, while production of ET by CYP2B6 was inhibited (Fig. 9B).

To investigate the inhibition and activation of metabolic activity of CYP isoforms (CYP2B6 and 3A4) by CPO for the production of metabolites in DEET metabolism, each isoform was pre-incubated with CPO and the metabolism of DEET subsequently added was observed. Both ET and BALC production from CYP2B6-mediated DEET metabolism was significantly decreased by CPO in a dose-

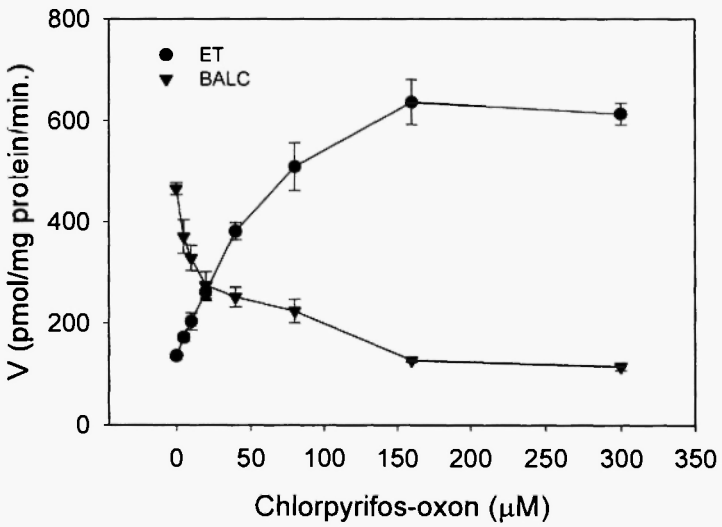

Fig. 8: Activation and inhibition of DEET (100 μM) metabolism by pHLM, which has been pre-incubated with CPO.

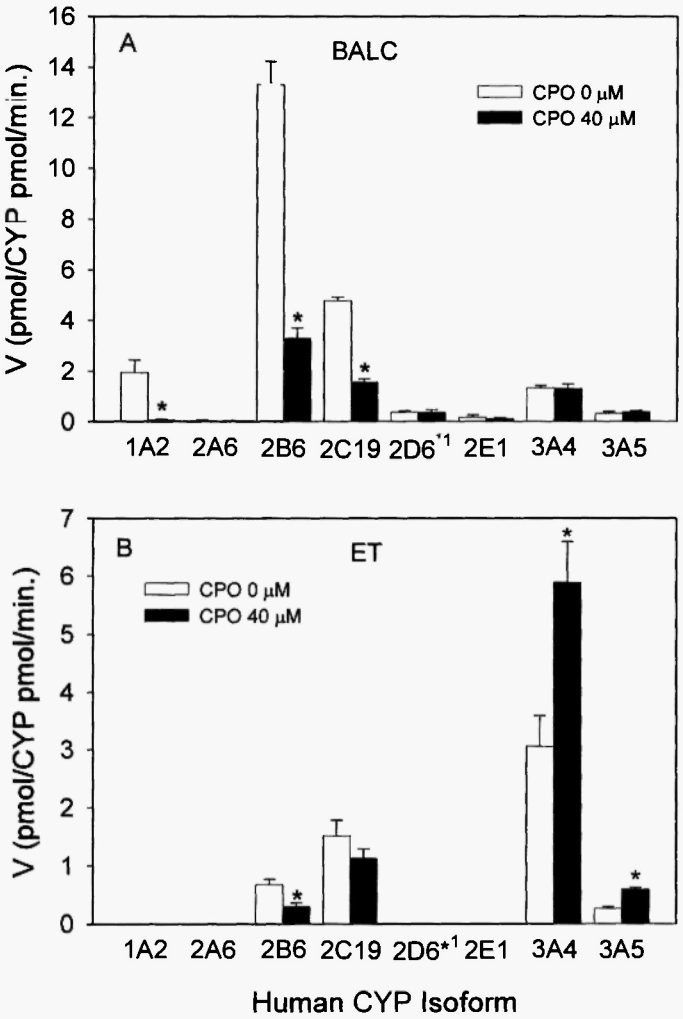

Fig. 9: Inhibition and activation of DEET (40 μM) metabolism by human CYP isoforms, pre-incubated with CPO (40 μM). Production of BALC (**A**) or ET (**B**) is shown. The asterisks indicate a significant difference compared to the corresponding control ($p < 0.05$).

dependant manner (Fig. 10A,B). While the production of BALC was significantly reduced by CPO, the production of ET was increased by CPO up to 80 μM and declined to 80% of the maximum velocity (Fig. 10C,D).

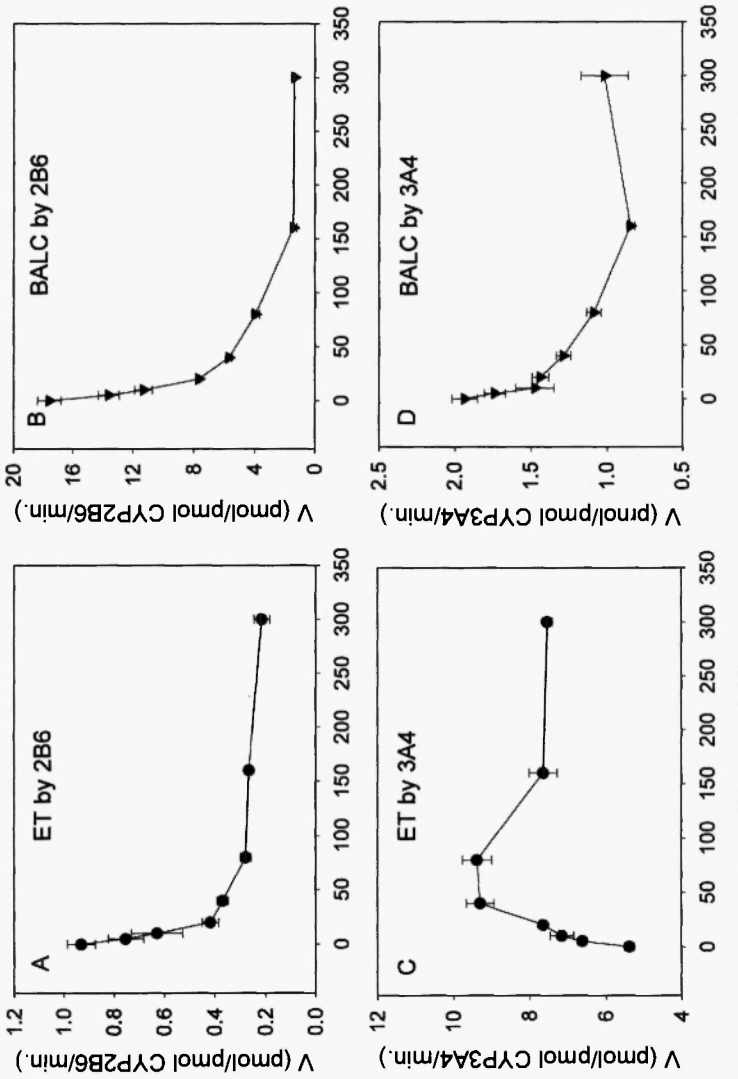

Fig. 10: CPO inhibition and activation of DEET (100 μM) metabolism by human CYP2B6 (**A** and **B**) or CYP3A4 (**C** and **D**). Production of ET (**A**) or BALC (**B**) by CYP2B6, and ET (**C**) or BALC (**D**) by CYP3A4 is shown.

DISCUSSION

This study investigated xenobiotic-associated stimulation or inhibition of naphthalene or DEET metabolism by human CYP isoforms. Metabolic inhibition of metabolism by CYP isoforms by xenobiotics other than the substrate is frequently observed and has been extensively studied, but stimulation of substrate metabolism is less well understood. Several human CYP isoforms including members of the 3A subfamily were stimulated by CPO for naphthalene metabolism, showing a similar pattern for the production of each metabolite. DEET metabolism by either pHLM or CYP3A4 pre-incubated with CPO showed increased production of ET and decreased production of BALC.

In contrast to CPO, CPS, the parent chemical of CPO, inhibited naphthalene metabolism in pHLM, and this inhibition is believed to be correlated with reactive sulfur dissociated from the parent chemical /19,20-22/. The 1:1 mixture of CPS and CPO caused relatively reduced stimulation of naphthalene metabolism. These observations indicate that, in all probability, CPS and CPO bind to CYP isoforms competitively at the binding sites, with CPS having an inhibitory and CPO a stimulatory effect on naphthalene metabolism by human CYP isoforms.

CPO inhibited naphthalene metabolism by CYP1A1, CYP1A2, CYP1B1, and CYP2B6, while stimulating the metabolism of naphthalene by CYPs of the subfamilies 2C, 3A, and CYP2D6*[1], with CYP3A4 being the most significantly affected isoform (Fig. 4). Calculation of the percentages of total normalized rate (%TNR) based on these CYP isoform screening assays is an integrated method to carry out *in vitro* CYP reaction phenotyping /18/. This analysis indicates that the major portion of naphthalene metabolism by pHLM pre-incubated with CPO is attributable to the metabolic contribution of CYP3A4. In contrast to CYP3A4, the contribution of CYP1A2 and 2B6 to naphthalene metabolism was significantly reduced or abolished following pre-incubation with CPO (Table 1). This example indicates that multiple xenobiotic exposures can cause modifications in the contributions of different CYPs to the overall metabolism of a substrate.

While CYP1A2 metabolism of naphthalene is inhibited by CPO (Fig. 5), CYP3A4 metabolism of the substrate is stimulated by CPO

(Fig. 6). Coincidently, α-naphthoflavone inhibited CYP1A2 metabolism of flavonoids /23/ and stimulated CYP3A4 metabolism of midazolam /24/. These different effects of xenobiotics on CYP1A2 and CYP3A4 are probably related to the characteristics of their active sites; size, shape, and hydrophobicity /25/. The hydrophobic cleft as well as the entire active site in CYP1A2 are presumably smaller than those in CYP3A4 /25/. The active site of CYP1A2 occupied by CPO is unlikely to have enough space for naphthalene, resulting in the inhibition of CYP1A2 metabolism of naphthalene by CPO, either competitively or non-competitively. Some CYP isoforms, including CYP3A4, are considered to have multiple binding sites and their active site is large enough to hold more than one substrate /26-30/.

The causes of activation or stimulation of CYP isoform metabolism of xenobiotics may include facilitation of electron transfer from cytochrome b_5 /31/, interaction of CYP and CYP reductase /32/, and/or conformational changes upon binding of substrates or effectors /33/. When the metabolic activity of CYP isoforms is stimulated by the enhanced interaction of CYP and the CYP reductase, V_{max} values are enhanced, but K_m values for the substrates do not generally change. For instance, 7,8-benzoflavone (or α-naphthoflavone) and flavone stimulate rabbit liver microsomal metabolism of benzo[*a*]-pyrene and show an enhanced V_{max} value but the K_m value of the substrate is not changed /32/, indicating that electron transport from the reductase to CYPs is facilitated. Cytochrome b_5 has a role in the transport of electrons to some CYPs /34/, and may also be involved in allosteric stimulation of CYPs with a role in conformational changes /35,36/. However, our preliminary results showed no involvement of cytochrome b_5. Conformational changes of CYP enzymes by effectors have been also reported /24,37/. Striking shifts of substrates toward the heme iron of CYP isoforms upon binding with the effectors have been observed /24,37/.

Stimulation of the metabolism of several CYP isoform substrates, such as naphthalene or DEET, was observed in these studies. Among those CYP isoforms, stimulation of CYP3A4 activity by CPO was most distinctive, and this stimulation was further characterized (Figs. 6, 7, 8, 10). The current observation of increased V_{max} and decreased K_m values (indicating increased affinity of the substrate) for naphthalene metabolism by CYP3A4 pre-incubated with CPO is similar to a previous report that dapsone produces stimulation of CYP2C9 meta-

bolism of flurbiprofen, which was interpreted as being caused by a shift of the substrate closer to the heme iron of CYP2C9 /37/. The observation that the substrate affinity of naphthalene for CYP3A4 is significantly enhanced by the presence of CPO indicates that electron transfer from CYP reductase or cytochrome b_5 to the CYP3A4 isoform is not likely to be involved. CPO also stimulated naphthalene metabolism of CYP3A4 in the absence of cytochrome b_5. This observation indicates that the stimulation by CPO of the metabolism of naphthalene by CYP3A4 is independent of the presence of cytochrome b_5. Therefore, this stimulation appears to be related to conformational change of CYP3A4 upon pre-incubation with CPO, possibly bringing the substrate closer to the heme iron of the enzyme, improving binding affinity, and facilitating reaction velocity. Topological alterations of the active site of CYP3A4 are also supported by the increased ratios of 1-naphthol to 2-naphthol production from naphthalene metabolism, indicating a changed position of the bound substrate in the active site. This increased value of the ratio of 1-naphthol to 2-naphthol production additionally indicates that the production of 1-naphthol and 2-naphthol from the chemically unstable intermediate, naphthalene-1,2-epoxide, may, at least in part, be influenced by the enzymatic environment /11/ in addition to generation due to spontaneous, non-enzymatic rearrangement /10,38,39/. The regioselectivity shown in the data of DEET metabolism in pHLM (Fig. 8) or CYP3A4 (Fig 10C,D) pre-incubated with CPO, namely increased ET and decreased BALC production, supports the hypothesis that the topological characteristics of the substrate at the active site of the enzyme are changed upon CPO binding. This stimulatory activity of CYP3A4 due to pre-incubation of the enzyme with CPO appears to be substrate-dependent because the stimulation pattern of DEET metabolism by CYP3A4 is different from that of naphthalene metabolism. While production of all metabolites was enhanced in the case of naphthalene metabolism, in the case of DEET production one metabolite was stimulated with the other being inhibited by CPO.

In summary, metabolic interactions of naphthalene and DEET with CPO in human CYP isoforms were studied. CPO, unlike its parent chemical CPS, stimulated the metabolism of naphthalene and DEET by several human CYP isoforms. Naphthalene metabolism was inhibited in CYP1A1, 1A2, 1B1, and 2B6 by CPO, but stimulated in CYP subfamilies 2C, 3A, and CYP2D6*[1] by the same effector. Metabolic

modification by CPO caused CYP3A4 to be the most contributory isoform for naphthalene metabolism. DEET metabolism in pHLM pre-incubated with CPO showed a biphasic mode; increased ET production and decreased BALC production. CYP1A2, 2B6, and 2C19 were inhibited by CPO for BALC production, while CYP3A4 and 3A5 were stimulated by this effector for ET production. Thus stimulation of CYP3A4 activity by CPO appears to be substrate dependent and associated with conformational changes of the active site on the binding of CPO.

ACKNOWLEDGEMENTS

We thank Dr. Alan R. Buckpitt (University of California, Davis, CA) for the generous gift of *trans*-1,2-dihydro-1,2-naphthalenediol. We also thank Dr. Wesley G. Taylor (Saskatoon Research Center, Saskatoon, Canada) for the generous gifts of *N,N*-diethyl-*m*-hydroxymethylbenamide (BALC) and *N*-ethyl-*m*-toluamide (ET). This study was supported by a grant from the U.S. Army (DAMD 17-00-2-008).

REFERENCES

1. Preuss R, Angerer J, Drexler H. Naphthalene–an environmental and occupational toxicant, Int Arch Occup Environ Health 2003; 76: 556-576.
2. Cho TM, Rose RL, Hodgson E. In vitro metabolism of naphthalene by human liver microsomal cytochrome P450 enzymes, Drug Metab Dispos 2006; 34: 176-183.
3. Usmani KA, Rose RL, Goldstein JA, Taylor WG, Brimfield AA, Hodgson E. In vitro human metabolism and interactions of repellent *N,N*-diethyl-*m*-toluamide, Drug Metab Dispos 2002; 30: 289-294.
4. Guengerich FP. Cytochromes P450, drugs, and diseases. Mol Interv 2003; 3: 194-204.
5. Guengerich FP, Wu Z, Bartleson CJ. Function of human cytochrome P450s: characterization of the orphans. Biochem Biophys Res Commun 2005; 338: 465-469.
6. Tang J, Cao Y, Rose RL, Brimfield AA, Dai D, Goldstein JA, Hodgson E. Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse, and rat liver microsomes. Drug Metab Dispos 2001; 29: 1201-1204.
7. Boocock DJ, Brown K, Gibbs AH, Sanchez E, Turteltaub KW, White INH. Identification of human CYP forms involved in the activation of tamoxifen and irreversible binding to DNA. Carcinogenesis 2002; 23: 1897-1901.

8. Gonzalez FJ, Yu A. Cytochrome P450 and xenobiotic receptor humanized mice. Annu Rev Pharmacol Toxicol 2006; 46: 41-64.
9. Bogaards JJ, Bertrand M, Jackson P, Oudshoorn MJ, Weaver RJ, vanBladeren PJ, Walther B. Determining the best animal model for human cytochrome P450 activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. Xenobiotica 2000; 30: 1131-1152.
10. Guengerich FP. Comparisons of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. Chem Biol Interact 1997; 106: 161-182.
11. Tracy TS, Hummel MA. Modeling kinetic data from in vitro drug metabolism enzyme experiments. Drug Metab Rev 2004; 36: 231-242.
12. Atkins WM. Implications of the allosteric kinetics of cytochrome P450s. Drug Discov Today 2004; 9: 478-484.
13. Usmani KA, Hodgson E, Rose RL. In vitro metabolism of carbofuran by human, mouse, and rat cytochrome P450 and interactions with chlorpyrifos, testosterone, and estradiol. Chem Biol Interact 2004; 150: 221-232.
14. Tang J, Cao Y, Rose RL, Hodgson E. In vitro metabolism of carbaryl by human cytochrome P450 and its inhibition by chlorpyrifos. Chem Biol Interact 2002; 141: 229-241.
15. Anders MW. Acetone enhancement of microsomal aniline para-hydroxylase activity. Arch Biochem Biophys 1968; 126: 269-275.
16. Huang M, Johnson EF, Muller-Eberhard U, Koop DR, Coon MJ, Conney AH. Specificity in the activation and inhibition by flavonoids of benzo[*a*]pyrene hydroxylation by cytochrome P-450 isozymes from rabbit liver microsomes. J Biol Chem 1981; 256: 10897-10901.
17. Usmani KA, Rose RL, Hodgson E. Inhibition and activation of the human liver microsomal and human cytochrome P450 3A4 metabolism of testosterone by deployment-related chemicals. Drug Metab Dispos 2003; 31: 384-391.
18. Rodrigues AD. Integrated cytochrome P450 reaction phenotyping, attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. Biochem Pharmacol 1999; 57: 465-480.
19. Norman BJ, Poore RE, Neal RA. Studies of the binding of sulfur released in the mixed-function oxidase-catalyzed metabolism of diethyl *p*-nitrophenyl phosphorothionate (parathion) to diethyl *p*-nitrophenyl phosphate (paraoxon), Biochem Pharmacol 1974; 23: 1733-1744.
20. Halpert J, Hammond D, Neal RA. Inactivation of purified rat liver cytochrome P-450 during the metabolism of parathion (diethyl *p*-nitrophenyl phosphorothionate). J Biol Chem 1980; 255: 1080-1089.
21. Neal RA, Halpert J. Toxicology of thiono-sulfur compounds. Annu Rev Pharmacol Toxicol 1982; 22: 321-339.
22. Butler AM, Murray M. Biotransformation of parathion in human liver: participation of CYP3A4 and its inactivation during microsomal parathion oxidation. J Pharmacol Exp Ther 1997; 280: 966-973.

23. Breinholt VM, Offord EA, Brouwer C, Nielsen SE, Brøsen K, Friedberg T. In vitro investigation of cytochrome P450-mediated metabolism of dietary flavonoids. Food Chem Toxicol 2002; 40: 609-616.
24. Cameron MD, Wen B, Allen KE, Roberts AG, Schuman JT, Campbell AP, Kunze KL, Nelson SD. Cooperative binding of midazolam with testosterone and α-naphthoflavone within the CYP3A4 active site: a NMR T_1 paramagnetic relaxation study. Biochemistry 2005; 44: 14143-14151.
25. Lewis DFV, Lake BG, Dickins M. Quantitative structure-activity relationships within a homologous series of 7-alkoxyresorufins exhibiting activity towards CYP1A and CYP2B enzymes: molecular modelling studies on key members of the resorufin series with CYP2C5-derived models of human CYP1A1, CYP1A2, CYP2B6 and CYP3A4. Xenobiotica 2004; 34: 501-513.
26. Yano JK, Wester MR, Schoch GA, Griffin KJ, Stout CD, Johnson EF. The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05-Å resolution. J Biol Chem 2004; 279: 38091-38094.
27. Tanaka T, Okuda T, Yamamoto Y. Characterization of the CYP3A4 active site by homology modeling, Chem Pharm Bull 2004; 52: 830-835.
28. Galetin A, Clarke SE, Houston JB. Multisite kinetic analysis of interactions between prototypical CYP3A4 subgroup substrates: midazolam, testosterone, and nifedipine. Drug Metab Dispos 2003; 31: 1108-1116.
29. Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ, Tracy TS. Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. Biochemistry 1998; 37: 4137-4147.
30. Shou M, Grogan J, Mancewicz JA, Krausz KW, Gonzalez FJ, Gelboin HV, Korzekwa KA. Activation of CYP3A4: evidence for the simultaneous binding of two substrates in a cytochrome P450 active site. Biochemistry 1994; 33: 6450-6455.
31. Lee CA, Manyike PT, Thummel KE, Nelson SD, Slattery JT. Mechanism of cytochrome P450 activation by caffeine and 7,8-benzoflavone in rat liver microsomes. Drug Metab Dispos 1997; 25: 1150-1156.
32. Huang M-T, Chang RL, Fortner JG, Conney AH. Studies on the mechanism of activation of microsomal benzo[*a*]pyrene hydroxylation by flavonoids. J Biol Chem 1981; 256: 6829-6836.
33. Ueng Y-F, Kuwabara T, Chun Y-J, Guengerich FP. Cooperativity in oxidations catalyzed by cytochrome P450 3A4. Biochemistry 1997; 36: 370-381.
34. Porter TD. The roles of cytochrome b_5 in cytochrome P450 reactions. J Biochem Mol Toxicol 2002; 16: 311-316.
35. Auchus RJ, Lee TC, Miller WL. Cytochrome b_5 augments the 17,20-lyase activity of human P450c17 without direct electron transfer. J Biol Chem 1998; 273: 3158-3165.
36. Yamazaki H, Nakamura M, Komatsu T, Ohyama K, Hatanaka N, Asahi S, Shimada N, Guengerich FP, Shimada T, Nakajima M, Yokoi T. Roles of NADPH-P450 reductase and apo- and holo-cytochrome b_5 on xenobiotic oxidations catalyzed by 12 recombinant human cytochrome P450s expressed in membranes of *Escherichia coli*. Protein Expr Purif 2002; 24: 329-337.

37. Hummel MA, Gannett PM, Aguilar JS, Tracy TS. Effector-mediated alteration of substrate orientation in cytochrome P450 2C9. Biochemistry 2004; 43: 7207-7214.
38. van Bladeren PJ, Vyas KP, Sayer JM, Ryan DE, Thomas PE, Levin W, Jerina DM. Stereoselectivity of cytochrome P-450c in the formation of naphthalene and anthracene 1,2-oxides. J Biol Chem 1984; 259: 8966-8973.
39. Buckpitt A, Boland B, Isbell M, Morin D, Shultz M, Baldwin R, Chan K, Karlsson A, Lin C, Taff A, West J, Fanucchi M, van Winkle L, Plopper C. Naphthalene-induced respiratory tract toxicity: metabolic mechanisms of toxicity. Drug Metab Rev 2002; 34: 791-820.