Liver-Selective Expression of Human Arylamine N-Acetyltransferase NAT2 in Transgenic Mice

Kim S. Sugamori, Debbie Brenneman, and Denis M. Grant

Department of Pharmacology and Toxicology, Faculty of Medicine (K.S.S., D.B., D.M.G.), and Leslie Dan Faculty of Pharmacy (D.M.G.), University of Toronto, Toronto, Ontario, Canada

Received January 13, 2011; accepted February 11, 2011

ABSTRACT:

Human arylamine N-acetyltransferase 2 (NAT2) mediates the biotransformation of arylamine drugs and procarcinogens into either innocuous or reactive DNA-damaging metabolites and is expressed predominantly in liver. Interspecies differences and incongruous results between in vitro, in vivo, and epidemiological studies make it difficult to extrapolate animal results to human risk. We have generated human NAT2 transgenic mice on both C57BL/6 (hNAT2tg) and Nat1/2 null backgrounds [hNAT2tgNat1/2(−/−)], in which liver-selective expression of human NAT2 is driven by the mouse albumin promoter. We detected expression of the human NAT2 transcript and protein in mouse liver by real-time PCR and Western blot analysis. NAT2 enzyme activity, measured using the human NAT2-selective substrate sulfamethazine (SMZ), was 40- to 80-fold higher in liver cytosols from hNAT2tgNat1/2(−/−) mice than in wild-type mice. An unexpected gender difference was observed, with males displaying 2-fold higher activity than females. Transgenic mice also had an increased in vivo plasma clearance of SMZ and higher levels of N-acetylated SMZ than wild-type mice. Liver expression of human NAT2 did not affect the disposition of the human NAT1-selective substrate p-aminosalicylic acid (PAS), because hNAT2tgNat1/2(−/−) mice displayed in vivo PAS pharmacokinetic profiles similar to those of Nat1/2(−/−) mice. The metabolism of 4-aminobiphenyl was similar between hNAT2tgNat1/2(−/−) and wild-type mice with the exception of a more liver-restricted pattern in hNAT2tgNat1/2(−/−) mice and lower activity in females. Overall, the hNAT2tgNat1/2(−/−) mouse mimics human expression of NAT2 and may thus be of value in clarifying the role of human NAT2 in arylamine clearance, detoxification, and bioactivation.

Introduction

Exposure to environmental chemicals such as arylamines and heterocyclic amines that require metabolic activation to DNA-damaging electrophiles is a causal factor leading to the production of tissue-specific tumors in both animal models and humans (Weisburger, 2002; Neumann, 2007). In humans, two arylamine N-acetyltransferase (NAT) enzymes, NAT1 and NAT2, may play important roles in either detoxifying or bioactivating chemicals such as the bladder carcinogen 4-aminobiphenyl (ABP), which is found in cigarette smoke (Stabbert et al., 2003). Whereas NAT1 is ubiquitously expressed, NAT2 is primarily localized to the liver, with some expression in the colon and small intestine (Husain et al., 2007). Because of the polymorphic nature of these enzymes (Sim et al., 2008; Hein, 2009), epidemiological studies have investigated the role of NAT genetic variation and cancers of the bladder, breast, prostate, and colon, particularly in relation to exposures to tobacco or to heterocyclic amines in cooked meats (Nöthlings et al., 2009; Rabstein et al., 2010; Sharma et al., 2010; Moore et al., 2011). However, these results are confounded by misclassification errors due to phenotype/genotype discordances and imprecise chemical exposure data.

A classic bioactivation model proposes that procarcinogenic arylamines are first N-oxidized in the liver to N-hydroxyarylamines. Owing to its hepatic localization and previous in vitro studies, CYP1A2 is postulated to be a primary enzyme involved in this reaction (Kim and Guengerich, 2005), whereas CYP1A1 or peroxi-
dases may be involved in activation within other tissues (Bendaly et al., 2009). Subsequent to N-oxidation, O-acetylation (OAT) by NATs yields an acetoxy ester, which can decompose into a highly reactive arylnitrenium ion that can bind to DNA, leading to the production of mutagenesis (Hein, 2002). Increases in C8-deoxyguanosinyl-ABP DNA adducts, hypoxanthine phosphoribosyltransferase (hprt) mutagenesis, and cytotoxicity to ABP exposure have been observed in Chinese hamster ovary cells upon coexpression of rapid acetylator NAT2*4 and CYP1A1 (Bendaly et al., 2009).

Competing with N-oxidation, at least for the monoamine ABP, is the protective NAT-mediated N-acetylation, which forms innocuous metabolites. However, N-acetylation of diamines such as benzidine appears to enhance its metabolic activation, possibly because the monoacetylated metabolite is efficiently bioactivated in subsequent

ABBREVIATIONS: NAT, N-acetyltransferase; ABP, 4-aminobiphenyl; OAT, O-acetyltransferase; SMZ, sulfamethazine; PCR, polymerase chain reaction; ASMZ, N-acetyl-sulfamethazine; PAS, p-aminosalicylic acid; APAS, N-acetyl-p-aminosalicylic acid; AABP, N-acetyl-4-aminobiphenyl; kb, kilobase; SV, simian virus; h, human; bp, base pair; BLAST, Basic Local Alignment Search Tool; Pde, phosphodiesterase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; AUC, area under the curve; ANOVA, analysis of variance; qPCR, quantitative polymerase chain reaction; PhiP, 2-amino-1-methyl-6-phenyl[4,5-b]pyridine.
reactions (Hein, 2006). Thus, it is not clear whether NATs are more important for the detoxification or the bioactivation of arylamine procarcinogens, and this may ultimately depend on their specific tissue localization, the balance of competing pathways, other organism-specific metabolism, the substrate characteristics, and overlapping substrate selectivities (Nebert and Dalton, 2006). In addition, oxidative stress that may arise from inflammation or environmental exposure has been shown to modulate NAT activity via a mechanism of oxidative modification of a key catalytic cysteine residue (Dairou et al., 2004, 2009).

Although in vitro studies may help to elucidate potential mechanistic information, such studies cannot take into consideration the influence of clearance, tissue-specific metabolism, and the route of administration. Studies using knockout mouse models have indicated that in vitro predictions do not necessarily extend to the intact animal. For instance, Cyp1a2-null mice are not protected from ABP-induced tumorigenesis or DNA adduct formation despite the bioactivation model mentioned above (Kimura et al., 1999; Tsuneoka et al., 2003), and Cyp1al-null mice are not protected from the toxicity of oral benzo[a]pyrene (Uno et al., 2004). Thus, it is imperative to conduct studies in vivo in which exposure can be controlled, the influence of tissue-specific metabolism may become apparent, and indices of toxicity such as DNA damage, cytotoxicity, and tumor incidence can be assessed.

Knockout mouse models have been instrumental in interrogating the role of specific enzymes in preventing or facilitating toxic responses. However, a major confounding factor in extrapolating these results to human risk assessment is species differences in metabolism (Cheung and Gonzalez, 2008). For instance, the human NAT2-selective substrate sulfamethazine (SMZ) is poorly metabolized in the mouse (Glowinski and Weber, 1982). In the mouse, ABP has been found to be metabolized exclusively by Nat2 (Loehle et al., 2006), the ortholog of human NAT1 (Kawamura et al., 2008), whereas in humans NAT2 and, to a lesser extent, NAT1 contribute to the N-acetylation of ABP and the O-acetylation of N-hydroxy-ABP in the liver (Doll et al., 2010). Thus, there are clear species differences with respect to NAT substrate selectivity and function. As such, our previously described Nat1/2 double null mouse model (Sugamori et al., 2003) may have limits in making human inferences.

To circumvent some of these species differences in function and selectivity, we have developed a transgenic mouse that expresses human NAT2 selectively in the liver, and we have also placed it on a Nat1/2-null background devoid of mouse Nat activity, giving it a more human-like acetylation profile. This model should help us to determine the role of liver-specific NAT2 acetylation, either detoxification or bioactivation, without the influence of endogenous mouse Nat activity and may thus serve as a useful model to predict human responses to arylamine or heterocyclic amine exposure.

Materials and Methods

Materials. The pSI and pBluescript SK+ vectors were from Promega (Madison, WI) and Stratagene (La Jolla, CA), respectively. The mouse albumin enhancer/promoter in pBluescript KS− was provided by Dr. Richard Palmiter (University of Washington, Seattle, WA). Restriction enzymes were purchased from Invitrogen (Burlington, ON, Canada). Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coraville, IA), and standard PCR reagents were purchased from MBI Fermentas (Burlington, ON, Canada). Power SYBR Green was supplied by Applied Biosystems (Streetsville, ON, Canada). Acetyl-DL-carnitine, carnitine acetyltransferase, acetyl-CoA sodium salt, SMZ, ASMZ, ABP, and PAS sodium salt used for NAT activity assays were from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). APAS was produced in definable quantities from PAS using recombinant human NAT1 as a catalyst, whereas AABP was generously provided by Dr. M. Novak (Miami University, Oxford, OH). Anti-rabbit IgG horseradish peroxidase-linked antibody was obtained from GE Healthcare (Baie D’urfe, QC, Canada). Polyclonal rabbit anti-human NAT2 antisera was produced previously (D.M.G.) in the laboratory of Dr. U. A. Meyer, University of Basel, Switzerland. C57BL/6 (B6) and SJL mice were obtained from Charles River Canada (Saint-Constant, QC, Canada). All procedures involving animals were performed in accordance with the Canadian Council for Animal Care guidelines for the use and care of animals.

Construction of Human NAT2 Transgene. The human NAT2 transgene construct consists of an ~2.3-kb mouse albumin enhancer/promoter region, a chimeric intron, the human NAT2*4 coding region, and the late SV40 poly(A) (Fig. 1A). The albumin enhancer/promoter was introduced to drive expression of the transgene selectively in the liver (Pinkert et al., 1987). The human NAT2 coding region was amplified by PCR with the primers hNAT2 5′ and hNAT2 3′ (Table 1), sequenced (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada) to ensure PCR fidelity and subcloned into the EcoRI and NotI sites of the vector pSI. An ~1.4-kb HindIII/ClaI fragment consisting of the chimeric intron, human NAT2, and late SV40 poly(A) was subcloned into the EcoRI and NotI sites of the vector pBluescript SK−.

![Fig. 1. Generation of hNAT2 transgenic mice. A, schematic representation of the 3.7-kb hNAT2 transgene construct injected into B6/SJL pronuclei. The location of the probe used for Southern blot analysis is indicated above. B, Southern blot analysis of BamHI-, EcoRI-, and KpnI-digested genomic DNA from the transgenic founder and a nontransgenic wild-type mouse. C, PCR genotyping from representative wild-type, hemizygous transgenic, and homozygous transgenic mice. Wild-type mice are identified by a single band at 499 bp. Hemizygotes have 499- and 652-bp bands, and homozygous transgenic mice have only the 652-bp band. D, chromosomal location and recombination of the hNAT2 transgene. The hNAT2 transgene was localized to intron 3 of the mouse Pde8b gene located on chromosome 13 (13D1). Most of the albumin enhancer region was found to be transposed to the 3′ end of the hNAT2 transgene. Genotyping primers used to distinguish between the wild-type allele (NAT2-F and NAT2-RGT) in Pde8b intron 3 and the transgenic allele (NAT2-F and NAT2-RGT) in Pde8b intron 3 and hNAT2 are indicated by the arrows. Alb E, albumin enhancer region; Alb P, albumin promoter; pA, SV40 late polyadenylation; wt, wild-type; tg, transgenic.](image-url)
excised from pBSI and subcloned into the HindIII and Clal sites of pBluescript. The 2.3-kb albumin enhancer/promoter region was then subcloned into the SstIII and EcoRV sites located 5′ to the chromic intron. Restriction digests and DNA sequencing confirmed insertion and correct orientation of the sequences.

**Generation of hNAT2 Transgenic Mice.** The 3.7-kb transgene was excised from the pBluescript vector backbone by restriction digestion with SstII and Clal to release the full-length transgene and with AvrII to digest the vector backbone into smaller pieces to facilitate excision of the transgene from the vector. The DNA was subjected to gel electrophoresis, purified by electroelution using an ElutaTube Protein Extraction Kit (MBI Fermentas), concentrated by butanol extraction, and subjected to one round of column purification using an ElutaTube Protein Extraction Kit (MBI Fermentas), and random hexamers as per the manufacturer’s instructions. For relative quantitative real-time PCR, triplicate reactions containing 0.5 μl of each cDNA were performed with 300 nM concentrations of either human NAT2, mouse Pde8B, or mouse GAPDH primers and power SYBR Green. Default cycling conditions were used on an Applied Biosystems 7500 real-time PCR machine. Standard curves with serial dilutions of the input cDNA were performed with the human NAT2 or mouse Pde8B primers and compared with those for mouse GAPDH to ensure equivalent primer efficiencies between the target and reference genes. The ΔΔC\text{\textsubscript{T}} method was used to determine the relative quantification of both the human NAT2 transcript and the mouse Pde8B transcript (n = 3 for each gender and genotype). The tissue with the highest expression was set to a relative quantification of 1.

**Western Blot Analysis.** Male and female liver cytosols (10 μg of protein) from hNAT2\textsuperscript{tgNat1/2} (−/−) and Nat1/2 (−/−) mice were run on a 12% SDS-polyacrylamide gel electrophoresis gel, transferred to a nitrocellulose membrane, probed with a 1:1000 dilution of rabbit anti-human NAT2 polyclonal antibody followed by a 1:10,000 dilution of anti-rabbit IgG secondary antibody, and visualized using an enhanced chemiluminescence kit (GE Healthcare) as per the manufacturer’s instructions.

**In Vivo NAT Activity.** Tissue cytosols were prepared from wild-type and transgenic animals as described previously (Sugamori et al., 2003). NAT activity for SMZ N-acetylation was determined using cytosols from three animals for each gender, genotype, and tissue. Initial reaction rates were determined in duplicate with either 0.5 mM SMZ, 0.1 mM PAS or 0.1 mM ABP, 0.1 mM acetyl-CoA, and 20 μl of an acetyl-CoA-regenerating system in a final reaction volume of 100 μl. The regenerating system consisted of 5 mM acetyl-CoA, acetyl-CoA-regenerating system

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNAT2 5′</td>
<td>Sense</td>
<td>TCCAGGATCCATTTGGAACAT</td>
</tr>
<tr>
<td>hNAT2 3′</td>
<td>Antisense</td>
<td>ATCCGCGCGCTCTTAAGTTGATC</td>
</tr>
<tr>
<td>NAT2-F</td>
<td>Sense</td>
<td>TCCCATGGAGAACAGGGT</td>
</tr>
<tr>
<td>NAT2-RWT</td>
<td>Antisense</td>
<td>CCGTGTGGGTAGAAACGACAA</td>
</tr>
<tr>
<td>NAT2-RTG</td>
<td>Antisense</td>
<td>TCTACTGTCATTCAGGGCTCC</td>
</tr>
<tr>
<td>hNAT2-PP</td>
<td>Sense</td>
<td>GTCCCTTTCCTCCACAGGTT</td>
</tr>
<tr>
<td>hNAT2-PR</td>
<td>Antisense</td>
<td>TCTGGAGCTGTTCCCCTCTA</td>
</tr>
<tr>
<td>hNAT2-FRT</td>
<td>Sense</td>
<td>GTCAGATCGTCTGTCGAGA</td>
</tr>
<tr>
<td>hNAT2-RRT</td>
<td>Antisense</td>
<td>GGCACCTGAGGCTGATCCT</td>
</tr>
<tr>
<td>mPde8B-RRT</td>
<td>Sense</td>
<td>GTGATGCGCCAAGCCTACA</td>
</tr>
<tr>
<td>mPde8B-RRT</td>
<td>Antisense</td>
<td>GGACCTGAGGCTGATCCT</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>Sense</td>
<td>CAGGCCTGCTGCTGGACA</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>Antisense</td>
<td>CCGGGGAATAACGCCAAA</td>
</tr>
</tbody>
</table>
(AUC) values were determined by the trapezoidal rule using GraphPad Prism (GraphPad Software Inc.).

Statistical Analyses. Data are presented as the mean ± S.D. Statistical differences between group mean values were determined using either a Student’s t test or a one-way ANOVA followed by the Bonferroni multiple comparison test using GraphPad Prism.

Results

Generation of hNAT2\textsuperscript{tg}Nat1/2(−/−) Transgenic Mouse Line. Using luciferase promoter plasmid constructs, we found the albumin promoter to be 5- to 10-fold more effective than the endogenous human NAT2 promoter in driving luciferase expression in HepG2 cells when coexpressed with the liver-enriched transcription factors CCAAT/enhancer-binding protein α and DBP (data not shown). We therefore selected the mouse 2.3-kb albumin enhancer/promoter region to drive the liver-selective expression of our human NAT2 transgene.

Initial PCR genotyping of seven pups generated from microinjection of B6/SJL F2 pronuclei identified one founder animal. Southern blot analysis of BamHI-, EcoRI-, and KpnI-digested genomic DNA and hybridization with a human NAT2 probe suggested that only one copy was inserted in this founder animal (Fig. 1B). This was revealed by the presence of only two fragments of ∼1.8 and ∼8 kb on the Southern blot of KpnI-digested DNA. A KpnI restriction endonuclease site is present within the human NAT2 coding region (Fig. 1A), and the presence of a single copy insertion would be reflected by the presence of two fragments of unknown sizes. A concatamer arrangement would have yielded a 3.7-kb KpnI-digested band, the intensity of which would be dependent on the number of copies inserted and two bands of unknown size.

The founder was bred to B6 mice, and transmission to offspring was 58%, indicating that the founder was not a mosaic. The hNAT2\textsuperscript{tg} mice were backcrossed to wild-type B6 mice for 10 generations to generate the transgenic line on a congenic B6 background. The hNAT2\textsuperscript{tg} mice are therefore selected the mouse 2.3-kb albumin enhancer/promoter region to drive the liver-selective expression of our human NAT2 transgene.

Identification of Human NAT2 Transgene Insertion Site. Inverse PCR of RsaI-digested DNA and BLAST analysis of the resultant sequence indicated that the human NAT2 transgene was inserted into intron 3 of the mouse phosphodiesterase 8B (Pde8B) gene located on chromosome 13 (Fig. 1D). Sequence analysis revealed that a recombination event had also occurred in which most of the albumin enhancer sequence was transposed to the 3’ end of the human NAT2 cDNA-SV40 late poly(A) sequence. The ∼1.8-kb band observed on the Southern blot could thus be explained by the presence of a KpnI site, which is located in intron 3 of the Pde8B gene upstream of the start of the recombinant transgene sequence, whereas the ∼8-kb band is due to the presence of a KpnI site located 5.4 kb downstream of the recombinant sequence in intron 3. Likewise, the ∼2.4-kb EcoRI band observed on the Southern blot can be accounted for by the presence of an EcoRI site at the 5’ end of the human NAT2 coding region and an EcoRI site located ∼1.2 kb within the albumin enhancer region (Fig. 1D) that was transposed to the 3’ end of the late SV40 poly(A) region.

The other high molecular weight BamHI, EcoRI, and KpnI bands that hybridized to the hNAT2 probe and were found in both the founder and a nontransgenic sibling represent known restriction fragments corresponding to the cross-hybridizing endogenous mouse Nat genes.

Human NAT2 and Pde8B Expression in Transgenic Mice. RNA was isolated from several tissues from hNAT2\textsuperscript{tg}Nat1/2(−/−) mice and subjected to real-time qPCR to determine the tissue specificity of hNAT2 transgene expression. The human NAT2 transcript was detected in the liver of transgenic mice (Fig. 2A) but not in wild-type mice (data not shown). However, hNAT2 transcript was approximately 2-fold higher in male liver than in female liver (p < 0.001). Human NAT2 transcript was detected at lower levels in other tissues.

To determine whether the insertion of the hNAT2 transgene in the Pde8B intron 3 region affected the expression of the Pde8B gene, real-time qPCR was performed on tissues from both wild-type and transgenic mice. The Pde8B transcript was found to be the highest in cerebrum in both wild-type and transgenic animals (Fig. 2B). Significant Pde8B expression was also found in the cerebellum, colon, and kidney. Liver and spleen had the lowest expression of Pde8B. Compared with wild-type animals, transgenic animals had an ∼40 to 55% decrease in the level of Pde8B expression in the two brain regions studied.

Human NAT2 Protein Expression in Transgenic Mice. Western blot analysis using liver cytosol prepared from hNAT2\textsuperscript{tg}Nat1/2(−/−) mice indicated the presence of a ∼33-kDa protein (Fig. 3). This protein was not detected in liver cytosols prepared from male or female Nat1/2(−/−) mice. Similar to the hNAT2 qPCR results, hNAT2 transgenic male liver had a higher level of hNAT2 protein than female liver.

![Figure 2](https://example.com/f2.png)

**FIG. 2.** Real-time qPCR detection of hNAT2 and Pde8B transcripts in tissues from hNAT2\textsuperscript{tg}Nat1/2(−/−) transgenic mice and/or wild-type mice. Levels of hNAT2 (A) and Pde8B (B) were normalized to mouse GAPDH, and relative quantification was determined using the ΔΔC\textsubscript{T} method. a, significantly different from male transgenic (p < 0.001); b, significantly different from wild-type (p < 0.001); c, significantly different from wild-type (p < 0.01); d, significantly different from female wild-type (p < 0.05). M, male; F, female.
**In Vitro NAT Activity.** The presence of the functional human NAT2 enzyme was assessed by measuring cytosolic SMZ-NAT activity. Homozygous transgenic animals on a mouse Nat1/2 null background had ~40- to 80-fold higher SMZ-NAT activity than wild-type mice (Fig. 4A). A gender difference in SMZ-N-acetylation was detected using liver cytosols prepared from the transgenic animals. Male liver cytosol had ~2-fold higher activity than female liver cytosol \( (p < 0.01) \). Of the other tissue cytosols assayed from the hNAT2\(^{2+}\)Nat1/2\((--)/--\) mice, only colon had detectable levels of SMZ-N-acetylation with a ~7-fold increase above wild-type levels, although they were still 15- to 20-fold lower than liver activities (Fig. 4A). Only low levels of SMZ activity \( (<0.03 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}) \) could be detected in the various tissue cytosols from wild-type mice. The apparent \( K_m \) values for SMZ-N-acetylation using male and female hNAT2\(^{2+}\)Nat1/2\((--)/--\) liver cytosol (Table 2) are similar to those reported previously for human liver and recombinant human NAT2 (Grant et al., 1991). The apparent \( V_{\text{max}} \) values for male and female transgenic liver cytosol are comparable to those observed for cytosols prepared from NAT2 rapid and intermediate acetylator cryopreserved human hepatocytes (Doll et al., 2010).

In contrast with SMZ, the N-acetylation of ABP was detected in all wild-type tissues assayed but was only detected in liver and colon cytosol from the hNAT2\(^{2+}\)Nat1/2\((--)/--\) animals (Fig. 4B). A gender difference in ABP-N-acetylation was also detected using liver cytosol \( (p < 0.001) \) from the transgenic animals, with male livers having ~2-fold higher activity than females (Fig. 4B; Table 2). This gender difference was not detected in livers from wild-type mice. However, a gender difference was observed in wild-type kidney cytosols, with males showing 2-fold higher ABP-NAT activity. The apparent \( K_m \) for ABP-N-acetylation was ~4-fold lower using liver cytosols from hNAT2\(^{2+}\)Nat1/2\((--)/--\) mice (Table 2), suggesting that ABP has a higher affinity for human NAT2 than for mouse Nat2. The apparent \( K_m \) values in the transgenic mice are similar to that reported for recombinant wild-type human NAT2 \( (15.9 \pm 1.3 \mu M) \) (Ferguson et al., 1994), whereas the apparent \( K_m \) values in wild-type liver cytosol are similar to that found for recombinant mouse Nat2 \( (42.6 \pm 3.0 \mu M) \) (M. Tychopoulos, unpublished observations). Liver ABP-NAT activities for hNAT2\(^{2+}\)Nat1/2\((--)/--\) male mice, wild-type male mice, and wild-type female mice were comparable to the level of activity \( (~2 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}) \) reported for NAT2 rapid acetylator human hepatocytes (Doll et al., 2010).

PAS N-acetylation activity was not detected (limit of detection, \( 0.003 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \)) in cytosols from any tissue of hNAT2\(^{2+}\)Nat1/2\((--)/--\) mice (data not shown). We have previously detected PAS-NAT activity in wild-type mouse liver with an apparent \( V_{\text{max}} \) of 5.3 to 6.0 nmol \cdot min\(^{-1}\) \cdot mg\(^{-1}\) and an apparent \( K_m \) of 3.9 to 4.0 \mu M (Sugamori et al., 2003).

**In Vivo NAT Activity and Plasma Pharmacokinetics.** The clearance of SMZ was drastically higher in hNAT2\(^{2+}\)Nat1/2\((--)/--\) mice than in wild-type mice, as reflected in a 97 to 98% decrease in AUC of SMZ (Sugamori et al., 1994). The AUC of SMZ was comparable in wild-type mice to that reported for NAT2 rapid acetylator human hepatocytes (Doll et al., 2010).

**FIG. 4.** Cytosolic N-acetylation rates from wild-type and hNAT2\(^{2+}\)Nat1/2\((--)/--\) transgenic mice. Tissue cytosols were assayed for NAT activity with either 0.5 mM SMZ (A) or 0.1 mM ABP (B) in the presence of 0.1 mM acetyl-CoA as described under Materials and Methods. ASMZ and AABP were separated and quantified by HPLC. Detection limits for N-acetylated product formation rates were 0.006 nmol \cdot min\(^{-1}\) \cdot mg\(^{-1}\) for ASMZ and 0.004 nmol \cdot min\(^{-1}\) \cdot mg\(^{-1}\) for AABP. Product formation rates represent the means ± S.D. from \( n = 3 \) animals per gender and genotype, respectively. a, significantly different from male and female wild-type \( (p < 0.001) \); b, significantly different from male transgenic \( (p < 0.001) \); c, significantly different from male and female transgenic \( (p < 0.01) \); d, significantly different from female wild-type \( (p < 0.05) \); e, significantly different from male and female wild-type \( (p < 0.05) \); f, significantly different from male wild-type \( (p < 0.001) \). M, male; F, female.

**TABLE 2**  
Kinetic constants for liver cytosolic SMZ-NAT and ABP-NAT activity  

<table>
<thead>
<tr>
<th>Gender</th>
<th>SMZ</th>
<th>ABP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_m ) ( \mu M )</td>
<td>( v_{\text{max}} ) ( \text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} )</td>
</tr>
<tr>
<td>Male wild-type</td>
<td>N.D.</td>
<td>48.3 ± 2.8</td>
</tr>
<tr>
<td>Female wild-type</td>
<td>N.D.</td>
<td>50.1 ± 0.8</td>
</tr>
<tr>
<td>Male hNAT2(^{2+})Nat1/2((--)/--)</td>
<td>96.5 ± 1.2</td>
<td>2.43 ± 0.21</td>
</tr>
<tr>
<td>Female hNAT2(^{2+})Nat1/2((--)/--)</td>
<td>93.5 ± 3.2</td>
<td>1.40 ± 0.11(^b)</td>
</tr>
</tbody>
</table>

N.D., not determined.  
\(^a\) Significantly different from male and female wild-type \( (p < 0.001) \).  
\(^b\) Significantly different from male transgenic \( (p < 0.001) \).  
\(^c\) Significantly different from male and female wild-type and male transgenic \( (p < 0.01) \).
The influence of the hNAT2 transgene on the in vivo disposition of PAS compared with our previously reported results in Nat1/2(-/-) mice, in which a complete absence of APAS and a 2- to 3-fold increase in the AUC for PAS were observed (Sugamori et al., 2003). The hNAT2 Nat1/2(-/-) mice also displayed no APAS upon the in vivo administration of PAS, suggesting that these mice were unable to N-acetylate PAS, consistent with the finding of no measurable liver cytosolic PAS-NAT activity in these animals. Thus, the AUC values for PAS were significantly increased in both male and female hNAT2 Nat1/2(-/-) mice compared with wild-type mice (p < 0.01), with a complete absence of APAS in plasma (Fig. 6; Table 3). Thus, as with Nat1/2(-/-) mice, the clearance of PAS remained impaired in the hNAT2 Nat1/2(-/-) mice, with a 2-fold increase in AUC (Fig. 7) over that in wild-type mice. In contrast, hNAT2 mice on a wild-type background displayed a profile similar to that of wild-type mice (Figs. 6 and 7). The acetylated metabolite APAS was detected in plasma samples from hNAT2 mice and was rapidly cleared from plasma.

**Discussion**

We have generated a transgenic mouse line that expresses human NAT2 selectively in liver using a construct containing the human NAT2 cDNA fused to the mouse albumin enhancer/promoter. The mouse albumin enhancer/promoter has been shown to direct efficient liver-specific transgene expression (Pinkert et al., 1987) and was chosen because we found it to drive expression of a luciferase reporter in HepG2 cells more effectively than the endogenous human NAT2 promoter. We identified only one viable founder animal, which was found to contain a single copy of the transgene, consistent with previous attempts by others to generate NAT transgenic mice in which only low-copy founders were identified and/or lethality was observed (Cao et al., 2005). It has been postulated that overexpression of human NAT transgenes, particularly human NAT1, may be detrimental dur-

![Fig. 5. Plasma pharmacokinetics of SMZ and ASMZ in wild-type and hNAT2 Nat1/2(-/-) transgenic mice. A, plasma kinetics of SMZ and ASMZ from a representative male wild-type mouse. AUC values for SMZ and ASMZ were 15.1 and 113.5 mmol x min per liter. B, plasma kinetics of SMZ and ASMZ from a representative hNAT2 Nat1/2(-/-) male mouse. AUC values for SMZ and ASMZ were 15.1 and 113.5 mmol x min per liter. C, plasma kinetics of SMZ and ASMZ from a representative wild-type female mouse. AUC values for SMZ and ASMZ were 461.5 and 16.1 mmol x min per liter, respectively. D, plasma kinetics of SMZ and ASMZ from a representative hNAT2 Nat1/2(-/-) female mouse. AUC values for SMZ and ASMZ were 16.4 and 61 mmol x min per liter. For all mice, SMZ (50 mg/kg) was administered by intraperitoneal injection, and four sequential blood samples were drawn from the saphenous vein of each mouse at the indicated time points. Plasma was isolated from each blood sample, diluted 1:50, and analyzed for SMZ and ASMZ by HPLC. AUC values were determined by the trapezoidal rule using the computer program GraphPad Prism. Detection limit for ASMZ was 2 μM.](https://www.aspetjournals.org/article/S0022-3050(07)06790-5/DC1/fig5.png)

TABLE 3

**AUC values for substrate-selective NAT elimination**

AUC values (mean ± S.D., n = 3) were determined by the trapezoidal rule for SMZ and ASMZ by HPLC. AUC values were determined by the trapezoidal rule using the computer program GraphPad Prism. Detection limit for ASMZ was 2 μM. Statistical differences between group mean values were determined by one-way ANOVA followed by the Bonferroni multiple comparison test.

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type hNAT2 Nat1/2(-/-)</th>
<th>Male</th>
<th>Female</th>
<th>Wild-Type hNAT2 Nat1/2(-/-)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMZ</td>
<td>538 ± 15.5 ± 1,200</td>
<td>15.5 ± 1.200</td>
<td>557 ± 92</td>
<td>11.1 ± 4.60</td>
<td>11.9 ± 4.60</td>
<td>17.5 ± 92</td>
</tr>
<tr>
<td>ASMZ</td>
<td>31.5 ± 2.8</td>
<td>107 ± 6.70</td>
<td>18.3 ± 2.4</td>
<td>50.0 ± 10.20</td>
<td>50.0 ± 10.20</td>
<td>70.0 ± 12.0</td>
</tr>
<tr>
<td>ABP</td>
<td>271 ± 39</td>
<td>253 ± 23</td>
<td>215 ± 28</td>
<td>175 ± 20</td>
<td>175 ± 20</td>
<td>175 ± 20</td>
</tr>
<tr>
<td>AABP</td>
<td>6.9 ± 1.3</td>
<td>7.7 ± 0.5</td>
<td>5.9 ± 0.6</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>PAS</td>
<td>15.4 ± 1.5</td>
<td>32.0 ± 4.30</td>
<td>15.7 ± 2.3</td>
<td>32.9 ± 5.3</td>
<td>32.9 ± 5.3</td>
<td>32.9 ± 5.3</td>
</tr>
<tr>
<td>APAS</td>
<td>10.3 ± 2.2</td>
<td>0.00</td>
<td>11.2 ± 1.7</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Significantly different from male and female wild-type (p < 0.001). Significantly different from male and female wild-type (p < 0.05). Significantly different from male transgenic (p < 0.001). Significantly different from male and female wild-type and male transgenic (p < 0.01). Significantly different from male and female wild-type (p < 0.01).
ing development by creating imbalances in the regulation of folate (Cao et al., 2010).

The human NAT2 transgene was found to be inserted into intron 3 of the phosphodiesterase 8B gene. Of interest, a recombination event occurred during insertion of the transgenic construct as determined by inverse PCR. This recombination event caused transposition of most of the albumin enhancer region to the 3′ end of the transgene construct, but it did not appear to markedly impair the expression of hNAT2 in the liver as demonstrated by real-time qPCR, with which hNAT2 transcript expression was found to be the highest in liver but absent in wild-type mouse liver, as well as by Western blot analysis of liver hNAT2 protein and functional in vitro and in vivo metabolism of SMZ.

Transcript levels of Pde8B were reduced by approximately 50%, particularly in the cerebrum and cerebellum. Little is known about the function of this particular phosphodiesterase isoform. Phosphodiesterases are a large family of enzymes that catalyze the degradation of the second messengers cAMP and cGMP and therefore participate in the regulation of signal transduction and subsequent downstream cellular events (Omori and Kotera, 2007). In humans, the Pde8B transcript has been found in several tissues but has the highest expression in the thyroid gland and brain (Lakics et al., 2010). In human liver Pde2A, Pde3B, and Pde8A are the most abundant isoforms present. The presence of multiple phosphodiesterases within human tissues makes it difficult to determine the relative importance of each isoform as there may be redundancy or compensation if multiple isoforms are present. Less is known about Pde8B in the mouse, and a knockout mouse model has not yet been created. However, our transgenic mice breed normally and appear to have no observable phenotype other than changes in drug acetylation.

With respect to in vitro drug metabolism, our hNAT2NAT1/2(−/−) mice appear to display a human-like NAT2 metabolic profile. In contrast to wild-type mice in which liver cytosolic SMZ-NAT
activity is low, SMZ-NAT activity was greatly enhanced in the livers of hNAT2*Nat1/2(−/−) mice to levels comparable to those found in rapid acetylator human livers and cryopreserved human hepatocytes. The apparent \( K_m \) value for SMZ N-acetylation was similar to those reported previously for human liver and recombinant human NAT2 (Grant et al., 1991), whereas the \( K_m \) for ABP was lower in hNAT2*Nat1/2(−/−) mice than in wild-type mice. Both SMZ- and ABP-NAT activities were primarily restricted to the liver of hNAT2*Nat1/2(−/−) mice, whereas all tissues assayed from wild-type mice displayed ABP-NAT activity. A gender difference in ABP-NAT activity was observed for wild-type kidney cytosol, with males displaying a higher activity. This gender difference in kidney has been reported previously for the acetylation of PAS (Sugamori et al., 2003), ABP, and 2-aminofluorene (Sugamori et al., 2006) and for p-amino- benzoic acid, which was shown to be modulated by testosterone (Smolen et al., 1993). Selectivity of the hNAT2 transgene was clearly evident by the absence of detectable enzyme activity toward the NAT1-selective substrate PAS in the livers of hNAT2*Nat1/2(−/−) mice.

We observed an unexpected gender difference in the apparent \( V_{\text{max}} \) for both SMZ and ABP N-acetylation in our transgenic mice, with males displaying almost 2-fold higher activity. Although it is unlikely that the use of the albumin promoter would account for this difference, gender-dependent alterations in hepatic H-ras12V have been observed in transgenic mice expressing the activated human H-ras12V transgene under control of the albumin enhancer/promoter (Wang et al., 2005). H-ras12V transcript expression was significantly higher in male livers from one transgenic mouse line with corresponding higher incidences of hepatocellular carcinoma. A second H-ras12V transgenic line did not exhibit this gender discrepancy. This finding suggests that the site of insertion of our transgene and possibly regulatory elements within this region may be regulated by sex hormone factors, and thus other transgenic hNAT2 mouse lines may not exhibit this gender difference. In any event, the gender difference in metabolism in our transgenic line should be considered when toxic outcomes are assessed.

Despite the presence of low levels of transcript in tissues other than liver, only colon cytosols displayed detectable human NAT2 function as assessed by measuring SMZ- and ABP-NAT activities, although still at 10- to 20-fold lower levels than in liver. This is similar to humans, in whom NAT2 mRNA has also been detected in the small intestine and colon with much lower levels in other tissues (Husain et al., 2000). For assessing the disposition and potential toxic effects of arylamine drugs and procarcinogens without the confounding influence of endogenous mouse NAT activity. These features suggest that our model may be very useful for assessing the disposition and potential toxic effects of arylamine drugs and procarcinogens without the confounding influence of endogenous mouse Nat activity.

Indeed, the utility of humanized transgenic mouse models has clearly been demonstrated with some cytochrome P450 transgenic mice in which species differences in metabolism have been circumvented (Cheung and Gonzalez, 2008). With the use of humanized CYP1A2 mice, the bioactivating N-\(^2\)-hydroxylation of the heterocyclic amine 2-amino-1-methyl-6-phenyl[4,5-b]pyridine (PhIP) was found to be the predominant metabolic pathway (Cheung et al., 2005), in contrast to detoxifying 4'-hydroxylation that occurs in wild-type mice. The 4'-hydroxylation reaction of PhIP that occurs as the primary pathway in wild-type mice would help explain why Cyp1a2-null mice were not protected in the neonatal mouse bioassay from PhIP-induced carcinogenesis (Kimura et al., 2003). Our hNAT2 mice may be further crossed to some of these cytochrome P450-humanized models such as the hCYP1A1/1A2_Cyp1a1/1a2(−/−) mouse (Dragan et al., 2007) to serve as an even better proxy for preclinical toxicity bioassays and human risk assessment.

An alternative animal approach for predicting human responses to chemical challenge are “humanized liver” chimeric mice generated by transplanting human hepatocytes into urokinase-type plasminogen activator-transgenic SCID mice, which are immunodeficient and un-
deger liver failure (Tateno et al., 2004). However, drawbacks to this approach include the ongoing presence of endogenous drug-metabolizing enzymes in other tissues of these animals, and animal-to-animal variability in the replacement index of the hepatocytes. Furthermore, human hepatocytes are in limited supply, making it difficult to generate sufficient chimeric mice from a single donor. Finally, the hepatocytes are transplanted after 20 to 30 days of birth, rendering these mice unsuitable for accelerated carcinogenicity assays such as the neonatal mouse bioassay (Flammang et al., 1997). In contrast, our humanized hNAT2©Nat1/2(–/–) mice can be used in carcinogenicity studies such as the neonatal mouse bioassay to determine tissue-specific tumor incidences after low-dose arylamine or heterocyclic amine exposures, and they can be bred to other humanized drug-metabolizing enzyme transgenic mouse models as mentioned above or to transgenic mutation assay reporter mice such as the MutaMouse (Lambert et al., 2005). Chemical-induced hepatotoxicity, levels of specific carcinogen-DNA adducts, and/or mutation frequencies can then be measured in these mice after controlled exposure to drugs and carcinogens. Thus, our humanized hNAT2©Nat1/2(–/–) mouse model, which mimics the pattern, expression, and metabolic profile of NAT2 in humans, can be used as an effective in vivo model to predict human responses to arylamine chemical challenge.

Acknowledgments
We thank Xiaoli Lu and Shuang Wang for their valuable and excellent technical assistance.

Authorship Contributions
Participated in research design: Sugamori and Grant.
Conducted experiments: Sugamori, Brennan, and Grant.
Performed data analysis: Sugamori and Grant.
Wrote or contributed to the writing of the manuscript: Sugamori and Grant.
Other: Grant acquired funding for the research.

References
Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.

Sugamori and Grant.