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Difference in desipramine metabolic profile between wild-type and CYP2D6-humanized mice

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Abstract

Desipramine (DMI), a CYP2D6 probe, was used as a model drug to test whether *CYP2D6*-humanized (Tg-*CYP2D6*) and wild-type control mice could be used as preclinical animal models to identify the effects of CYP2D6 genotype/phenotype on drug metabolic profiles. After the analyses by liquid chromatography coupled with tandem mass spectrometry, DMI biotransformations were compared in Tg-*CYP2D6* and wild-type mouse liver microsomes (MLM), and in human CYP2D6 extensive and poor metabolizer liver microsomes. Furthermore, urinary DMI metabolic profiles in Tg-*CYP2D6* and wild-type mice were evaluated. Three metabolites, 2-hydroxyl-, 10-hydroxyl, and *N*-desmethyl-desipramine were identified in the incubations of DMI with both wild-type and Tg-*CYP2D6* MLM, as well as in human CYP2D6 extensive metabolizer liver microsomes. Three additional metabolites were found in mouse urine samples, and their chemical structures were elucidated. Although the ratio of individual metabolites produced in Tg-*CYP2D6* MLM was closer to that in human CYP2D6 extensive metabolizer liver microsomes, the urinary DMI metabolic profiles did not show much difference between wild-type and Tg-*CYP2D6* mice. The results suggest that other mouse enzymes have significant contribution to DMI metabolism.

Keywords

Desipramine; metabolism; CYP2D6; mass spectrometry; transgenic; mouse

Introduction

Species difference in metabolic elimination of drugs is one of the major challenges for the extrapolation of pharmacokinetic data from animals to humans towards reliable risk assessment in drug discovery [1,2]. The preclinical animal models and human subjects may differ in the composition, expression and function of drug-metabolizing enzymes [3]. The cytochrome P450 2D6 (CYP2D6) metabolizes 20-30% of drugs, and its genetic variations can cause considerable interindividual variability in drug metabolism, pharmacokinetics and pharmacodynamics [4,5]. Over 9 and 6 CYP2D genes have been identified in mice and rats, respectively, whereas none of the encoded proteins seems to have the same drug-metabolizing activity as human CYP2D6. For instance, both debrisoquine 4-hydroxylation and bufuralol 1'-hydroxylation are mediated by CYP2D6 in humans. However, the liver microsomes from three different strains (C57BL/6, DBA/2 and ddY) of mice and the purified mouse cyp2d9, cyp2d10, and cyp2d11 enzymes all show an impaired debrisoquine 4-hydroxylase activity [6]. In contrast, bufuralol 1'-hydroxylase activity in the B6C3F1 mouse liver microsomes (MLM) is

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over 80-fold higher than that in human liver microsomes (HLM), although the activity in MLM can not be inhibited by an anti-human CYP2D6 antibody or a potent CYP2D6 inhibitor quinidine [2]. Furthermore, despite mouse *cyp2d22* and human CYP2D6 share more than 70% of identity in their amino acid sequences, *cyp2d22* drug-metabolizing activity looks more like human CYP3A4 rather than CYP2D6 [7,8].

One possible approach to overcome the species difference in drug metabolism is to use transgenic mouse models that express human drug-metabolizing enzymes [3,9]. The *CYP2D6*-humanized (Tg-*CYP2D6*) mice [10] exhibit similar CYP2D6 tissue expression pattern as that in humans. Indeed, use of Tg-*CYP2D6* and wild-type control mice have successfully revealed the effects of CYP2D6 status on the metabolism, pharmacokinetics and dynamics of a number of CYP2D6 substrate drugs including desipramine and indoleamines [10-13]. Nevertheless, there are limited studies on the metabolic profiles of drugs in transgenic mouse models [14,15] while the safety assessment of uniquely or disproportionately produced metabolites in humans is necessary in preclinical studies, and the use of an appropriate humanized animal models may offer some benefits [16]. Therefore, this study aimed to test whether Tg-*CYP2D6* and wild-type control mice could be used as preclinical animal models for the investigation of metabolic profiles of CYP2D6-metabolized drugs vis-à-vis CYP2D6 status. Desipramine (DMI) was chosen as a model drug because it is a well-known probe substrate for CYP2D6, and CYP2D6 genotype/phenotype significantly affects desipramine pharmacokinetics in humans [17-19]. To our surprise, our liquid chromatography coupled with mass spectrometry (LC-MS) analytical data revealed that DMI urinary metabolic profiles were comparable between wild-type and Tg-*CYP2D6* mice, albeit the ratio of individual metabolites produced in Tg-*CYP2D6* MLM was closer to that in human CYP2D6 extensive metabolizer liver microsomes.

Materials and Methods

Chemicals and Materials

Desipramine (DMI) was purchased from Sigma-Aldrich (St. Louis, MO). 2-Hydroxyl-*N*-desmethylinipramine (2-OH-DMI) was kindly provided by the National Institute of Mental Health Chemical Synthesis and Drug Supply Program. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was bought from EMD Biosciences (Darmstadt, Germany). Individual HLMs, purchased from BD Biosciences (Woburn, MA), were pooled to generate CYP2D6 poor metabolizer (PM) HLM (HG06, HG30, HH31 and HH35) and extensive metabolizer (EM) HLM (H056, H066, H77 and H88). The Tg-*CYP2D6* and wild-type MLM were prepared according to the method described [20]. All organic solvents were either analytical or high-performance liquid chromatography (HPLC) grade.

Microsomal incubation

Incubations were carried out in potassium phosphate buffer (0.1 M, pH 7.4) containing 0.5 mg protein/mL of HLM or 0.1 mg protein/mL of MLM, 1 μ M of DMI, and 1 mM of NADPH in a final volume of 200 μ L at 37°C, as described [12,20]. The reactions were stopped with equal volume of ice-cold acetonitrile (containing 20 nM of nortriptyline as internal standard) after 120 min (HLM) or 90 min (MLM). All reactions were conducted in triplicate. The samples were centrifuged at 14,000 rpm for 10 min, and the supernatants were subjected to LC-MS analyses.

Mouse urine collection and processing

Age-matched adult wild-type FVB/N (five totally) and Tg-*CYP2D6* (six) mice [10] were used. Mouse genotypes were confirmed by PCR analysis, as described [10]. Each mouse was treated intraperitoneally (i.p.) with 30 mg/kg of desipramine that was able to significantly alter mouse

behaviors (unpublished data). Mice were placed in metabolic cages, and urine samples were collected over 24 hr after drug administration. The animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at University at Buffalo. One hundred microliters of urine samples were added with equal volume of acetonitrile, and the supernatants were injected for LC-MS analyses.

LC-MS analyses

The LC-MS system consisted of a Shimadzu prominence HPLC system (Kyoto, Japan) and an API 3000 turbo ionspray ionization triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The system was controlled by Analyst 1.4.2 software (Applied Biosystems). Separation of DMI and its metabolites was achieved by using a Waters Symmetry C₁₈ column (30 × 2.1 mm, 3.5 μm), and at a flow rate of 0.2 mL/min. The mobile phases included 0.1% formic acid (Buffer A), and 90% acetonitrile containing 0.1% formic acid (Buffer B). The gradient cycle used for metabolite identification consisted of an isocratic elution with 5% Buffer B for 2 min, followed by a linear increase of Buffer B to 65% from 2 to 20 min. The mobile phase was then maintained at 65% Buffer B for 5 min before being changed back to 5% at 26 min for a 4-min equilibration. The mass spectrometer was operated in positive full scan mode for metabolite identification, and the fragmentation patterns of possible metabolites were further examined at a collision energy of 25 eV.

Results and Discussion

We first investigated the metabolic profiles of DMI in CYP2D6 PM and EM HLM. The incubations lacking NADPH were used as controls. Following the full-scan acquisition, ions for DMI and its possible oxidative metabolites were extracted (Fig. 1), which was based on the predicted mass changes after common biotransformations. The parent drug DMI was eluted around 11 min, with a protonated molecular ion (MH⁺) at *m/z* 267. Two hydroxylated metabolites (MH⁺, *m/z* 283), M1 and M2, were eluted at 8.1 and 8.9 min, respectively. The predicted desmethyl-desipramine (DDMI, M3), with a MH⁺ at *m/z* 253, was also found in the incubations. None of the metabolites were detected in the control incubation without NADPH (data not shown). In addition, we did not find any other oxidative metabolites in the incubations with PM or EM HLM.

The chemical structures of individual metabolites were readily elucidated by the analyses of their corresponding MS fragmentation patterns (Fig. 2). M2, with a MH⁺ at *m/z* 283, was revealed as 2-OH-DMI because its retention time and MS spectrum were the same as those of authentic standard. The predominant ion *m/z* 72 was from the *N*-methylpropan-1-amine group, and the prominent ions *m/z* 252, 238, and 224 arose from the loss of CH₃NH₂, CH₃NHCH₃, and CH₃NHC₂H₅, respectively (Fig. 2B). M1 showed a MH⁺ at *m/z* 283 and a number of major product ions at *m/z* 265, 234, 206, and 72. The formation of predominant ion *m/z* 72 was similar as that from M2, suggesting that the hydroxylation occurred at the dibenzoazepine moiety. The characteristic ion at *m/z* 265, loss of a water molecule (*m/z* 18) from the protonated molecular ion, indicated the presence of an alcoholic group. Taken together, M1 was identified as 10-hydroxyl-desipramine (10-OH-DMI) (Fig. 2A). The presence of product ions at *m/z* 236, 208, 196 and 58 from the MH⁺ at *m/z* 253 further supported that M3 was the demethylated metabolite (DDMI) (Fig. 2C). It is noteworthy that all the three metabolites have been observed in humans [17-19]. Furthermore, our data (Fig. 1 and 2) showed that 2-OH-DMI was the major metabolite formed in EM HLM, as manifested by a 2-OH-DMI/DDMI ratio of 5.8 and a limited level of 10-OH-DMI. In contrast, production of 2-OH-DMI was minimal in the incubation with PM HLM, with a 2-OH-DMI/DDMI ratio of 0.3. The results are consistent with previous observations [17-19] that 2-hydroxylation is the major metabolic pathway of DMI in humans, and the biotransformation is mainly mediated by CYP2D6.

We then compared the metabolic profiles of DMI in wild-type and Tg-*CYP2D6* MLM (Fig. 3). Although many strains of mice are known to have impaired debrisoquine 4-hydroxylase activities [6], our data showed that 2-OH-DMI was extensively produced by the FVB/N wild-type MLM (Fig. 3A), and the level of 2-OH-DMI was around 2-fold higher than DDMI. The result is consistent with previous findings that 2-OH-DMI is the principle metabolite formed by CD-1 MLM [19]. As expected, incubation of DMI with Tg-*CYP2D6* MLM led to a higher level of 2-OH-DMI production (Fig. 3B), as indicated by the 2-OH-DMI/DDMI ratio of 4.1. Given the 2-OH-DMI/DDMI ratio in wild-type MLM (1.9) and Tg-*CYP2D6* MLM (4.1), and the ratio (5.8) in human CYP2D6 EM HLM, the Tg-*CYP2D6* mouse might be better mouse model for the prediction of metabolic profiles of CYP2D6 substrate drugs in humans.

We, therefore, further examined the urinary DMI metabolic profiles in the two genotyped mice treated with 30mg/kg of DMI. Beside the detection of all three oxidative metabolites (M1, M2 and M3) in both wild-type and Tg-*CYP2D6* mouse urine samples, extracted ion chromatograms showed that there were three additional metabolites, M4, M5 and M6 (Fig. 4). M4, a glucuronide conjugate that showed a MH^+ at m/z 459 and similar fragmentations (m/z 283, 252, 224; Fig. 5A) as 2-OH-DMI (M2, Fig. 2B) after the loss of glucuronic acid moiety, was identified as desipramine-2-*O*-glucuronide (DMI-2-*O*-Gluc). This secondary metabolite was likely formed from 2-OH-DMI (Fig. 6). M5 and M6 were eluted at 7.9 and 8.5 min, respectively. Given the MH^+ at m/z 269 and the same product ions (m/z 234 and 206) (Fig. 5B) as 10-OH-DMI, M5 was identified as 10-hydroxyl-desmethyl-desipramine (10-OH-DDMI). Likewise, M6 was identified as 2-hydroxyl-desmethyl-desipramine (2-OH-DDMI). The two metabolites are presumably produced from 10-OH-DMI and 2-OH-DMI, respectively (Fig. 6). In addition, the estimated urinary DMI:2-OH-DMI:DMI-2-*O*-Gluc:2-OH-DDMI:10-OH-DMI:10-OH-DDMI:DDMI values were 1.0:1.1:0.97:0.89:0.05:0.10:0.06 and 1.0:0.95:0.99:0.83:0.04:0.07:0.05 in wild-type and Tg-*CYP2D6* mice, respectively. The *in vivo* metabolic data (Fig. 4) agreed with *in vitro* observations (Fig. 1) that 2-hydroxylation was the major metabolic pathway of DMI in mice. However, the urinary metabolic ratio did not show much difference between the two genotyped mice although DMI 2-hydroxylation is known to be affected by CYP2D6 status in humans [17,21]. This observation is also in contrast to previous findings that use of wild-type and Tg-*CYP2D6* mouse model readily elucidates the effects of CYP2D6 status on the metabolism of CYP2D6 substrate drugs *in vivo* [10,12]. One possible interpretation is that other mouse enzymes have significant contribution to DMI 2-hydroxylation, which is evident in the production of 2-OH-DMI from DMI by wild-type MLM *in vitro* (Fig. 3) although the underlying enzymes have not been disclosed. Nevertheless, these results suggest that the value of transgenic mouse model in understanding drug metabolism may be substrate dependent, and caution need be advised when interpreting data obtained from the transgenic mouse model, same as other animal models.

In summary, the metabolism of DMI was compared in wild-type and Tg-*CYP2D6* mice *in vitro* and *in vivo*. Our data showed that DMI metabolite ratios in transgenic mouse liver microsomes were closer to those in human CYP2D6 EM liver microsomes, whereas the urinary metabolic profiles were comparable in wild-type and Tg-*CYP2D6* mice. These results suggest other drug-metabolizing enzymes may be largely involved in DMI 2-hydroxylation in mice, although CYP2D6 plays important role in DMI metabolism in humans.

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(A) Incubation in PM HLM **(B) Incubation in EM HLM** **Fig 1.**

Extracted ion chromatograms of desipramine and its oxidative metabolites obtained after full-scan data acquisition. (A) Incubation with CYP2D6 poor metabolizer HLM. (B) Incubation with CYP2D6 extensive metabolizer HLM. All incubations were conducted at 37°C for 120 min.

Fig 2.
Individual MS/MS spectra of M1 (A), M2 (B), and M3 (C) found in microsomal incubations with desipramine.

(A) Incubation in wild-type MLM **(B) Incubation in Tg-CYP2D6 MLM**

Fig 3. Extracted ion chromatograms of desipramine and its oxidative metabolites produced by wild-type (A) and Tg-CYP2D6 (B) mouse liver microsomes after 90-min incubation.

(A) Wild-type mouse urine **(B) Tg-CYP2D6 mouse urine** **Fig 4.**

Extracted ion chromatograms of desipramine and its urinary metabolites in wild-type (A) and Tg-CYP2D6 (B) mice treated i.p. with 30 mg/kg of desipramine.

Fig 5.
Individual MS/MS spectra of M4 (A), M5 (B), and M6 (C) found in mouse urine samples.

Fig 6.

Proposed metabolic pathways of desipramine in wild-type and Tg-CYP2D6 mice. DMI is primarily oxidized to 2-hydroxyl-desipramine (2-OH-DMI, M2), which is subjected to *O*-glucuronidation to produce desipramine-2-*O*-glucuronide (DMI-2-*O*-Gluc, M4). A small fraction of DMI is converted to 10-hydroxyl-desipramine (10-OH-DMI, M1) and desmethyl-desipramine (DDMI, M3). In addition, M1 and M2 might be further *N*-demethylated to M5 (10-OH-DDMI) and M6 (2-OH-DDMI), respectively.