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In Vitro Human Naphthalene Metabolism

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List of abbreviations: Dihydrodiol, *trans*-1,2-dihydro-1,2-naphthalenediol;

pHLM, pooled human liver microsomes;

CYP, cytochromes P450;

HPLC, high performance liquid chromatography;

AUC, area under curve.

Abstract

The polycyclic aromatic hydrocarbon naphthalene is an environmental pollutant, a component of jet fuel and, since 2000, has been reclassified as a potential human carcinogen. Few studies of the *in vitro* human metabolism of naphthalene are available and these focus on lung metabolism. The current studies were performed to characterize naphthalene metabolism by human cytochromes P450 (CYP). Naphthalene metabolites from pooled human liver microsomes (pHLM) were *trans*-1,2-dihydro-1,2-naphthalenediol (dihydrodiol), 1-naphthol, and 2-naphthol. Metabolite production generated Kms of 23, 40, and 116 μM and Vmaxs of 2860, 268, and 22 pmol/mg protein/min., respectively. CYP isoform screening of naphthalene metabolism identified CYP1A2 as the most efficient isoform for producing dihydrodiol and 1-naphthol and CYP3A4 as the most effective for 2-naphthol production. Metabolism of the primary metabolites of naphthalene was also studied to identify secondary metabolites. While 2-naphthol was readily metabolized by pHLM to produce 2,6- and 1,7-dihydroxynaphthalene, dihydrodiol and 1-naphthol were inefficient substrates for pHLM. A series of human CYP isoforms was used to further explore the metabolism of dihydrodiol and 1-naphthol. 1,4-naphthoquinone and four minor unknown metabolites from 1-naphthol were observed, and 1A2 and 2D6*1 were identified as the most active isoforms for the production of 1,4-naphthoquinone. Dihydrodiol was metabolized by CYP isoforms to three minor unidentified metabolites with CYP3A4 and CYP2A6 having the greatest activity toward this substrate. The metabolism of dihydrodiol by CYP isoforms was lower than that of 1-naphthol. These studies identify primary and

secondary metabolites of naphthalene produced by pHLM and CYP isoforms. The dihydrodiol is a potential biomarker of human exposure to naphthalene.

Introduction

The polycyclic aromatic hydrocarbon naphthalene is an environmental pollutant, a component of jet fuel and, since 2000, has been reclassified as a potential human carcinogen. (White, 1999; Riviere *et al.,* 1999; McDougal *et al.,* 2000; Preuss *et al.,* 2003). Naphthalene has been also used in the production of phthalate plasticisers and resins, azo-dyes, dispersants, and tanning agents in the rubber and leather industries (Preuss *et al.,* 2003). Naphthalene is volatile and is discharged into the environment through incomplete burning of fossil fuels as well as domestic and industrial uses of products containing this chemical.

The toxicity of naphthalene has been studied *in vitro* and *in vivo*. In the presence of NADPH and human liver microsomes, 100 μM naphthalene produced significant cytotoxicity in human blood mononuclear leucocytes, but not genotoxicity (Tingle *et al.*, 1993). However, naphthalene has been reclassified as a potential human carcinogen due to evidence of its carcinogenic activity in rats (Preuss *et al*, 2003). Naphthalene has also been reported to induce oxidative stress, resulting in lipid peroxidation and DNA damage in a cultured macrophage cell line, J774A.1 (Bagchi *et al.*, 1998). Lipid peroxidation in mitochondria and glutathione decreases in hepatic and brain tissues are observed in naphthalene-dosed rats (Vuchetich *et al.*, 1996). DNA-single strand breaks are caused by naphthalene in hepatic tissues in the same studies (Vuchetich *et al.*, 1996). In addition, the p53 tumor suppressor gene may be related to toxicity of naphthalene, including enhanced production of superoxide anion and DNA fragmentation (Bagchi *et al.*, 2000). Early stage toxicological indicators of naphthalene exposure in the mouse include

perturbation of nonciliated bronchiolar (Clara) epithelial cell membranes, changes of cell ultrastructure including swollen smooth endoplasmic reticulum and cytoplasmic blebbing and intracellular glutathione depletion (Van Winkle *et al.*, 1999; Plopper *et al.*, 2001).

Since the toxicity of naphthalene in cell culture and animal models is closely related to the metabolism of the compound, CYP monooxygenases may play an important role in its toxicological effects. High concentrations of naphthalene (>500 μM) cause significant toxicity and alter viability in isolated murine Clara cells, but a CYP inhibitor, piperonyl butoxide, blocks the decreased cell viability on preincubation with Clara cells (Chichester *et al.*, 1994). Naphthalene metabolism to naphthalene 1R,2S-oxide stereoselectively mediated by CYP2F2 is suggested to be closely related to species-specific and tissue-selective cytotoxicity of this chemical (Buckpitt *et al.*, 1995). Metabolic formation of 1,2- and/or 1,4-naphthoquinone from 1-naphthol may be a direct cause (Stohs *et al.*, 2002) or an intermediate step in the production of naphthosemiquinone radicals for the toxicity of 1-naphthol (Doherty *et al.*, 1984). Susceptibility to naphthalene-induced injury is gender dependent in the mouse with female mice producing more dihydrodiol in primary injury sites than male mice (Van Winkle *et al.*, 2002).

The metabolism of naphthalene has been studied primarily in experimental animals (Buckpitt *et al*, 1984; 1986; 1987; 1995; 2000; Chichester *et al*, 1994). Metabolic characterization in humans has been investigated in only a few studies (Buckpitt and Bahnson, 1986; Tingle *et al*, 1993). Dihydrodiol and three glutathione conjugates are generated by human lung microsomes in the presence of glutathione and glutathione transferases (Buckpitt and Bahnson, 1986). In naphthalene metabolism using

human liver microsomes, *trans*-1,2-dihydrodiol and 1-naphthol are generated (Tingle *et al*, 1993). However, detailed biochemical characterization as well as identification of the CYP isoforms most responsible for human naphthalene metabolism have not been reported.

In the present studies, we provide biochemical data for characterizing naphthalene metabolism by both human liver microsomes and a wide spectrum of human CYP isoforms. The secondary metabolism of naphthalene primary metabolites was also studied to identify metabolic pathways of naphthalene. A potential biomarker for exposure to naphthalene was identified in these studies.

Materials and Methods

Chemicals. Naphthalene, 1-naphthol, 2-naphthol, 1,4-naphthoquinone, 2,6-dihydroxynaphthalene, and 1,7-dihydroxynaphthalene 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). Acetonitrile, tetrahydrofuran, and phosphoric acid were purchased from the 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) (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9*[1](Arg$_{114}$), 2C18, 2C19, 2D6*[1](Val$_{374}$), 2E1, 3A4, 3A5, 3A7, 4A11) and microsomal epoxide hydrolase (mEH) were purchased from the BD Gentest (Woburn, MA).

***In vitro* Naphthalene Metabolism by Pooled Human Liver Microsomes.** Naphthalene metabolism mediated by pHLM was tested *in vitro.* 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 the substrate was preincubated at 37°C for 5 minutes, the enzymatic reactions were initiated by the addition of cold pHLM (0.48 mg/ml) and incubated at 37°C for 10 minutes. Metabolism of metabolites of naphthalene, 1-naphthol, 2-naphthol and *trans*-1,2-dihydro-1,2-naphthalenediol, in pHLM was also studied in the same conditions stated above for investigating the metabolic pathways of naphthalene with a series of substrate

concentrations. For controls, each substrate was incubated in the same buffer system containing pHLM without the NADPH-generating system.

***In vitro* Screening and Enzyme Kinetics for the Metabolic Activity of Human Cytochrome P450 Isoforms.** The metabolic activity of the human CYP isoforms (50 pmol/ml) listed above was determined at a substrate concentration of 300 μM naphthalene. The enzymatic assays were performed in a same manner as above with a modified incubation time of 15 minutes. 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 a control for these assays.

Based on the screening for metabolic activity of human CYP isoforms, enzyme kinetics of the most efficient isoforms (CYP1A1, 1A2, 2B6, 2E1 and 3A4; 40 pmol/ml) for naphthalene metabolism were also studied using a series of substrate concentrations and an incubation time of 10 minutes.

The metabolic activity of human CYP isoforms (40 pmol/ml) for 80 μM 1-naphthol or *trans*-1,2-dihydro-1,2-naphthalenediol (dihydrodiol) was also screened. In order to identify metabolites, the retention time and the spectra of each metabolite were closely compared. A small number of minor metabolites could not be identified in these studies.

Because pooled human liver microsomes generated a higher ratio of *trans*-1,2-dihydro-1,2-naphthalenediol to 1-naphthol than was observed using CYP isoforms, the potential contribution of naturally occurring epoxide hydrolase to the generation of this product was explored using purified CYP1A2. The kinetics of the formation of 1-

naphthol, 2-naphthol, and *trans*-1,2-dihydro-1,2-naphthalenediol were examined by incubating various concentrations of naphthalene with CYP1A2 (40 pmol/ml) in the absence or presence of human microsomal epoxide hydrolase (0.2 mg/ml).

In order to compare the metabolic efficiency of human CYP isoforms for 1-naphthol with that for *trans*-1,2-dihydro-1,2-naphthalenediol, the metabolic activities for two of the most efficient isoforms, based on the isoform screening (CYP1A2 and 2D6*[1] for 1-naphthol, and CYP2A6 and 3A4 for *trans*-1,2-dihydro-1,2-naphthalenediol), were compared by measuring residual parent chemical. The controls did not include the NADPH-generating system.

All assay reactions were terminated by addition of an equal volume (250 μl) of acetonitrile and vortexing. After 5-minute 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 from 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 composed of 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 designed 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, 1,7-dihydroxynaphthalene and 2,6-dihydroxynaphthalene were selected as 232.7, 225.6, 262.2, 251.6, 239.8, and 228 nm, respectively. Standards of metabolites were prepared in acetonitrile and 50 μl of standard or sample was injected into the HPLC system.

Sample Preparation for GC/MS Analysis. Naphthalene (300 μM) was incubated in a total volume of 500 μl 100 mM potassium phosphate buffer containing 3.3 mM $MgCl_2$ (pH 7.4) with pHLM (0.96 mg/ml) and the NADPH generating system mentioned above for 10 minutes at 37°C after 5-minute preincubation. Immediately after incubation, sample tubes were centrifuged at 15,000 rpm (21,000 g) for 5 minutes and 470 μl of supernatant from each tube was transferred into a fresh tube. 100 μl Dichloromethane (DCM) was added to the fresh tube containing supernatant, and each tube was vigorously shaken for 1 minute. The lower (DCM) layer was then collected for analysis after the tubes were centrifuged at 5,000 rpm for 3.5 minutes. This extraction process with DCM was performed three more times, and the supernatants were combined for the GC/MS analysis.

Analysis of Metabolites by GC/MS. The generation of naphthalene metabolites by pHLM was confirmed by analysis with an Agilent GC/MS system equipped with 6890 GC and 5973 Mass Selective Detector (Palo Alto, CA). A 30-meter capillary column with 0.25-mm nominal diameter (Restek Rtx-5MS, Bellefonte, PA) was used for the analyses with an injection volume of 2 μl and a constant flow of helium gas (1 ml/min. carrier gas). The oven temperature was programmed as follows: initially 40°C with a 1-

min. hold, increased to 100°C at a rate of 25°C/min., followed by an increase to 300°C at a rate of 10°C/min. followed by a 10-min. hold. The total running time was 33.4 minutes and electron impact was used for the ionization of metabolites.

These analyses were performed as a confirmatory process for the HPLC analysis for the production of primary metabolites of naphthalene metabolism by pHLM. Throughout the GC/MS analyses, naphthalene, 1-naphthol, and *trans*-1,2-dihydro-1,2-naphthalenediol were detected at retention times of 7.7, 11.6, and 12.0, respectively, and their fragmentation patterns were compared with those of standards. Detection of 2-naphthol was not successful in these analyses probably due to the combination of its low level of production and potential loss during the extraction process.

Data Analysis and Statistics. The apparent Vmax and Km parameters were calculated using a nonlinear regression curve fitted to the Michaelis-Menton equation. The coefficient of determination (R^2), a measure of how well a regression model describes the data, is shown in the tables. Data means were obtained by at least three determinations. The percentages of total normalized rate (%TNR) were determined as described previously (Rodrigues,1999). The nominal specific contents of individual CYP proteins in native human livers (10 donors) for calculating the %TNR were obtained from BD Gentest (2003 product catalog) except for the contents of CYP2C8 and CYP2C18, which were from Rodrigues (1999).

Statistical significance of the data was determined with one-way ANOVA followed by the Tukey's multiple comparisons.

Results

Three metabolites were detected in metabolism studies of naphthalene by pooled human liver microsomes, 1-naphthol, 2-naphthol and *trans*-1,2-dihydro-1,2-naphthalenediol (dihydrodiol). As presented in table 1, dihydrodiol was the most abundant metabolite followed in order by 1-naphthol and 2-naphthol. The Km value for dihydrodiol was in the same range as that for 1-naphthol, but the Km for 2-naphthol was significantly higher than those for the other two metabolites. The intrinsic clearance (CLint) of dihydrodiol was significantly higher than those for 1-naphthol and 2-naphthol (Table 1). The Michaelis-Menton fitting curves and metabolic rates for productions of the three naphthalene metabolites are shown in figure 1.

Metabolic activities of 15 human CYP isoforms for naphthalene were evaluated (Fig. 2). Among those tested, CYP1A2 was found to be the most efficient for the production of 1-naphthol and dihydrodiol, while CYP3A4 was the most efficient for the production of 2-naphthol. The individual isoforms showed varying degrees of efficiency for the production of each metabolite. CYP isoforms such as 2C8, 2C9*[1], 2C18, 3A5, 3A7 and 4A11 showed minimal or no activity for naphthalene metabolism (Fig. 2). CYP1A2 was the only isoform to generate 1,4-naphthoquinone from naphthalene in a detectable amount (data not shown). CYP1A2, 3A4, and 2E1 showed the highest total normalized rates (%TNR) for 1-naphthol and 2-naphthol generated in naphthalene metabolism, and CYP1A2, 2A6, and 3A4 showed the highest %TNR for dihydrodiol (Table 2).

The five most efficient human CYP isoforms for naphthalene metabolism as shown in figure 2 were selected to further characterize their metabolic activity for

naphthalene. As expected, CYP1A2 was identified as the most efficient isoform for generating 1-naphthol and dihydrodiol, showing the highest Vmax values for these metabolites (Table 3). The Vmax and Km for the production of 1,4-naphthoquinone from naphthalene by this CYP1A2 isoform were 2.3 pmol/pmol/min and 29 μM, respectively. In general, more 1-naphthol than dihydrodiol was produced from naphthalene by these isoforms, which is in contrast to naphthalene metabolism by pHLM. CYP2E1 has higher affinity (i.e. lower Km values) for naphthalene in the production of 1- or 2-naphthol compared to other isoforms. For CLint of 1-naphthol, CYP2E1, 1A2, and 2B6 in that order showed higher values than 3A4 and 1A1. CYP2E1 and 3A4 were higher for the intrinsic clearance of 2-naphthol than other isoforms. CYP3A4 had the highest Vmax for the production of 2-naphthol and CYP2E1 had the lowest Km, accounting for their greater CLint values observed relative to the other isoforms. The Vmax and CLint values of 1A2 and 1A1 were higher for dihydrodiol production than those of the other isoforms (Table 3). Naphthalene metabolism by CYP1A2 produced one unknown minor metabolite (RT=14.3 min.), for which the area under the curve (AUC) was less than 1% of the total metabolite AUC.

In order to investigate apparent discrepancies between amounts of dihydrodiol and 1-naphthol as observed in pHLM- compared to CYP isoform-mediated naphthalene metabolism, naphthalene metabolism by CYP1A2 in the presence of human microsomal epoxide hydrolase (mEH) was studied. These results were compared with the naphthalene metabolism mediated only by CYP1A2 (Table 4 and Fig. 3). The production of 1-naphthol and 2-naphthol were significantly reduced in the presence of mEH, while the production of dihydrodiol was increased based on Vmax and CLint values. Km

values for 1- and 2-naphthol production were significantly increased in the presence of mEH, although the Km value for dihydrodiol production did not change (Table 4). The significant changes in the catalytic velocities by the addition of mEH are also shown in the fitted curves in figure 3.

The secondary metabolism of naphthalene was tested by incubating 1-naphthol, 2-naphthol, or dihydrodiol with either pHLM or CYP1A2. 1-naphthol was not readily metabolized by pHLM, while 2-naphthol was metabolized to produce 2,6- and 1,7-dihydroxynaphthalene, and two unknown minor metabolites (about 3% based on the AUC). In contrast with pHLM, however, 1-naphthol was metabolized by CYP1A2 to generate 1,4-naphthoquinone and four unknown metabolites (about 56% based on the AUC). 2-naphthol metabolism by CYP1A2 also produced the same metabolites as those by pHLM and three additional unknown metabolites (about 6% based on AUC). More 2,6-dihydroxynaphthalene than 1,7-dihydroxynaphthalene was generated by both pHLM and CYP1A2. Neither pHLM nor CYP1A2 metabolized dihydrodiol. The kinetic parameters for this secondary metabolism are shown in the table 5.

To further investigate the unknown metabolites from 1-naphthol or dihydrodiol, and to determine which human CYP isoforms are efficient in secondary metabolism, a series of human CYP isoforms were utilized for 1-naphthol or dihydrodiol metabolism. 1-naphthol was metabolized to 1,4-naphthoquinone and four unknown metabolites by most CYP isoforms (Fig. 4). Dihydrodiol metabolism generated three unknown metabolites primarily due to activity of CYP 2A6 and 3A4. Based on the total AUC of metabolites for 1-naphthol or dihydrodiol, the total normalized rate (%TNR) of their metabolites for each CYP isoform was calculated (Table 6). CYP3A4, 1A2, and 2C19

showed the highest %TNR for 1-naphthol metabolite, and CYP3A4, 2A6, and 2C8 had the highest %TNR for dihydrodiol metabolite (Table 6). Identification of unknown metabolites was not successful because of the lack of potential standards. To investigate which substrate, 1-naphthol or dihydrodiol, is more effectively metabolized by individual CYP isoforms, two isoforms among the most efficient for each substrate, CYP1A2 and 2D6*[1] for 1-naphthol, and 2A6 and 3A4 for dihydrodiol, were selected. Because there were unknown metabolites from this metabolism, substrate disappearance after metabolism by each CYP enzyme was compared for evaluating the metabolic efficiency of each isoform. For 1-naphthol, 16.3 ± 0.5 and 19.6 ± 1.1% (mean ± S.E.M) of the parent chemical were metabolized by 1A2 and 2D6*[1], while for dihydrodiol, 0.4 ± 0.1 and 2.7 ± 0.2% were metabolized by 2A6 and 3A4, respectively.

Discussion

In naphthalene metabolism by pooled human liver microsomes, about 10 times more *trans*-1,2-dihydro-1,2-naphthalenediol (dihydrodiol) was generated than 1-naphthol, and generation of the latter was about 10 times higher than that of 2-naphthol. The observation of the predominant production of the dihydrodiol metabolite in these studies agrees with a previous report, in which about 8.6 times more dihydrodiol was generated than 1-naphthol in human liver microsomes (Tingle *et al*, 1993). While the previous studies of human naphthalene metabolism were performed with microsomes obtained from a limited number of organ donor(s) (1 to 6) (Buckpitt and Bahnson, 1986; Tingle *et al*, 1993; Wilson *et al*, 1996), the human liver microsomes used in these studies were commercially prepared from organs donated from as many as 46 people. Therefore, the potential bias due to individual variation was significantly reduced in these studies. Although the generation of 2-naphthol in naphthalene metabolism has been known, the observation of 2-naphthol generation by human liver microsomes has not been previously reported. The predominant generation of the *trans*-form of dihydrodiol is probably due to its chemical stability compared to the *cis*-form.

The CYP isoform screen in the current studies revealed the most efficient isoforms for producing naphthalene metabolites. Although there have been a few metabolic studies of naphthalene using human microsomes (Buckpitt and Bahnson, 1986; Tingle *et al*, 1993; Wilson *et al*, 1996), naphthalene metabolism using a series of individual human CYP isoforms has not been previously studied. CPY1A2 was identified as the most effective isoform for naphthalene metabolism. Total CYP protein content of the 1A2 isoform in human liver ranges from approximately 8 to 13 %

(Shimada *et al*, 1994; Rodrigues, 1999). Using the mean specific protein contents of CYP isoforms obtained from BD Gentest (2003) and Rodrigues (1999), the calculated %TNR of CYP1A2 demonstrates its important role in naphthalene metabolism in human liver along with CYP3A4, 2E1, and 2A6. Although CYP3A4 showed generally lower metabolic activity toward naphthalene than CYP1A2 (Table 3 and Fig. 2), the %TNR of CYP3A4 was approximately 50 and 25% of those for CYP1A2 for 1-naphthol and dihydrodiol generation, respectively due to its highest abundance in the human liver microsomes (Table 2). Furthermore, CYP3A4 was the dominant isoform for 2-naphthol formation not only in the absolute generation of this metabolite but also the %TNR value shows that about three fourths of 2-naphthol formed from naphthalene in human liver is associated with CYP3A4. It is known that formation of 1-naphthol and 2-naphthol can be achieved by spontaneous, nonenzymatic rearrangement from the chemically unstable intermediate, naphthalene-1,2-epoxide (Preuss *et al*, 2003; Buckpitt *et al*, 2002; Van Bladeren *et al*, 1984). In the current studies, however, the CYP isoforms tested showed various metabolite ratios produced from naphthalene. These results lead to the conclusion that the production of 1-naphthol and 2-naphthol may be, at least in part, either enzymatic or influenced by the enzyme environment.

Kinetic parameters were obtained for the five CYP isoforms showing the most efficient metabolism of naphthalene. In contrast to naphthalene metabolism in pHLM, more 1-naphthol was produced by several CYP isoforms than was 2-naphthol or dihydrodiol. In pHLM dihydrodiol formation was higher than either 1- or 2-naphthol. Epoxide hydrolase is generally known to be involved in the production of dihydrodiol from naphthalene epoxide. Naphthalene assays with a mixture of CYP1A2 and human

microsomal epoxide hydrolase showed that microsomal epoxide hydrolase in pHLM contributes to the higher production of dihydrodiol. Epoxide hydrolase may not be the sole contributor for converting naphthalene-1,2-epoxide into the dihydrodiol, because purified CYP isoforms also produced the dihydrodiol metabolite from naphthalene, possibly by non-enzymatic hydrolysis. However, since individual isoforms vary in the production of the dihydrodiol, the possibility exists for CYP involvement directly or indirectly in this transformation into dihydrodiol.

In the secondary metabolism of naphthalene, primary metabolites from naphthalene were used as substrates and their metabolic reactions in pHLM and CYP isoforms were investigated. Although 1-naphthol was readily metabolized by most CYP isoforms, this substrate was not metabolized by pHLM. These results contrast with a previous report, in which 1-naphthol showed metabolism-dependent cytotoxicity to human mononuclear leucocytes in the presence of NADPH and human liver microsomes prepared from a kidney transplant donor (Wilson *et al*, 1996). Methodology differences, including direct HPLC method for metabolite detection, shorter incubation time (10 minutes vs. 2 hours), and sample size (46 pooled individuals vs. 1 individual), make it difficult to reconcile this difference. In contrast to our results with pooled HLM, individual CYP isoforms, including 1A2 and 2D6*[1], were effective in metabolizing 1-naphthol to produce 1,4-naphthoquinone and unknown metabolites. Although it is unclear why pHLM did not readily metabolize 1-naphthol while individual CYP isoforms did, 1-naphthol is a less favorable substrate for pHLM than its parent chemical, naphthalene.

Differently from 1-naphthol, 2-naphthol was readily metabolized by pHLM and CYP1A2. More abundant production of 2,6- rather than 1,7-dihydroxynaphthalene from 2-naphthol indicates that hydroxylation at the carbon 6 position is kinetically more favorable than at the carbon 8 position after carbon 2 is hydroxylated. The product hydroxylated at carbons 2 and 8 is also named 1,7-dihydroxynaphthalene. Metabolism of 2-naphthol by pHLM, which was more active than those of 1-naphthol and dihydrodiol, may be a factor influencing the higher apparent Km value for 2-naphthol production from naphthalene than those for 1-naphthol or dihydrodiol production in the metabolic system mediated by pHLM.

Trans-1,2-dihydro-1,2-naphthalenediol (dihydrodiol) was not readily metabolized by either pHLM or CYP1A2. Dihydrodiol is known to be converted into 1,2-dihydroxynaphthalene by dihydrodiol dehydrogenase and 1,2-naphthoquinone by further oxidation or 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydronaphthalene by CYP (Penning *et al*, 1999; Buckpitt *et al*, 2002). However, neither 1,2-naphthoquinone nor 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydronaphthalene were identified in this study. Because dihydrodiol dehydrogenase is a cytosolic enzyme, the absence of this enzyme in liver microsomes may explain why 1,2-naphthoquinone was not detected in the current study. Naphthalene metabolites, including dihydrodiol, can be further transformed into conjugation products including glutathione (Smart and Buckpitt, 1983; Buckpitt *et al*, 1987), mercapturic acid (Pakenham *et al*, 2002), and glucuronide/sulphate conjugates (Preuss *et al*, 2003).

As the major metabolites, naphthols can be utilized as biomarkers for exposure to naphthalene. 1- and 2-naphthol are detected in urine of Wistar rats administered

intraperitoneally with naphthalene (Elovaara *et al*, 2003). However, these naphthols have also been detected in cases of exposure to environmental polycyclic aromatic hydrocarbons in human and animals. A study of the urinary naphthol contents in Japanese male workers, for instance, suggests that 1- and 2-naphthol can be used as biomarkers for exposure to airborne polycyclic aromatic hydrocarbons (Yang *et al*, 1999). Additionally, personal preferences in lifestyle, including smoking, can provide significant variation in urinary naphthol contents (Lee *et al*, 2001) and 1-naphthol is also generated as a metabolite when humans are exposed to the insecticide, carbaryl (Shealy *et al*, 1997). Furthermore, in the present studies, these naphthols were more readily metabolized by CYP isoforms than dihydrodiol. Therefore, dihydrodiol may be a better and more specific biomarker for exposure to naphthalene in humans due to the abundant generation and less effective conversion in human liver metabolism. The amount of dihydrodiol formed from naphthalene in mouse lung or liver microsomes in the presence of cytosolic proteins is not changed much over the range of 0-2 mg of cytosolic protein concentration (Buckpitt *et al*, 1984). This observation indicates that cytosolic enzymes may have minimal effects in the formation of dihydrodiol from naphthalene and in the conversion into downstream metabolites.

In summary, human naphthalene metabolism was extensively studied. The metabolic pathway of naphthalene by human liver microsomes and CYP isoforms is shown in figure 5. Naphthalene metabolism in pooled human liver microsomes produced *trans*-1,2-dihydro-1,2-naphthalenediol, 1-naphthol, and 2-naphthol in order of production. The most efficient and important isoforms in human naphthalene metabolism were identified through human CYP isoform screening. Based on the total normalized

rates (%TNR), CYP1A2, 3A4, 2E1, and 2A6 are considered to be the most important isoforms in human liver naphthalene metabolism. In these studies, the secondary metabolism of naphthalene was investigated using the primary metabolites as substrates for pHLM and CYP isozymes. CYP1A2 and 2D6*[1], and CYP2A6 and 3A4 were identified as the most efficient isoforms for metabolizing 1-naphthol and dihydrodiol, respectively. Based on the protein contents in human liver, CYP3A4, 1A2, and 2C19 are considered the important isoforms for 1-naphthol metabolism, and CYP3A4, 2A6, and 2C8 were for dihydrodiol. Dihydrodiol was less favorable for being metabolized by CYP isoforms than naphthalene and other primary metabolites, so that this metabolite may be a good biomarker for exposure to naphthalene.

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Footnotes

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Figure Legends

Figure 1. Naphthalene metabolism by pooled human liver microsomes. Each metabolite was curve-fitted to the Michaelis-Menton equation. Specific activities are expressed as picomoles of product generated per mg of liver microsomal protein per minute. The data shown are the mean ± S.E.M (n=3).

Figure 2. Human cytochromes P450 isoform activity screening in naphthalene metabolism. Naphthalene (300 μM) was metabolized by individual CYP isoforms (50 pmol/ml) in a NADPH generating system at 37°C for 15 minutes. Specific activities are expressed as picomoles of product generated per picomole of CYP isoform per minute. The data shown are the mean ± S.E.M (n=3).

Figure 3. Naphthalene metabolism by CYP1A2 isoform in the presence of human microsomal epoxide hydrolase (0.2 mg/ml). These data are compared with those of naphthalene metabolism by CYP1A2 only. Specific activities are expressed as picomoles of product generated per picomole of CYP1A2 isoform per minute. The data shown are the mean ± S.E.M (n=3).

Figure 4. Human cytochromes P450 isoform activity screening by the production of 1,4-naphthoquinone in 1-naphthol metabolism. 1-naphthol (80 μM) was metabolized by individual CYP isoforms (40 pmol/ml) in a NADPH generating system at 37°C for 15 minutes. Specific activities are expressed as picomoles of product generated per picomole of CYP isoform per minute. The data shown are the mean ± S.E.M (n=3).

Figure 5. Metabolic pathway of naphthalene in humans.

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Table 1. Metabolism of naphthalene by pooled human liver microsomes.

Metabolite	Human Liver Microsomes			
	V_{max}	K_m	CLint (V_{max}/K_m)	R^2
	pmol/mg Prot./min.	μM	μl/mg Prot./min.	
1-Naphthol	268.2 ± 11.1^a	40.2 ± 2.2^a	6699^a	0.99
2-Naphthol	22.3 ± 0.3^b	116.1 ± 9.0^b	194^a	0.99
Dihydrodiol	2860.2 ± 48.3^c	22.9 ± 0.7^a	125278^b	0.99

Dihydrodiol indicates *trans*-1,2-dihydro-1,2-naphthalenediol. Means with a different letter in the same column are significantly different ($p<0.05$). Data shown are the mean ± S.E.M. (n=3).

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Table 2. Total normalized rate (%TNR) for naphthalene metabolites by individual CYP isoforms 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	
1A1	ND	ND	ND	NA
1A2	40.4	9.2	48.8	55
1B1	ND	ND	ND	NA
2A6	9.5	3.2	24.7	52
2B6	6.9	3.2	3.3	21
2C8†	1.0	0.0	0.0	64
2C9*[1]	1.4	0.0	0.0	76
2C18†	0.02	0.03	0.0	2.5
2C19	6.0	3.0	5.4	39
2D6*[1]	1.2	0.5	3.3	12
2E1	11.7	4.3	2.3	52
3A4	21.8	76.5	12.3	133
3A5	0.02	0.03	0.0	1.2
3A7	ND	ND	0.0	NA
4A11	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 indicates not determined. NA indicates not available.

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Table 3. Metabolism of naphthalene by human CYP isoforms.

	1-Naphthol			
	V_{max}	K_m	CL_{int} (V_{max}/K_m)	R^2
CYP Isoform	pmol/pmol/min.	μM	μl/nmol/min.	
1A1	9.1 ± 0.6^{a}	111.0 ± 13.0^{a}	84^{a}	0.99
1A2	35.8 ± 4.4^{b}	72.7 ± 18.5^{a}	522^{b}	0.98
2B6	20.2 ± 2.2^{c}	$58.6 \pm 10.4^{a,b}$	361^{b}	0.99
2E1	8.4 ± 0.1^{a}	10.1 ± 0.7^{b}	841^{c}	0.98
3A4	8.1 ± 1.0^{a}	$60.7 \pm 17.6^{a,b}$	146^{a}	0.99

	2-Naphthol			
	V_{max}	K_m	CL_{int} (V_{max}/K_m)	R^2
CYP Isoform	pmol/pmol/min.	μM	μl/nmol/min.	
1A1	0.3 ± 0.0^{a}	109.5 ± 25.4^{a}	3^{a}	0.97
1A2	0.9 ± 0.0^{b}	116.2 ± 16.0^{a}	8^{a}	0.99
2B6	$0.8 \pm 0.1^{a,b}$	$93.8 \pm 22.4^{a,b}$	9^{a}	0.99
2E1	0.2 ± 0.0^{a}	9.9 ± 0.3^{b}	22^{b}	0.95
3A4	1.6 ± 0.2^{c}	$65.4 \pm 20.3^{a,b}$	27^{b}	0.99

	trans-1,2-Dihydro-1,2-naphthalenediol			
	V_{max}	K_m	CL_{int} (V_{max}/K_m)	R^2
CYP Isoform	pmol/pmol/min.	μM	μl/nmol/min.	
1A1	7.4 ± 0.9^{a}	53.9 ± 5.9^{a}	140^{a}	0.96
1A2	7.7 ± 0.5^{a}	33.5 ± 8.6^{a}	250^{b}	0.98
2B6	2.2 ± 0.2^{b}	49.5 ± 9.3^{a}	47^{c}	0.99
2E1	0.4 ± 0.1^{b}	33.8 ± 15.0^{a}	14^{c}	0.90
3A4	1.0 ± 0.1^{b}	55.8 ± 13.0^{a}	19^{c}	0.96

	1,4-Naphthoquinone			
	V_{max}	K_m	CL_{int} (V_{max}/K_m)	R^2
CYP Isoform	pmol/pmol/min.	μM	μl/nmol/min.	
1A2	2.3 ± 0.0	28.9 ± 0.9	79	0.95

Means with a different letter in the same column are significantly different ($p<0.05$).

Data shown are the mean ± S.E.M. (n≥3).

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Table 4. Metabolism of naphthalene by human CYP1A2 in the presence of human microsomal epoxide hydrolase (mEH: 0.2 mg/ml).

Metabolites	CYP1A2 with mEH (0.2 mg/ml)			
	V_{max}	K_m	CLint (V_{max}/K_m)	R^2
	pmol/pmol/min.	μM	μl/nmol/min.	
1-Naphthol	20.2 ± 1.0**	163.5 ± 14.8*	125*	0.99
2-Naphthol	0.6 ± 0.0	195.7 ± 17.6*	3	0.99
Dihydrodiol	56.4 ± 0.9**	38.5 ± 3.6	1490**	0.99

Dihydrodiol indicates *trans*-1,2-dihydro-1,2-naphthalenediol. Symbols * and ** indicate $p<0.05$ and $p<0.001$, respectively, when compared to their counterparts in the absence of mEH. Data shown are the mean ± S.E.M. (n=3).

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Table 5. Metabolism of 1-naphthol and 2-naphthol by pooled human liver microsomes (pHLM) or CYP1A2.

Parent Chemical	Metabolite	pHLM			
		V_{max}	K_m	CLint (V_{max}/K_m)	R^2
		pmol/mg Protein/min.	μM	μl/mg Prot./min.	
1-Naphthol	1,4-NapQ	ND	ND	ND	
2-Naphthol	2,6-DiOH	322.6 ± 25.0	7.0 ± 1.1	47459	0.98
	1,7-DiOH	132.1 ± 6.5	12.3 ± 1.2	10978	0.99

Parent Chemical	Metabolite	CYP1A2			
		V_{max}	K_m	CLint (V_{max}/K_m)	R^2
		pmol/pmol 1A2/min.	μM	μl/nmol 1A2/min.	
1-Naphthol	1,4-NapQ	10.8 ± 0.4	28.7 ± 1.4	378	0.99
2-Naphthol	2,6-DiOH	45.2 ± 1.3	25.2 ± 2.0	1805	0.98
	1,7-DiOH	6.4 ± 0.3	43.2 ± 4.1	149	0.97

1,4-NapQ, 2,6-DiOH, and 1,7-DiOH indicate 1,4-naphthoquinone, 2,6-dihydroxynaphthalene, and 1,7-dihydroxynaphthalene, respectively. ND indicates not determined. Data shown are the mean ± S.E.M. (n=3).

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Table 6. Total normalized rate (%TNR) for 1-naphthol or *trans*-1,2-dihydro-1,2-naphthalenediol (dihydrodiol) metabolites by individual human CYP isoforms and the specific content of each CYP protein in native human liver microsomes.

CYP Isoform	%TNR		Mean content of CYP¶ (pmol CYP/mg Prot.)
	1-Naphthol	Dihydrodiol	
1A1	ND	ND	NA
1A2	19.5	0.0	55
1B1	ND	0.0	NA
2A6	0.4	17.0	52
2B6	7.5	2.3	21
2C8†	5.6	14.0	64
2C9*[1]	1.9	6.3	76
2C18†	0.2	0.02	2.5
2C19	9.9	3.1	39
2D6*[1]	4.5	0.01	12
2E1	7.1	1.2	52
3A4	43.3	56.2	133
3A5	0.2	0.01	1.2
3A7	ND	ND	NA
4A11	ND	ND	NA

¶ Mean content data were obtained from the BD Gentest (2003).

† Mean content data for 2C8 and 2C18 were obtained from Rodrigues (1999).

The %TNR values for metabolites of 1-naphthol or dihydrodiol were calculated with the total area of metabolites. ND indicates not determined. NA indicates not available.

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Fig. 1. Naphthalene metabolism by pooled human liver microsomes.

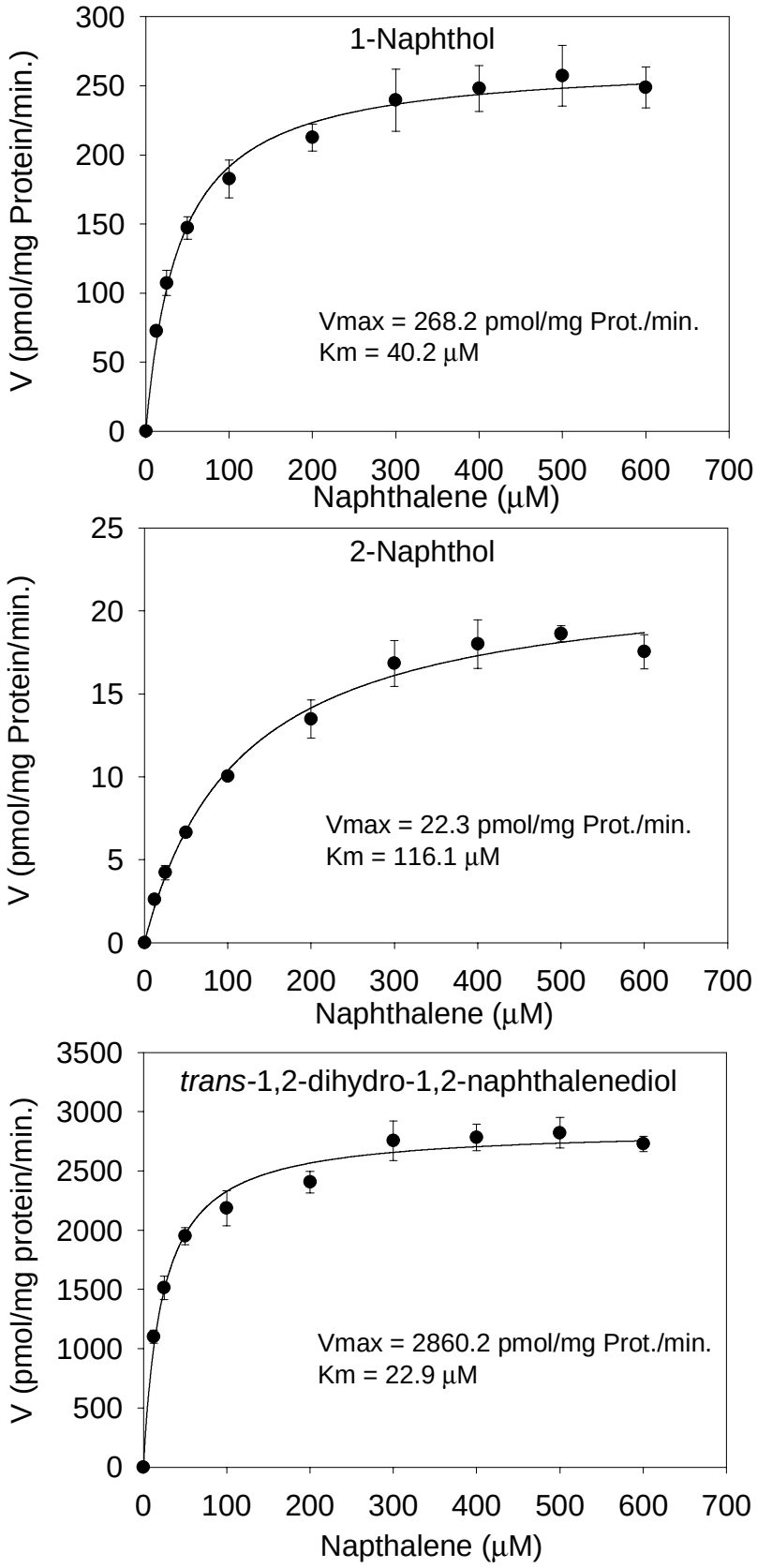

Fig. 2. Human cytochromes P450 isoform activity screening in naphthalene metabolism.

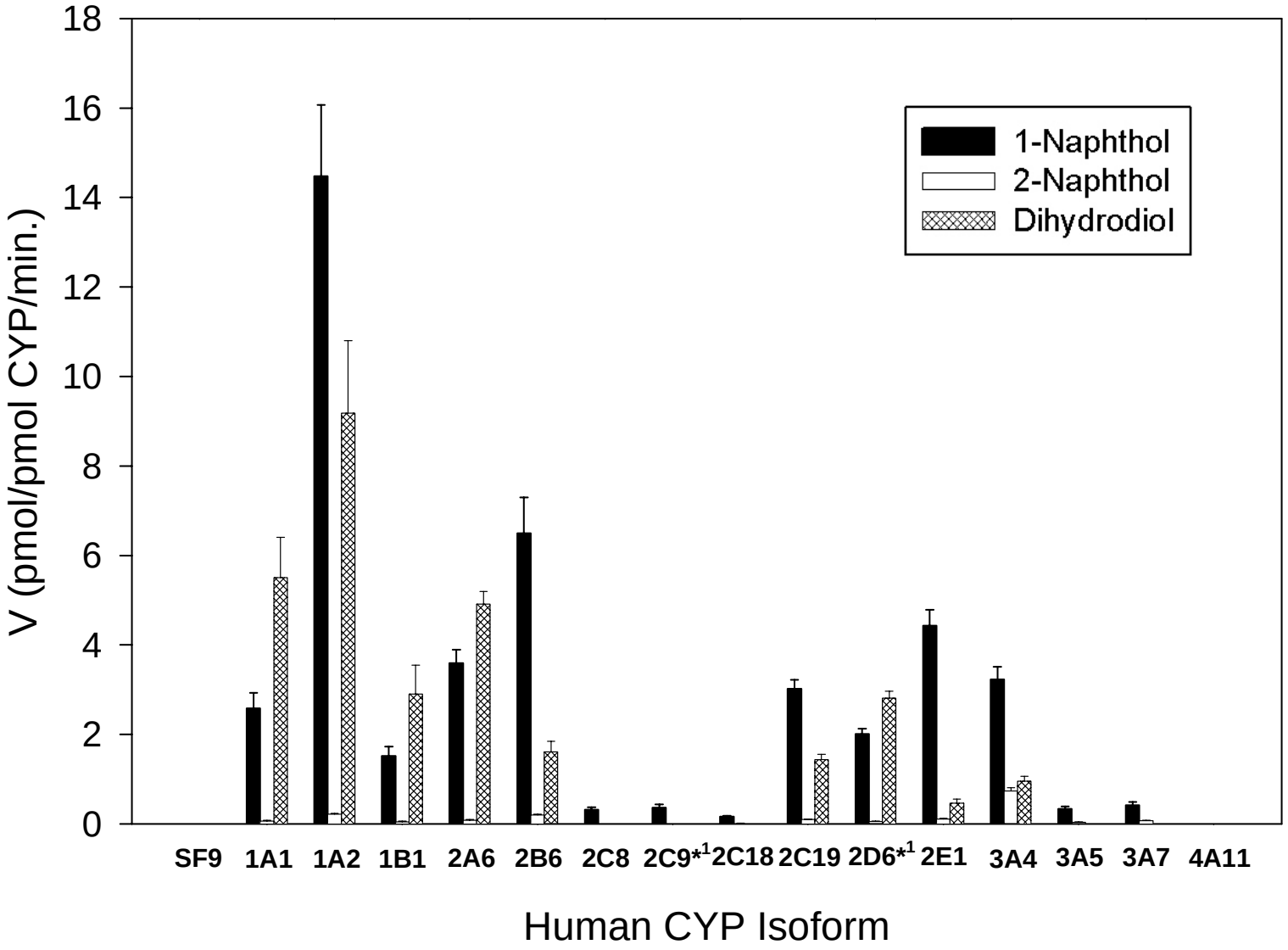

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Fig. 3. Naphthalene metabolism by CYP1A2 isoform in the presence of human microsomal epoxide hydrolase.

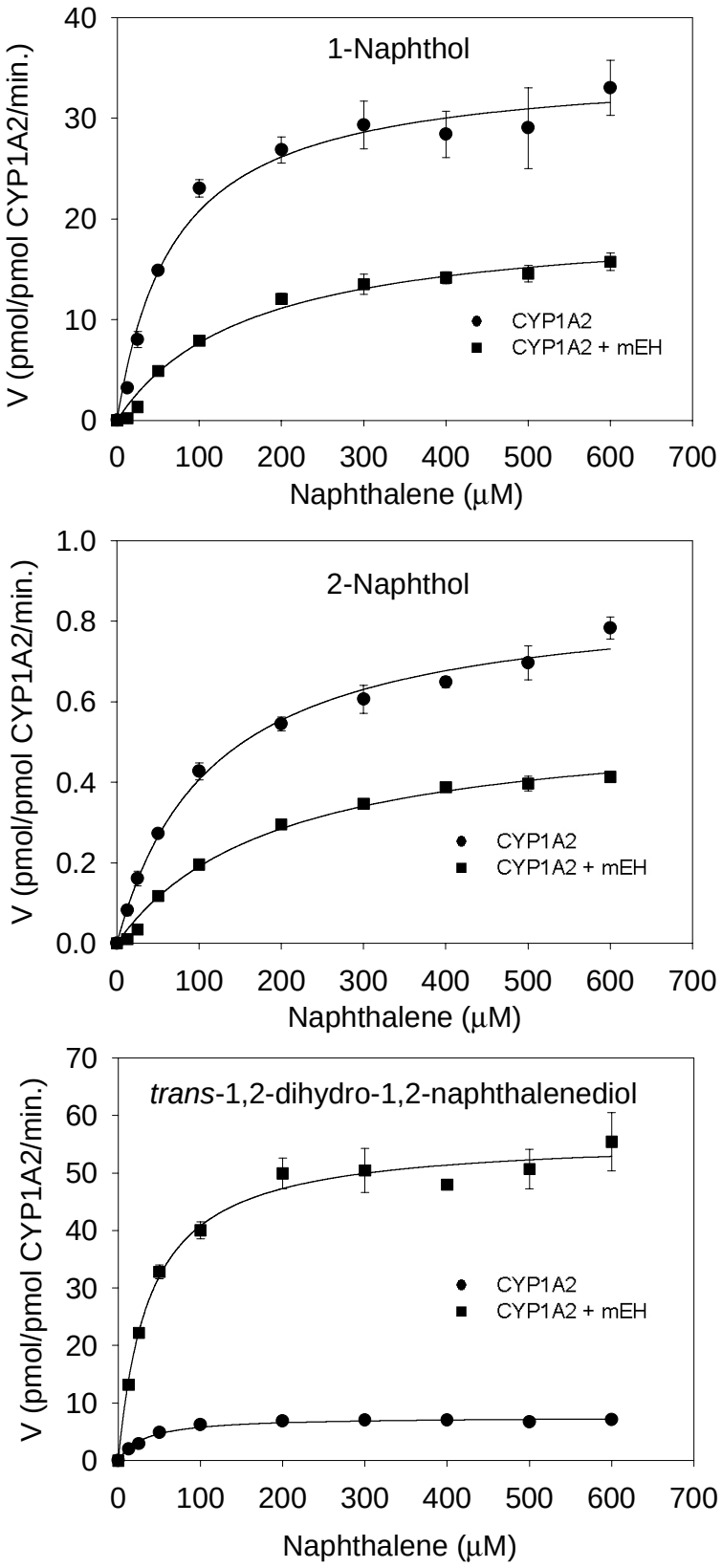

Fig. 4. Human cytochromes P450 isoform activity screening by the production of 1,4-naphthoquinone in 1-naphthol metabolism.

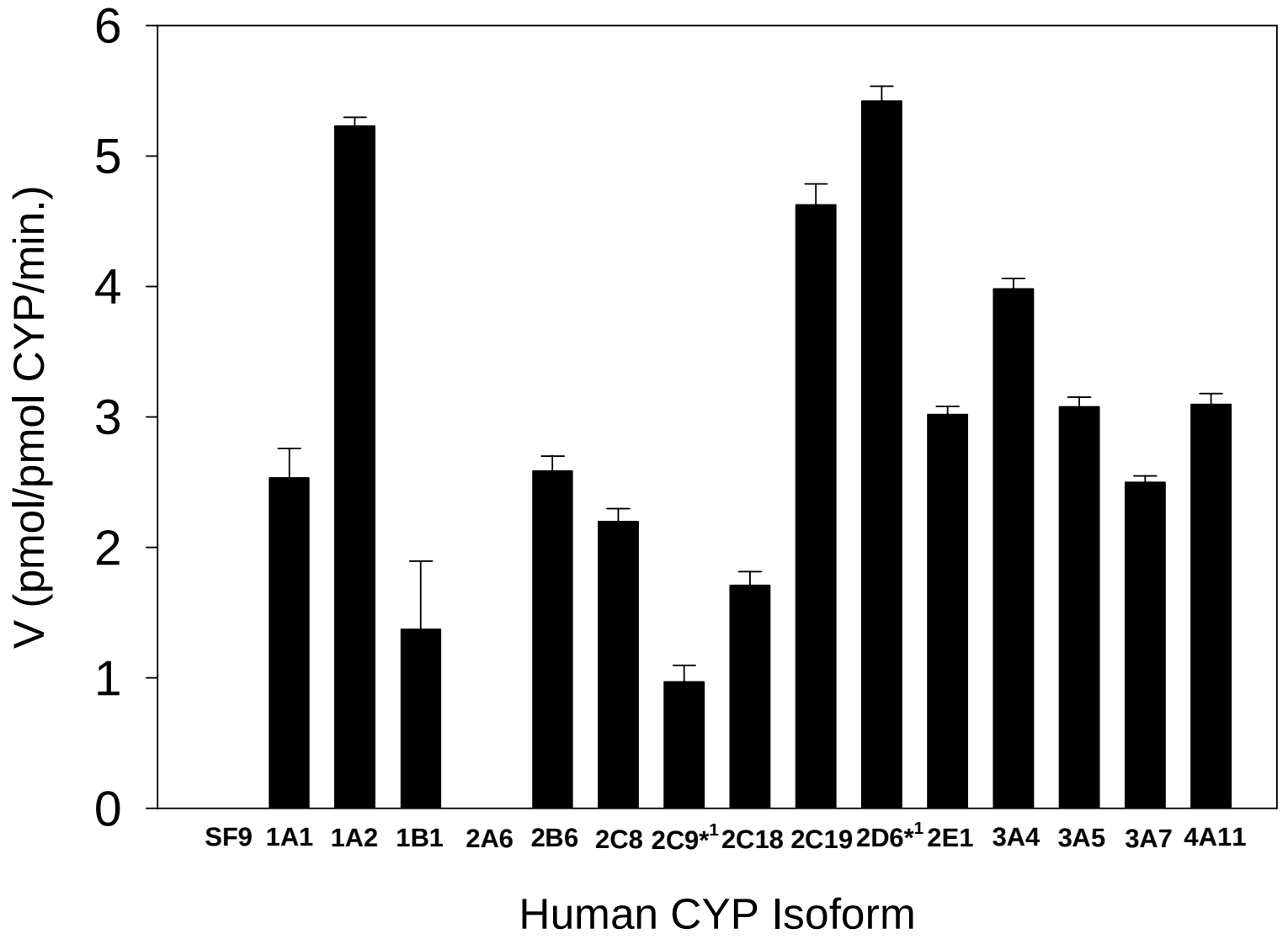

Fig. 5. Metabolic pathway of naphthalene in human.