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Cytochrome P450 expression and regulation in CYP3A4/CYP2D6 double transgenic humanized mice

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Abbreviations: CYP, Cytochrome P450; DDIs, drug-drug interactions; DXM, dextromethorphan; DXO, dextrorphan; MEM, 3-methoxymorphinan; MDZ, midazolam; TEST, testosterone; PCN, pregnenolone 16α-carbonitrile; TCPOBOP, 1,4-bis-2-(3, 5-dichloropyridyloxy)-benzene; HPLC, high-performance liquid chromatography.

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Abstract

Analysis of the developmental and sexual expression of cytochrome P450 (CYP) drug-metabolizing enzymes is impeded by multiple and varied external factors that influence its regulation. In the present study, a *CYP2D6/CYP3A4*-double transgenic (Tg-*CYP2D6/CYP3A4*) mouse model was employed to investigate hepatic CYP2D6 and CYP3A4 ontogeny and sexual dimorphism. Both age and sex have considerable effects on hepatic CYP3A4 protein expression in 3- to 8-week-old transgenic mice, whereas neither factor alters CYP2D6 content. Constitutive CYP2D6 expression resulted in 2- to 3-fold higher dextromethorphan *O*-demethylase activity in Tg-*CYP2D6/CYP3A4* mouse liver microsomes when compared to wild-type mice. In contrast, expression of CYP3A4 in transgenic mouse livers did not increase dextromethorphan *N*-demethylase and midazolam 1'-hydroxylase activities. Pretreatment with pregnenolone 16α-carbonitrile (PCN) and 1,4-bis-2-(3, 5-dichloropyridyloxy)-benzene (TCPOBOP) elevated CYP3A4 expression in double transgenic mice. Interestingly, induction of hepatic CYP3A4 was greater in females than age- and treatment-matched males. Consequently, the increase in midazolam 1'-hydroxylase activity was markedly higher in 8-week-old female mice than in corresponding males (8-fold vs. 6-fold for PCN treatment and 6-fold vs. 5-fold for TCPOBOP). Furthermore, increases in testosterone 6β-hydroxylase activity after CYP3A induction were relatively lower when compared to those in midazolam 1'-hydroxlyation for age-, sex- and treatment-matched mice. The difference in CYP3A4 expression and induction between male and female mice suggests that women may be more susceptible to CYP3A4-mediated drug-drug interactions, and the extent of drug-drug interactions could be substrate dependent.

Introduction

The cytochrome P450 (CYP) enzymes are the most important phase I drug-metabolizing enzymes responsible for the biotransformations of numerous xenobiotics including pharmaceutical agents. Human CYP2D6 and CYP3A4 collectively metabolize over 70% of drugs on the market (Nebert and Russell, 2002; Guengerich, 2003). Of particular note, many opioids such as codeine, oxycodone and dextromethorphan are oxidized by both CYP2D6 and CYP3A4 in humans (Gonzalez and Yu, 2006). Maturation of individual CYP enzymes during development follows characteristic patterns with adult levels typically reached within the first year. As a result, infants may have lower metabolic capacity and clearance of CYP substrate drugs (Alcorn and McNamara, 2002; Stevens, 2006). Nonetheless, discrepancies still exist in understanding the mechanisms of developmental regulation and sex-related differences in the expression of CYP3A4 and CYP2D6 enzymes (Beierle et al., 1999; Hines and McCarver, 2002; Gandhi et al., 2004; Parkinson et al., 2004). While some studies (George et al., 1995; McCune et al., 2001; He et al., 2006) have shown that CYP3A4 and CYP2D6 levels are not different between women and men, others (Watkins et al., 1989; Schmidt et al., 2001; Wolbold et al., 2003) have demonstrated that women have higher CYP3A4 levels or metabolic activities than men.

Analysis of these differences in human populations is confounded by the fact that many environmental factors such as concomitant medication, dietary supplements and toxicant exposure can considerably alter CYP3A4 and CYP2D6 expression or activities via enzyme induction and/or inhibition. Inevitably, animals or cells maintained under defined environmental conditions are useful for understanding *CYP* gene regulation and the underlying mechanisms.

Transgenic mouse models are an invaluable addition to these in vivo and in vitro models towards delineation of *CYP* regulation in a whole body system (Gonzalez and Yu, 2006). In addition, transgenic animals overcome functional differences between human CYPs and the corresponding animal isoforms (Gonzalez and Kimura, 2003; Henderson and Wolf, 2003; Yu et al., 2004; Yu and Haining, 2006; Gonzalez, 2007). Indeed, the development and utilization of *CYP*-transgenic animal models have offered important clues for *CYP* gene regulation and functional responses to xenobiotics (Gonzalez and Yu, 2006; Gonzalez, 2007). Studies with *CYP2D6*-transgenic (Tg-*CYP2D6*) mice (Corchero et al., 2001; Yu et al., 2003) (and unpublished data) have provided a mechanistic understanding for the role of CYP2D6 in drug metabolism, pharmaco/toxicokinetics and dynamics, and results from these animal models may better predict the difference in the fate and response of CYP2D6 substrate drugs in relation to CYP2D6 status. Recent studies (Granvil et al., 2003) have also demonstrated that midazolam (MDZ) first-pass metabolism is significantly enhanced in *CYP3A4*-transgenic (Tg-*CYP3A4*) mice. Ultimately, a mouse model expressing both CYP2D6 and CYP3A4 would provide an *in vivo* animal system to understand their ontogeny and contribution of individual isoforms to drug metabolism.

To study the developmental and sexual expression and regulation of the two major human drug-metabolizing CYPs, a double transgenic mouse line carrying both *CYP2D6* and *CYP3A4* transgenes (Tg-*CYP2D6/CYP3A4*) was generated by breeding Tg-*CYP2D6* to Tg-*CYP3A4* mice. CYP2D6 expression led to 2-3 fold increase in hepatic dextromethorphan (DXM) *O*-demethylase activity in Tg-*CYP2D6/CYP3A4* mice. Consistent with previous studies, sexual dimorphism in CYP3A4 expression was uncovered in Tg-*CYP2D6/CYP3A4* mouse livers. Furthermore, pregnenolone 16α-carbonitrile (PCN) and 1,4-bis-2-(3, 5-dichloropyridyloxy)-benzene

(TCPOBOP) were used to assess CYP3A4 induction and consequent impact on MDZ and testosterone (TEST) metabolism. These results indicate that CYP3A4 protein level was induced to a greater extent in adult female mouse livers than corresponding males, resulting in a markedly higher level of drug-metabolizing capacity in these females that may be dependent on drug substrate. These findings could provide insight into potential differences in CYP3A4 expression and induction and effects on drug-drug interactions (DDIs) between men and women.

Materials and Methods

Chemicals, Enzymes and Other Reagents. Dextromethorphan hydrobromide and dextrorphan-D-tartrate were purchased from ICN Biomedicals (Aurora, OH). 3-hydroxymorphinan hydrobromide, 3-methoxymorphinan hydrochloride, PCN, and TCPOBOP were purchased from Sigma Chemical Co. (St. Louis, MO). Testosterone and 6β-hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI). Midazolam and 1'-hydroxymidazolam were purchased from Cerilliant Corp. (Round Rock, TX). All additional chemicals and solvents used were of the highest chemical grade available. Pooled human liver microsomes (coded H161) were purchased from BD Discovery Labware, Inc. (Woburn, MA). Selective immunoblot antibodies included those against human CYP2D6 (MAB 2D6) (BD Discovery Labware, Inc., Woburn, MA), CYP3A4 (MAb 275-1-2), mouse Cyp3a (MAb 2-3-2), rat Cyp2d1, rat cyp2c7, rat cyp2b1 (Yu et al., 2005), and glycerylaldehyde-3-phosphate dehydrogenase (Gapdh) (Chemicon International Inc., Temecular, CA).

Animals and Treatments. All mice described in these studies (wild-type, Tg-*CYP2D6*, Tg-*CYP3A4* and Tg-*CYP2D6/CYP3A4* mice) were maintained under controlled temperature and lighting conditions. Food and water were provided *ad libitum.* For CYP3A4 induction studies, Tg-*CYP2D6/CYP3A4* mice were treated with PCN (100 mg/kg i.p. in corn oil for two days) or TCPOBOP (3 mg/kg i.p. in corn oil for two days). Control mice were administered with vehicle (corn oil) for two days. Four mice were used in each group. All animal studies were approved by the Institutional Animal Care and Use Committee at the University at Buffalo, The State University of New York.

Generation of Tg-*CYP2D6/CYP3A4* Mice. Tg-*CYP2D6* mice (Corchero et al., 2001) were mated with Tg-*CYP3A4* mice (Granvil et al., 2003) to produce the Tg-*CYP2D6/CYP3A4* mouse line. Offspring carrying both *CYP2D6* and *CYP3A4* transgenes were crossed to each other; the resultant double transgenic mice were interbred for 5-6 generations to establish mice homozygous for both transgenes. Wild-type, Tg-*CYP2D6*, Tg-*CYP3A4* and Tg-*CYP2D6/CYP3A4* mice were bred and maintained by brother-sister mating under the same environmental conditions.

Genotype Analysis. Genotype analysis was carried out to verify the presence of the transgenes in the Tg-*CYP2D6/CYP3A4* mouse model. Genomic DNA was isolated from mouse tails and PCR was conducted using the REDExtract-N-Amp Tissue PCR kit purchased from Sigma (St. Louis, MO). Tail DNA was amplified with microsomal epoxide hydrolase (mEH) (internal control), CYP2D6 and CYP3A4 gene-specific primers (Corchero et al., 2001; Granvil et al., 2003). PCR products (406 bp for CYP3A4, 341 bp for mEH and 234 bp for CYP2D6) were separated on a 2% agarose gel in Tris-Borate-EDTA buffer.

Preparation of Microsomes. Microsomes of mouse liver, small intestine, colon, kidney, heart, lung, brain and spleen were prepared as described previously (Yu et al., 2005; Cheung et al., 2006). Tissues were homogenized in ice-cold buffer A [320 mM sucrose, 10 mM KH_2PO_4, 1 mM EDTA and Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), pH7.4] and microsomes were harvested by differential centrifugation. The microsomal pellet was resuspended in buffer B [100 mM KH_2PO_4, 1 mM EDTA, 20% (v/v) glycerol, Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), pH 7.4], aliquoted and

stored at -80^{o}C until analysis. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) following the manufacturer's instructions.

Western Blot Analysis. Microsomal protein (20 μg) was denatured in loading buffer (1:50 β-mercaptoethanol:Laemlli's sample buffer) at 90^{o}C for 8 minutes, resolved by 12.5% SDS-PAGE and electro-transferred to a nitrocellulose membrane using a Mini-PROTEAN II Cell System (BioRad, Hercules, CA). Following incubation with blocking buffer (2-5% nonfat milk) for 1 hour at room temperature, the membrane was probed using protein-selective monoclonal antibodies for two hours. After washing, the membrane was incubated with the appropriate alkaline phosphatase- (AP) or peroxidase-conjugated (PER) antibodies. Blots were visualized using NBT/BCIP visualizing solution (AP-conjugated antibodies) (Sigma, St. Louis, MO) or an Enhanced Chemiluminescence Detection Kit (PER-conjugated antibodies) (Pierce Chemical Co., Rockford, IL).

Drug Metabolism in Liver Microsomes. Incubations for DXM, TEST and MDZ were modified from previously described methods (Yu and Haining, 2001; Yu and Haining, 2006). Briefly, incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) containing microsomal protein (20 μg for TEST and DXM; 1.5-20 μg for MDZ) and individual substrates (DXM 0-2000 μM; TEST 10 μM; MDZ 5 μM) with a final volume of 200 μl. The mixtures were pre-incubated for five minutes at 37^{o}C in a shaking water bath, and the reactions were initiated by the addition of 20 μl of 10 mM NADPH. Metabolite formation was linear up to 25 minutes for all substrates in mouse liver microsomes. Microsomal protein concentrations were selected from the linear range of metabolite formation with less than 10% substrate depletion. Incubations

were stopped after 5 minutes (MDZ and DXM) or 10 minutes (TEST) by the addition of 8 µl 70% ice-cold perchloric acid (DXM) or 100 µl acetonitrile (MDZ and TEST), followed by immersion in ice for 10 minutes. Enzyme activity was evaluated over a range of concentrations (DXM 0-2000 µM) or at a single concentration for each probe substrate (MDZ 5 µM; TEST 10 µM). MDZ and TEST concentrations were selected based on the K_m value for human CYP3A4 to ensure sensitivity in measuring changes in induction of enzyme activity and eliminate the possibility of product inhibition. All reactions were performed in triplicate.

HPLC Quantification of Drugs and Metabolites. All HPLC analyses were conducted on an Agilent 1100 series HPLC consisting of the online vacuum degasser, quaternary pump, well plate autosampler, thermostat controlled column compartment, fluorescence detector, and diode-array detector. For the quantitation of DXM, dextrorphan (DXO) and 3-methoxymorphinan (MEM), a Zorbax phenyl column (5 µm, 250 mm x 4.6 mm i.d.; Agilent Technologies, Palo Alto, CA) was used. The mobile phase consisted of 60% acetonitrile with 0.1% trifluoroacetic acid. The flow rate through the column at 25°C was 1 mL/min. The excitation and emission wavelengths of the fluorescence detector were 280 and 310 nm, respectively. Under these conditions, DXM, MEM, DXO and the secondary metabolite (3-hydroxymorphinan) were detected at 15.9, 13.1, 8.8 and 7.7 min, respectively. For the quantitation of TEST and 6β-hydroxytestosterone, a Symmetry C18 column (3.5 µm, 75 mm x 4.6 mm i.d.; Waters Corporation, Milford, MA) was used. The mobile phase was changed from 50:50 (v/v) to 90:10 (v/v) methanol:water linearly over 11 minutes with a flow rate of 1 mL/min at 25°C. A three minute post-run wash was used to re-equilibrate the column to initial conditions. TEST and its metabolite detected at 240 nm were eluted at 8.7 and 5.0 min, respectively. For the quantitation

of MDZ and 1'-hydroxymidazolam, a Symmetry C18 column (3.5 μm, 75 mm x 4.6 mm i.d.; Waters Corporation, Milford, MA) was used. The mobile phase consisting of 50:50 (v/v) acetonitrile:10 mM phosphate buffer, pH 6.5 with a flow rate of 1 mL/min at 25°C nicely separated 1'-hydroxymidazolam (2.0 min) from MDZ (3.0 min) which were monitored at 245 nm. All drugs and metabolites were quantified with the external standard method. Calibration curves were linear for DXM, MEM, DXO and 3-hydroxymorphinan ranging from 10 to 200 pmol; MDZ and 1'-hydroxymidazolam ranging from 25 to 500 pmol; TEST and 6β-hydroxytestosterone ranging from 20 to 200 and from 5 to 200 pmol, respectively. Intra-day and inter-day coefficients of variation were less than 10% for each analyte.

Data Analysis. All values are expressed as mean ± standard error of mean (S.E.M.) unless otherwise indicated. Michaelis-Menten parameters, K_m and V_{max}, were estimated by nonlinear regression (GraphPad Prism 4.00, San Diego, CA).

Results

Expression of Human CYP2D6, CYP3A4 and Murine Cyp Enzymes in Transgenic Mice. The presence of *CYP2D6* and *CYP3A4* transgenes in mice was confirmed by PCR genotyping at each breeding step. Genomic DNA from wild-type, Tg-*CYP2D6*, Tg-*CYP3A4* and Tg-*CYP2D6/CYP3A4* mice was amplified with three gene-specific primers for *CYP2D6*, *CYP3A4* and *mEH*, respectively (Corchero et al., 2001; Granvil et al., 2003). While the amplification of *mEH* fragments (341 bp) in all strains served as a positive control, the *CYP2D6* PCR product (234 bp) was only present in Tg-*CYP2D6* and Tg-*CYP2D6/CYP3A4* mice and the *CYP3A4* PCR product (406 bp) was only amplified in Tg-*CYP3A4* and Tg-*CYP2D6/CYP3A4* mice (Fig. 1). Tg-*CYP2D6/CY3A4* mice were fertile and produced average size litters; however the pups were generally smaller than age- and sex-matched wild-type mice (see Supplemental Data). The decrease in body weight is consistent with observations in the Tg-*CYP3A4* mice (Yu et al., 2005).

As expected, age and sex had an important impact on the expression of CYP3A4 protein in Tg-*CYP2D6/CYP3A4* mouse liver microsomes. Similar to Tg-*CYP3A4* mice (Yu et al., 2005), CYP3A4 protein became undetectable in the livers of male Tg-*CYP2D6/CYP3A4* mice greater than five weeks of age (Fig. 2), whereas it was constitutively expressed in small intestines of both male and female double-transgenic mice at all ages examined (see Supplemental Data). On the other hand, no marked impact of age and sex was shown for hepatic CYP2D6, which was actually expressed at similar levels in both double and single transgenic mice (Fig. 2). Furthermore, CYP2D6 expression was readily observed in Tg-*CYP2D6/CYP3A4* small intestines and kidneys (data not shown), two major extrahepatic sites of CYP2D6 protein expression reported in humans.

Additionally, the introduction of human *CYP2D6* transgenes did not alter the expression of corresponding murine Cyp2d proteins in the livers of both single and double transgenic mice (Fig. 3). However, the expression of some intrinsic Cyp3a proteins appeared to be altered in double transgenic mouse livers, which is presumably due to CYP3A4 expression (Yu et al., 2005). In particular, Tg-*CYP2D6/CYP3A4* female mice aged three weeks appeared to have lower Cyp3a levels and eight-week-old Tg-*CYP2D6/CYP3A4* male mice exhibited higher Cyp3a contents when compared to age- and gender-matched wild-type mice (Fig. 3). However, further quantitative analysis is required to determine the magnitude of these differences. Expression of other mouse Cyp isoforms evaluated in Tg-*CYP2D6,* Tg-*CYP3A4* and Tg-*CYP2D6/CYP3A4* mice was consistent with expression in wild-type mice (Fig. 3).

Impact of CYP2D6 and CYP3A4 on Dextromethorphan Metabolism in Transgenic Mouse Liver Microsomes. Dextromethorphan may be utilized as a dual probe for the assessment of both CYP2D6 and CYP3A4 activity (Yu and Haining, 2001). To determine whether CYP2D6 and CYP3A4 present in Tg-*CYP2D6/CYP3A4* mice are active and how they affect drug metabolism, the formation of DXO (*O*-demethylase activity) and MEM (*N*-demethylase) from DXM was compared between wild-type and Tg-*CYP2D6/CYP3A4* mouse liver microsomes. The estimated intrinsic clearance (V_{max}/K_m or Cl_{int}) for DXM *O*-demethylase activity in Tg-*CYP2D6/CYP3A4* mouse hepatic microsomes was 2-3 fold higher in comparison to wild-type mice for all age groups evaluated (Table 1). However, DXM *N*-demethylase activity was not increased (Table 1) in spite of the presence of CYP3A4 in three- and five-week-old Tg-*CYP2D6/CYP3A4* mice (Fig. 2). The potential reasons for this discrepancy may include a

relatively low overall expression of CYP3A4 and the decrease in the levels of murine Cyp3a in Tg-*CYP2D6/CYP3A4* mice (Fig. 3) that largely contributes to DXM *N*-demethylation. In addition, the calculated intrinsic clearance was greater in eight-week-old male transgenic mice than wild-type mice, which may be attributable to the increased Cyp3a expression (Fig. 3). The absence of enhanced drug metabolism in liver microsomes from transgenic mice expressing CYP3A4 was further confirmed using MDZ and TEST hydroxylations as index reactions (data not shown), which may be similarly confounded by the significant contribution to their metabolism from murine Cyp enzymes.

Effects of PCN and TCPOBOP on Human CYP3A4 and Murine Cyp3a Expression in Transgenic Mice. While the expression of human CYP2D6 and murine Cyp2d was not altered, both PCN and TCPOBOP treatments resulted in marked increases in the expression of human CYP3A4 and murine Cyp3a drug-metabolizing enzymes in transgenic mouse livers (Fig. 4) and small intestine (data not shown). Under the doses tested, the extents of hepatic CYP3A4 induction by PCN were relatively higher than those by TCPOBOP (e.g. 7-fold vs. 3-fold in three-week mice, Fig. 4B). Similarly, murine Cyp3a levels were relatively lower in mice treated with TCPOBOP when compared to PCN. Interestingly, liver CYP3A4 contents in eight-week-old female mice treated with PCN and TCPOBOP were markedly higher than corresponding male mice (Fig. 4), which is presumably due to the difference in basal levels of CYP3A4 expression. This finding may suggest a possible difference in the extent of CYP3A4 induction between women and men.

Impact of Hepatic CYP3A Induction on Midazolam and Testosterone Metabolism. To assess the effects of elevated human CYP3A4 and mouse Cyp3a expression on drug metabolism, MDZ 1'-hydroxylation and TEST 6β-hydroxylation in mouse liver microsomes were examined. Consistent with sharply-increased CYP3A levels (Fig. 4), PCN treatment resulted in a marked increase of MDZ 1'-hydroxlase activity in all Tg-*CYP2D6/CYP3A4* mice (Fig. 5A). Fold changes were also distinctly higher in mice treated with PCN when compared to age- and sex-matched mice treated with TCPOBOP. Furthermore, eight-week-old female Tg-*CYP2D6/CYP3A4* mice demonstrated markedly higher MDZ 1'-hydroxylase activity than age-matched male mice (40 vs. 30 nmol/mg protein/min), suggesting an increased potential for DDIs in females. However, the magnitude of interindividual variability in CYP3A4 activity needs to be assessed to further elucidate the impact of sex differences in CYP3A4 activity on metabolic DDIs because a large degree of interindividual would abrogate the sex dependent changes in induction of CYP3A4 activity.

A sharp impact on TEST 6β-hydroxylase activity was also observed for PCN treatment (Fig. 5B). In contrast, production of 6β-hydroxytestosterone did not exhibit marked differences between mice treated with TCPOBOP and corn oil controls. These results are in agreement with lower CYP3A4 induction (Fig. 4) and slower MDZ 1'-hydroxylation in mice treated with TCPOBOP than PCN (Fig. 5A), indicating a stronger effect by PCN at the doses tested. Nonetheless, sex difference was also shown in TEST 6β-hydroxylase activity after CYP3A induction in eight-week-old mice (Fig. 5B). Furthermore, the fold changes in MDZ 1'-hydroxylation (Fig. 5A) were substantially higher than those shown in TEST 6β-hydroxylation (Fig. 5B) in age-, gender-, and treatment-matched mice (e.g. 6-fold vs. 2.5-fold in 8-week-old male mice treated with PCN).

Discussion

Assessment of constitutive expression of transgenes in the Tg-*CYP2D6/CYP3A4* mice maintained in defined environment revealed that both age and sex had considerable impact on hepatic CYP3A4 expression. In contrast, CYP2D6 was expressed at similar levels in transgenic mice regardless of age or sex. Moreover, the extent of CYP3A4 induction was markedly higher in females than age- and treatment-matched males, and the degree of impact on metabolic DDIs may be dependent on the substrate drug.

Different degrees of CYP3A4 expression, governed transcriptionally by nuclear receptors, are known to cause considerable inter-individual variability in the metabolism, pharmacokinetics and drug response of CYP3A4 substrate drugs. CYP3A4 content increases during development from child to adulthood (Stevens et al., 2003; Stevens, 2006). In contrast, there is considerable debate about whether women have higher CYP3A4 levels than men, as examined *in vitro* using human liver preparations and assessed *in vivo* with CYP3A4 probe drugs and summarized in recent reviews (Hines and McCarver, 2002; Gandhi et al., 2004; Parkinson et al., 2004). Given the similarity in physiology and biochemistry between animals and humans, investigation with transgenic mice that are housed in a controlled environment would provide important insights into CYP3A4 developmental and sexual expression. Indeed, results observed in Tg-*CYP2D6/CYP3A4* mice and recent studies using other transgenic mouse lines (Corchero et al., 2001; Granvil et al., 2003; Yu et al., 2005; Cheung et al., 2006) have revealed that both age and sex have an important impact on CYP3A4 expression. Although another *CYP3A4*-transgenic mouse line, generated with a liver promoter driven expression cassette containing human CYP3A4 cDNA, exhibits stable CYP3A4 expression in adult transgenic male mouse livers (van

Herwaarden et al., 2005), CYP3A4 protein is consistently undetectable in our transgenic male adult mice. Nonetheless, the observed impact of sex and age on hepatic CYP3A4 protein expression in Tg-*CYP2D6/CYP3A4* mice does not translate into any remarkable change in hepatic CYP3A4 enzymatic activity as measured by DXM, MDZ and TEST oxidations. This may be due to significant contribution from mouse Cyp3a and even Cyp2c enzymes. In contrast, *CYP2D6*-humanization results in 2-3 fold increase in DXM *O*-demethylation, suggesting that transgenic mice expressing CYP2D6 are useful in assessing the significance of CYP2D6-catalyzed drug metabolism at the systemic level.

The higher CYP3A4 protein levels in female adult mice treated with nuclear receptor activators, in comparison to corresponding males, indicate that a sexual difference could also apply to CYP3A4 induction. This leads to distinctly faster drug metabolism in female mice as demonstrated by both MDZ and TEST hydroxylation. These findings suggest that gender may be an important factor leading to interindividual variability in CYP3A4 induction (Tang et al., 2005), and women may be subject to stronger CYP3A4 induction-mediated DDIs, which is amenable to clinical investigation. An additional consideration when assessing CYP3A4-based DDIs is the selection of an appropriate probe substrate. Previous studies have demonstrated the importance of CYP3A4 probe selection when assessing inhibition potential (Wang et al., 2000; Galetin et al., 2005). Discrepancy in the prediction of potential DDIs may be due to simultaneous binding of two or more substrate and inhibitor molecules to CYP3A4 enzyme (Williams et al., 2004; Pearson et al., 2006). In this study, the data reveal that the impact of CYP3A4 induction on drug metabolism is also dependent upon substrate drug selection. Elevation of CYP3A protein levels may show limited effects on the metabolism of one drug (e.g. TEST 6β-hydroxylation)

while having markedly greater effects on another drug (e.g. MDZ 1'-hydroxylation). This suggests that inappropriate probe substrate selection (e.g. TEST only) may lead to incorrect prediction of the extent of DDIs caused not only by CYP3A4 inhibition but also by induction, and multiple substrates are desired to yield precise assessment of potential DDIs. Additionally, large interindividual variability in CYP3A4 activity may negate the observed differences between hydroxylation index reactions, as well as between males and females, which awaits further critical assessment.

CYP-humanized mouse models are anticipated to overcome species differences caused by the *CYP* genes and provide a mechanistic understanding for the role of individual CYP enzyme in drug metabolism in the whole body system. These animal models have also shown value in understanding *CYP* gene regulation and in predicting the significance of individual CYP drug-metabolizing enzyme in pharmacokinetics and drug response (Gonzalez and Yu, 2006; Gonzalez, 2007). In agreement with recent studies (Yu et al., 2005), some murine genes are actually altered by human *CYP3A4* transgene in mice. Therefore, changes in the expression of relative mouse drug-metabolizing enzymes may become confounding factors when assessing the potential effects of *CYP3A4* transgene expression on drug metabolism (Table 1). Indeed, the present studies have also shown that, in comparison to the wild-type mice, disruption of *Cyp1a2* in mice not only leads to a sharp reduction of melatonin 6-hydroxylation (mainly catalyzed by CYP1A2) but also results in a significant increase in melatonin *O*-demethylation (by CYP2C) in liver microsomes (data not shown). A thorough characterization of the expression of related genes in genetically-modified animals would provide better understanding of the benefits and limitations of these animal models, which should not be overlooked in transgenic animal studies.

Studies with growth hormone (Cheung et al., 2006) have delineated its important role in CYP3A4 sexual dimorphism in mouse liver. On the other hand, the underlying mechanisms for reduced or absent hepatic CYP3A4 expression with aging are unknown. Because intestinal CYP3A4 expression is constitutively expressed in these mice, suppression of hepatic CYP3A4 expression is likely related to cell specific regulatory factors at certain developmental stages. Furthermore, given the opposite developmental expression patterns for mouse Cyp3a in transgenic and wild-type males, and the opposite development of CYP3A4 and mouse Cyp3a in transgenic mice (Yu et al., 2005), nuclear receptors unlikely provide complete explanation for CYP3A developmental suppression. Other regulatory pathways may be triggered to selectively inhibit *CYP3A4* transgene expression in males and to suppress both *CYP3A4* and mouse *Cyp3a* gene expression in females. Delineation of these regulatory factors for CYP3A orthologs, which could have been ignored, will not only provide mechanistic understanding of CYP3A4 developmental expression in transgenic mice but also provide novel insights into understanding *CYP3A* gene regulation in humans. In particular, microRNA molecules (miRNAs) have been identified as an extensive class of endogenous small interfering RNA (siRNA) molecules that control the expression of targeted genes through the inhibition of protein synthesis or the cleavage of mRNA in specific cells at certain developmental stages among different species (Ambros, 2004; He and Hannon, 2004). It has been estimated that at least 30% of all human genes are regulated by those conserved vertebrate miRNAs. Therefore, miRNA-mediated mechanisms cannot be ruled out for the observed CYP3A4 suppression in transgenic mice carrying the whole *CYP3A4* gene sequence, which warrants further investigation (Yu, 2007).

In conclusion, CYP3A4, but not CYP2D6, exhibits developmental and sexual expression in the Tg-*CYP2D6/CYP3A4* mouse livers. Marked differences in hepatic microsomal CYP3A expression and drug-metabolizing activity are also shown between male and female mice after treatment with xenobiotic receptor activators. Furthermore, the extent of induction in CYP3A4 activity is dependent on CYP3A4 probe substrate selection. These findings demonstrate potential differences in CYP3A4 expression, induction and the resultant drug-metabolizing capacity between females and males, and emphasize the critical need for appropriate substrate drugs towards accurate evaluation of potential DDIs.

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Footnotes

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Figure Legends

Fig. 1. PCR genotyping of wild-type, Tg-*CYP2D6*, Tg-*CYP3A4*, and Tg-*CYP2D6/CYP3A4* mice. Each lane represents a sample obtained from an individual mouse. Tail DNA was amplified with mEH (internal control), CYP2D6 and CYP3A4 gene-specific primers. PCR products (406 bp for CYP3A4, 341 bp for mEH and 234 bp for CYP2D6) were separated on a 2% agarose gel.

Fig. 2. Effects of sex and age on hepatic CYP2D6 and CYP3A4 protein expression in Tg-*CYP2D6/CYP3A4* mice. Pooled (N = 4) mouse liver microsomes were prepared from male and female mice at 3, 5, and 8 weeks (W) of age. 20 μg of microsomal proteins from each sample were separated by SDS-PAGE, and immunoblot analyses were carried out with selective antibody against CYP2D6, CYP3A4 and Gapdh, respectively. HLM, human liver microsomes.

Fig. 3. Expression of relevant mouse Cyp proteins in wild-type, Tg-*CYP2D6*, Tg-*CYP3A4* and Tg-*CYP2D6/CYP3A4* mice, aged 3, 5 and 8 weeks (W). Western blots were conducted using 20 μg of pooled (N = 4) mouse liver microsomal proteins and selective antibodies against individual proteins. Note that a number of murine Cyp2d isoforms were detected. HLM, human liver microsomes.

Fig. 4. Induction of CYP3A4 transgene expression in Tg-*CYP2D6/CYP3A4* mice. (A) Immunoblot analyses of CYP3A4 protein in mouse liver microsomes. Mice were treated i.p. with corn oil (CO), PCN (100 mg/kg) or TCPOBOP (3 mg/kg) for 2 days. Pooled microsomes (N = 4 in each group) were prepared, and 20 μg of proteins were subjected to immunoblot analyses.

HLM, human liver microsomes. (B). Semi-quantitation of hepatic CYP3A4 expression based on densiometric analysis of bands.

Fig. 5. Formation of 1'-hydroxymidazolam (A) and 6β-hydroxytestosterone (B) in pooled hepatic microsomes from Tg-*CYP2D6/CYP3A4* mice (N = 4 in each group) treated i.p. with PCN (100 mg/kg) and TCPOBOP (3 mg/kg) for two days. Control mice were treated with the vehicle corn oil (CO). Values are mean ± S.E.M. from triplicate experiment. Note that both PCN and TCPOBOP treatments led to remarkable increase in MDZ 1'-hydroxylase activity in comparison to corresponding controls, whereas only PCN treatment resulted in marked increase in TEST 6β-hydroxylation. Furthermore, MDZ 1'-hydroxylase activities were obviously higher in 8-week-old females in comparison to age- and treatment-matched males.

Table 1. *Apparent enzyme kinetic parameters of dextromethorphan metabolism in pooled hepatic microsomes from wild-type and Tg-CYP2D6/CYP3A4 mice aged 3, 5 and 8 weeks (W) (N = 4 in each group).*

Values are mean ± S.E.M. from triplicate experiments. Incubations were performed in 100 mM phosphate buffer, pH 7.4 at 37^{o}C for 5 min. Dextromethorphan concentrations ranged from 0 to 2000 μM. The rates of dextrorphan (DXO) and 3-methoxymorphinan (MEM) formation were measured by HPLC with fluorescence detection. Michaelis-Menten parameters, K_m and V_{max}, were estimated by nonlinear regression.

Mouse liver microsomes			DXO formation			MEM formation		
			K_m	V_{max}	CL_{int} (V_{max}/K_m)	K_m	V_{max}	CL_{int} (V_{max}/K_m)
			μM	*nmol/min/mg protein*	*μL/min/mg protein*	*μM*	*nmol/min/mg protein*	*μL/min/mg protein*
Male	3W	Wild-type	3.80 ± 0.48	1.19 ± 0.03	313	237 ± 21.2	2.92 ± 0.12	12.3
		Transgenic	3.60 ± 0.34	3.51 ± 0.06	975	523 ± 37.8	3.85 ± 0.11	7.36
	5W	Wild-type	3.50 ± 0.46	1.82 ± 0.04	520	503 ± 34.3	7.68 ± 0.21	15.3
		Transgenic	3.20 ± 0.37	3.54 ± 0.08	1110	534 ± 40.5	7.06 ± 0.21	13.2
	8W	Wild-type	5.10 ± 0.72	1.17 ± 0.03	229	412 ± 51.9	2.79 ± 0.16	6.79
		Transgenic	3.10 ± 0.23	2.73 ± 0.03	880	374 ± 36.3	4.23 ± 0.15	11.3
Female	3W	Wild-type	4.40 ± 0.75	1.35 ± 0.04	307	237 ± 21.2	2.92 ± 0.12	8.75
		Transgenic	2.50 ± 0.27	2.73 ± 0.05	1090	523 ± 37.8	3.85 ± 0.11	5.45
	5W	Wild-type	3.70 ± 0.47	2.33 ± 0.05	630	503 ± 34.3	7.68 ± 0.21	17.1
		Transgenic	3.20 ± 0.43	4.71 ± 0.13	1470	534 ± 40.5	7.06 ± 0.21	18.6
	8W	Wild-type	4.30 ± 0.46	1.79 ± 0.03	416	412 ± 51.9	2.79 ± 0.16	12.8
		Transgenic	3.00 ± 0.21	2.79 ± 0.03	930	374 ± 36.3	4.23 ± 0.15	10.3

Figure 1

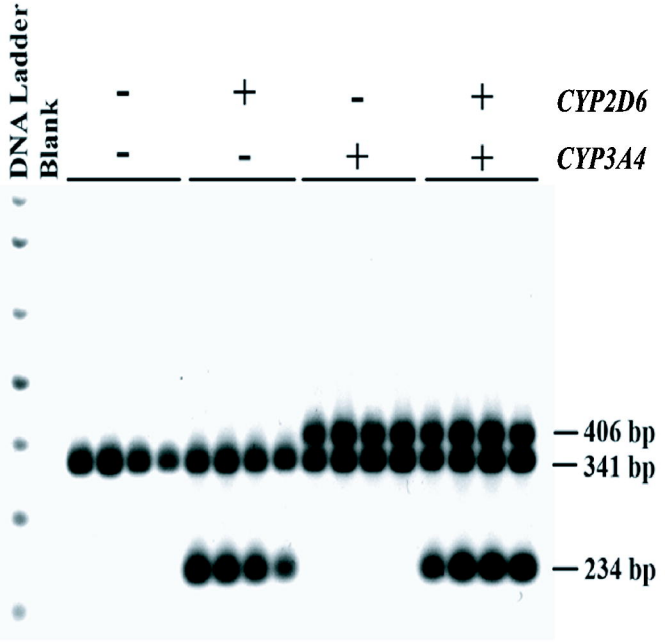


Figure 2

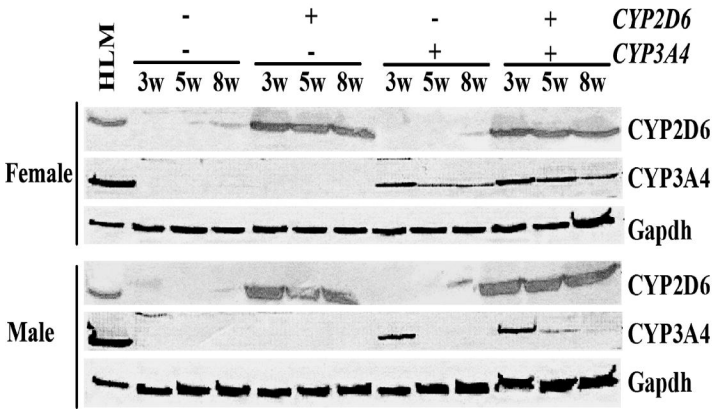

HLM
-
-
+
-
-
+
+
CYP2D6
CYP3A4
3w 5w 8w 3w 5w 8w 3w 5w 8w 3w 5w 8w
Female
Male
CYP2D6
CYP3A4
Gapdh
CYP2D6
CYP3A4
Gapdh

Figure 3

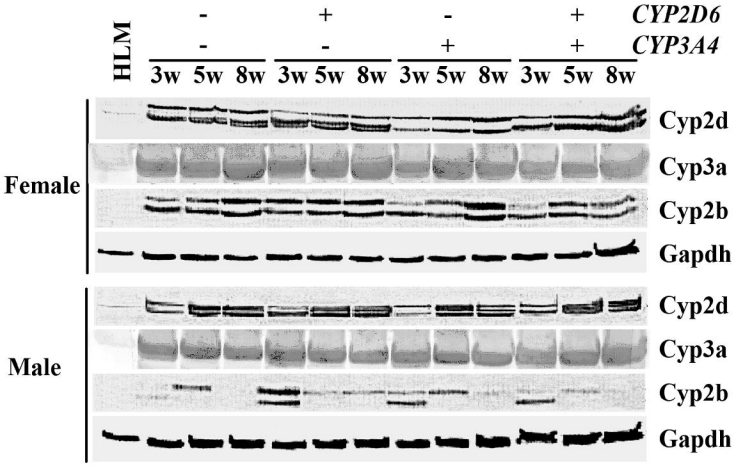
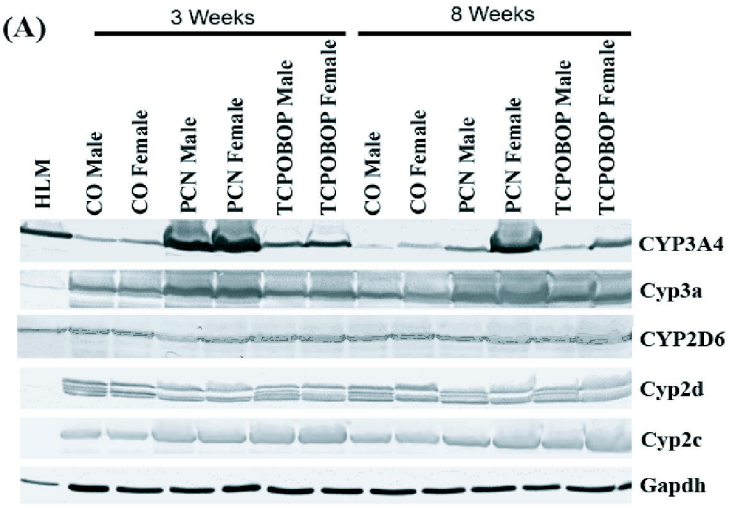

HLM
- + - + CYP2D6
- - + + CYP3A4
3w 5w 8w 3w 5w 8w 3w 5w 8w 3w 5w 8w
Female
Cyp2d
Cyp3a
Cyp2b
Gapdh
Male
Cyp2d
Cyp3a
Cyp2b
Gapdh

Figure 4


(A)
3 Weeks
8 Weeks
HLM
CO Male
CO Female
PCN Male
PCN Female
TCPOBOP Male
TCPOBOP Female
CO Male
CO Female
PCN Male
PCN Female
TCPOBOP Male
TCPOBOP Female
CYP3A4
Cyp3a
CYP2D6
Cyp2d
Cyp2c
Gapdh

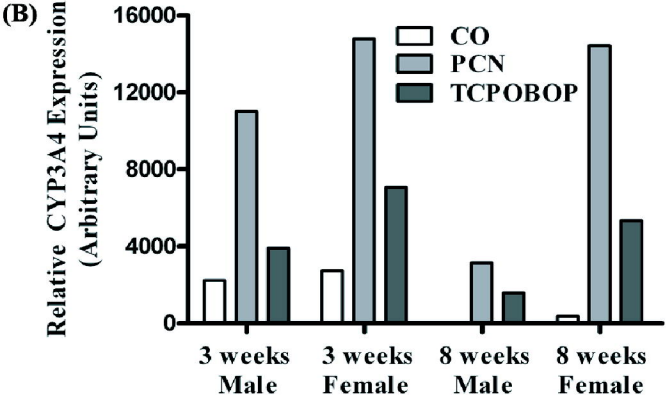
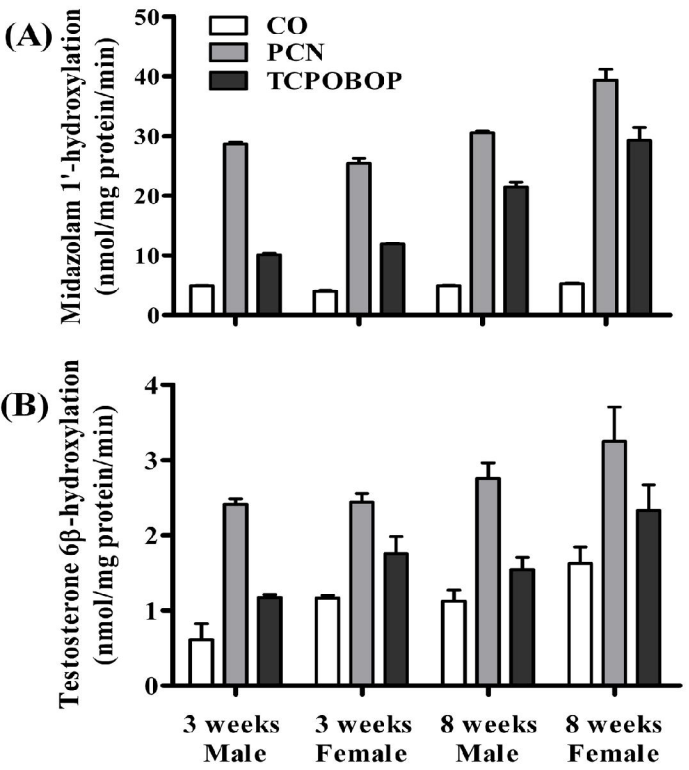

(B)
Relative CYP3A4 Expression (Arbitrary Units)
16000
12000
8000
4000
0
CO
PCN
TCPOBOP
3 weeks Male
3 weeks Female
8 weeks Male
8 weeks Female

Figure 5


(A)
Midazolam 1'-hydroxylation (nmol/mg protein/min)
50
40
30
20
10
0
CO
PCN
TCPOBOP
(B)
Testosterone 6β-hydroxylation (nmol/mg protein/min)
4
3
2
1
0
3 weeks Male
3 weeks Female
8 weeks Male
8 weeks Female