DMD Fast Forward. Published on May 19, 2009 as doi:10.1124/dmd.109.026997

FURTHER ASSESSMENT OF 17α-ETHINYL ESTRADIOL AS AN INHIBITOR OF DIFFERENT HUMAN CYTOCHROME P450 FORMS IN VITRO

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Running title: In vitro inhibition of P450 by 17α-ethinyl estradiol

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Number of text pages:	36
Number of Tables:	3
Number of Figures:	4
Number of references:	44
Number of words in *Abstract*:	240
Number of words in *Introduction*:	761
Number of words in *Discussion*:	1,862
Total number of words:	8,184

[1]*Abbreviations*: P450, cytochrome P450; IC_{50}, concentration of inhibitor required to decrease activity by 50%; $IC_{50(total)}$, IC_{50} not corrected for $f_{u,inc}$; $IC_{50(free)}$, IC_{50} corrected for $f_{u,inc}$; $IC_{50(t)}$, concentration of inhibitor required to decrease activity by 50% after a pre-incubation time (t); [I], concentration of inhibitor; K_i, inhibition constant; AUC, area under the plasma concentration versus. time curve; [S], substrate concentration; EE, 17α-ethinyl estradiol; OC, oral contraceptive; PK, pharmacokinetics; K_m, Michaelis constant; V_{max}, maximal rate of reaction; *v*, rate of reaction at a given substrate concentration; K_I, concentration of inhibitor that supports half the rate of maximal inactivation; k_{inact},

maximal rate of inactivation; HLM, human liver microsomes; HIM, human intestine microsomes; $f_{u,inc}$, free fraction in the incubation. rCYP1A1, recombinant CYP1A1; rCYP1A2, recombinant CYP1A2; rCYP, recombinant cytochrome P450; AhR, aryl hydrocarbon receptor; 2-hydroxy EE, 2-hydroxy 17α-ethinyl estradiol; 2-methoxy EE, 2-methoxy 17α-ethinyl estradiol; EE 3-*O*-sulfate, 3-*O*-sulfate conjugate of 17α-ethinyl estradiol; EE 3-*O*-glucuronide, 3-*O*-glucuronide of 17α-ethinyl estradiol; POD, phenacetin *O*-deethylation; MEL, melatonin; MEL 6-OH, 6-hydroxy melatonin; ANF, α-naphthoflavone; 7-EFC, 7-ethoxy-fluoro coumarin.

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Abstract

17α-ethinyl estradiol (EE) was systematically evaluated as a reversible and time-dependent inhibitor of eleven human drug-metabolizing P450s (CYP1A1, 1A2, 1B1, 2B6, 2C8, 2C9, 2C19, 2D6, 2J2, 3A4 and 3A5) in vitro. When ranked, the lowest IC_{50} (concentration of inhibitor required to decrease activity by 50%) values were obtained with recombinant CYP1A1 (rCYP1A1, $IC_{50(total)}$ = $IC_{50(free)}$ = 2.7 μM) and CYP2C19 activity in human liver microsomes (HLM, $IC_{50(total)}$ = 4.4 μM; $IC_{50(free)}$ = 2.8 μM). For rCYP1A1, formal inhibition studies revealed that EE was a competitive inhibitor ($K_{i(free)}$ = 1.4 μM). All other IC_{50} values were greater than 8.0 μM and the weakest inhibition was observed with CYP1A2 activity in HLM ($IC_{50(free)}$ > 39 μM). In agreement, the IC_{50} characterizing the inhibition of melatonin (MEL) 6-hydroxylation in human intestine microsomes (CYP1A1 catalyzed) was lower than that of HLM (0.91 μM versus >40 μM). Because EE is known to impact the PK of CYP2C19 probe drugs, this raises the possibility that the concentration of EE during first pass may exceed 1000 nM, sufficient to impact CYP1A1 and CYP2C19, with less impact on CYP3A4 and other P450s. The results implicate intestinal CYP1A1, and possibly CYP2C19, as the loci of EE drug interactions with highly extracted drugs like MEL. Overall, it is very difficult to rationalize drug interactions involving EE based on direct inhibition of CYP2B6 (e.g., selegiline) and hepatic CYP1A2 (e.g., MEL, tizanidine, caffeine and theophylline).

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Introduction

Reports continue to appear describing drug interactions involving oral contraceptive (OC) formulations containing EE. For example, Hilli *et al* (2008) reported recently that MEL AUC is increased (~5-fold) and the MEL 6-OH/MEL AUC ratio is decreased (88%). CYP1A2 is known to play a major role in the metabolism of MEL in HLM, so the authors deduced that the enzyme was the locus of the interaction (Hartter et al., 2001; Facciola et al., 2001; Ma et al., 2005). EE-containing OCs exert a similar effect on the PK of tizanidine (Granfors et al., 2005). Both tizanidine and MEL are low oral bioavailability (<25%) CYP1A2 substrates because of first pass metabolism (Granfors et al., 2005; Hartter et al., 2001). Although caffeine and theophylline also serve as CYP1A2 substrates, they undergo minimal first pass, are highly bioavailable and the impact of EE is less marked (~40% decrease in clearance) (Balogh et al., 1995; Roberts et al., 1983). Despite these clinical data, it is only very recently that assessment of CYP1A2 inhibition in vitro has been described (Karjalainen et al., 2008). In fact, Karjalainen et al. reported EE as a relatively weak inhibitor of POD activity in HLM (low K_m component; IC_{50} = 24 μM).

Drug interactions with EE-containing OC formulations have also included a number of CYP2C19 substrates, such as omeprazole, mephenytoin, and proguanil (Hagg et al., 2001; Shelepova et al., 2005; Rodrigues and Lu, 2004, references therein). Similarly, drug interactions with imipramine and selegiline have been described (Laine et al., 1999; Abernethy et al., 1984). The latter is a CYP2B6 and CYP2C19 substrate, also with a low oral bioavailability (<10%), and greater than 10-fold increases in AUC have been reported with OCs (Benetton et al., 2007; Laine et al., 1999). Importantly, the

contribution of CYP2C19 after oral dosing of selegiline is thought to be minimal (Laine et al., 2001). Although CYP2B6 is implicated, there are no reports describing the impact of *CYP2B6* genotype (or phenotype) on the oral PK of selegeline and its contribution in vivo is not known. Moreover, the clinical drug interaction between EE and a CYP2B6 probe (bupropion) is not significant despite evidence for mechanism-based inhibition in vitro (Palovaara et al., 2003; Kent et al., 2002). The same can also be said for the observed mechanism-based inhibition of CYP3A4 and CYP3A5 in vitro, because the effect of EE on the PK of CYP3A substrates (e.g., midazolam and nifedipine) is minimal (Shelepova et al., 2005; Belle et al, 2002; Lin et al., 2002; Atkinson et al., 2005; Lin and Hollenberg, 2007; Palovaara et al., 2000; Balogh et al., 1998).

Overall, available clinical data suggest that EE exerts a differential inhibitory effect across the various human P450s. Such a hypothesis is supported by the data of Shelepova et al (2005), who conducted an EE interaction study employing the Cooperstown ("5+1") drug cocktail. In this instance, EE did not impact the PK of the CYP2C9, CYP2D6, and CYP3A4 probes, whereas the effect on the putative CYP1A2 and CYP2C19 trait measures was statistically significant. The reports of Hagg et al (2001) and Hatorp et al (2003) further support that EE-containing OCs do not impact certain P450s like CYP2D6 and CYP2C8.

Importantly, the *total* concentration of EE in enterocytes (~6.0 nM) and the hepatic portal vein (~ 0.4 nM) has been estimated using accepted methods and found to be low at clinically relevant doses (Zhang et al., 2007). For reference, the free fraction of EE in human serum is 1.7% (Karjalainen et al., 2008 and references therein). So why does EE elicit a significant interaction (>2-fold increase in AUC) with MEL and

tizanidine? Why the observed interactions with selegiline and various CYP2C19 substrates? Based on current in vitro extrapolation methods, one would not anticipate clinically relevant reversible or mechanism-based inhibition of *any* P450s (Zhang et al., 2007; Rodrigues and Lu, 2004).

As a first step to address such questions, EE was systematically evaluated as a reversible and time-dependent inhibitor of numerous drug-metabolizing P450s in pooled HLM (CYP1A2, CYP3A4, CYP2C9, CYP2C8, CYP2C19, CYP2D6, and CYP2B6). CYP2A6 and CYP2E1 were not part of the study; Draper et al (1997) have reported that EE is a weak inhibitor of the former (coumarin 7-hydroxylase) in HLM. Inhibition of rCYP1A1, rCYP1A2, rCYP1B1, rCYP3A4, rCYP3A5 rCYP2B6, and rCYP2J2 was evaluated also. Some of these latter P450s (CYP1A1, CYP1B1 and CYP2J2) are expressed in the gut, and are less prominent in the liver (Paine et al., 2006). Additional inhibition studies (MEL 6-OH formation) were conducted with pooled HIM and HLM. As much as a possible, the different P450s were assessed under similar incubation conditions so that the generated IC_{50} values could be ranked.

Materials and Methods

Materials. Furafylline, ANF, ticlopidine, troleandomycin, phencyclidine, paroxetine, phenelzine, phenacetin, acetaminophen, MEL 6-OH, 6-chloromelatonin, MEL, dextromethorphan, dextrophan, diclofenac, 4'-hydroxydiclofenac, paclitaxel, midazolam, 1'-hydroxymidazolam, terfenadine, (*S*)-mephenytoin, 4'-hydroxymephenytoin, propranolol, trazodone, chlorzoxazone, 6-hydroxychlorzoxazone, saccharic acid 1,4-lactone, potassium phosphate dibasic, potassium phosphate monobasic, EDTA and

NADPH were purchased from Sigma-Aldrich (St Louis, MO). 10-Deacetyltaxol-C was purchased from LKT Laboratories (St. Paul, MN). Tienilic acid was purchased from Toronto Research Chemicals (North York, Canada). Acetaminophen-D_4, 6β-hydroxytestosterone-D_3 and α-hydroxymidazolam-D_4 were purchased from the Cerilliant Corporation (Round Rock, TX). 4'-hydroxydiclofenac-D_4 was purchased from SynFine Research (Richmond Hill, Ontario, Canada). Testosterone, 6β-hydroxytestosterone, EE 3-*O*-sulfate and EE 3-*O*-glucuronide were obtained from Steraloids (Newport, RI). Pooled HLM (n = 27 different organ donors), baculovirus-infected insect cell microsomes (Supersomes®) containing various (individual) cDNA-expressed human P450s (co-expressed with P450 oxidoreductase), pooled HIM (n = 15 different organ donors), and 6α-hydroxypaclitaxel were purchased from BD Gentest (Woburn, MA). 2-hydroxy EE and 2-methoxy EE were kindly provided by Drs G. Newport and W. Slikker (National Center for Toxicology, NCTR, Jefferson, AR). All other reagents and chemicals were of analytical grade and of the highest quality available commercially.

Instrumentation. All the P450 assays were LC-MS/MS based (Table 1 and 2), employing a API4000 QTrap triple quadrupole mass spectrometer equipped with a turbo-V ionization source (Applies Biosystems, Concord, Ontario, Canada), two LC-10ADvp pumps with a SCL-10ADvp system controller and DGU-14 solvent degasser (Shimadzu, Columbia, MD), a LEAP CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC). Incubations were carried out in 96-well Thermowell Gold plates (Corning Inc., Corning, NY) using an 8-tip Genesis 150 liquid handler (Tecan Group Ltd, Research Triangle Park, NC) equipped with a temperature-controlled shaker.

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Incubation and evaluation of EE as a P450 inhibitor. The incubation mixture (final volume of 0.2 mL) consisted of the following: 0.1 M potassium phosphate buffer (100 mM, pH 7.4), protein (HLM, HIM, or rCYP), EDTA (1 mM) and EE (final concentration of 10 nM to 45 μM) dissolved in DMSO (0.2%, v/v, final concentration). For each assay, the incubation conditions are described in Tables 1 and 2. When studying reversible inhibition, substrate was added to the reaction mixture and warmed to 37°C. Reactions were then initiated with NADPH (1 mM). After incubation, the reaction mixtures were transferred to a filter plate pre-loaded with cold methanol (POD and testosterone 6β-hydroxylase assays) or acetonitrile (all other assays) containing the internal standard. Assessment of time-dependent inhibition involved pre-incubation of EE (same concentration range) with NADPH-fortified (1 mM) enzyme (30 minutes), followed by substrate addition without dilution of incubate. Following the requisite incubation period, reactions were terminated by transfer of the assay contents to a pre-loaded filter plate as described above. For all assays, the filter plate was stacked with a 2 mL injection plate, centrifuged and the filtrates were subjected to LC/MS analysis (Table 1 and 2).

Where possible, the k_{inact}/K_I ratio was estimated from the IC_{50} at 30 minutes ($IC_{50(t)}$) and compared to positive controls (Maurer et al., 2000; Berry and Zhao, 2008). Only estimates of k_{inact}/K_I ratio are reported herein and no attempt was made to obtain the actual values of k_{inact} and K_I. Inhibition studies with rCYP1A1 and rCYP1A2 were also extended to include four metabolites of EE (2-hydroxy EE, 2-methoxy EE, EE 3-*O*-sulfate and EE 3-*O*-glucuronide). All incubations were as described above, except that saccharic acid 1,4-lactone (0.1 mM) was included in the incubations with EE 3-*O*-glucuronide.

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Determination of $f_{u,inc}$. Equilibrium dialysis was used to determine the binding of EE (2.0 μM) to microsomal (0.05, 0.1 and 0.25 mg/mL) and rCYP (5 pmol/mL) protein. Protein was prepared in the assay buffer (100 mM potassium phosphate, pH 7.4;1 mM EDTA) and added to a 96-well equilibrium dialysis apparatus (HTDialysis, Gales Ferry, CT). After loading protein and buffer on each side of the dialysis membrane (HTDialysis, molecular weight cut off: 12,000 to 14,000 Daltons), the apparatus was incubated in a 37°C oven with a reciprocating shaker for six hours. Protein samples removed from the dialysis wells were diluted (ten-fold) with buffer and buffer samples were diluted (2-fold) with protein. Two volumes of methanol (containing 0.1% formic acid and internal standard, 6β-hydroxytestosterone) were then added to each sample. A portion of the supernatant (10 μL) was subjected to LC-MS/MS, employing a QTrap triple quadrupole mass spectrometer (as described above). Chromatographic separation was accomplished using a Phenominex Luna Phenyl-hexyl column (2x150 mm, 5 μm). For the mobile phase, a gradient system was used at a flow rate 0.3 mL/min. Initially, the mobile phase was comprised of methanol-water (40/60, v/v) containing 0.1% formic acid. The mobile phase was then programmed, so that the amount of methanol was 100% (containing 0.1% formic acid) in 2 minutes. At which time, the mobile phase was held constant for another 2 minutes. Detection of EE was achieved in the electrospray positive ion (ESI) multiple reaction monitoring (MRM) mode, by monitoring *m/z* transitions of 279 → 133 (EE) and 305 → 269 (internal standard). The declustering potential was set at 51 volts (EE) and 61 volts (internal standard), the collision energy was set at 25 volts (EE) and 23 volts (internal standard) and the turbo-V source temperature was set at 400 °C.

Results

Reversible (Non-Time Dependent) Inhibition of P450s. EE was evaluated as an inhibitor of seven different P450s in HLMs (CYP3A4, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP1A2, and CYP2B6). Inhibition was assessed also with seven different rCYP proteins (CYP3A4, CYP3A5, CYP1A1, CYP1A2, CYP1B1, CYP2J2 and CYP2B6). IC_{50}s were generated and were then ranked (Table 3). In all cases, EE was found not to be a sub-micromolar inhibitor of P450 activity catalyzed by rCYPs and HLM preparations. Binding of EE to HLM was low ($f_{u,inc}$, 0.84 to 0.64), and on the basis of the $IC_{50(total)}$ and $IC_{50(free)}$ values obtained, CYP2C19 was ranked the lowest ($IC_{50(total)}$ = 4.1 μM and $IC_{50(free)}$ = 2.8 μM). The next highest IC_{50} in HLM was CYP2C9, followed by CYP2C8, CYP2B6, CYP2D6, and CYP3A4. The weakest inhibition in HLM was observed with CYP1A2 (POD) activity ($IC_{50(total)}$ > 45 μM and $IC_{50(free)}$ >39 μM). Under the same incubation conditions, ANF behaved as a potent inhibitor of POD activity (IC_{50} ~ 7 nM) in HLM (data not shown). Therefore, the POD assay was able to detect inhibition of CYP1A2.

Perhaps the most important finding in the present study was the relatively potent inhibition of rCYP1A1-catalyzed POD by EE (Table 3). Because assays were run at the K_m of the substrate, the $K_{i(free)}$ was estimated to be ~1000 nM (assuming competitive inhibition; $IC_{50(free)}/2$). The results of more formal kinetic studies revealed that EE was in fact a competitive inhibitor (K_i = 1.4 ± 0.12 μM) of rCYP1A1-catalyzed POD (Fig. 1). Binding of EE to the rCYP1A1 preparation was not detectable ($f_{u,inc}$ ~1.0) and so the K_i generated is regarded as a "free K_i.". In comparison, EE was found to be a weaker

inhibitor of rCYP3A4, rCYP3A5, rCYP1B1 (IC_{50} ~ 9.0 μM), rCYP1A2 (IC_{50} = 14 μM), rCYP2J2 (IC_{50} = 31 μM), and rCYP2B6 (IC_{50} = 41 μM) (Table 3).

Time-Dependent Inhibition. After 30 minutes of pre-incubation, IC_{50} shifts (≥2-fold) were only observed for CYP2C8 and CYP3A4 activity in HLM, and rCYP2J2, rCYP3A4 and rCYP3A5 (Table 3). Therefore, no time-dependent inhibition of either CYP1A2 or CYP1A1 was observed. Although the moderate (~2-fold) IC_{50} shift for rCYP2C8 and rCYP2J2 precluded estimates of a k_{inact}/K_I ratio, the expected greater than 4-fold shift enabled estimates of the k_{inact}/K_I ratio for HLM- (0.005 $min^{-1}\mu M^{-1}$) and rCYP3A4-catalyzed (0.031 $min^{-1}\mu M^{-1}$) midazolam 1'-hydroxylase activity. There was some evidence for the time-dependent inhibition of rCYP3A5, but the IC_{50} shift was lower versus rCYP3A4 (6-fold versus ~2-fold). Such an observation is consistent with reports indicating that the k_{inact}/K_I ratio is lower for rCYP3A5 versus rCYP3A4 (Lin et al., 2002; Lin and Hollenberg, 2007). Despite the report of Kent et al (2002), no time-dependent inhibition of CYP2B6 activity was observed with HLM or recombinant protein (Table 3, see *Discussion*).

EE Metabolites as CYP1A Inhibitors. EE is known to be extensively metabolized and undergoes first pass extraction in the gut and liver. Sulfation at the 3-hydroxy position is major in the gut, although the products of 2-hydroxylation (followed by methylation) and 3-*O*-glucuronidation are also present in the excreta following a radiolabeled dose (Zhang et al., 2007, references therein). Therefore, four metabolites (EE 3-*O*-sulfate, EE 3-*O*-glucuronide, 2-hydroxy EE and 3-methoxy EE) were evaluated as inhibitors of rCYP1A1 and rCYP1A2 (Fig. 2). For both the sulfate and glucuronide conjugates, relatively minimal inhibition of POD activity (IC_{50} > 45 μM) was observed (<30% inhibition at the

highest concentration tested) and no time-dependent inhibition was evident (data not shown). The 2-hydroxy and 2-methoxy EE derivatives were no more potent than parent EE as rCYP1A1 and rCYP1A2 inhibitors (Fig. 2). So despite extensive EE sulfation during first pass, the sulfate conjugate is unlikely to contribute to the observed drug interactions with CYP1A substrates.

MEL as CYP1A1 and CYP1A2 Substrate. Ma et al (2005) have reported MEL 6-OH formation in the presence of rCYP1A1 and the authors described K_m and V_{max} values similar to those of rCYP1A2. The data presented in Figure 3 show that it is possible to normalize the turnover rates reported by Ma et al (2005), the most complete recombinant panel described to date, and predict that CYP1A2 dominates (66%) in HLM, with contribution from CYP3A4 (13%), CYP2C9 (6%) and CYP2C19 (7%) (Fig. 3B). Because no reaction phenotype data exist for HIM, the recombinant P450 data reported by the authors were also used to estimate the contribution of different P450s in HIM. In this instance, CYP1A1 (52%) and CYP3A4 (43%) were predicted to account for the majority of MEL 6-OH formation in HIM (Fig. 3C).

Although not shown in Figure 3, the P450 reaction phenotype of MEL *O*-demethylation was considered also. Based on the data of Ma et al (2005), it was estimated that CYP1A1, CYP2C19, CYP2C9 and CYP3A4 contribute to 24%, 53%, 9% and 11% of total *O*-demethylase activity in HIM, respectively. For HLM, CYP1A2, CYP2C19, CYP2C9 and CYP3A4 are estimated to contribute 50%, 35%, 6% and 8%, respectively. Overall, the data suggest that CYP1A1 and CYP2C19 (~80%) may play a significant role in the gut.

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EE as Inhibitor of MEL 6-OH Formation in HIM and HLM. As described above, MEL 6-OH formation is predicted to be catalyzed by CYP1A1 (52%) and CYP1A2 (66%) in HIM and HLM, respectively. To confirm the reaction phenotype in HIM and HLM, ANF was used as a CYP1A reaction phenotyping tool. The compound is a potent inhibitor of both CYP1A1 and CYP1A2 (~10-fold lower IC_{50} for CYP1A2) and does not inhibit other drug-metabolizing P450s at concentrations below 1.0 μM (Tassaneeyakul et al., 1993; Shimada et al., 2007; Bourrie et al., 1996). In the presence of human microsomes, the formation of MEL 6-OH was characterized by a single K_m of ~20 μM (data not shown), similar to that of rCYP1A1 and rCYP1A2 reported by Ma et al (2005). As expected, however, a differential pattern was observed for the inhibition of MEL 6-OH formation by ANF in HIM versus HLM (Fig. 4A). For example, ANF was not able to completely inhibit the reaction in HIM and the residual (uninhibited) activity (44%) could be attributed to CYP3A4 (Fig. 3C). In comparison, ANF (1 μM) was able to inhibit MEL 6-OH formation in HLM by 66%, consistent with the furafylline data presented by Facciola et al (2001).

As expected for EE, the IC_{50} value for the inhibition of MEL 6-OH formation in HIM was low compared to HLM (IC_{50} = 0.91 ± 0.06 versus >40 μM) (Fig. 4B) and was similar to the IC_{50} value obtained with rCYP1A1 (Table 3). For EE, the I_{max} (maximal percent inhibition) of 61 ± 2.5% was similar to that of ANF (56 ± 3.8%) in HIM, consistent with the CYP1A1-catalyzed formation of MEL 6-OH therein.

Discussion

Despite eliciting interactions with a number of drugs, relatively little effort has been made to assess the inhibition profile of EE across numerous P450s in vitro (Zhang et al, 2007; Rodrigues and Lu, 2004). Some of the observed drug interactions are quite marked, with greater than 2-fold increases in AUC. For example, OCs containing EE increase the AUC of selegiline, MEL, and tizanidine >10-fold, ~5-fold, and ~4-fold, respectively (Hilli et al., 2008; Laine et al., 1999; Granfors et al., 2005). Such effects on AUC cannot be rationalized based on the known dose and exposure of EE (Zhang et al., 2007; Rodrigues and Lu, 2004). Therefore, an attempt was made to systematically evaluate EE as a reversible and time-dependent inhibitor of numerous human P450s in vitro.

Based on the results described herein, it is concluded that EE is not a sub-micromolar inhibitor of human P450s in vitro (Table 3). The lack of sub-micromolar inhibition of CYP2C19 (Rodrigues and Lu, 2004; Laine et al., 2003; Di Marco et al., 2007), CYP2C8 (Walsky et al., 2005), CYP2C9 (Laine et al., 2003), CYP2B6 (Walsky et al., 2006), and CYP1A2 (Karjalainen et al., 2008) activity in vitro is consistent with the literature. To our knowledge, however, this is the first report describing the assessment of EE as an in vitro inhibitor of CYP2J2, CYP2D6, CYP1B1 and CYP1A1. Pepper et al (1991) have reported the inhibition of CYP2D6-catalyzed metoprolol oxidation in HLM (83%), although no IC_{50} was reported and only a single high EE concentration (100 μM) was evaluated. Also consistent with the literature, there was evidence for time-dependent inhibition of CYP3A4 and CYP3A5, although the inhibition is not considered to be clinically meaningful given the minimal effect on the PK of oral midazolam and

nifedipine (Belle et al., 2002; Balogh et al., 1998; Palovaara et al., 2000; Shelepova et al., 2005; Lin and Hollenberg, 2007).

Unexpectedly, it was not possible to show time-dependent inhibition of rCYP2B6 with EE, under conditions where phencyclidine elicited a marked IC_{50} shift (Table 3 legend). Consequently, a number of additional incubation formats were attempted (e.g., change of substrate concentration, increased protein concentration during pre-incubation with subsequent dilution of incubate) and still it was not possible to observe an IC_{50} shift with EE. Finally, additional studies were conducted with 7-EFC as substrate and there was no further evidence for time-dependent inhibition of rCYP2B6. In fact, the IC_{50} generated (~20 μM) was similar to that reported by Walsky et al (2006) (data not shown). Therefore, the results are contrary to those of Kent et al (2002), who were able to observe mechanism-based inhibition of 7-EFC *O*-deethylation. It is worth noting, however, that commercially available (insect cell-expressed) rCYP2B6 was used in the present study whereas the studies of Kent et al employed rCYP2B6, expressed in *E. coli* and purified to homogeneity, incubated in a standard reconstitution system (Kent et al., 2002). It is possible that mechanism-based inhibition by EE may be sensitive to assay conditions (Dr. Paul Hollenberg, University of Michigan, personal communication). EE did not behave as a time-dependent inhibitor of CYP2B6 activity in native (pooled) HLM also and it is assumed that such a preparation expresses the requisite ratio of P450-to-P450 reductase and cytochrome b_5. Importantly, under the same assay conditions, phencyclidine did exhibit time-dependent inhibition of bupropion hydroxylation in HLM (IC_{50} >120 μM; $IC_{50(t)}$ = 5.5 ± 1.6 μM). Furthermore, the IC_{50} for EE generated with either rCYP2B6 or

HLM (Table 3), and subsequent estimate of $[I]/K_i$, would correctly predict a minimal drug interaction with bupropion (Palovaara et al., 2003).

Perhaps the most important finding in the present study was the relatively potent inhibition of CYP1A1 by EE (Fig. 1). In reality, the interaction with rCYP1A1 was not completely unexpected, given that the enzyme has been shown to metabolize EE (Wang et al., 2004). When compared to other P450s, the $IC_{50(total)}$ values for rCYP1A1 (2.7 μM) and HLM CYP2C19 (4.4 μM) were ranked the lowest, yielding a K_i of 1.4 μM (determined) and ~2.2 μM (estimated), respectively. The latter estimate compliments the recent report of Foti and Wahlstrom (2008), employing (*S*)-omeprazole, (*R*)-omeprazole and (*S*)-fluoxetine as rCYP2C19 substrates. However, the same authors reported a lower K_i (289 nM) with (*S*)-mephenytoin. When adjusted for HLM binding, the CYP2C19 $K_{i(free)}$ ($IC_{50(free)}$ = 2.8 μM) in the present study is estimated to be similar to that of CYP1A1 (1.4 μM). Other authors have reported IC_{50}s for (*S*)-mephenytoin with HLM in the range of ~3.5 to 20 μM, although no attempt was made to correct for microsomal binding (DiMarco et al., 2007, Rodrigues and Lu, 2004). To date, all reported K_i and IC_{50} values for CYP2C19 are still considerably higher than the calculated total concentration of EE in enterocytes (~6.0 nM) and hepatic portal vein (~0.4 nM). Karjalainen et al. (2008) have come to the same conclusion for CYP1A2 and have suggested that accumulation of EE in tissues is a possibility.

Because of the marked interaction with MEL, and because most reports have focused on the metabolism of MEL by HLM and liver P450s (Hartter et al., 2001; Ma et al., 2005; Hilli et al, 2008; Facciola et al., 2001), the present study also focused on the P450s involved in the metabolism of MEL. CYP1A2 is expressed in the liver and one

would expect the enzyme to play a major role in HLM. In agreement, reaction phenotyping of MEL with furafylline indicates that CYP1A2 plays a significant role (~60%) therein, although some inhibition by CYP2C9-selective sulfaphenazole (20%) and CYP3A-selective ketoconazole (~15%) can be observed (Facciola et al., 2001). The data presented in Figure 3 show that it is possible to normalize the turnover rates reported by Ma et al (2005) and predict that CYP1A2 dominates (66%) in HLM, with contribution from CYP3A4 (13%), CYP2C9 (6%) and CYP2C19 (7%). For HIM, CYP1A1 (52%) and CYP3A4 (43%) are predicted to account for the majority of MEL 6-OH formation. MEL *O*-demethylation was predicted to be catalyzed by CYP1A2 (50%), CYP2C19 (35%), CYP2C9 (6%) and CYP3A4 (8%) in HLM (see *Results*). The contribution of CYP1A2 and CYP2C19 is consistent with the data of Facciola et al (2001), who reported up to 40% inhibition of HLM-catalyzed MEL *O*-demethylation with furafylline and up to 40% inhibition with omeprazole (used as a CYP2C19 inhibitor). On the other hand, CYP1A1 (24%) and CYP2C19 (53%) would be expected to contribute to the majority of MEL *O*-demethylase activity in HIM. This implies that CYP1A1 (52%) and CYP1A2 (66%) contribute to MEL 6-OH formation in HIM and HLM, respectively. For MEL *O*-demethylation, CYP2C19 (~40%) plays a role in HLM, whereas CYP2C19 and CYP1A1 (~80%) dominate in HIM. Given the differences in the IC_{50} for CYP1A1 and CYP1A2, therefore, one would expect EE to be a more potent inhibitor of MEL 6-OH in HIM (versus HLM) (Fig. 4B). Although not determined, inhibition of HIM-catalyzed MEL *O*-demethylation by EE is implicated.

The results of the present study raise some important questions. EE is a more potent inhibitor of CYP1A1 (versus CYP1A2), which is expressed in the gut (Paine et al.,

2006). Because rCYP1A1 also metabolizes MEL (Fig. 3), is it possible that the interaction between EE and MEL involves the inhibition of gut CYP1A1 during first pass (Fig. 4B)? Caffeine and theophylline also serve as rCYP1A1 substrates (Ha et al., 1995, 1996), so could EE impact their metabolism in the gut also? Is it possible that the concentrations of EE exceed 1000 nM in the gut during first pass to the point that CYP2C19 (in addition to CYP1A1) is inhibited with a relatively minimal effect on other P450s? CYP2C19 is present in the intestine and likely contributes to the first pass metabolism of its probe drugs (Paine et al., 2006; Gelatin and Houston, 2006). Moreover, the interaction of EE-containing OCs and CYP2C19 substrates appears significant. For example, the increased (0.28 versus 0.11) ratio of (*S*)-mephenytoin to (*R*)-mephenytoin in urine (*S*/*R* ratio) with EE is similar to that observed with *CYP2C19*1/*2* (heterozygous) subjects and is suggestive of up to 50% inhibition of the enzyme (Rodrigues and Lu, 2004, references therein).

It is worth noting that the ranked IC_{50}s described in Table 3 are consistent with the results of Shelepova et al (2005), who evaluated the impact of an EE-containing OC formulation on five P450s using a "5+1" drug cocktail. In the study, no significant inhibitory effect was seen with the CYP3A (midazolam), CYP2C9 ((*S*)-warfarin) and CYP2D6 (dextromethorphan) probes. On the other hand, the effect on the putative CYP2C19 (100% increase in the omeprazole/5-hydroxy omeprazole AUC ratio) and CYP1A2 (23% decrease in the caffeine demethylation ratio of [5-acetyl-amino-6-formylamino-3-methyluracil + 1-methylxanthine + 1-methylurate]/1,7-dimethylurate in urine) trait measures was statistically significant. Given the arguments above, is it

possible that the effect on the caffeine demethylation ratio is more reflective of CYP1A1 inhibition (versus CYP1A2 inhibition)?

In light of the absence of sub-micromolar P450 inhibition in vitro, the magnitude of the effect on MEL, tizanidine, and selegiline PK, and the low dose of 30 μg, it is apparent that additional in vitro and in vivo studies with EE are necessary. Specifically, for drugs like MEL that undergo considerable first pass, and are metabolized by CYP1A1, the possibility that EE directly inhibits CYP1A1 and impacts gut extraction should be investigated and it cannot be assumed that interactions simply involve the direct inhibition of liver CYP1A2. Importantly, the majority of the P450s evaluated in the present study (e.g., CYP3A4, CYP2D6, CYP2C8, and CYP2C9) exhibited lower IC_{50}s than CYP1A2 and clinical drug interactions with the same P450s are minor. For MEL in particular, the inhibition CYP2C19-catalyzed *O*-demethylation may also contribute to the observed interaction with EE.

Although co-dosed with EE, the observed interactions with MEL cannot be ascribed to gestodene. Like Karjalainen et al (2008), gestodene was shown to be a very weak inhibitor (IC_{50} and $IC_{50(t)}$ >100 μM) of POD activity in HLM (data not shown). Likewise, the interaction with MEL likely does not involve the conjugate metabolites of EE. Neither the EE 3-*O*-sulfate or 3-*O*-glucuronide behaved as potent inhibitors of CYP1A1 or CYP1A2 (Fig. 2). On the other hand, both the 2-hydroxy and 2-methoxy metabolites of EE were shown to inhibit CYP1A1 (similar IC_{50} to parent EE). Therefore, the possible contribution of both should be considered in vivo. Unfortunately, there is limited data on the exposure of both of these metabolites in humans (Zhang et al., 2007).

One also has to consider the possibility that chronic EE dosing may affect specific forms of P450 (e.g., CYP1A1) at the transcriptional level. Such effects may involve transcription factors, AhR antagonism (or transrepression), and could be direct or involve the estrogen receptor (Beischlag and Perdew, 2005). For example, preliminary data show that EE may behave as an AhR antagonist in HepG2 cells stably transfected with the CYP1A1 promoter upstream of the luciferase reporter gene. In this instance, EE decreases the induction of luciferase by 2,3,7,8-tetrachlorodibenzo-p-dioxin (up to ~40%) in a concentration-dependent manner (0.1 nM to 10 μM). In comparison, 3',4'-dimethoxyflavone (5 μM) decreases the induction by 95% (Swanson and Choi, unpublished). The chronic effects of AhR antagonism are not known.

From the viewpoint of P450 drug interactions, EE continues to be an enigmatic drug. Additional research is needed in order to enable a better understanding of such interactions at a mechanistic level. This is important because most, if not all, new chemical entities are evaluated as perpetrators and victims of OC interactions. The results of such studies appear in the product label which, in some cases, can influence competitive marketing of OCs to women of child bearing potential (Zhang et al., 2007).

Acknowledgements

The authors would like to thank Drs. G. Newport and W. Slikker (National Center for Toxicology, NCTR, Jefferson, AR) for making the 2-hydroxy and 3-methoxy metabolites of EE available. Gratitude is extend also to Drs. H. Swanson and E.-Y. Choi (University of Kentucky, Lexington, KY) for conducting the luciferase induction study with HepG2 cells stably transfected with the CYP1A1 promoter.

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Figure Legends:

Fig. 1. EE behaves as a competitive inhibitor of recombinant CYP1A1-catalyzed POD activity

(A) EE (0.06, 0.19, 0.56, 1.7 and 5.0 μM) was incubated with phenacetin (10, 30, 50 and 100 μM) and the rate of POD was determined. Data were simultaneously fit to an equation describing competitive inhibition (SAAM II software, version 1.0.2, University of Washington, Seattle, WA); $v = \frac{V_{max} \bullet [S]}{K_m \bullet \left(1 + \frac{[I]}{K_i}\right) + [S]}$.

The following parameters (± the standard deviation of the parameter fit) were determined: K_i = 1.4 ± 0.12 μM, K_m = 31.8 ± 2.1 μM, and V_{max} = 16.3 ± 0.4 pmol/min per pmol CYP1A1. Figures (B) and (C) represent Dixon (1/rate versus EE concentration) and Cornish-Bowden (phenacetin concentration/rate versus EE concentration) plots, respectively, and are consistent with reversible competitive inhibition. Binding of EE to the rCYP1A1 preparation was negligible ($f_{u,inc}$ ~1.0), so the $K_{i(free)}$ = 1.4 μM.

Fig. 2. Evaluation of EE and its metabolites as inhibitors of POD catalyzed by rCYP1A1 and rCYP1A2

POD activity was measure after incubation with rCYP1A1 and rCYP1A2. In both cases, the IC_{50} for each of the metabolites was determined at a phenacetin concentration approaching the K_m. The IC_{50} values represent the mean (± standard deviation) of three determinations.

DMD Fast Forward. Published on May 19, 2009 as DOI: 10.1124/dmd.109.026997
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Fig. 3. Formation of MEL 6-OH catalyzed by different P450s

(A) Rates of MEL 6-OH formation were determined for a panel of recombinant P450s (Ma et al., 2005). The contribution of each P450, defined as % TNR (% total normalized rate), in HLM (B) and HIM (C) was determined as described by Chang et al (2008). Mean specific content for each P450 in HIM and HLM has been reported in the literature (Paine et al., 2006; Chang et al., 2008). For HLM, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were assigned a specific content of 45, 41, 30, 96, 19, 10, 49 and 108 pmol/mg, respectively (CYP1A1 not detected). For HIM, CYP1A1, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 were assigned a specific content of 5.6, 8.4, 1.0, 0.5 and 43 pmol/mg, respectively (CYP1A2, CYP2A6, CYP2B6, CYP2E1 not detected).

Fig. 4. Evaluation of EE and ANF as inhibitors of MEL 6-OH formation in HIM and HLM

The effect of ANF (A) and EE (B) on MEL 6-OH formation was measured after incubation of MEL (10 μM) with HLM and HIM. Where possible, the line represents the best fit to the equation: $\%\ \text{of Control} = A_0 - \frac{I_{\max} \bullet [I]^n}{IC_{50}{}^n + [I]^n}$

where % of Control is the percentage of activity remaining in the presence of inhibitor (relative to activity in the presence of vehicle alone), A_0 is the percentage of activity in the absence of inhibitor, I_{max} is the maximal percent inhibition, n is the Hill factor, and [I] is the inhibitor concentration. Where possible, the IC_{50} was estimated by fitting the % of control versus [I] and a standard error of the parameter estimate was obtained in each case (KaleidaGraph, Synergy Software, v.3.6, Reading, PA). For ANF, IC_{50}s of 270 ± 29

nM (HLM) and 970 ± 110 nM (HIM) were determined. A_0 in HLM and HIM was 94.5 ± 3.8 and 96.6 ± 2.9%, respectively. The corresponding I_{max} for ANF was 65.6 ± 5.4 and 55.9 ± 3.8%, with a Hill factor n of 3.6 ± 1.8 and 3.2 ± 0.9, respectively. For EE with HIM, parameter estimates were 0.91 ± 0.06 μM (IC_{50}), 97.9 ± 1.7% (A_0), 60.5 ± 2.5% (I_{max}) and 3.2 ± 0.5 (n). Because of relatively weak inhibition (IC_{50} > 40 μM), no attempt was made to fit the data for EE with HLM.

Table 1

Method for POD, MEL 6-OH, bupropion 6-hydroxylase, paclitaxel 6α-hydroxylase and diclofenac 4'-hydroxylase assays

Parameter(s)	Phenacetin *O*-Deethylase (CYP1A)	Melatonin 6-Hydroxylase (CYP1A, 1B1)	Bupropion Hydroxylase (CYP2B6)	Paclitaxel 6α-Hydroxylase (CYP2C8)	Diclofenac 4'-Hydroxylase (CYP2C9)
Microsomal Protein concentration (mg/mL)	0.15	0.5	0.05	0.05	0.15
Recombinant P450 concentration (pmol/mL)	5	10	5	Not conducted	Not conducted
Incubation time (min)	5	10	5	5	5
Substrate (final concentration, μM)	Phenacetin (45)	Melatonin (20)	Bupropion (80)	Paclitaxel (5)	Diclofenac (10)
Analyte	Acetaminophen	6-Hydroxymelatonin	Hydroxybupropion	6α-Hydroxypaclitaxel	4'-Hydroxydiclofenac
Internal standard	Acetaminophen-D_4	6-Chloromelatonin	Trazodone	10-Deacetyltaxol-C	4'-Hydroxydiclofenac-D_4
Column	Luna Pheny-hexyl 2x150 mm, 5 μm	YMC ODS-AQ 2.0 x 50 mm, 3 μm	Gemini 2x50 mm, 5 μm	Luna Pheny-hexyl 2x150 mm, 5 μm	Gemini 2x50 mm, 5 μm
Flow rate	0.3 mL/min	0.3 mL/min	0.3 mL/min	0.3 mL/min	0.3 mL/min
Mobile phase A	0.1% formic acid	10 mM Ammonium acetate with 0.1% formic acid	0.1% formic acid	0.1% formic acid	0.1% formic acid
Mobile phase B	ACN containing 0.1% formic acid	Acetonitrile	ACN containing 0.1% formic acid	ACN containing 0.1% formic acid	ACN containing 0.1% formic acid
Mass spectrometer conditions					
Mode	Positive	Positive	Positive	Negative	Negative
Declustering potential (Volt)	61	35	46	-55	-65
Collision energy (Volt)	25	15	19	-20	-18
Turbo-V Source temperature (°C)	400	350	350	300	400
Analyte *m/z* transition	152 → 110	240 → 190	256 → 238	915 → 541	310 → 266
Internal standard *m/z* transition	156 → 114	267 → 208	372 → 148	851 → 501	314 → 270
Standard curve range (nM)	5 to 1000	6 to 3200	0.5 to 100	0.2 to 100	25 to 5,000

Table 2

Method for (*S*)-mephenytoin 4'-hydroxylase, dextromethorphan *O*-demethylase, terfenadine hydroxylase, midazolam 1'-hydroxylase, and testosterone 6β-hydroxylase assays

Parameter(s)	(*S*)-Mephenytoin 4'-Hydroxylase (CYP2C19)	Dextromethorphan *O*-Demethylase (CYP2D6)	Terfenadine Hydroxylase (CYP2J2)	Midazolam 1'-Hydroxylase (CYP3A)	Testosterone 6β-Hydroxylase (CYP3A)
Microsomal Protein concentration (mg/mL)	0.25	0.15	Not conducted	0.1	0.15
Recombinant P450 concentration (pmol/mL)	Not conducted	Not conducted	2	5	Not conducted
Incubation time (min)	5	5	5	5	5
Substrate (final concentration, μM)	(*S*)-Mephenytoin (55)	Dextromethorphan (10)	Terfenadine (1)	Midazolam (5)	Testosterone (75)
Analyte	4'-Hydroxymephenytoin	Dextrorphan	Terfenadine alcohol	1'-Hydroxymidazolam	6β-Hydroxytestosterone
Internal standard	6-Hydroxychlozoxazone	Propanolol	Trazodone	α-Hydroxymidazolam-D_4	6β-Hydroxytestosterone-D_3
Column	Luna Pheny-hexyl 2x150 mm, 5 μm	Luna Pheny-hexyl 2x150 mm, 5 μm	Luna Pheny-hexyl 2x150 mm, 5 μm	Zorbax SB-C18 150x2.1 mm, 5 μm	Zorbax SB-C18 150x2.1 mm, 5 μm
Flow rate	0.3 mL/min	0.3 mL/min	0.3 mL/min	0.6 mL/min	0.6 mL/min
Mobile phase A	0.1% formic acid	0.1% formic acid	0.1% formic acid	0.1% formic acid	0.1% formic acid
Mobile phase B	ACN containing 0.1% formic acid	ACN containing 0.1% formic acid	ACN containing 0.1% formic acid	ACN containing 0.1% formic acid	Methanol containing 0.1% formic acid
Mass spectrometer conditions					
Mode	Negative	Positive	Positive	Positive	Positive
Declustering potential (Volt)	-55	81	106	76	71
Collision energy (Volt)	-22	53	39	31	21
Turbo-V Source temperature (°C)	400	400	350	500	350
Analyte *m/z* transition	233 → 190	258 → 157	488 → 452	342 → 324	305 → 269
Internal standard *m/z* transition	184 → 120	260 → 183	372 → 148	346 → 328	308 → 272
Standard curve range (nM)	2 to 100	25 to 10,000	0.1 to 500	2 to 1000	5 to 5,000

Table 3

Evaluation of EE as an inhibitor of human P450s in vitro

P450	Incubation[a]	Substrate (activity)	$IC_{50(total)}$ (μM)		$IC_{50(free)}$ (μM)
			No pre-incubation	Pre-incubation ($IC_{50(t)}$)[d]	No pre-incubation[f]
1A1	Recombinant	Phenacetin (*O*-deethylase)	2.7 ± 0.3	2.1 ± 0.5	2.7 ± 0.3
3A4	Recombinant	Midazolam (1'-hydroxylase)	8.5 ± 0.7	1.5 ± 0.3 (0.031)[c]	8.5 ± 0.7
3A5	Recombinant	Midazolam (1'-hydroxylase)	8.9 ± 2.8	3.3 ± 0.3 (0.014)	8.9 ± 2.8
1B1	Recombinant	Melatonin (6-hydroxylase)	9.2 ± 2.2	11 ± 0.5	9.2 ± 2.2
1A2	Recombinant	Phenacetin (*O*-deethylase)	14 ± 0.4	41 ± 0.7	14 ± 0.4
2J2	Recombinant	Terfenadine (hydroxylase)	31 ± 5.0	15 ± 4.0[b]	31 ± 5.0
2B6	Recombinant	Bupropion (hydroxylase)	41 ± 2.4	38 ± 4.4	41 ± 2.4
2C19	HLM	Mephenytoin (4'-hydroxylase)	4.4 ± 1.6	9.7 ± 1.4	2.8 ± 1.0
2C9	HLM	Diclofenac (4'-hydroxylase)	13 ± 3.2	9.2 ± 0.3	11 ± 0.3
2C8	HLM	Paclitaxel (6α-hydroxylase)	16 ± 2.0	8.3 ± 0.5[b]	13 ± 1.7
2B6	HLM	Bupropion (hydroxylase)	23 ± 1.5	23 ± 3.5	19 ± 1.3
2D6	HLM	Dextromethorphan (*O*-demethylase)	28 ± 2.0	32 ± 12	24 ± 1.7
3A4	HLM	Testosterone (6β-hydroxylase)	39 ± 9.0	8.9 ± 0.8 (0.005)	34 ± 7.8
3A4	HLM	Midazolam (1'-Hydroxylase)	42 ± 1.3	8.0 ± 0.7 (0.006)	37 ± 1.1
1A2	HLM	Phenacetin (*O*-deethylase)	>45 (~40%)[e]	>45 (~40%)[e]	>39

Legend to Table 3

[a]Incubations were performed in 0.1M potassium phosphate buffer (pH 7.4) containing EDTA (1 mM), HLM protein (0.05 to 0.15 mg/mL) or recombinant P450 (2 to 5 pmol/mL). EE was dissolved in DMSO (final concentration range of 10 nM to 45 μM). The final concentration of DMSO was < 0.2% (v/v). Incubations and pre-incubations were initiated with NADPH (1.0 mM). Data represent mean ± standard deviation of three determinations.

[b]$IC_{50} > IC_{50(t)}$ (~2-fold) for CYP2C9 activity (HLM) and recombinant CYP2J2, but no attempt was made to calculate a k_{inact}/K_I ratio.

[c]Data in parentheses represent the k_{inact}/K_I ratio calculated from the IC_{50} after 30 minutes of pre-incubation ($IC_{50(t)}$), where $k_{inact}/K_I = [0.693 \bullet (1 + [S]/K_m)]/[IC_{50(t)} \bullet t]$ (Maurer et al 2000, and Berry and Zhao 2008). There was no dilution of incubate. For each P450 assay, the substrate concentration approximated the K_m ($[S]/K_m$ ~1.0) and the pre-incubation time was 30 mins (t = 30). Troleandomycin was used as a positive control for recombinant CYP3A4 (IC_{50} = 2.5 μM, $IC_{50(t)}$ = 0.20 μM) and CYP3A5 (IC_{50} = 11 μM, $IC_{50(t)}$ = 2.4 μM) and k_{inact}/K_I ratios of 0.23 and 0.019 $min^{-1}\mu M^{-1}$ were calculated, respectively. The k_{inact}/K_I ratio for troleandomycin in HLM (IC_{50} = 19 μM, $IC_{50(t)}$ = 0.55 μM) was 0.084 $min^{-1}\mu M^{-1}$.

[d]Furafylline (CYP1A1, IC_{50} = 2.8 μM , $IC_{50(t)}$ = 0.46 μM; and CYP1A2, IC_{50} = 1.8 μM , $IC_{50(t)}$ = 0.11 μM), phencyclidine (CYP2B6, IC_{50} = 44 μM, $IC_{50(t)}$ = 0.67 μM), paroxetine (CYP2D6, IC_{50} = 1.0 μM , $IC_{50(t)}$ = 0.09 μM), phenelzine (CYP2C8, IC_{50} = 308 μM , $IC_{50(t)}$ = 114 μM), tienilic acid (CYP2C9, IC_{50} = 2.1 μM , $IC_{50(t)}$ = 0.21 μM), and ticlopidine (CYP2C19, IC_{50} = 2.1 μM , $IC_{50(t)}$ = 0.91 μM) were also served as positive controls (k_{inact}/K_I = 0.10, 0.42, 0.07, 0.54, 0.0004, 0.22, and 0.051 $min^{-1}\mu M^{-1}$, respectively).

Legend to Table 3 (Contd.)

[e]Percent inhibition at the highest concentration of EE tested (45 μM).

[f]Total IC_{50} corrected for EE free fraction in the incubation ($f_{u,inc}$), where IC_{50} free = IC_{50} total • $f_{u,inc}$. For recombinant P450s, binding of EE to total protein was negligible ($f_{u,inc}$ ~1.0). For HLM, $f_{u,inc}$ was 0.63 (0.25 mg/mL), 0.87 (0.1 mg/mL) and 0.84 (0.05 mg/mL).

Figure 1

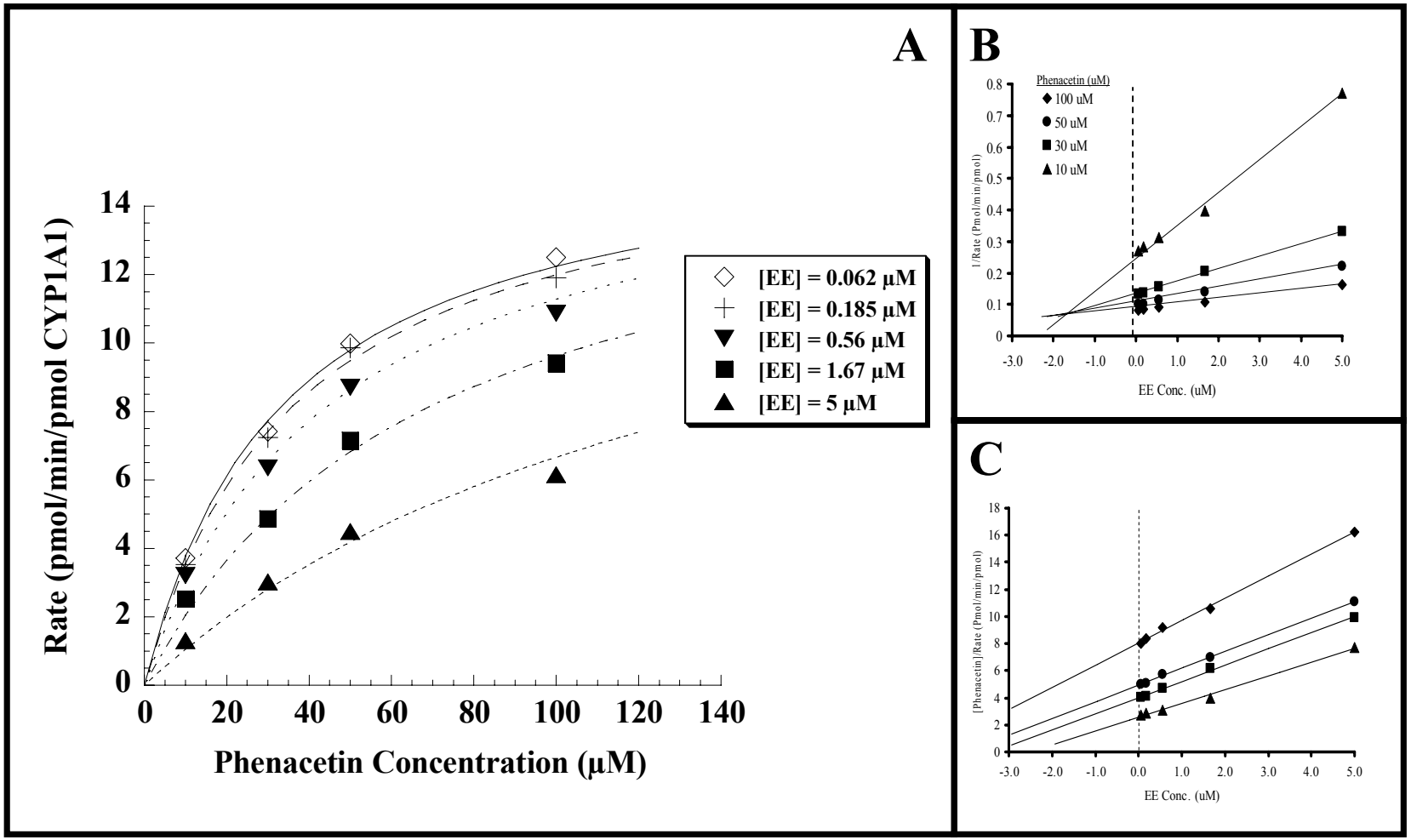


Figure 2

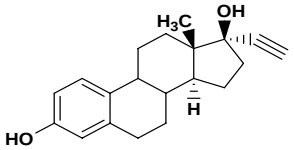
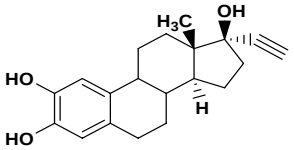
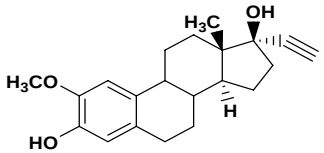
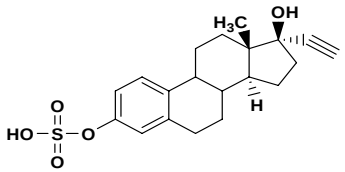
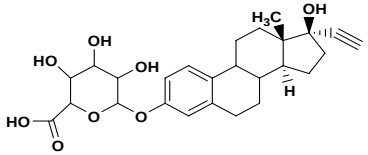
EE or Metabolite of EE	Structure	IC_{50} (μM)	
		rCYP1A1	rCYP1A2
EE		2.7 ± 0.3	14 ± 0.4
2-Hydroxy EE		2.5 ± 0.5	18 ± 1.4
2-Methoxy EE		2.2 ± 0.1	28 ± 0.5
EE 3-*O*-sulfate		>45	>45
EE 3-*O*-Glucuronide		>45	>45

Figure 3

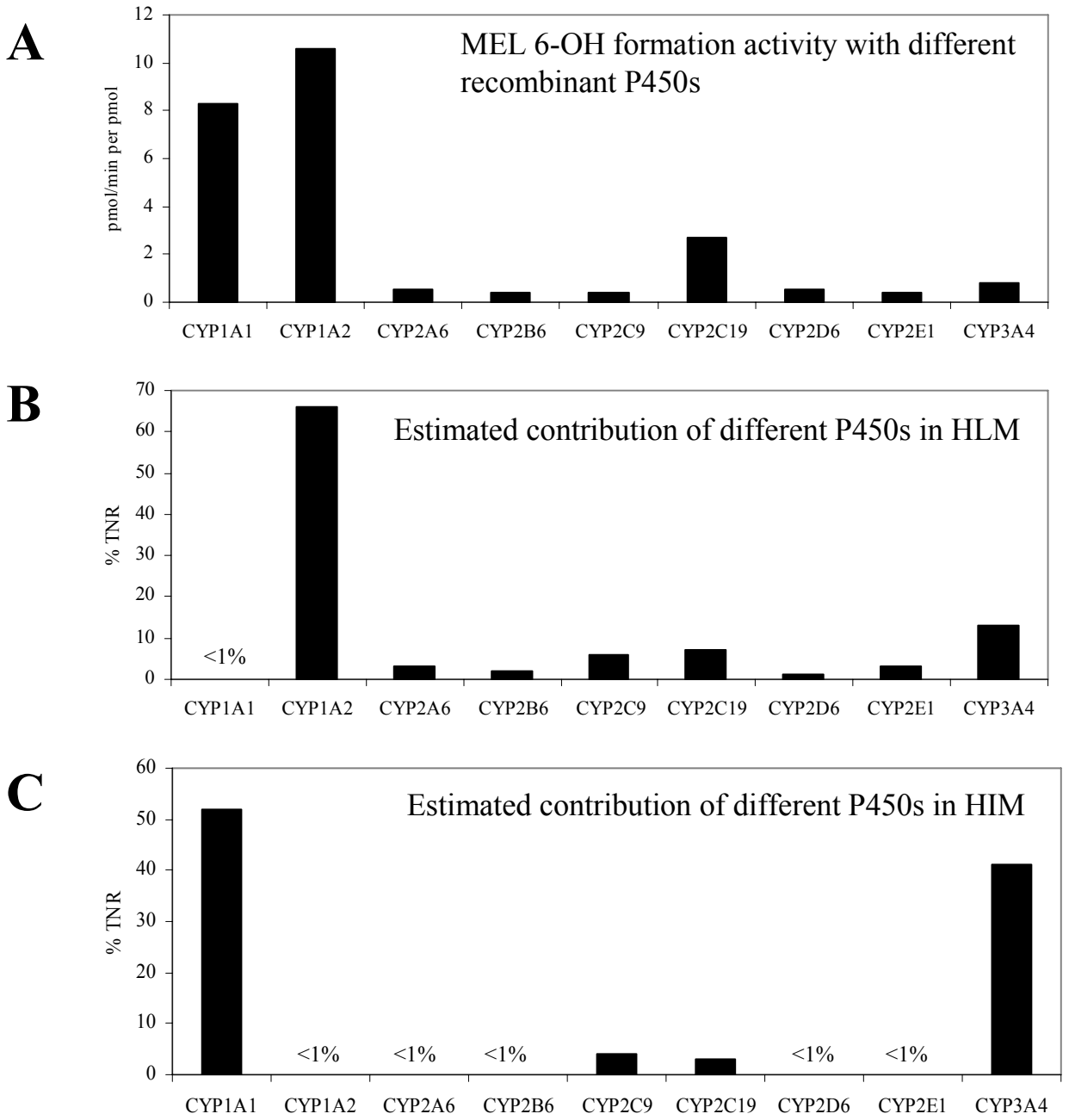


Figure 4