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The *CYP2D6* Humanized Mouse: Effect of the Human *CYP2D6* Transgene and *HNF4α* on the Disposition of Debrisoquine in the Mouse

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ABSTRACT

CYP2D6 is a highly polymorphic human gene responsible for a large variability in the disposition of more than 100 drugs to which humans may be exposed. Animal models are inadequate for preclinical pharmacological evaluation of CYP2D6 substrates because of marked species differences in CYP2D isoforms. To overcome this issue, a transgenic mouse line expressing the human *CYP2D6* gene was generated. The complete wild-type *CYP2D6* gene, including its regulatory sequence, was microinjected into a fertilized FVB/N mouse egg, and the resultant offspring were genotyped by both polymerase chain reaction and Southern blotting. CYP2D6-specific protein expression was detected in the liver, intestine, and kidney from only the *CYP2D6* humanized mice. Pharmacokinetic analysis revealed that debrisoquine (DEB) clearance was markedly higher (94.1 ± 22.3 l/h/kg), and its half-life significantly reduced (6.9 ± 1.6 h), in *CYP2D6* humanized mice compared with wild-type animals (15.2 ± 0.9 l/h/kg and 16.5 ± 4.5 h, respectively). Mutations in hepatic nuclear factor 4α (*HNF4α*), a hepatic transcription factor known to regulate in vitro expression of the *CYP2D6* gene, could affect the disposition of CYP2D6 drug substrates. To determine whether the *HNF4α* gene modulates in vivo pharmacokinetics of CYP2D6 substrates, a mouse line carrying both the *CYP2D6* gene and the *HNF4α* conditional mutation was generated and phenotyped using DEB. After deletion of *HNF4α*, DEB 4-hydroxylase activity in *CYP2D6* humanized mice decreased more than 50%. The data presented in this study show that only *CYP2D6* humanized mice but not wild-type mice display significant DEB 4-hydroxylase activity and that HNF4α regulates CYP2D6 activity in vivo. The *CYP2D6* humanized mice represent an attractive model for future preclinical studies on the pharmacology, toxicology, and physiology of CYP2D6-mediated metabolism.

CYP2D6 is responsible for the DEB 4-hydroxylase polymorphism, which has important clinical consequences, including pronounced interindividual variation in the disposition of many important drugs such as β-adrenergic blocking agents, antiarrhythmics, antidepressants, and analgesics. Since it was first discovered, CYP2D6 has been the most studied human genetic polymorphism in drug metabolism with more than 75 identified alleles within most human populations and racial groups (http://www.imm.ki.se/CYPalleles/cyp2d6.htm). Approximately 7 to 10% of the Caucasian population inherit mutant *CYP2D6* alleles as an autosomal recessive trait (Mahgoub et al., 1977). This polymorphism stratifies the population depending on the copy number of wild-type alleles: poor (PM, zero), intermediate (one), extensive (EM, two), and ultra-rapid metabolizers (multiple copies) (Gonzalez, 1996; Wolf and Smith, 1999). The *CYP2D* gene locus has been isolated, sequenced, and mapped to human chromosome 22q13.1, and contains the only active *CYP2D6* gene downstream of two inactive pseudogenes *CYP2D7P* and *CYP2D8P* (Gonzalez et al., 1988; Kimura et al., 1989).

Clinical studies are fundamental to the identification of human polymorphisms, to the establishment of pharmacokinetic profiles, and to drug-drug interaction effects. However,

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ABBREVIATIONS: PM, poor metabolizer; EM, extensive metabolizer; DEB, debrisoquine; HNF4α, hepatic nuclear factor 4α; bp, base pair(s); P450, cytochrome P450; fl, flanked by lox P; AlbCre, to albumin-Cre heterozygous; PCR, polymerase chain reaction; mEH, microsomal epoxide hydrolase; SSC, standard saline citrate; MOPS, 4-morpholinepropanesulfonic acid; LC/MS/MS, liquid chromatography/tandem mass spectrometry; 4-OH-DEB, 4-hydroxydebrisoquine; AUC, area under the curve.

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to determine how a drug is metabolized, what toxic effects it may produce, or how pathophysiological conditions affect drug metabolism, animal models or in vitro systems must be developed.

Because of marked differences between humans and experimental animals, the results from animal studies can be misleading and need to be interpreted very cautiously. For example, the *CYP2D* family in humans has a single active member, *CYP2D6*, whereas rats and mice have at least five genes (Gonzalez and Nebert, 1990; Nelson et al., 1996). DEB is hydroxylated to 4-hydroxydebrisoquine (4-OH-DEB) by humans and by Sprague-Dawley rats. However, Dark Agouti rats have been found to posses a low capacity to metabolize DEB (Al-Dabbagh et al., 1981). Similarly, no significant formation of 4-hydroxy DEB was detected by liver microsomes from three strains of mice and by purified cyp2d9–11 (Masubuchi et al., 1997).

The transcriptional factor hepatic nuclear factor 4α (HNF4α) is known to play a major role in liver organogenesis, maintenance, and gene transcription (Sladek, 1994). Several lines of evidence suggest that HNF4α controls constitutive levels of expression of the *CYP2D6* gene. Indeed, it was shown that cotransfection of the minimal *CYP2D6* promoter-CAT construct (−392 bp) with a mammalian *HNF4* expression vector produced a 30-fold induction of CAT activity in COS-7 cells (Cairns et al., 1996). Moreover, using adenovirus-mediated antisense targeting that selectively diminishes HNF4α content in human hepatocytes, *CYP2D6* gene expression, among other P450s, is down-regulated by the depletion of HNF4α (Jover et al., 2001).

In addition, the R154X mutation in the human *HNF4α* gene provokes maturity onset diabetes of the young, a genetically and clinically heterogeneous subtype of non-insulin-dependent diabetes mellitus. Persons carrying this mutation in *HNF4α* are predicted to have reduced levels of this transcription factor in the tissues where it is expressed (Yamagata et al., 1996; Lindner et al., 1997). Because this pathology occurs concomitantly with other diseases like obesity, hypertension, coronary insufficiency, and heart failure among others, multiple drug regimes are common within this population (Scheen and Lefebvre, 1995). Because CYP2D6 metabolizes a large number of clinically prescribed drugs, it would be relevant to determine whether the lack of HNF4α could modulate CYP2D6 metabolic activity in vivo. However, because of the embryonic lethality of a standard gene HNF4α knock-out approach, preclinical evaluation of this hypothesis using a intact animal model is not possible. Fortunately, the generation of a conditional liver-specific knock-out of the HNF4α gene mouse line has overcome this issue (Hayhurst et al., 2001).

To circumvent all of these methodological problems, a humanized mouse line expressing CYP2D6 would offer a unique approach to answering fundamental questions about the specific role of CYP2D6 in drug metabolism and drug interactions. Such experiments would be performed in the context of the entire animal and would overcome many limitations inherent in in vitro experiments. To this end, the complete wild-type allele of the human *CYP2D6* gene, including its regulatory sequence, was microinjected into a fertilized FVB/N mouse egg to produce a humanized transgenic mouse line. In addition, to explore the in vivo regulation of CYP2D6 expression by HNF4α, a mouse line carrying both the *CYP2D6* gene and the conditional *HNF4α* mutation has been generated. Using DEB as a substrate, only the humanized CYP2D6 mice are able extensively to convert DEB to its 4-hydroxy metabolite and to display a pharmacokinetic profile similar to humans.

Materials and Methods

Animals. Adult males from all the genotypes described in this work (25–30 g, 2–4 months) were maintained under conditions of controlled temperature (23 ± 1°C) and lighting (lights on 6:00 AM–6:00 PM), with food and water provided ad libitum. All animal experiments were conducted under National Institutes of Health guidelines for the use and care of laboratory animals, and approved by National Institutes of Health Animal Care and Use Committee.

Generation of the Humanized Mouse. The *CYP2D6* gene (GenBank accession number M33388) previously isolated and sequenced (Kimura et al., 1989) was microinjected into a fertilized FVB/N mouse egg to produce a transgenic mouse line. Incorporation of the *CYP2D6* DNA within the mouse genome was determined by both PCR and Southern blot analysis. The transgenic founder was mated to a nontransgenic FVB/N (wild-type), and animals from this cross were subsequently crossed to each other to produce homozygous mice. Mice homozygous for the transgene were confirmed by crossing them with wild-type mice and testing the progeny for transgene transmission. Heterozygous animals were generated by crossing homozygous and wild-type mice. Wild-type and homozygous littermates were bred and maintained by brother-sister mating.

Generation of Liver-Specific Deletion of *HNF4α* in the *CYP2D6* Transgenic Line. *HNF4α* conditional knock-out mice had been generated previously (Hayhurst et al., 2001). The breeding scheme to produce liver-specific deletion of *HNF4α* in the *CYP2D6* transgenic line was designed using two different mice genotypes: a) *HNF4α* fl/wt, AlbCre +/− mice generated by crossing *HNF4α* fl/fl homozygous to albumin-Cre heterozygous (Alb-Cre) kindly provided by Dr. Derek LeRoith (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) (Yakar et al., 1999); and b) *HNF4α* fl/fl, *CYP2D6* +/− mice: generated by crossing *HNF4α* fl/fl homozygous mice to *CYP2D6* homozygous animals and back-crossing the resultant offspring to homozygous *HNF4α* fl/fl mice.

The breeding of mice carrying genotypes a and b generates *HNF4α* knock-out mice carrying the *CYP2D6* transgene (*HNF4α* fl/fl, AlbCre+/−, *CYP2D6* +/−), and littermate control mice for AlbCre, and CYP2D6, as described under *Results*.

PCR Genotyping Procedures. Genomic DNA was isolated from tails as described previously (Laird et al., 1991). For *CYP2D6* PCR analysis, approximately 50 ng of tail DNA was amplified in 25 μl of reaction mixture containing 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1.25 U of AmpliTaq (PerkinElmer, Foster City, CA), and 20 pmol of *CYP2D6* gene-specific primers CYP2D6F 5′-AGAAGGGGAAGCAGGTTTG-3′ and CYP2D6R 5′-CGGCACTCAGGACTA ACTCATC-3′, and microsomal epoxide hydrolase (mEH) gene-specific primers MEHF 5′-AAGTGAGTTTGCATGGCGCAGC-3′ and MEHR 5′-CCCTTTAGCCCCTTCCCTCTG-3′. Cycling conditions were 94°C for 5 min, and then 33 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a 5-min extension at 72°C. mEH primers served as a positive control for amplification, yielding a fragment of 341 bp in all samples (Miyata et al., 1999). An additional band of 241 bp was amplified exclusively in *CYP2D6* humanized animals. For *HNF4α* fl/fl mice and AlbCre transgenic PCR procedures were described previously (Hayhurst et al., 2001).

Southern Blot Analysis and Determination of Transgene Copy Number. Tail genomic DNA (15 μg/lane) was digested with *Bam*HI and subjected to electrophoresis on a 0.5% agarose gel containing 0.5× Tris/borate/EDTA. The DNA was hydrolyzed in 0.2 M HCl and transferred on to a Gene Screen Plus nylon membrane (DuPont, Wilmington, DE) by capillary blotting in 0.4 M NaOH.

Blots hybridized with random-primer ^{32}P-labeled *CYP2D6* cDNA probe (Gonzalez et al., 1988) at 42°C overnight, washed twice in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) and 0.5% SDS at 65°C for 15 min, twice in 0.1 × SSC and 0.5% SDS for 5 min, and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for 2 to 4 h.

Transgene copy number was determined by Southern blotting using the purified genomic clone DNA as a standard. Cloned DNA was diluted with mouse DNA to yield the equivalent of 1, 2, 5, and 10 copies of the gene per haploid genome (based on 3×10^9 base pairs per haploid genome). The DNA was digested with *Bam*HI and subjected to Southern blot analysis with DNA isolated from heterozygous and homozygous CYP2D6 humanized mice. The signals were quantified by use of a PhosphorImager, and the copy number was determined from a standard curve of the standard.

Northern Blot Analysis. Total RNA was isolated from the livers of all the *HNF4* fl/fl, AlbCre, and *CYP2D6* humanized mice used in this study by the acidic guanidine isocyanate/phenol/chloroform extraction method using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX). Ten micrograms of total RNA per sample was subjected to electrophoresis on a 1% agarose gel containing 220 mM formaldehyde in 20 mM MOPS, 8 mM sodium acetate, and 1 mM EDTA buffer, pH 7.0, and transferred to Gene Screen Plus membranes by capillary blotting in 20× SSC. Blots were hybridized with a random-primer ^{32} P-labeled probe specific for *HNF4α* exon 4 and 5 probe (Hayhurst et al., 2001) at 42°C overnight, washed twice in 2× SSC and 0.5% SDS at 65°C for 15 min, twice in 0.1× SSC and 0.5% SDS for 5 min, and exposed to a PhosphorImager screen for 2 to 4 h.

Western Blot Analysis. Tissues were homogenized in ice-cold buffer (1.15% KCl, 50 mM Tris-HCl, and 1 mM EDTA, pH 7.4) and microsomes were prepared by differential centrifugation as described previously (Sinal et al., 1999). Microsomal protein concentration was determined using a bicinchoninic acid protein kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis and Western blot analysis of microsomal proteins (40 μg) were performed with a 4% stack and 10% separating gel and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) using standard methods. The primary mouse monoclonal anti-CYP2D6 antibody (Gentest Corp., Woburn, MA) was diluted 3,000-fold in 0.5% nonfat dry milk, 1× phosphate-buffered saline. The primary monoclonal antibody against rat CYP2E1 (kindly provided by Dr. Harry V. Gelboin) was diluted 500-fold in 3% milk and 1× phosphate-buffered saline. Bound antibody was detected with a horseradish peroxidase-conjugated secondary antibody anti-mouse IgG, that was diluted 2,000- and 10,000-fold for CYP2D6 and CYP2E1 blotting, respectively. P450 proteins were visualized by use of the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

Drug Administration and Blood and Urine Samples. DEB hemisulfate (ICN, Irvine, CA) was dissolved in sterile water and administered orally by gavage at 2.5 mg/kg. For pharmacokinetic evaluations, blood samples were collected from suborbital veins 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after DEB administration (2.5 mg/kg). Each time point was analyzed with three to four animals. Serum was separated by centrifugation at 1000*g*, 4°C, for 10 min. For the urine excretion analysis, three to four animals per group were dosed with DEB (2.5 mg/kg) and placed in metabolic chambers (Jencons, Leighton Buzzard, UK). Total urinary excretion from individual mice was collected for 24 h. Serum aliquots (0.2–0.5 ml) or urine volumes (0.8–1.5 ml) were stored at −80°C until analyzed. At the end of experiments, mice were euthanized by carbon dioxide.

Quantification of DEB and 4-OH-DEB by Liquid Chromatography/Tandem Mass Spectrometry. Serum and urine concentrations of the DEB and 4-OH-DEB were determined using a previously described LC/MS/MS method with a minor modification using liquid-liquid extraction (Scott et al., 1999) and solid phase extraction (Pereira et al., 2000). Briefly, 100 μl of serum or urine (supplemented with 30 mg of sodium chloride) was spiked with 20 μl of internal standard (phenacetin, 30 μM in methanol), 500 μl of isopropanol, 50 μl of aqueous sodium hydroxide (400 mM), and 3 ml of methyl-*t*-butyl ether were added. The mixture was vortex-mixed for 1 min and the phases were separated by 10 min of centrifugation at 1000*g*, 4°C. The aqueous layer was frozen in dry ice and the organic phase was transferred to a fresh borosilicate tube and evaporated to dryness under a gentle stream of air at 30°C using a heating block (Pierce, Rockford, IL). The residue was reconstituted in 100 μl of acetonitrile-water (20:80, v/v) and transferred to polypropylene autosampler vials and 10 to 25 μl of sample was injected into the LC/MS/MS system.

The high-performance liquid chromatography system consisted on a PerkinElmer Series 200 quaternary pump, a vacuum degasser, and a series 200 autosampler with a 100-μl loop (PerkinElmer) both interfaced to a triple-quadrupole tandem mass spectrometer (API2000; PE SCIEX). DEB, 4-OH-DEB, and the internal standard were separated on a LUNA RX-Polar C18 column (2.0 × 50 mm, 3 μm; Phenomenex, Torrance, CA). The mobile phases consisted of the following: solvent A, 0.1% formic acid in water, and solvent B, 0.1% formic acid in acetonitrile. A linear gradient of solvent B from 5 to 95% over 4 min was applied on the column and was returned to the original condition and equilibrated for 1 min before the next injection. The samples were delivered with a flow rate of 0.20 ml/min and the run time was 5.0 min.

The mass spectrometer was operated in the turbo ion spray mode with positive ion detection. The turbo ion spray temperature was maintained at 300°C and a voltage of 4.8 kV was applied to the sprayer needle. Nitrogen was used as the turbo ion spray and nebulizing gas. The detection and quantification of compounds were performed using MS/MS in the multiple reaction monitoring mode. Multiple reaction monitoring data acquisition, monitoring the transitions m/z 176/134 for DEB, 192/132 for 4-OH-DEB, and 180/110 for the internal standard, was employed. All raw data were processed with PerkinElmer SCIEX Analyst Software, version 1.2.

The method was linear for DEB and 4-OH-DEB concentrations ranging from 2.5 to 5000 nM. Calibration curves were constructed in duplicate and were completed using $1/\chi^2$ weighting. Good linearity was achieved with correlation coefficients greater than 0.995 for both the analytes. The lower limit of quantitation was 2.5 nM for DEB and 4-OH-DEB in both serum and urine, where the coefficient of variation was less than 20%. The recoveries for DEB and 4-OH-DEB ranged between 80 and 102% in serum and urine. Intraday and interday coefficients of variation were less than 10% at a concentration of 30 nM for DEB and 4-OH-DEB in serum and urine.

Pharmacokinetic Analysis. Pharmacokinetics parameters for DEB and its metabolite 4-OH-DEB were estimated from the serum concentration-time data by a noncompartmental approach with the software package WinNonlin Standard version 1.5 (Scientific Consulting Inc., Cary, NC). The peak concentration in serum (C_{max}) and the time to reach serum concentration (T_{max}) were obtained from the original data. The area under the serum concentration versus time curves from 0 to 24 h ($AUC_{0-24\ h}$) was determined with a combination of linear and logarithmic trapezoidal methods. The elimination rate constant (β) was determined by use of log-linear regression of the terminal portion of the concentration versus time curve using at least four data points. The elimination half-life ($t_{1/2}$) was calculated from $0.693/\beta$. The apparent oral clearance was calculated from dose/$AUC_{0-24\ h}$. From urine concentrations, the percentage of the total dose excreted was determined.

Statistics. Statistical analyses were performed with SigmaStat software (Jandel Corporation, San Rafael, CA) using one-way or two-way analysis of variance followed by the Student-Newman-Keul's test when comparing three or four groups, respectively. Differences were considered significant if the probability that they were due to chance was less than 5%.

Results

Generation of a *CYP2D6* Humanized Mouse Line. The *CYP2D6* gene was isolated and sequenced in an earlier study (Kimura et al., 1989). The sequence indicated that it was a wild-type allele. The complete *CYP2D6* gene (Fig. 1A), including its promoter and regulatory elements, was microinjected into a fertilized FVB/N mouse egg to produce a transgenic mouse line. Successful incorporation of the *CYP2D6* DNA into the germ line was assessed by Southern blot analysis. Genomic DNA from wild-type and *CYP2D6* humanized mice was digested with *Bam*HI, and probed with the *CYP2D6* cDNA. Hybridization signal was found only in the lanes with transgenic DNA but not in the lanes with wild-type DNA. The size of the bands corresponds exactly with the predicted sizes calculated from the sequence of the *CYP2D6* gene (Fig. 1B). Furthermore, the results of Southern blot analysis were corroborated by PCR. Genomic DNA from wild-type and *CYP2D6* humanized mice was amplified with two different sets of specific primers: *CYP2D6*, and *mEH* (Miyata et al., 1999). Figure 1C shows the amplification of *mEH* PCR product (341 bp) in both wild-type and *CYP2D6* humanized animals, which served as a positive control for amplification. *CYP2D6* PCR product (241 bp) is only present in *CYP2D6* humanized mice. The transgene was present at 5 ± 1 copies per haploid genome.

Expression of the transgenic protein was assessed by Western blotting. Microsomal protein preparations from wild-type and *CYP2D6* humanized animals were analyzed with a CYP2D6-specific monoclonal antibody, which did not cross react with the CYP2D proteins present in the wild-type mice. CYP2D6 was expressed in liver, intestine, and kidney microsomes only in humanized mice (Fig. 1D).

DEB Pharmacokinetics in CYP2D6 Humanized Mice. Nonlinear pharmacokinetics in the elimination of CYP2D6 substrates has been reported (Brøsen and Gram, 1988; Brøsen, 1990). To determine a dose within the linear range of DEB elimination, a dose-effect study (up to 30 mg/kg) on DEB AUC was performed. We observed nonlinear pharmacokinetics of DEB elimination at doses of 10 mg/kg or higher. Therefore, the characterization of *CYP2D6* humanized mouse pharmacokinetics was performed using a dose of 2.5 mg/kg, which is within the linear range of DEB elimination (data not shown). The average DEB serum concentration versus time curves in wild-type, *CYP2D6* heterozygous and *CYP2D6* homozygous humanized mice were determined (Fig. 2A). After a single oral dose of DEB (2.5 mg/kg), both *CYP2D6* heterozygous and homozygous mice had DEB serum levels significantly lower than in wild type. Consistently, 4-OH-DEB levels are highest in *CYP2D6* homozygous, intermediate in *CYP2D6* heterozygous and extremely low in the wild-type (Fig. 2B).

The pharmacokinetic data was calculated from the three groups (Table 1). The AUC was 3- and 6-fold higher in wild-type mice than in heterozygous and homozygous *CYP2D6* humanized mice, respectively. This is illustrated by differences in the elimination half-life of DEB, which was 2.1 and 1.4 times shorter in the heterozygous and homozygous *CYP2D6* humanized mice than in wild-type mice. Accordingly, CYP2D6 humanized mice showed a clearance 6- and 4-fold higher than wild-type mice. All the phenotypic differences were statistically significant.

Urinary Metabolic Balance in the CYP2D6 Humanized Mice. *CYP2D6* integration in the mouse genome did not affect any of the physiological parameters concerning renal

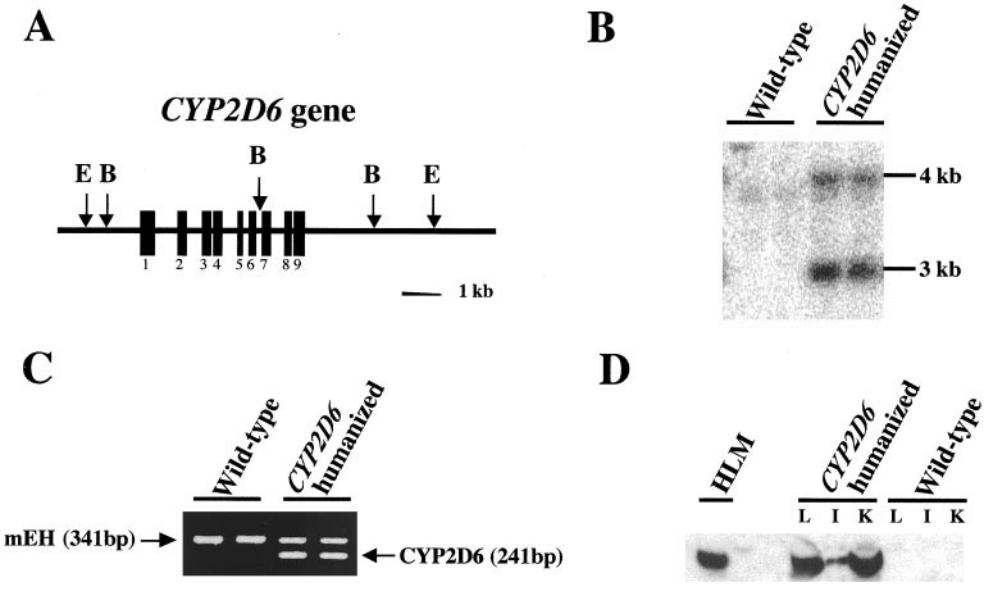

Fig. 1. Generation and analysis of the *CYP2D6* humanized mouse. A, schematic diagram of the wild-type *CYP2D6* gene used for microinjection (GenBank accession number M33388). Restriction sites for *Eco*RI (E) and *Bam*HI (B) are depicted. Black boxes represent *CYP2D6* exons. The bar represents 1 kb. B, Southern blot genotyping of wild-type and *CYP2D6* humanized mice. Tail DNA (15 μg) was digested with *Bam*HI and probed with *CYP2D6* cDNA. Hybridization signals were present only in *CYP2D6* humanized mice, and their sizes were as expected from the *CYP2D6* sequence. C, PCR genotyping of wild-type and *CYP2D6* humanized mice. Tail DNA was amplified with *mEH* (internal PCR control) and *CYP2D6* gene-specific primers. The PCR products (341 bp for *mEH*, 241 bp for *CYP2D6*) were separated on a 1.5% agarose gel. D, Western blot analysis of CYP2D6 protein expression in wild-type and *CYP2D6* humanized mice. Liver (L), intestine (I), and kidney (K) microsomal proteins (40 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. A CYP2D6-specific monoclonal antibody was used to assess CYP2D6 protein expression. The antibody only reacted against CYP2D6 expressed protein but did not recognize any of the mouse CYP2D proteins. Human liver microsomes (HLM) were used as a control.

function (data not shown). Twenty four hours after a single oral dose of DEB, *CYP2D6* humanized mice excreted significantly higher amounts of 4-OH-DEB (28.9 ± 12.5% of dose) and lower amounts of DEB (14.6 ± 6.4%) than the wild-type mice (6.2 ± 3.1% and 61.0 ± 9.0%, respectively). Total recoveries of DEB + 4-OH-DEB were 67.2 ± 10.7% and 43.5 ± 18.9% for the wild-type and *CYP2D6* humanized mice, respectively (Fig. 3). This latter finding perhaps indicates that the human *CYP2D6* gene provokes metabolism of DEB to additional metabolites not detected in this study. The metabolic ratio (Mahgoub et al., 1977) for the wild-type mice fell from 9.8 to 0.5 after insertion of the human transgene.

Regulation of CYP2D6 Activity by Conditional Knock-Out of the *HNF4α* Allele. To determine the mechanism of regulation of the *CYP2D6* gene, the transgenic mouse was bred with a HNF4α conditional null mouse line. The HNF4α conditional null mouse allows the study of the role of this hepatic factor in an intact model, and circumvents the embryonic lethality of a standard HNF4α null mouse (Hayhurst et al., 2001). A mouse line carrying the *CYP2D6* gene and the conditional *HNF4α* mutation was generated (see *Materials and Methods*). To this end, *HNF4α* fl/+; AlbCre +/− mice, previously produced, were crossed with *HNF4α* fl/fl; *CYP2D6* +/− obtained in the present work. The F1 generation of this cross yielded *HNF4α* fl/fl; AlbCre +/−; *CYP2D6* +/− and littermate control mice, *HNF4α* fl/fl; AlbCre −/−; *CYP2D6* +/−, *HNF4α* fl/fl; AlbCre +/−; *CYP2D6* −/− and *HNF4α* fl/fl; AlbCre −/−; *CYP2D6* −/−. Genotypes of all mice were assessed by PCR of tail DNA (data not shown).

The effect of Cre-mediated recombination at the *HNF4α* locus was determined to be optimal at 45 days of age (Hayhurst et al., 2001). Therefore, all of these experiments were conducted using 45-day-old mice. Deletion of *HNF4α* mRNA in AlbCre +/− mice was confirmed by Northern blotting using a cDNA probe for exons 4 and 5 (Hayhurst et al., 2001).

Disruption of *HNF4α* expression resulted in a 50% decrease in CYP2D6 as analyzed by Western blotting. Because the antibody used is CYP2D6 specific, there was no signal detected in any of the wild-type mice. On the other hand, CYP2E1 protein levels were not affected by *HNF4α* deletion. The lack of effect of *HNF4α* on CYP2E1 protein levels is in agreement with a previous reports (Jover et al., 2001) and suggests that the hepatic lesions produced by the conditional mutation do not prevent the synthesis of nontarget proteins (Fig. 4B).

The phenotypic results of *HNF4α* mutation on CYP2D6 metabolic activity in intact animals were assessed by measuring the metabolic ratio after 24 h of an oral dose of DEB. As expected, intact *CYP2D6* humanized animals showed the

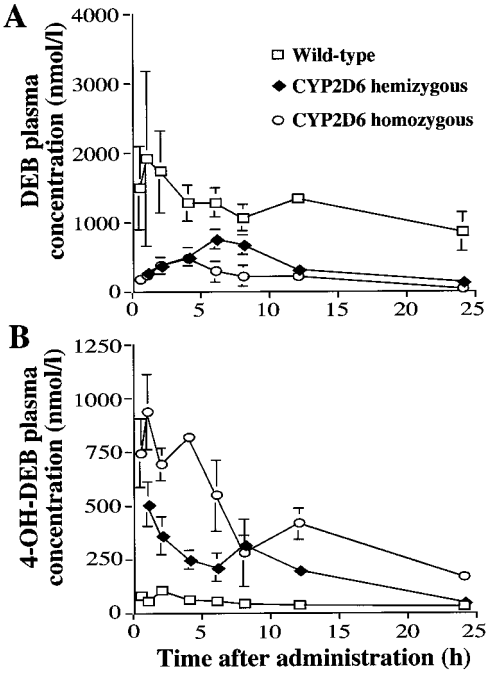

Fig. 2. Time course of serum concentrations of DEB (A) and 4-OH-DEB (B) from wild-type, *CYP2D6* humanized heterozygous, and *CYP2D6* humanized homozygous mice after one single oral administration of DEB (2.5 mg/kg). Venus blood was obtained 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after DEB administration. Values represent the mean and the vertical lines (±S.E.M.) of DEB and 4-OH-DEB from three to four mice.

TABLE 1

Pharmacokinetic parameters for DEB and 4-OH-DEB after a single oral administration of DEB (2.5 mg/kg) in wild-type, *CYP2D6* humanized heterozygous, and *CYP2D6* humanized homozygous mice

Values represent the mean and the S.E.M. from three to four mice.

Parameter	Wild Type	Heterozygous	Homozygous
DEB			
T_{max} (h)	2.5 ± 1.8	6.7 ± 0.7	4.6 ± 1.8
C_{max} (nM)	2,937 ± 795	879 ± 128*	467 ± 61*,#
$AUC_{0-24\ hrs}$ (nM ·)	28,393 ± 1840	8,757 ± 1215*	4,634 ± 1348*,#
CL (l/h/kg)	15.2 ± 0.9	48.9 ± 6.4*	94.1 ± 22.3*,#
$T_{1/2}$ (h)	16.5 ± 4.5	8.9 ± 2.1*	6.9 ± 1.6*
4-OH-DEB			
T_{max} (h)	1.7 ± 0.3	3.3 ± 2.3	0.8 ± 1.2
C_{max} (nM)	110 ± 12	535 ± 79*	1075 ± 97*,#
$AUC_{0-24\ hrs}$ (nM ·)	1,086 ± 28	4,627 ± 377*	9,289 ± 931*,#

*, values from *CYP2D6* humanized mice that are significantly different ($p < 0.05$) from wild-type mice. #, values from *CYP2D6* humanized homozygous mice that are significantly different ($p < 0.05$) from *CYP2D6* humanized heterozygous mice ($p < 0.05$).

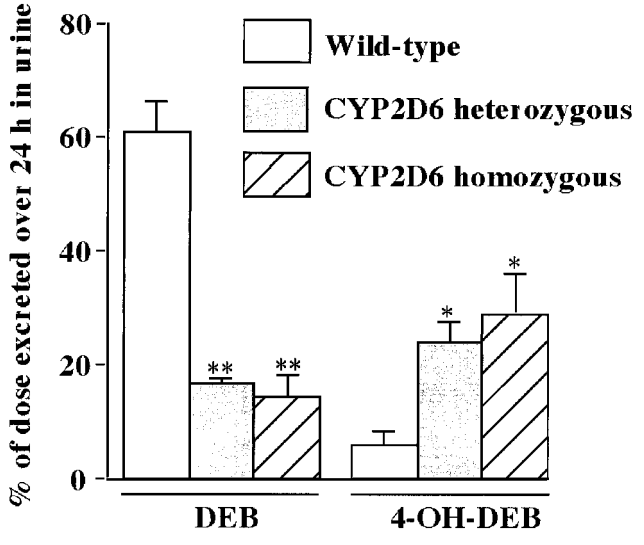

Fig. 3. Percentage of a single oral dose of DEB (2.5 mg/kg) excreted in urine over 24 h from wild-type, *CYP2D6* humanized heterozygous and *CYP2D6* humanized homozygous mice. Columns represent the mean and S.E.M of DEB and 4-OH-DEB from three to four mice. *, values of 4-OH-DEB levels from *CYP2D6* humanized mice that are significantly different ($p < 0.05$) form wild-type mice. **, values of DEB levels from *CYP2D6* humanized mice that are significantly different ($p < 0.05$) form wild-type mice.

greatest metabolism of DEB (18.3 ± 3.7% dose as DEB, 7.2 ± 1.4% dose as 4-OH-DEB, MR 2.6 ± 0.2; see Table 3). However, in the absence of *HNF4α,* the metabolism of these animals decreased more than 50% (10.1 ± 0.3% DEB, 2.9 ± 0.5% 4-OH-DEB, MR 3.6 ± 0.6; $P < 0.005$). In control animals, basal DEB metabolic ratio levels were 5-fold lower than intact CYP2D6 humanized mice (51.6 ± 9.5% DEB, 2.9 ± 0.4% 4-OH-DEB, MR 18.3 ± 6.2); after HNF4α deletion, the metabolic ratio was also decreased (25.8 ± 1.9% DEB, 0.8 ± 0.1% 4-OH-DEB, MR 32.0 ± 3.0; $P < 0.005$) (Table 3).

Discussion

Although DEB has been extensively and successfully used as a clinical probe for the CYP2D6 polymorphism, the knowledge about its disposition in humans is limited. Most phenotyping studies have been carried out using urine excretion analysis; and during the last 20 years, only three studies of DEB pharmacokinetics have been reported (Silas et al., 1978; Sloan et al., 1983; Dalen et al., 1999). Human disposition of DEB depends on the number of functional *CYP2D6* alleles present in each subject. Using individuals that carried 0, 1, 2, 3 to 4, and 13 functional alleles, it was shown that there is a gene dose effect on DEB and 4-OH DEB pharmacokinetic profile. When the number of *CYP2D6* active copies increases, 4-hydroxylase activity also increases, and the plasma levels of DEB decrease, so the drug is eliminated faster (Dalen et al., 1999).

In vitro, DEB 4-hydroxylase activity in wild-type mice is almost absent (Masubuchi et al., 1997). The data presented in this study provides an in vivo confirmation for this previous finding and show that, once the *CYP2D6* gene is integrated, constitutively expressed, and transmitted into the germ line, DEB 4-hydroxylase activity is clearly present. After a single oral dose of DEB, the half-life, clearance, AUC values for wild-type, heterozygous and homozygous *CYP2D6* humanized mice reflect the gene dose effect found in the human population. All these data show that the humanized *CYP2D6* mouse model mimics the pharmacokinetic and metabolic properties of human CYP2D6-mediated metabolism.

Clinical studies on CYP2D6-mediated metabolism are based on phenotype and genotype tests. Although phenotyping analysis reflects the real metabolic status of the patient, and genotyping analysis offers pure genetically based prediction of individual metabolic profile, there are a number of nongenetic factors (age, sex, smoking and drinking habits, and pathological status) that can affect drug metabolism and interfere with an experimentally determined phenotype or genotype (Wolf and Smith, 1999). *CYP2D6* humanized mice have a tremendous potential in phenotype-genotype correlations, because these animals share the same genetic background except for the presence or absence of the human *CYP2D6* gene. Because the environmental conditions through the present work have been identical for all the animals used, the phenotypic differences determined in this study are caused by the presence/absence of the *CYP2D6* gene.

An intriguing and apparently unaccountable observation is the major decline in the recovery of DEB plus 4-OH-DEB in the transgenic mice compared with the wild-type mice (Table 2). The wild-type mice had a total recovery of 67.2 ± 10.7% dose, which fell to 40.9 ± 6.6% and 43.5 ± 18.9% in the heterozygous and homozygous humanized mice, respectively (unpaired t test, $t = 2.90$, df = 7, $P = 0.02$). Apparently, the fall in excretion of DEB due to CYP2D6 metabolism is not compensated by a concomitant rise in excreted 4-OH-DEB; some 25% of the dose is unaccounted for, a similar proportion of the dose as is excreted as 4-OH-DEB. The answer lies in alternative metabolic products produced by CYP2D6. Early studies (Allen et al., 1976; Idle et al., 1979) showed that DEB, in addition to 4-hydroxylation, was also metabolized to 5-, 6-, 7- and 8-hydroxy-DEB (phenolic metabolites) in humans, together with two ring-opened products, which are thought to arise from 1- and 3-hydroxylation of the drug and also are mediated by CYP2D6 (Eiermann et al., 1998). Therefore, DEB is metabolized at positions 1, 3, 4, 5, 6, 7, and 8 (i.e., at every C-H bond in the ring system). Quantitatively, the phenols represented 7.1 ± 5.8% dose eliminated in the 0- to 24-h human urine, with 7-hydroxylation predominating (Idle et al., 1979). In the female Wistar rat (Al-Dabbagh et al., 1981), 6-hydroxy-DEB (14.3 ± 1.5% dose) and an unidentified phenolic metabolite (11.3 ± 2.3% dose, calculated as a phenol; probably a dihydroxy metabolite) accounted for significant

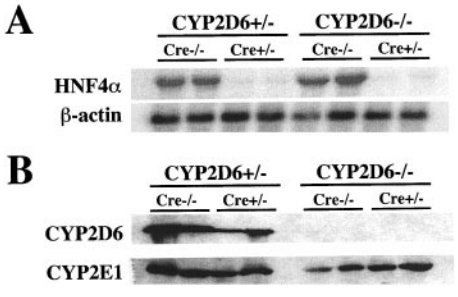

Fig. 4. Regulation of CYP2D6 by conditional knock-out of *HNF4α.* A, Northern blot analysis of mouse liver *HNF4α* expression. Hepatic mRNA (10 μg) from *HNF4α*; AlbCre +/−; *CYP2D6* +/− and littermate control mice, *HNF4α* fl/fl; AlbCre −/−; *CYP2D6* +/−, *HNF4α* fl/fl; AlbCre +/−; *CYP2D6* −/− and *HNF4α* fl/fl; AlbCre −/−; *CYP2D6* −/− was separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with an *HNF4α* exon 4 and 5 cDNA probe. *HNF4α* was completely deleted after Cre-mediated recombination. β-actin was used as a loading control. B, Western blot analysis of CYP2D6 protein expression in liver microsomal protein (40 μg) from the same animal groups of A. Deletion of *HNF4α* resulted in a more than 50% of CYP2D6 protein expression. The blots were re-probed with an antibody to CYP2E1 to verify loading and the presence of microsomal proteins in all the samples.

TABLE 2

Urinary excretion (0–24 h) of percentage dose as DEB, 4-OH-DEB, and DEB + 4-OH-DEB, together with the metabolic ratio (MR; percentage dose as DEB/percentage dose as 4-OH-DEB) for wild-type (WT), heterozygous *CYP2D6* humanized (HT), and homozygous *CYP2D6* humanized mice (HM)

Mouse	DEB	4-OH-DEB	Total	MR
WT-1	60.7	3.0	63.7	20.5
WT-2	52.2	6.5	58.7	8.0
WT-3	70.1	9.1	79.2	7.7
MEAN	61.0	6.2	67.2	12.1
S.D.	9.0	3.1	10.7	7.3
HT-1	16.9	25.5	42.4	0.7
HT-2	15.9	17.7	33.6	0.9
HT-3	17.9	28.7	46.6	0.6
MEAN	16.9	24.0	40.9	0.7
S.D.	1.0	5.7	6.6	0.2
HM-1	8.5	17.4	25.8	0.5
HM-2	14.1	27.2	41.2	0.5
HM-3	21.2	42.2	63.4	0.5
MEAN	14.6	29.0	43.5	0.5
S.D.	6.4	12.5	18.9	0

proportions of the 0- to 24-h excreted dose, compared with DEB (3 ± 0%) and 4-OH-DEB (23.7 ± 2.5%). The hydroxylations in positions 1 and 3 may contribute significantly to the excretion profile. Swedish investigators (Dalen et al., 1999) found that, as the number of functional *CYP2D6* alleles increased in their human study population from 0 to 13, the total recovery of DEB and 4-OH-DEB decreased, a situation analogous to that reported here in the mouse. The early studies (Allen et al., 1976; Idle et al., 1979) had observed up to 15% dose excreted in 24 h as the ring-opened metabolites, and the Swedish study (Eiermann et al., 1998) suggests that the 1- and 3-hydroxylations may account for even more of the dose than this. However, until this point, it has not been possible to gain a true insight in vivo into relative quantitative importance of 4-hydroxylation and non-4-hydroxylation by CYP2D6. There were indications from older literature that the non-4-hydroxylation pathways may be major routes of DEB metabolism in man, rat, and dog (Allen et al., 1976; Idle et al., 1979; Al-Dabbagh et al., 1981). Using the *CYP2D6* humanized mouse, it is possible to visualize that the non-4-hydroxylation of DEB by CYP2D6 may comprise about 50% of the total metabolism of this drug.

In addition to the pharmacokinetic study of CYP2D6 substrates, of which DEB is an archetype, the *CYP2D6* humanized mouse offers us an opportunity to understand better the genetic foundation of the CYP2D6 polymorphism. The EM trait was recognized at the outset (Mahgoub et al., 1977) as the dominant phenotype, but few occasions have presented themselves for the quantification of the degree of dominance of the EM over the PM trait. The first determinations were made in family pedigrees that permitted identification of obligate heterozygotes (e.g., EM children of PM probands). The mean MR values for homozygous EMs (calculated), heterozygous EMs (observed), and PMs (observed) were 0.4, 1.9, and 33.9, respectively. These data permitted calculation of the degree of dominance of EM over PM to be approximately 30% (Evans et al., 1980). These findings were thought to explain the major phenotypic overlap between the homozygous and heterozygous EM genotypes and demonstrated just why molecular genetic methods would be required to determine the genotype reliably. However, the expression of the human transgene in the mouse background offers yet further insights. The MR values in the mouse for 0, 1, and 2 human *CYP2D6* genes are 15.2 ± 7.0 (n = 6), 0.7 ± 0.2 (n = 3), and 0.5 ± 0 (n = 3). Using log-transformed data according to the method of Evans et al. (1980), these results yield a degree of dominance of EM over PM, for the human gene in the mouse, of 78% (95% confidence interval, 60 to 100%) (Fig. 5). This is significantly greater than the human estimate of 30% and may reflect the fact that transcription and translation of the *CYP2D6* transgene proceeds very efficiently. Perhaps the mouse hepatic background provides a more transcriptionally active environment than the endogenous human one.

TABLE 3

DEB urine metabolic ratios from the animal groups in Figure 4A

Animals were dosed with DEB (2.5 mg/kg) and placed in urine metabolic chambers for 24 h. Values from *HNF4α*-deleted, *CYP2D6* humanized mice that are significantly different ($p < 0.05$) from *HNF4α*-nondeleted, *CYP2D6* humanized mice (see Table 2 for key).

Mouse	Genotype	DEB	4-OH-DEB	Total	MR
1	fl/fl,CRE−/−,2D6+/−	22.8	8.8	31.7	2.6
2	fl/fl,CRE−/−,2D6+/−	19.6	7.2	26.8	2.7
3	fl/fl,CRE−/−,2D6+/−	16.0	7.2	23.2	2.2
4	fl/fl,CRE−/−,2D6+/−	14.8	5.5	20.3	2.7
MEAN		18.3	7.2	25.5	2.6
S.D.		3.7	1.4	4.9	0.2
1	fl/fl,CRE+/−,2D6+/−	10.4	2.8	13.2	3.7
2	fl/fl,CRE+/−,2D6+/−	9.7	2.5	12.3	3.8
3	fl/fl,CRE+/−,2D6+/−	10.1	3.7	13.7	2.8
4	fl/fl,CRE+/−,2D6+/−	10.2	2.5	12.7	4.1
MEAN		10.1	2.9	13.0	3.6
S.D.		0.3	0.5	0.6	0.6
1	fl/fl,CRE−/−,2D6−/−	60.5	2.4	62.9	25.1
2	fl/fl,CRE−/−,2D6−/−	41.7	3.2	44.9	12.8
3	fl/fl,CRE−/−,2D6−/−	52.6	3.1	55.7	16.9
MEAN		51.6	2.9	54.5	18.3
S.D.		9.5	0.4	9.1	6.2
1	fl/fl,CRE+/−,2D6−/−	26.2	0.9	27.1	29.2
2	fl/fl,CRE+/−,2D6−/−	23.0	0.8	23.7	29.8
3	fl/fl,CRE+/−,2D6−/−	27.2	0.8	28.0	35.3
4	fl/fl,CRE+/−,2D6−/−	26.7	0.8	27.5	33.8
MEAN		25.8	0.8	26.6	32.0
S.D.		1.9	0.1	1.9	3.0

HNF4α is known to regulate the basal levels of expression of a several P450s. Promoter activity, mobility shift, and antisense targeting assays have shown that HNF4α regulates, among others, the expression of human *CYP2D6* (Cairns et al., 1996; Jover et al., 2001) but not rat *CYP2D5* (Lee et al., 1994) or human *CYP2E1* (Liu and Gonzalez, 1995). The results from the present study provide, for the first time, in vivo confirmation of these earlier findings.

The most intriguing finding with the *HNF4a* conditional knock-out experiments is not that in the wild-type and *CYP2D6* humanized mice the metabolic ratio (MR) rises from 18.2 ± 6.2 to 32.0 ± 2.9 (t = 3.93, df = 5, P = 0.011) and from 2.6 ± 0.2 to 3.6 ± 0.6 (t = 3.31, df = 6, P = 0.016), respectively, but that the percentage dose recovered in urine as DEB plus 4-OH-DEB fell from 54.5 ± 9.1% to 26.6 ± 2.0% (t = 6.17, df = 5, P = 0.002) and from 25.5 ± 4.9% to 13.0 ± 0.6% (t = 5.06, df = 6, P = 0.002), respectively. This situation is somewhat similar to that described above for the decrease in urinary recovery associated with insertion of the *CYP2D6* transgene into the mouse. However, it is inconceivable that the conditional knock-out of *HNF4a* in the mouse liver, either in wild-type or humanized mice, could result in switching to alternative and undisclosed pathways of metabolism in the mouse. There is no evidence that DEB is metabolized by any P450s other than the CYP2D family. Conditional *HNF4a* knock-out mice harbor several biochemical abnormalities, together with hepatomegaly (Hayhurst et al., 2001). The single most dramatic biochemical change in these mice was an 18-fold elevation in serum bile acids from 16.7 ± 7 μM in wild-type mice to 283 ± 61 μM (t = 8.70, df = 6, P = 0.0001)

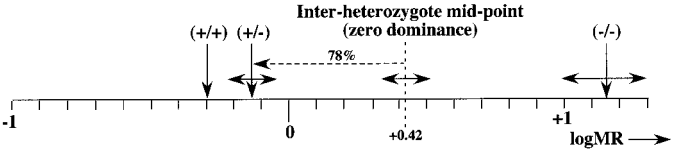

Fig. 5. Estimation of the mean degree of dominance of the EM [transgenic] over the PM [endogenous] phenotype in mice. Horizontal double-headed arrows represent 95% confidence intervals. The vertical dotted line shows the position of the mid-point between the two homozygous genotypes (+/+) and (−/−), the position of zero dominance. When the mean heterozygote (+/−) value falls to the left of this dotted line, then (+/+) is dominant over (−/−), the excursion to the left of this mid-point revealing the degree of dominance. For all calculations, only log-transformed data were used, because logMR is normally distributed, unlike its arithmetic values that are generally skewed to the right (Evans et al., 1980).

in the HNF4α conditional null mice (conditionally hepatic *HNF4a*-null mice). Elevated bile acids, as is seen in biliary obstruction, for example, are known to impair renal function (Shen et al., 1990; Bomzon et al., 1997) and thus reduce urine output. The reduced urinary recovery of DEB and 4-OH-DEB, relative to controls, reported here in both the heterozygous *CYP2D6* humanized mice with conditional hepatic *HNF4a* knock-out (fl/fl, CRE+/−, 2D6+/−) and wild-type mice with the same *HNF4a* knock-out (fl/fl, CRE+/−, 2D6−/−), is almost certainly secondary to the massively elevated serum bile acid concentration and concomitantly reduced renal function. In human populations with reduced urine volumes caused by dehydration, for example in Saudis and Egyptians (Islam et al., 1980), the 0- to 8-h urinary recovery of DEB plus 4-OH-DEB fell from 41% in British subjects to 15 to 16% in the Arab populations.

In conclusion, we report here the first humanized P450 model that predicts human drug metabolism and reflects the polymorphic status of the gene in the human population. Moreover, we show in vivo regulation of *CYP2D6* by *HNF4a*. This preclinical model may be of considerable interest for the identification of in vivo drug interactions and for the preclinical evaluation of novel CYP2D6 substrates or inhibitors. The *CYP2D6* humanized mouse will also permit investigation into the physiological significance of CYP2D6 and its polymorphism.

Acknowledgment

We thank John Buckley for technical assistance.

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Correction to "The *CYP2D6* humanized mouse: effect of the human *CYP2D6* transgene and *HNF4α* on the disposition of debrisoquine in the mouse"

Table 1 of this article [Corchero J, Granvil CP, Akiyama TE, Hayhurst GP, Pimprale S, Feigenbaum L, Idle JR, Gonzalez FJ (2001) *Mol Pharmacol* **60:**1260–1267] contains a printing error. The corrected table appears below.

We regret this error and apologize for any confusion or inconvenience it may have caused.

TABLE 1

Pharmacokinetic parameters for DEB and 4-OH-DEB after a single oral administration of DEB (2.5 mg/kg) in wild-type, *CYP2D6* humanized heterozygous, and *CYP2D6* humanized homozygous mice

Values represent the mean and the S.E.M. from three to four mice.

Parameter	Wild Type	Heterozygous	Homozygous
DEB			
T_{max} (h)	2.5 ± 1.8	6.7 ± 0.7	4.6 ± 1.8
C_{max} (nM)	2,937 ± 795	879 ± 128*	467 ± 61*,#
$AUC_{0-24\ h}$ (nM · h)	28,393 ± 1840	8,757 ± 1215*	4,634 ± 1348*,#
CL (l/h/kg)	15.2 ± 0.9	48.9 ± 6.4*	94.1 ± 22.3*,#
$T_{1/2}$ (h)	16.5 ± 4.5	8.9 ± 2.1*	6.9 ± 1.6*
4-OH-DEB			
T_{max} (h)	1.7 ± 0.3	3.3 ± 2.3	0.8 ± 1.2
C_{max} (nM)	110 ± 12	535 ± 79*	1075 ± 97*,#
$AUC_{0-24\ h}$ (nM · h)	1,086 ± 28	4,627 ± 377*	9,289 ± 931*,#

*, values from *CYP2D6* humanized mice that are significantly different ($p < 0.05$) from wild-type mice.

#, values from *CYP2D6* humanized homozygous mice that are significantly different ($p < 0.05$) from *CYP2D6* humanized heterozygous mice ($p < 0.05$).