Quantitative prediction of CYP2B6 induction by estradiol during pregnancy, Potential explanation for increased methadone clearance during pregnancy

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Running title: Estradiol induces CYP2B6

Short communication

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Abbreviations: EC_{50}, concentration at which 50% of maximum induction is reached; f_{ins}, the fraction of total clearance of the drug to which the affected enzyme contributes.
Abstract

There is considerable evidence that pregnancy changes the disposition of drugs in an enzyme and gestational stage specific manner. Based on probe drug studies the activity of CYP3A4 and CYP2D6 increases and CYP1A2 decreases during human pregnancy. However, no studies of CYP2B6 activity during human pregnancy have been conducted. In rodent models and in HepG2 cells, CYP2B isoforms have been shown to be regulated by estradiol. As estradiol concentrations increase about 50-fold during human pregnancy, it was hypothesized that the increasing estradiol concentrations during human pregnancy would result in induction of CYP2B6 activity. Hepatocytes from three female donors were treated with estradiol and the EC₅₀ and E₉₉ measured for CYP2B6 mRNA and bupropion hydroxylation activity. The measured values were used to predict the magnitude of CYP2B6 induction during human pregnancy. At 100 nM total estradiol, a concentration achievable during the third trimester of pregnancy, CYP2B6 activity was predicted to increase between 1.5 and 3-fold based on increased CYP2B6 activity and mRNA, respectively. When the E₉₉ and EC₅₀ values were compared to carbamazepine and rifampin, estradiol was found to be as potent an inducer of CYP2B6 as rifampin and carbamazepine. These data suggest that during human pregnancy, the increasing estradiol concentrations will result in increased clearance of drugs that have CYP2B6 mediated clearance pathways. This could in part explain the observed increase in methadone clearance during pregnancy.
Introduction

CYP2B6 contributes to the clearance of 3-12% of common drugs, including 25-30% of known CYP3A4 substrates (Xie and Evans, 2001; Walsky et al., 2006). CYP2B6 is one of the primary enzymes responsible for the clearance of bupropion and methadone, (Totah et al., 2007; Wang and Tompkins, 2008), and in an uninduced state it contributes to the clearance of sertraline, diazepam, ketamine, propofol, selegiline and various designer drugs (Kreth et al., 2000; Yanagihara et al., 2001; Wang and DeVane, 2003; Maurer et al., 2004; Obach et al., 2005; Walsky et al., 2006; Wang and Tompkins, 2008) as well as to the clearance of efavirenz and nevirapine (Erickson et al., 1999; Ward et al., 2003), two drugs used to treat HIV infection. Due to its importance as one of the clearance pathways of many drugs taken by pregnant women, increased CYP2B6 activity during human pregnancy may have a large impact in the therapy of pregnant women. Despite CYP2B6’s importance in clearance of drugs given to pregnant women, the CYP2B6 activity during human pregnancy has not been studied. Two studies have evaluated the clearance of methadone during human pregnancy in women receiving methadone maintenance, and lower plasma methadone concentrations and increased clearance of methadone were reported (Pond et al., 1985; Wolff et al., 2005). This could be explained by either CYP3A4 or CYP2B6 induction or increased renal clearance during pregnancy. In a small study of sertraline pharmacokinetics during pregnancy, three subjects were studied both post partum and during pregnancy (Freeman et al., 2008). In all three subjects sertraline concentrations were higher post partum than during second trimester, and in two subjects the third trimester sertraline concentrations were lower than post partum. The magnitude of change in sertraline concentrations varied between 29% decrease to 90% decrease (Freeman et al., 2008). In contrast to these observations, the oral clearance of nevirapine was not different during the third trimester.
of pregnancy and postpartum when evaluated in a population of unknown CYP2B6 genotypes (Capparelli et al., 2008). As all of these drugs are eliminated by multiple cytochrome P450 enzymes, the effect of pregnancy on CYP2B6 activity cannot be conclusively evaluated from these studies.

There is considerable evidence from rodent studies that estradiol induces the expression of Cyp2b genes. In female and male mouse hepatocytes, Cyp2b9 and Cyp2b10 were induced in an estradiol and estrone concentration dependent manner (Nemoto and Sakurai, 1995), and in vivo in male mice, treatment with 0.5 mg/kg/day estradiol resulted in a significant increase of Cyp2b9 mRNA (Nemoto and Sakurai, 1995). In aromatase−/− mice, which are deficient in estrogen biosynthesis, Cyp2b9 expression was eliminated, suggesting a role of estradiol in constitutive Cyp2b9 regulation (Yamada et al., 2002). In primary mouse hepatocytes, the Cyp2b10 promoter was activated by estradiol and an estradiol responsive DNA element was identified (Yamamoto et al., 2001). In HepG2 cells, reporter assays showed that estradiol activates constitutive androstan receptor (CAR) and enhances CYP2B6 promoter activity (Koh et al., 2012). In addition, in rat hepatocytes estradiol was shown to cause nuclear translocation of CAR (Koh et al., 2012). Together these studies demonstrate an important role of estrogens in CYP2B regulation, but the potential magnitude of CYP2B6 induction resulting from the increasing circulating estrogen concentrations during pregnancy has not been previously predicted. The aim of this study was to determine, in human hepatocytes, whether CYP2B6 mRNA and activity is increased by estradiol and to predict the magnitude of CYP2B6 induction during human pregnancy from the hepatocyte data. This is, to our knowledge, the first report of mechanistic quantitative predictions of magnitude of change of drug clearance during pregnancy.
Materials and Methods

Reagents and Chemicals. William’s E Medium, Dubelcco’s Modified Eagle’s Medium (DMEM), dexamethasone, rifampicin, estradiol, carbamazepine, and bupropion, were purchased from Sigma-Aldrich (St. Louis, MO). Cryopreserved Recovery Medium (CHRM), and media supplements were purchased from Invitrogen (Carlsbad, CA). Matrigel and hydroxybupropion was purchased from BD Biosciences (San Jose, CA) and Kreb-Henseleit buffer (KHB) from Celsis/In Vitro Technologies (Chicago, IL). Nuclease free water, RNA later, MagMax 96 RNA isolation kit, High Capacity cDNA Transcription Kit, TaqMan assays and all TaqMan reagents and consumables were purchased from Applied Biosystems (Foster City, CA).

Cell culture. Cryopreserved human hepatocytes (see Supplemental Table 1 for demographics) were purchased from Invitrogen (Carlsbad, CA). Hepatocytes were plated and cultured according to previously described methods (Dickmann et al., 2011). Cells were treated the day after plating and media with an appropriate concentration of drug was replaced daily for cytochrome P450 induction studies. Estradiol, carbamazepine and rifampin were dissolved in ethanol and cells were treated with 10 different concentrations (0.39-200 µM for carbamazepine and 0.025-25 µM for rifampicin and estradiol) of the inducer or vehicle control for 48 hours. All treatments at each concentration were conducted in triplicate in all three donors. The concentration of ethanol in treatments and vehicle controls was 0.1%.

mRNA analysis. Immediately following activity assay, cells were lysed and RNA stabilized with lysis buffer from the MagMax96 RNA isolation kit. Total RNA was isolated using this kit according to manufacturer’s protocol and RNA quantity was assessed by determining the ratio of absorbance at 280 and 260 nm using a NanoDrop spectrophotometer (Thermo Scientific). All
RNA samples were then adjusted to 10 ng/µL with nuclease free water. cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit with a final volume of 40 µL and 132 ng total RNA according to manufacturer’s protocol. After synthesis, the cDNA reactions were diluted to 160 µL total volume with nuclease free water. TaqMan reactions were run on a 7900HT Real-time PCR system (Applied Biosystems, Foster City, CA) in a 384-well optical reaction plate. Each reaction contained 10 µL 2x Gene Expression Master Mix, 5 µL nuclease free water, 1 µL 20x Primer and Probe mix, and 4 µL of cDNA. All reactions were run in duplicate using default cycling parameters. The TaqMan assay used for CYP2B6 measurement was Hs03044634_m1 and for the housekeeping gene (18s) Hs03928985_g1.

**Cytochrome P450 Activity Assays in Hepatocyte Culture.** After a 48 hour incubation with either rifampicin, carbamazepine, or estradiol, cells were washed with 100 µL of Krebs-Hanseleit buffer (KHB). Cells were then incubated with KHB containing 250 µM bupropion for 30 minutes. KHB containing parent and metabolite was removed and frozen at -70°C until analysis. Hydroxybupropion formation from bupropion was measured by LC-MS/MS with an API4000 Q-trap mass spectrometer (Applied Biosystems, Foster City, CA), two LC-20AD pumps with an in-line CBM-20A controller and DGU-20A5 solvent degasser (Shimadzu, Columbia, MD) and a Leap CTC HTS PAL autosampler (CTC Analytics, Carrboro, NC). The injection volume was 20 µl. LC separation was achieved using a Gemini C18 2.0 * 30 mm 5-µm column (Phenomenex, Torrance, CA). Gradient elution (flow rate 500 µl/min) was performed using a mobile phase system consisting of (A) 5 mM ammonium formate with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid with the following gradient: 0 to 0.5 min, 5% B; 0.5 to 1.75 min, 100% B; and 1.75 to 2 min, 5% B. Source and gas parameters were as follows: curtain gas, 10; collision gas, medium; ionspray voltage, 4500 V; and temperature, 450°C.
Analyte was detected with positive ion multiple reaction monitoring with the following conditions: Q1, 256.2 m/z; Q3, 139 m/z; DP, 60; CE, 30; CXP, 13. Tolbutamide was used as an internal standard. The lower limit of detection of the assay was 2.5 nM and the day to day variability was less than 10%.

**Data analysis and prediction of increased CYP2B6 activity during pregnancy.** Data was fit to both a 3- (fixed slope) and 4-parameter (variable slope) dose response model using GraphPad Prism 5, version 5.04 (La Jolla, CA). The 4-parameter model resulted in ambiguous fits for induction by carbamazepine. Therefore, for consistency between donors and inducers, only 3-parameter fits \( \text{CYP2B6 mRNA/activity} = E_{\text{min}} \left( 1 + 10^{(\text{Log}[EC50] - \text{Log}[\text{Drug}])} \right) \) are shown for all donors and compounds tested. The goodness of fit was determined from the R squared values generated in GraphPad Prism. R-squared values were equal to or greater than 0.89 for mRNA and activity fits in all donors tested. Significance of the magnitude of induction in individual treatments was tested by ANOVA followed by Dunnett’s test as a post hoc test using GraphPad Prism. A p-value of <0.05 was considered significant. Significance of differences between the \( E_{\text{max}} \) and \( EC_{50} \) values between estradiol, carbamazepine and rifampicin treatments was tested using paired (each donor considered as a paired set) t-test and a p-value <0.05 was considered significant.

To predict the effect of rifampicin and carbamazepine on CYP2B6 activity, in vivo average plasma concentrations of 8 μM for rifampicin and 34 μM for carbamazepine were used (Thummel et al., 2011). Since estradiol concentrations vary considerably between women during pregnancy and between gestational stages, estradiol concentrations between 10 and 1000 nM were used for predictions. The fold induction was predicted as described previously (Ripp et al., 2006; Sinz et al., 2008) according to equation:
fold induction = \[1 + \frac{[I] \cdot E_{\text{max}}}{[I] + EC_{50}}\]

in which [I] is the inducer concentration, \(E_{\text{max}}\) is the maximum fold induction observed in vitro and \(EC_{50}\) is the inducer concentration at which 50% of maximum fold induction is reached. The increase in CYP2B6 expression and activity was predicted separately from each hepatocyte donor using mRNA and activity data.
Results and Discussion

Estradiol, as well carbamazepine and rifampicin resulted in significant (p<0.05), concentration dependent induction of CYP2B6 mRNA and activity in human hepatocyte donors (Table 1 and Figure 1). In all donors the increase in CYP2B6 activity was significant (p<0.05) following treatment with $\geq 12.5\mu M$ carbamazepine, $\geq 0.78\mu M$ estradiol and $\geq 0.78\mu M$ rifampicin. The increase in CYP2B6 mRNA was significant (p<0.05) in all donors following treatment with $\geq 6.5\mu M$ carbamazepine, $\geq 2\mu M$ estradiol and $\geq 2\mu M$ rifampicin. In each individual donor the E_max of CYP2B6 mRNA induction by estradiol was greater than that observed following carbamazepine and rifampicin treatment but the difference in E_max values for all donors combined was not significant (p>0.05). Based on CYP2B6 activity, the maximum effect of estradiol on CYP2B6 activity was similar to that of rifampicin. While the average EC_{50} value of CYP2B6 induction by estradiol is above the circulating concentrations of estradiol (Table 1), estradiol had similar potency (p>0.05 for EC_{50} values) with rifampicin and carbamazepine as CYP2B6 inducer.

To test whether the CYP2B6 induction observed in these hepatocytes could be used to predict the magnitude of CYP2B6 induction in vivo, the magnitude of increase in CYP2B6 mediated clearance caused by rifampicin and carbamazepine was predicted. Based on the CYP2B6 mRNA data, carbamazepine and rifampicin were predicted to increase CYP2B6 mRNA 9-11 fold (mean 10-fold) and 7-20 fold (mean 15-fold), respectively. Based on the CYP2B6 activity data, the fold increase in CYP2B6 activity was predicted to be 2.3-5.8 fold (mean 3.9-fold) and 4-24 fold (mean 14-fold) by carbamazepine and rifampicin, respectively. These predictions are in good agreement with the observed increase in bupropion clearance following carbamazepine and rifampicin administration. Carbamazepine decreases bupropion AUC by 87% and increases OH-
bupropion to bupropion AUC ratio 11.5-fold (Ketter et al., 1995) suggesting an 11-fold increase in hydroxybupropion formation clearance assuming the clearance of hydroxybupropion is unaffected by carbamazepine. Rifampicin decreases bupropion AUC by 46-67% (Loboz et al., 2006), and increases hydroxybupropion formation clearance (CLf) 4-fold (2.3-6.8 fold) (Kirby et al., 2011), an effect slightly lower than that predicted from the hepatocyte data.

Based on the good predictions with carbamazepine and rifampicin, the effect of increasing estradiol concentrations on CYP2B6 activity during pregnancy was predicted without using an additional scaling factor (Figure 2). At 50 nM and 100 nM estradiol, predicted CYP2B6 mRNA induction was 1.9 (± 0.72) -fold and 2.8 (± 1.4) -fold, respectively, while CYP2B6 activity induction was 1.2 (± 0.15) -fold and 1.4 (0.30) -fold, respectively. As circulating estradiol concentrations reach up to 50 nM during pregnancy (Soldin et al., 2005), based on this data a 2-fold increase in CYP2B6 expression is predicted during pregnancy based on mRNA data. As estradiol concentrations increase gradually during pregnancy (O'Leary et al., 1991), based on this data it is expected that CYP2B6 activity also increases gradually throughout pregnancy reaching the maximum induction during the third trimester. It is noteworthy that estradiol concentrations vary considerably between individuals during pregnancy (O'Leary et al., 1991) and as such the magnitude of CYP2B6 induction is expected to vary largely between women during pregnancy. Although these predictions based on methods used for xenobiotics suggest that CYP2B6 activity is increased only 2-fold during pregnancy, it is possible that a greater effect would be observed in vivo because estradiol concentrations are high consistently and do not fluctuate like commonly administered xenobiotic inducers. The effect of inducer concentration fluctuations is not well characterized and depends on whether fluctuation is sufficient to cause inducer concentrations to drop below receptor saturation.
It is well established that CYP2B6 is inducible via CAR activation although PXR also appears to play a role in CYP2B6 regulation (Faucette et al., 2007; Mo et al., 2009). It has also been shown that estradiol activates CAR directly (Kawamoto et al., 2000), and that activation of CAR by estradiol results in CYP2B6 induction (Koh et al., 2012). While it is possible that activation of PXR or ER by estradiol also contributes to increased CYP2B6 mRNA and activity, it is expected that a similar in vitro to in vivo prediction is applicable for induction of CYP2B6 by estradiol as is shown for rifampicin and carbamazepine. However, it is not clear whether circulating concentrations of estradiol accurately reflect the concentrations inside the hepatocytes. The 100 nM concentration of estradiol appears appropriate and physiologically relevant for evaluating effects of estradiol in human hepatocytes, as the same concentration was shown to down-regulate CYP2C19 in human hepatocytes via activation of ER (Mwinyi et al., 2010). This down-regulation provides a potential explanation for the mechanisms by which CYP2C19 activity is decreased during pregnancy. The agreement between the down-regulation of CYP2C19 by 100 nM estradiol in human hepatocytes and decreased clearance during pregnancy suggests that this concentration reflects the exposure in the liver during human pregnancy.

The predicted magnitude of increase in CYP2B6 activity (2-fold) during pregnancy is in excellent agreement with the observed 2-fold increase in methadone clearance during pregnancy (Pond et al., 1985; Wolff et al., 2005). While in vivo DDI studies and in vitro experiments have shown that methadone is cleared predominantly by CYP2B6 (Totah et al., 2008), CYP3A4 and renal clearance also contribute to methadone clearance. Based on the data presented here, it is likely that the increased methadone clearance is due to increased CYP2B6 activity together with increased CYP3A4 and renal clearance during pregnancy. Based on studies on midazolam oral
clearance during pregnancy, CYP3A4 activity increases approximately 2-fold during the third trimester of pregnancy (Hebert et al., 2008). Since CYP3A4 is only a minor elimination pathway of methadone, a two-fold increase in CYP3A4 activity alone is not sufficient to explain the magnitude of increase in methadone clearance during pregnancy. Unfortunately no other studies of disposition of CYP2B6 substrates/probes such as bupropion have been reported to further evaluate the magnitude of CYP2B6 induction during pregnancy. As CYP2B6 is a minor elimination pathway for number of drugs, induction of CYP2B6 during human pregnancy may have important clinical implications, especially when it is combined with increased CYP3A4 and CYP2D6 activity during pregnancy.
Authorship Contribution

Participated in Research Design: Dickmann, Isoherranen

Conducted Experiments: Dickmann

Contributed New Reagents or analytic tools: None

Performed Data analysis: Dickmann, Isoherranen

Wrote or contributed to the writing of the manuscript: Dickmann, Isoherranen
References


Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, and Desta Z (2003) The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary


Legends to Figures

Figure 1. Representative CYP2B6 mRNA and activity dose response curves for primary human hepatocytes from two donors treated with rifampicin, carbamazepine, and estradiol. Cells were treated in triplicate with rifampicin, carbamazepine, and estradiol for 48 hours and CYP2B6 mRNA and activity was assessed as described in the Materials and Methods section. Data was fit to a 3-parameter (fixed slope) dose response model as described in the Materials and Methods section. A) Donor Hu8127, CYP2B6 mRNA; B) Donor Hu8127, CYP2B6 activity; C) Donor Hu4069, CYP2B6 mRNA; and D) Donor Hu4069, CYP2B6 activity. Error bars indicate the standard deviation of triplicate treatments.

Figure 2. Prediction of in vivo increase of CYP2B6 mRNA and activity with increasing concentrations of estradiol. Predictions were done as described in the Materials and Methods section for each donor using $E_{\text{max}}$ and $EC_{50}$ values in Table 1.
Table 1. Characterization of the potency and magnitude of increase in CYP2B6 mRNA (top) and activity (bottom) by rifampicin, carbamazepine, and estradiol treatment in three cultured human hepatocyte donors (Hu1399, Hu8127 and Hu4069). EC$_{50}$ and E$_{max}$ values for rifampicin, carbamazepine, and estradiol were determined as described in Materials and Methods.

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<th>mRNA</th>
<th>Rifampicin</th>
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<th>Estradiol</th>
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<td>EC$_{50}$ (μM)</td>
<td>E$_{max}$ (fold increase)</td>
<td>EC$_{50}$ (μM)</td>
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<table>
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<th>Estradiol</th>
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<td>Donor</td>
<td>EC$_{50}$ (μM)</td>
<td>E$_{max}$ (fold increase)</td>
<td>EC$_{50}$ (μM)</td>
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<td>Hu1399</td>
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<td>145±65</td>
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</table>
Figure 1

A) Fold increase in CYP2B6 mRNA:
- Carbamazepine
- Estradiol
- Rifampicin

B) Fold increase in CYP2B6 activity:
- Carbamazepine
- Estradiol
- Rifampicin

C) CYP2B6 mRNA (relative quantitation):
- Carbamazepine
- Estradiol
- Rifampicin

D) Fold increase in CYP2B6 activity:
- Carbamazepine
- Estradiol
- Rifampicin
Figure 2

(A) Estradiol (μM) vs. Fold increase in CYP2B6 mRNA

(B) Estradiol (μM) vs. Fold increase in CYP2B6 activity