Liver-selective expression of human arylamine N-acetyltransferase NAT2 in transgenic mice

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List of Abbreviations: ABP, 4-aminobiphenyl; AABP, N-acetyl-4-aminobiphenyl; AUC, area-under-the-curve; CYP, cytochrome P450; NAT, N-acetyltransferase; OAT, O-acetyltransferase; PAS, p-aminosalicylic acid; APAS, N-acetyl-p-aminosalicylic acid; Pde, phosphodiesterase; SMZ, sulfamethazine; ASMZ, N-acetyl-sulfamethazine.
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 (hNAT2tg Nat1/2-/-), in which liver-selective expression of human NAT2 is driven by the mouse albumin promoter. We detected expression of 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 40-80-fold higher in liver cytosols from hNAT2tg Nat1/2-/- 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), since hNAT2tg Nat1/2-/- mice displayed similar in vivo PAS pharmacokinetic profiles to Nat1/2-/- mice. The metabolism of 4-aminobiphenyl (ABP) was similar between hNAT2tg Nat1/2-/- and wild-type mice with the exception of a more liver-restricted pattern in hNAT2tg Nat1/2-/- mice and lower activity in females. Overall, the hNAT2tg Nat1/2-/- 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 (Neumann, 2007; Weisburger, 2002). 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). While NAT1 is ubiquitously expressed, NAT2 is primarily localized to the liver, with some expression in the colon and small intestine (Husain et al., 2007). Due to the polymorphic nature of these enzymes (Hein, 2009; Sim et al., 2008), 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 (Moore et al., 2011; Nothlings et al., 2009; Rabstein et al., 2010; Sharma et al., 2010). However, these results are confounded by misclassification errors due to phenotype/genotype discordances and imprecise chemical exposure data.

A classical 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 as a primary enzyme involved in this reaction (Kim and Guengerich, 2005), whereas CYP1A1 or peroxidases 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 mutations (Hein, 2002). Increases in C8-deoxyguanosinyl-ABP DNA adducts, hypoxanthine phosphoribosyltransferase (hp rt) mutagenesis and cytotoxicity
to ABP exposure have been observed in CHO cells upon co-expression 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 reactions (Hein, 2006). Thus it is unclear 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 organ-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; Dairou et al., 2009).

While \textit{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 \textit{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 Cyp1a1 null mice are not protected from the toxicity of oral benzo[a]pyrene (Uno et al., 2004). Thus it is imperative to conduct studies \textit{in vivo} where exposure can be controlled, where the influence of tissue-specific metabolism may become apparent, and where indices of toxicity such as DNA damage, cytotoxicity and tumor incidence can be assessed.
Knockout mouse models have thus 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 orthologue of human NAT1 (Kawamura et al., 2008), while 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 IDT (Coralville, 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, AcCoA 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, while AABP was generously provided by Dr. M. Novak (Miami University, Oxford, OH). Anti-rabbit IgG HRP-linked antibody was obtained from GE Healthcare (Baie D’urfe, QC, Canada). Polyclonal rabbit anti-human NAT2 antiserum was previously produced (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 a ~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. A ~1.4 kb HindIII/ClaI fragment consisting of the chimeric intron, human NAT2 and late SV40 poly A was excised from pSI and subcloned into the HindIII and ClaI sites of pBluescript. The 2.3 kb albumin enhancer/promoter region was then subcloned into the SstII and EcoRV sites located 5’ to the chimeric 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 ClaI to release the full-length transgene and with AlwNI 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 Invitrogen Purelink PCR purification kit. The transgene DNA was provided to the Transgenic Mouse Facility at the Hospital for Sick Children for pronuclear microinjection into B6/SJL F2 zygotes by standard techniques. One founder mouse was identified (see below) and the line was backcrossed for 10 generations onto a B6 background to generate the congenic hNAT2<sup>tg</sup> mouse strain. This line was then crossed with our Nat1/2<sup>−/−</sup> double null line (Sugamori et al., 2003) to generate a human NAT2 transgenic line (hNAT2<sup>tg</sup>)
Nat1/2−/−) on a background devoid of endogenous mouse Nat activity. B6 mice served as wild-type controls in all experiments.

**Genotyping of transgenic mice**

Genomic DNA was isolated from mouse tail biopsies as described previously (Sugamori et al., 2003). To identify potential founders and subsequent transgenic progeny, Southern blot analysis with *Bam*H I-, *Eco*R I- and *Kpn* I-digested genomic DNA and/or PCR genotyping were performed. For Southern blotting an 870 bp screening probe corresponding to the human NAT2 sequence was generated by PCR using the primers listed in Table 1 and 30 cycles of amplification consisting of 94°C 10 s, 57°C 10 s, 72°C 30 s. An AlkPhos Direct™ labeling kit (GE Healthcare, Baie D’urfe, QC, Canada) was used for the labeling and hybridization of the probe as per the manufacturer’s instructions. For PCR genotyping and to determine the zygosity of transgenic mice, a duplex PCR method with one forward primer (NAT2-F) and two reverse primers (NAT2-RWT and NAT2-RTG) was subsequently developed (Fig. 1C). The wild-type allele sequence was identified by inverse PCR (see below). The presence of one band of 499 bp indicates a wild-type animal, two bands of 499 bp (wild-type) and 652 bp (transgenic) indicate a hemizygous transgenic animal, and the presence of only the 652 bp band indicates the presence of two transgenic alleles, and thus a transgenic homozygote.

**Inverse PCR**

An inverse PCR strategy was used to identify the transgene insert location in the mouse genome. Genomic DNA from a transgenic animal was digested with the frequent cutter *Rsa* I, self-ligated with T4 DNA ligase to circularize products, digested with *Bam*H I to linearize, and
subjected to PCR amplification with primers (hNAT2-IPF and hNAT2-IPR) located within the
RsaI-digested transgenic sequence but annealing in opposite directions so as to amplify unknown
sequence flanking the insertion site. A 970 bp fragment was amplified, gel-purified using an
Invitrogen Purelink gel purification kit according to the manufacturer’s instructions, and sent for
dNA sequencing. The resulting sequence was compared to known mouse DNA sequences using

**RT-PCR determination of human NAT2 expression**

Total RNA was prepared from various tissue sources (liver, kidney, colon, cerebrum,
cerebellum and spleen) using Trizol (Invitrogen). Approximately 1 μg of total RNA was treated
with DNase for 30 min at 37°C, heat-inactivated in the presence of 2.5 mM EDTA for 10 min at
65°C, and reverse transcribed using a RevertAid cDNA synthesis 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 of either human
NAT2, mouse Pde8B or mouse GAPDH primers and 1x 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 to mouse GAPDH to ensure equivalent primer efficiencies between the
target and reference genes. The ΔΔCₜ method was used to determine the relative quantification
(RQ) 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 RQ of 1.
**Western Blot Analysis**

Male and female liver cytosols (10 μg protein) from hNAT2tg Nat1/2−/− and Nat1/2−/− mice were run on a 12% SDS-PAGE 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 vitro NAT activity**

Tissue cytosols were prepared from wild-type and transgenic animals as described (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 performed 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 AcCoA regenerating system in a final reaction volume of 100 μl. The regenerating system consisted of 5 mM acetyl-DL-carnitine and 1 U carnitine acetyltransferase per milliliter of assay buffer (250 mM triethanolamine-HCl, 5 mM EDTA, 5 mM dithiothreitol, pH 7.5). Reactions were preincubated for 3 min at 37°C and initiated with the addition of 50 μl of cytosol diluted to a protein concentration of 1 mg/ml. Reactions were incubated for 10 min (ABP, PAS) or 30 min (SMZ) at 37°C and terminated by the addition of 10 μl of 15% perchloric acid. After precipitation of the denatured protein, the supernatant fractions were assayed for the N-acetylated product by HPLC using a Shimadzu LC-2010A System (Mandel Scientific Company Inc., Guelph, ON, Canada) and a reverse-phase Beckman Ultrasphere ODS 5 μM column (15 cm x 4.6 mm I.D.; Beckman Instruments, Fullerton, CA) as described previously (Sugamori et al., 2006).
For kinetic determinations with SMZ (10-1500 μM), 0.1 mM AcCoA and 20 μl of regenerating system were incubated in duplicate with liver cytosol (~100 μg of total protein) as described above. For ABP, 5-1000 μM (B6) or 2.5-400 μM (hNAT2tgxNAT1/2/-/-) and ~20-25 μg of total protein was used. Apparent $K_m$ and $V_{max}$ values were determined by nonlinear regression of the standard Michaelis-Menten kinetic equation using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

**In Vivo Drug Administration and Plasma Elimination Kinetics**

PAS (50 mg/kg) or SMZ (50 mg/kg) dissolved in saline, or ABP (50 mg/kg) dissolved in DMSO was administered by i.p. injection to 8 week-old age- and sex-matched transgenic or wild-type mice (n=3). Serial blood samples were drawn from the saphenous vein using heparinized microvettes (Sartstedt Inc., Montreal, QC, Canada) at four time points for each animal and centrifuged to separate plasma. The time points for ABP and SMZ plasma sampling were 2, 6, 22 and 24 hr, and PAS plasma sampling times were 15, 30, 60 and 90 min. Plasma samples were diluted 1:50 in HPLC mobile phase and analyzed for parent and acetylated metabolites by HPLC as described above. Blank plasma samples were spiked with known amounts of SMZ and ASMZ, ABP and AABP or PAS and APAS to quantify the amount of parent and acetylated products present in the samples. Area-under-the-curve (AUC) values were determined by the trapezoidal rule using GraphPad Prism (GraphPad Software Inc., San Diego, CA).
Statistical Analyses

Data are presented as 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 Bonferroni’s multiple comparison test using GraphPad Prism (GraphPad Software Inc., San Diego, CA).
RESULTS

Generation of hNAT2tg Nat1/2-/- transgenic mouse line

Using luciferase promoter plasmid constructs, we found the albumin promoter to be 5-10-fold more effective than the endogenous human NAT2 promoter in driving luciferase expression in HepG2 cells when co-expressed with the liver-enriched transcription factors C/EBPα 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 7 pups generated from microinjection of B6/SJL F2 pronuclei identified one founder animal. Southern blot analysis of BamH I-, EcoR I- and Kpn I-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 kb and ~8 kb on the Southern blot of Kpn I-digested DNA. A Kpn I 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 Kpn I-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 hNAT2tg mice were backcrossed to wild-type B6 mice for 10 generations to generate the transgenic line on a congenic B6 background. The congenic hNAT2tg mice were subsequently crossed to our Nat1/2-double null mouse line (Nat1/2-/-) to generate hNAT2tg Nat1/2-/- mice on a background devoid of endogenous mouse Nat activity, since mouse Nat3 does not contribute to N-acetylation in the mouse (Sugamori et al., 2007).
Identification of human NAT2 transgene insertion site

Inverse PCR of Rsa I-digested DNA and BLAST analysis of the resultant sequence indicated that the human NAT2 transgene was inserted into intron 3 of the mouse phosphodieserase 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 Kpn I site which is located in intron 3 of the Pde8B gene upstream of the start of the recombined transgene sequence, whereas the ~8 kb band is due to the presence of a Kpn I site located 5.4 kb downstream of the recombined sequence in intron 3. Similarly, the ~2.4 kb EcoR I band observed on the Southern blot can be accounted for by the presence of an EcoR I site at the 5’ end of the human NAT2 coding region and an EcoR I site located ~1.2 kb within the albumin enhancer region (Fig. 1D) that was transposed to the 3’ end of the SV40 late poly A region. The other high molecular weight BamH I, EcoR I and Kpn I bands that hybridized to the hNAT2 probe and were found in both the founder and a non-transgenic 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 hNAT2tg Nat1/2-/- mice and subjected to real-time qPCR to determine the tissue specificity of hNAT2 transgene expression. 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 about two-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 \textit{Pde8B} intron 3 region affected the expression of the \textit{Pde8B} gene, real-time qPCR was performed on tissues from both wild-type and transgenic mice. \textit{Pde8B} transcript was found to be highest in cerebrum in both wild-type and transgenic animals (Fig. 2B). Significant \textit{Pde8B} expression was also found in the cerebellum, colon and kidney. Liver and spleen had the lowest expression of \textit{Pde8B}. Compared to wild-type animals, transgenic animals had a ~40-55\% decrease in the level of \textit{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\textsuperscript{-/-} 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\textsuperscript{-/-} mice. Similar to the hNAT2 qPCR results, hNAT2 transgenic male liver had a higher level of hNAT2 protein than female liver.

**In vitro NAT activity**

The presence of functional human NAT2 enzyme was assessed by measuring cytosolic SMZ-NAT activity. Homozygous transgenic animals on a mouse Nat1/2 null background had a ~40-80 fold higher SMZ-NAT activity than wild-type mice (Fig. 4A). A gender difference in SMZ \textit{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\textsuperscript{tg} Nat1/2\textsuperscript{-/-} mice, only colon had detectable levels of SMZ \textit{N}-acetylation with a ~7-fold increase above wild-type levels, although they were still 15-20-fold lower than liver activities (Fig. 4A). Only low levels of SMZ activity (<0.03 nmol/min/mg...
protein) 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$^{tg}$ 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 $V_{max}$ 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 to 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$^{tg}$ 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$^{tg}$ 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±1.3 μM; (Ferguson et al., 1994), while the apparent $K_m$ values in wild-type liver cytosol are similar to that found for recombinant mouse Nat2 (42.6±3.0 μM; unpublished results). Liver ABP-NAT activities for hNAT2$^{tg}$ Nat1/2$^{−/−}$ male mice, wild-type male and wild-type female mice were comparable to the level of activity (~2 nmol/min/mg) reported for NAT2 rapid acetylator human hepatocytes (Doll et al., 2010).

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

**In vivo NAT activity and plasma pharmacokinetics**

The clearance of SMZ was drastically higher in hNAT2tg Nat1/2−/− mice than in wild-type mice, as reflected in a 97-98% decrease in AUC values (Table 3, Fig. 7). AUC values for ASMZ were also increased compared to wild-type (Fig. 5), indicating that the transgenic mice were clearing SMZ by $N$-acetylation more efficiently than wild-type animals.

The influence of the hNAT2 transgene on the *in vivo* disposition of ABP and AABP was less apparent. AUC values for ABP and AABP were similar in wild-type and hNAT2tg Nat1/2−/− mice, although female transgenic animals did have a smaller AUC for AABP ($p < 0.01$). The clearance of ABP appeared to be less dependent on the formation of AABP, as determined by the small AUC values for AABP compared to ABP in both the wild-type and transgenic animals and the lack of a significant change in the AUC values for ABP in the transgenic compared to wild-type animals (Table 3). The lack of a significant difference between wild-type and transgenic animals for ABP AUC values indicates that $N$-acetylation does not appear to play a major role in the *in vivo* elimination of ABP even when human NAT2 is expressed in mouse liver at levels comparable to those observed in human liver.

In contrast, the presence of hNAT2 in the liver of Nat1/2−/− mice (hNAT2tg Nat1/2−/−) was unable to influence the *in vivo* disposition of PAS compared to our previously reported results in Nat1/2−/− mice in which a complete absence of APAS and a two to three-fold increase in the AUC for PAS were observed (Sugamori et al., 2003). The hNAT2tg 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. As such, the AUC values for PAS were significantly increased in both male and female hNAT2<sup>tg</sup> Nat1/2<sup>-/-</sup> mice compared to wild-type mice (<i>p</i> < 0.01), with a complete absence of APAS in plasma (Fig. 6, Table 3). Thus as with Nat1/2<sup>-/-</sup> mice, the clearance of PAS remained impaired in the hNAT2<sup>tg</sup> Nat1/2<sup>-/-</sup> mice, with a two-fold increase in AUC (Fig. 7) over wild-type. In contrast, hNAT2<sup>tg</sup> mice on a wild-type background displayed a profile similar to wild-type mice (Fig 6, Fig 7). The acetylated metabolite APAS was detected in plasma samples from hNAT2<sup>tg</sup> 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 since 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, which is 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 over-expression of human NAT transgenes, particularly human NAT1, may be detrimental during 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. Interestingly, 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, where 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 about 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, 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. Our transgenic mice, however, breed normally and appear to have no observable phenotype other than changes in drug acetylation.

With respect to in vitro drug metabolism, our hNAT2\textsuperscript{tg} Nat1/2\textsuperscript{-/-} 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\textsuperscript{tg} Nat1/2\textsuperscript{-/-} 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), while the $K_m$ for ABP was lower in hNAT2\textsuperscript{tg} Nat1/2\textsuperscript{-/-} mice than in wild-type mice. Both SMZ- and ABP-NAT activities were primarily restricted to the liver of hNAT2\textsuperscript{tg} Nat1/2\textsuperscript{-/-} 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 AF (Sugamori et al., 2006), and for PABA, 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 towards the NAT1-selective substrate PAS in the livers of hNAT2\textsuperscript{tg} Nat1/2\textsuperscript{-/-} 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 two-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-ras\textsuperscript{12V} have been observed in transgenic mice expressing the activated human H-ras\textsuperscript{12V} transgene under control of the albumin enhancer/promoter (Wang et al., 2005). H-ras\textsuperscript{12V} transcript expression was significantly higher in male livers from one transgenic mouse line with corresponding higher incidences of hepatocellular carcinoma. A second H-ras\textsuperscript{12V} transgenic line did not exhibit this gender discrepancy. This 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 assessing toxic outcomes.

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 10-20-fold lower levels than in liver. This is similar to humans, where NAT2 mRNA has also been detected in the small intestine and colon with much lower levels in other tissues (Husain et al., 2007), and where SMZ-NAT enzyme activity has been detected in intestine (Hickman et al., 1998). It is unclear why there would be human NAT2 expression in the colon in our mice. However, it would not be unusual for some ectopic expression to occur in other tissues, perhaps depending on the site of integration. Also, the tissue selectivity of some presumably tissue-specific promoters is not absolute, and will depend on the
relative tissue levels of normally liver-enriched transcription factors such as DBP and C/EBPα, which are known to govern the activity of the mouse albumin promoter and likely drive the human NAT2 promoter as well (unpublished observations).

To assess the effect of liver-selective hNAT2 expression on the disposition of NAT-selective substrates without the influence of endogenous mouse Nat1 and Nat2, we performed in vivo pharmacokinetic studies using various NAT-selective substrates. Similar to our in vitro characterization of hNAT2 liver cytosolic enzyme activity, SMZ was the substrate whose disposition was most affected by the presence of human NAT2 in mouse liver. The clearance of SMZ was greatly enhanced in the transgenic mice and appeared to resemble the in vivo pharmacokinetic profile of SMZ given to human NAT2 rapid acetylators (Chapron et al., 1980). The increase in the AUC for ASMZ in these mice indicates that the N-acetylation of SMZ plays a more significant role in its elimination than it does in wild-type mice.

The in vivo pharmacokinetics of ABP, however, were not significantly affected by human NAT2 in our hNAT2$^{\ast4}$ Nat1/2$^{-/-}$ mice compared to wild-type mice, indicating that human NAT2 in our model behaves similarly to mouse Nat2, the orthologue to human NAT1. It will be interesting to determine whether human NAT2 differs from mouse Nat2 with respect to OAT activity in cytosols prepared from these animals, or whether biomarkers of DNA damage such as ABP-DNA adducts levels differ between ABP-dosed wild-type and hNAT2$^{\ast4}$ Nat1/2$^{-/-}$ animals. Increased cytotoxicity and ABP-DNA adducts were observed when human NAT2*4 was co-expressed with CYP1A1 in repair-deficient ABP-treated CHO cells (Bendaly et al., 2009), and ABP-NAT and $N$-hydroxy-ABP-OAT activities have been predominantly associated with human NAT2 acetylator phenotype (Doll et al., 2010).
The human NAT2 selectivity of our model was further confirmed by the disposition of the human NAT1-selective substrate PAS in our hNAT2tg Nat1/2-/- mice. No APAS was detected in the plasma of hNAT2tg Nat1/2-/- mice, and we saw an increased AUC for PAS, similar to Nat1/2-/- mice (Sugamori et al., 2003). This indicates that our model is selective for human NAT2 enzyme activity, and that the acetylation of PAS in the mouse is dependent on the presence of endogenous mouse Nat2.

Other human NAT transgenic mouse models have been reported, including a human NAT2 prostate-expressing transgenic mouse that was generated to test the role of human NAT2 in the localized bioactivation of heterocyclic amines within the prostate (Leff et al., 1999). Another human NAT2 transgenic mouse model, constructed using the CMV promoter, yielded only a two-fold increase in NAT activity for isoniazid (Cao et al., 2005). Studies attempting to express human NAT1 in transgenic mice have succeeded in producing only low levels of expression, suggesting that high levels of human NAT1 either alone or in combination with endogenous mouse Nat2 activity may be detrimental to the animal (Cao et al., 2010). Our transgenic mouse expresses human NAT2 selectively in the liver, and at levels very similar to those seen in the livers of human rapid acetylators. 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 (CYP) transgenic mice in which species differences in metabolism have been circumvented (Cheung and Gonzalez, 2008). Using humanized CYP1A2 mice, the bioactivating $N^2$-hydroxylation of the heterocyclic amine 2-amino-1-methyl-6-phenyl[4,5-\textit{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
CYP-humianized models such as the hCYP1A1_1A2 Cyp1a1/1a2(-/-) mouse (Dragin 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 undergo 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 20-30 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 hNAT2tg Nat1/2+/- mice can be used in carcinogenicity
studies such as the neonatal mouse bioassay to determine tissue-specific tumor incidences
following 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. As such, our humanized hNAT2tg 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.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Sugamori, Grant

Conducted experiments: Sugamori, Brenneman, Grant

Performed data analysis: Sugamori, Grant

Wrote or contributed to the writing of the manuscript: Sugamori, Grant

Other: Grant acquired funding for the research
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FOOTNOTES

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**LEGENDS FOR FIGURES**

**Fig. 1.** Generation of hNAT2 transgenic mice.  
A, Schematic representation of the 3.7 kb hNAT2 transgene construct injected into B6/SJL pronuclei. Alb E, albumin enhancer region; Alb P, albumin promoter; pA, SV40 late polyadenylation. The location of the probe used for Southern blot analysis is indicated above.  
B, Southern blot analysis of *BamH I-*, *EcoR I-* and *Kpn I*-digested genomic DNA from the transgenic founder and a non-transgenic 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 bp 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-RWT) in *Pde8B* intron 3 and the transgenic allele (NAT2-F and NAT2-RTG) in the *Pde8B* intron 3 and hNAT2 are indicated by the arrows.

**Fig. 2.** Real-time qPCR detection of hNAT2 and Pde8 transcripts in tissues from hNAT2tg 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_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).
**Fig. 3.** Western blot analysis using mouse liver cytosol (10 μg) from male and female hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> transgenic or Nat1/2<sup>−/−</sup> mice.

**Fig. 4.** Cytosolic N-acetylation rates from wild-type and hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> 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 AcCoA, as described in Materials and Methods. ASMZ and AABP were separated and quantified by HPLC. Detection limits for N-acetylated product formation rates were 0.006 nmol/min/mg for ASMZ and 0.004 nmol/min/mg for AABP. Product formation rates represent the means ± SD from n=3 animals per gender and genotype, respectively. <sup>a</sup>, significantly different from male and female wild-type (p < 0.001); <sup>b</sup>, significantly different from male transgenic (p < 0.001); <sup>c</sup>, significantly different from male and female wild-type (p < 0.01); <sup>d</sup>, significantly different from female wild-type (p < 0.05); <sup>e</sup>, significantly different from male and female wild-type (p < 0.05); <sup>f</sup>, significantly different from male wild-type (p < 0.001).

**Fig. 5.** Plasma pharmacokinetics of SMZ and ASMZ in wild-type and hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> transgenic mice. A, Plasma kinetics of SMZ and ASMZ from a representative male wild-type mouse. AUC values for SMZ and ASMZ were 519.6 and 30.4 mmol min/l. B, plasma kinetics of SMZ and ASMZ for a representative hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> male mouse. AUC for SMZ and ASMZ were 15.1 and 113.5 mmol min/l. 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 min/l, respectively. D, plasma kinetics of SMZ and ASMZ from a representative hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> female mouse. AUC values for SMZ and ASMZ were 16.4 and 61 mmol min/l. For all mice, SMZ (50 mg/kg) was administered by i.p. 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 (GraphPad Software Inc., San Diego, CA). Detection limit for ASMZ was 2 μM.

Fig. 6. Plasma pharmacokinetics of PAS and APAS in wild-type, hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> and hNAT2<sup>tg</sup> transgenic mice. A, Plasma kinetics of PAS and APAS from a representative wild-type mouse. AUC values for PAS and APAS were 13.9 and 9.3 mmol min/l. B, plasma kinetics of PAS and APAS for a representative hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> male mouse. AUC for PAS was 27.7 mmol min/l. C, plasma kinetics of PAS and APAS from a representative hNAT2<sup>tg</sup> male mouse. AUC values for PAS and APAS were 16.7 and 11.8 mmol min/l. D, plasma kinetics of PAS and APAS from a representative wild-type female mouse. AUC values for PAS and APAS were 13.3 and 9.5 mmol min/l, respectively. E, plasma kinetics of PAS and APAS from a representative hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> female mouse. AUC value for PAS was 31.6 mmol min/l. F, plasma kinetics of PAS and APAS for a representative hNAT2<sup>tg</sup> female mouse. AUC values for PAS and APAS were 14.5 and 10.9 mmol min/l. For all mice, PAS (50 mg/kg) was administered by i.p. 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 PAS and APAS by HPLC. Detection limit for APAS was 9 μM.

Fig. 7. Graphical representation of AUC values for hNAT2<sup>tg</sup> Nat1/2<sup>−/−</sup> and hNAT2<sup>tg</sup> mice plotted as a percent of wild-type AUC.
### TABLE 1

**PCR Primers**

<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>ATCGAATTGGCATATGGACATTGAAGCAT</td>
</tr>
<tr>
<td>hNAT2 3’</td>
<td>antisense</td>
<td>ATTCGCCCTAAATAGTAAGGGATCCATCAC</td>
</tr>
<tr>
<td>NAT2-F</td>
<td>sense</td>
<td>TCCCTGACCTGAAGAAGGTG</td>
</tr>
<tr>
<td>NAT2-RWT</td>
<td>antisense</td>
<td>CCTGTGTAAAGAACGACAAAAACC</td>
</tr>
<tr>
<td>NAT2-RTG</td>
<td>antisense</td>
<td>TACTTACCTGCCCAGTGCCCT</td>
</tr>
<tr>
<td>hNAT2-IPF</td>
<td>sense</td>
<td>TTGCCTTTCTCTTGACAGGT</td>
</tr>
<tr>
<td>hNAT2-IPR</td>
<td>antisense</td>
<td>TCTGGAGCTGTTCCTCTCTCTGTTTCCCTCTA</td>
</tr>
</tbody>
</table>

**Real-time Primers:**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNAT2 F RT</td>
<td>sense</td>
<td>GTCGATGCTGTTGCTGATGAA</td>
</tr>
<tr>
<td>hNAT2 R RT</td>
<td>antisense</td>
<td>GGCACCTGAGGCTGATCTCC</td>
</tr>
<tr>
<td>mPde8B F RT</td>
<td>sense</td>
<td>GTGATGCCTAGAGCGAGCTACA</td>
</tr>
<tr>
<td>mPde8B R RT</td>
<td>antisense</td>
<td>GATGGGAGCTCAATGCCATCAT</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>sense</td>
<td>CAGCCTCGTCCCGTACTAAGAAA</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>antisense</td>
<td>CGCCCAATACGGGCCAAA</td>
</tr>
</tbody>
</table>

PCR primers were used to generate the hNAT2 cDNA for the transgene construction, Southern blotting screening probe, for PCR genotyping hNAT2 transgene-expressing mice, for inverse PCR to determine the site of transgene integration, and for RT-PCR of hNAT2-, mPde8B-, GAPDH-specific products from the various tissue sources. Conditions used for PCR amplification are described in the text.
## 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>\text{Apparent}</td>
<td>\text{Apparent}</td>
</tr>
<tr>
<td></td>
<td>\text{(K_m)}</td>
<td>\text{(V_{max})}</td>
</tr>
<tr>
<td>Male wild-type</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Female wild-type</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Male hNAT2\textsuperscript{tg} Nat1/2\textsuperscript{-/-}</td>
<td>96.5±1.2</td>
<td>2.43±0.21</td>
</tr>
<tr>
<td>Female hNAT2\textsuperscript{tg} Nat1/2\textsuperscript{-/-}</td>
<td>93.5±3.2</td>
<td>1.40±0.11\textsuperscript{c}</td>
</tr>
</tbody>
</table>

Liver cytosols (n=3-4) were incubated with an AcCoA regenerating system, 0.1 mM acetyl-CoA and SMZ (10-1500 μM), or ABP (5-1000 μM) for wild-type or (2.5-400 μM) for hNAT2\textsuperscript{tg} Nat1/2\textsuperscript{-/-} mice as described under Material and Methods. The values represent the means ± S.D. Apparent \(K_m\) (μM) and \(V_{max}\) (nmol/min/mg) values were determined using nonlinear regression of the standard Michaelis-Menten kinetic equation with GraphPad Prism. N.D., not determined. \(a\), significantly different from male and female wild-type and male transgenic (\(p < 0.01\)); \(b\), significantly different from male and female wild-type (\(p < 0.001\)); \(c\), significantly different from male transgenic (\(p < 0.001\)). Statistical differences between group mean values were determined using either a Student’s \(t\)-test or a one-way ANOVA followed by Bonferroni’s Multiple Comparison Test.
### TABLE 3

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

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild-type</td>
<td>hNAT2&lt;sup&gt;tg&lt;/sup&gt; Nat1/2&lt;sup&gt;-/-&lt;/sup&gt;</td>
</tr>
<tr>
<td>SMZ</td>
<td>538±17</td>
<td>15.5±1.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ASMZ</td>
<td>31.5±2.8</td>
<td>107±6.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ABP</td>
<td>271±39</td>
<td>253±23</td>
</tr>
<tr>
<td>AABP</td>
<td>6.9±1.3</td>
<td>7.7±0.5</td>
</tr>
<tr>
<td>PAS</td>
<td>15.4±1.5</td>
<td>32.0±4.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>APAS</td>
<td>10.3±2.2</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

AUC values (mmol min l<sup>-1</sup>, mean ± SD, n=3) were determine by the trapezoidal rule. ABP, SMZ or PAS (50 mg/kg) was dosed by i.p. injection to age- and sex-matched wild-type and hNAT2<sup>tg</sup> Nat1/2<sup>-/-</sup> mice. Four sequential blood samples were drawn and analyzed for parent and metabolite compounds as described under Materials and Methods.  

- <sup>a</sup>, significantly different from male and female wild-type (<i>p</i> < 0.05);  
- <sup>b</sup>, significantly different from male and female wild-type (<i>p</i> < 0.01);  
- <sup>c</sup>, significantly different from male and female wild-type (<i>p</i> < 0.001);  
- <sup>d</sup>, significantly different from male transgenic (<i>p</i> < 0.001);  
- <sup>e</sup>, significantly different from male and female wild-type and male transgenic (<i>p</i> < 0.01). Statistical differences between group mean values were determined by one-way ANOVA followed by Bonferroni’s Multiple Comparison Test.
Fig. 1
Fig. 2
Fig. 3
Figure 4

(A) ASMZ formation rate (nmol/min/mg) for wild-type and hNAT2°Nat1/2± mice in different tissues: liver, kidney, colon, cerebrum, and spleen. Significant differences are indicated by subscripts a, b, c, d, e, f, and g.

(B) AABP formation rate (nmol/min/mg) for wild-type and hNAT2°Nat1/2± mice in different tissues. Significant differences are indicated by subscripts a, b, and c.
Fig. 5
Fig. 6