Phenotype of the Most Common “Slow Acetylator” Arylamine *N*-Acetyltransferase 1 Genetic Variant (*NAT1*14B*) Is Substrate-Dependent

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Received July 20, 2011; accepted October 18, 2011

ABSTRACT:

Human arylamine *N*-acetyltransferase 1 (*NAT1*) is a phase II cytosolic enzyme responsible for the activation or deactivation of many arylamine compounds including pharmaceuticals and environmental carcinogens. *NAT1* is highly polymorphic and has been associated with altered risk toward many cancers. *NAT1*14B* is characterized by a single nucleotide polymorphism in the coding region (rs4986782; 560G>A; R187Q). *NAT1*14B* is associated with higher frequency of smoking-induced lung cancer and is the most common “slow acetylator” arylamine *NAT1* genetic variant. Previous studies have reported decreased *N*- and *O*-acetylation capacity

and increased proteasomal degradation of *NAT1* 14B compared with the referent, *NAT1* 4. The current study is the first to investigate *NAT1*14B* expression using constructs that completely mimic *NAT1* mRNA by including the 5'- and 3'-untranslated regions, together with the open reading frame of the referent, *NAT1*4*, or variant, *NAT1*14B*. Our results show that *NAT1* 14B is not simply associated with “slow acetylation.” *NAT1* 14B-catalyzed acetylation phenotype is substrate-dependent, and *NAT1* 14B exhibits higher *N*- and *O*-acetylation catalytic efficiency as well as DNA adducts after exposure to the human carcinogen 4-aminobiphenyl.

Introduction

Human arylamine *N*-acetyltransferase 1 (*NAT1*) is a phase II cytosolic enzyme responsible for the biotransformation of many arylamine compounds including pharmaceuticals and environmental carcinogens (Hein et al., 2000). *NAT1* catalyzes both arylamine *N*-acetylation and hydroxyarylamine *O*-acetylation. Genetic polymorphisms in *NAT1* can alter the amount of *NAT1* protein and result in modified enzymatic activity. In addition to bioactivation of arylamines, studies have provided evidence that *NAT1* is involved in density-dependent cell growth and survival. Studies have also shown that overexpression of *NAT1* increased density-dependent cell proliferation, whereas knockdown of *NAT1* resulted in marked change in cell morphology, an increase in cell-cell contact inhibition, and a loss of cell viability at confluence (Adam et al., 2003; Tiang et al., 2011).

This work was supported by the National Institutes of Health National Cancer Institute [Grant R01-CA034627]; the National Institutes of Health National Institute for Environmental Health Sciences [Grants T32-ES011564, P30-ES014443]; and the Department of Defense Breast Cancer Research Program [Grant BC083107].

Portions of this work constitute partial fulfillment of the requirements for the degree of Doctor of Philosophy in pharmacology and toxicology: Millner LM (2011) *Functional Analysis of N-Acetyltransferase (NAT1*14B and NAT1*10) in Complete NATb and NATa mRNA*. Doctoral dissertation, University of Louisville, Louisville, KY.

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

<http://dx.doi.org/10.1124/dmd.111.041855>.

Molecular epidemiological studies have reported associations between *NAT1* genetic polymorphisms and altered risk for developing several types of cancer including urinary bladder (Gago-Dominguez et al., 2003), breast (Millikan et al., 1998; Zheng et al., 1999; Ambrosone et al., 2007), colorectal (Bell et al., 1995; Lilla et al., 2006), lung (Wikman et al., 2001), non-Hodgkin lymphoma (Morton et al., 2006), and pancreatic (Li et al., 2006). The only known endogenous *NAT1* substrate is *p*-aminobenzoyl-glutamate, a catabolite of folate (Wakefield et al., 2007). *NAT1* has been associated with various birth defects (Lammer et al., 2004; Jensen et al., 2005) that may be related to deficiencies in folate metabolism. The most common *NAT1* variant allele associated with reduced acetylator phenotype is *NAT1*14B*. The allelic frequency for *NAT1*14B* in the Lebanese population was determined to be 23.8% (Dhaini and Levy, 2000), whereas American, German, French, and Canadian *NAT1*14B* allelic frequencies are less than 5% (Doll and Hein, 2002). *NAT1*14B* is likely to be very prevalent in other countries in the Middle East; however, allelic frequencies for many of those populations are not available. *NAT1*14B* has been associated with an increased risk of smoking-induced lung cancer (Bouchardy et al., 1998).

*NAT1*14B* is characterized by a single nucleotide polymorphism G560A (rs4986782) located in the open reading frame (ORF). G560A results in an amino acid substitution R187Q. Computational homology modeling based on the *NAT1* crystal structure indicates that the side chain of R187 is partially exposed to the domain II beta barrel, the protein surface, and the active site pocket (Walraven et al., 2008).

ABBREVIATIONS: *NAT1*, *N*-acetyltransferase 1; CoASAc, acetyl CoA; PABA, *p*-aminobenzoic acid; UTR, untranslated region; ABP, 4-aminobiphenyl; HPLC, high-performance liquid chromatography; *N*-OH-ABP, *N*-hydroxy-4-aminobiphenyl; ORF, open reading frame; CHO, Chinese hamster ovary; FRT, Flp recombination target; α -MEM, α -modified minimal essential medium; ACN, acetonitrile; dG, deoxyguanosine; PBS, phosphate-buffered saline.

Interactions with these domains serve to stabilize the protein and help shape the active site pocket. The substitution of arginine for glutamine results in at least partial loss of these stabilizing hydrogen bonds, resulting in destabilization of the NAT1 structure. Therefore, homology modeling predicts that NAT1 binding of CoASAc, active site acetylation, substrate specificity, and catalytic activity could be affected by the R187Q substitution (Walraven et al., 2008).

Previous studies have reported NAT1*14B to be associated with a reduced N-acetylation phenotype. For example, in peripheral blood mononuclear cells, NAT1 14B was reported to result in reduced N-acetyltransferase activities and protein levels (Hughes et al., 1998). Recombinant NAT1 14B expression in yeast demonstrated reduced N- and O-acetylation, reduced protein levels, and increased proteasomal degradation (Fretland et al., 2001, 2002; Butcher et al., 2004). NAT1 14 expressed in mammalian cells also resulted in decreased V_{max} but increased substrate K_m toward *p*-aminobenzoic acid (PABA) (Zhu and Hein, 2008).

Modifications in NAT1 protein activity are biologically relevant because formation of DNA adducts, tumor growth, and drug resistance could be altered by differences in enzymatic activity. This study reports findings in constructs that completely mimic NAT1 mRNA by including the 5'-untranslated region (UTR) transcribed by the major promoter (NATb), 3'-UTR and ORF of the referent, NAT1*4, and of the most common allele associated with reduced acetylation, NAT1*14B. This report describes NAT1 14B N-acetylation of the urinary bladder carcinogen 4-aminobiphenyl (ABP) and O-acetylation of *N*-hydroxy-4-aminobiphenyl (*N*-OH-ABP). Initial pilot experiments were conducted after recombinant expression in yeast (*Schizosaccharomyces pombe*) followed by more detailed studies using recombinant expression in Chinese hamster ovary (CHO) cells. ABP is present in both mainstream (up to 23 ng/cigarette) and side-stream (up to 140 ng/cigarette) smoke (Hoffmann et al., 1997). Although strict federal regulations have banned industrial uses of ABP (IARC, 1987), it can still be found as a contaminant in color additives, paints, food colors, leather, textile dyes, diesel-exhaust particles, cooking oil fumes, and commercial hair dyes (Nauwelaers et al., 2011).

Materials and Methods

Experiments in Yeast. *In situ* N-acetylation after recombinant expression of human NAT1 in yeast. The ORFs of NAT1*14B and NAT1*4 were recombinantly expressed in the pESP-3 yeast (*S. pombe*) expression system (Agilent Technologies, Santa Clara, CA). They were cultured in YES media (Teknova, Hollister, CA; 0.5% yeast extract, 3.0% glucose, 0.0225% adenine, 0.0225% histidine, 0.0225% leucine, 0.0225% uracil, and 0.0225% lysine). To ensure that the amount of cells expressing NAT1*4 and NAT1*14B was the same, both cell cultures were grown to an optical density (OD) of 0.40. Cell numbers were calculated on the basis of OD, using the conversion of 1.0 OD (600 nm) corresponds to 2×10^7 cells (Agilent Technologies). Aliquots (10 ml) from both the NAT1*4- and the NAT1*14B-expressing cultures were each treated with ABP to make total volume concentrations of 10, 50, and 100 μ M ABP. Samples (100 μ l) were collected after a 30-min incubation with ABP. N-acetyl-ABP was separated and quantified by high-performance liquid chromatography (HPLC) as described previously (Hein et al., 2006).

Experiments in CHO Cells. *Polyadenylation site removal.* The bovine growth hormone polyadenylation site from the pcDNA5/Flp recombination target (FRT) (Invitrogen, Carlsbad, CA) vector was removed to allow the endogenous NAT1 polyadenylation sites to be active. This was accomplished by digestion of pcDNA5/FRT at 37°C with restriction endonucleases, ApaI and SphI (New England Biolabs, Ipswich, MA), followed by overhang digestion with T4 DNA polymerase (New England Biolabs) and ligation with T4 ligase (New England Biolabs).

*Preparation of NATb/NAT1*4 construct.* NATb/NAT1*4 construct was created using gene splicing via overlap extension (Horton et al., 1989) by amplifying the 5'-UTR and the coding region/3'-UTR separately and then fusing the two regions together. Beginning with a frequently used transcription start site of the NATb promoter, the 5'-UTR (Husain et al., 2004; Barker et al., 2006) was amplified from cDNA prepared from RNA isolated from homozygous NAT1*4 HepG2 cells. All primer sequences used are shown in Table 1. The primers used to amplify the NATb 5'-UTR region were Lkm40P1 and NAT1 (3') ORF Rev. The coding region and 3'-UTR were amplified as one piece from NAT1*4 human genomic DNA with NAT1*4/NAT1*4 genotype. The forward primer used to amplify the coding region/3'-UTR was NAT1 (3') ORF Forward, whereas the reverse primer was pcDNA5distal Reverse. The two sections, the 5'-UTR and the coding region/3'UTR, were fused together via overlap extension and amplification of the entire product using nested primers. The forward nested primer was P1 Fwd Inr NheI and the reverse nested primer was NAT1 Kpn Rev. The forward nested primer included the KpnI endonuclease restriction site, and the reverse nested primer contained the NheI endonuclease restriction site to facilitate cloning. The pcDNA5/FRT vector and NATb/NAT1*4 allelic segments were digested at 37°C with restriction endonucleases KpnI and NheI (New England Biolabs). The NATb/NAT1*4 construct was then ligated into pcDNA5/FRT using T4 ligase (New England Biolabs).

*Preparation of NATb/NAT1*14B.* To construct the NATb/NAT1*14B pcDNA5/FRT plasmid, the NATb/NAT1*4 pcDNA5/FRT and a previously constructed NAT1*14B allelic construct expressed in a yeast vector, pESP-3 (Agilent Technologies) (Fretland et al., 2001), were both incubated at 37°C with restriction enzymes SbfI and AflII (New England Biolabs). After restriction digestion, the NATb/NAT1*4 pcDNA5/FRT and the 476-base pair segment of NAT1*14B (including G560A) were gel purified and ligated using T4 ligase (New England Biolabs). All constructs were sequenced to ensure integrity of allelic segments and junction sites. These constructs that contain NATb 5'-UTR, coding region of NAT1*4 or NAT1*14B, and 3'-UTR are illustrated in Fig. 1 and are referred to as NAT1*4 and NAT1*14B throughout this manuscript.

Cell Culture. UV5/CHO cells, a nuclease excision repair-deficient derivative of AA8 that are hypersensitive to bulky DNA lesions, were obtained from the American Type Culture Collection. Unless otherwise noted, cells were incubated at 37°C in 5% CO₂ in complete α -modified minimal essential medium (α -MEM; Lonza Walkersville, Inc., Walkersville, MD) without L-glutamine, ribosides, and deoxyribosides supplemented with 10% fetal bovine serum (Hyclone; Thermo Fisher Scientific, Waltham, MA), 100 units/ml penicillin (Lonza Walkersville, Inc.), 100 μ g/ml streptomycin (Lonza Walkersville, Inc.), and 2 mM L-glutamine (Lonza Walkersville). The UV5/CHO cells used in this study were previously stably transfected with a single FRT integration site (Bendaly et al., 2007). The FRT site allowed stable transfections to use the Flp-In System (Invitrogen). When cotransfected with pOG44 (Invitrogen), an Flp recombinase expression plasmid, a site-specific, conserved recombination event of pcDNA5/FRT (containing either NATa/NAT1*4 or

TABLE 1

Primers used to amplify NATb/NAT1*4 construct

Primer Name	Use	Sequence
Lkm40P1	NATb 5'-UTR forward specific PCR	5'-GGCCGCGGCATTTCAGTCTAGTCTCTGGTGGCC-3'
P1 Fwd Inr NheI	NATb 5'-UTR forward specific nested PCR	5'-TTTAAAGCTAGCATTTCAGTCTAGTCTAGTCTCTGGTTGCCGGCT-3'
NAT1 (3') ORF Rev	NATa/NATb 5'-UTR reverse PCR	5'-TTCTCTACTCAGAGTCTTGAACCTATT-3'
NAT1 (3') ORF For	NAT1 coding region forward PCR	5'-AGACATCTCCATCATCTGTGTTTACTAGT-3'
pcDNA5 FRTdistal Rev	NAT1 3'-UTR reverse PCR	5'-CGTGGGGATACCCCTAGA-3'
NAT1 KPN-Rev	NAT1 3'-UTR reverse nested PCR	5'-ATAGTAGGTACCTCTGAATTATAGATAAGCAAAGATTTCAGATTCT-3'

PCR, polymerase chain reaction.

FIG. 1. NATb/NAT1*4 and NATb/NAT1*14B constructs. a, schematic of NAT1 genomic structure and most common RNA transcribed by the NATb promoter. b, constructs including 5'-UTR, ORF (exon 9), and 3'-UTR.

NATb/NAT1*4) occurs at the FRT site. The FRT site allows recombination to occur immediately downstream of the hygromycin resistance gene, allowing for hygromycin selectivity only after Flp-recombinase-mediated integration. The UV5/FRT cells were further modified by stable integration of human *CYP1A1* and NADPH-cytochrome P450 reductase gene (Bendaly et al., 2007). They are referred to in this manuscript as UV5/IA1 cells.

Stable Transfections. Stable transfections were performed using the Flp-In System (Invitrogen) into UV5/IA1 cells that were previously stably transfected with an FRT site (as noted above). The pcDNA5/FRT plasmids containing human NATb/NAT1*4 or NATb/NAT1*14B were cotransfected with pOG44 (Invitrogen), an Flp recombinase expression plasmid. UV5/IA1 cells were stably transfected with pcDNA5/FRT containing NATb/NAT1*4 and NATb/NAT1*14B constructs using Effectene transfection reagent (QIAGEN, Valencia, CA) following the manufacturer's recommendations. Because the pcDNA5/FRT vector contains a hygromycin resistance cassette, cells were passaged in complete α -MEM containing 600 μ g/ml hygromycin (Invitrogen) to select for cells containing the pcDNA5/FRT plasmid. Hygromycin-resistant colonies were selected approximately 10 days after transfection and were isolated with cloning cylinders.

Determination of In Vitro (in Solution Biochemistry) Kinetic Parameters of N-Acetylation for NAT1 4 and NAT1 14B. Cells were collected by washing with 1 $\times$ phosphate-buffered saline (PBS) followed by addition of 1 ml of 0.25% trypsin-EDTA and were allowed to incubate for 5 min (Invitrogen). Cells were then collected, centrifuged at 3000g, and washed with 1 $\times$ PBS. Lysate was prepared by centrifuging the cells and resuspending pellet in homogenization buffer (20 mM NaPO₄, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, and 2 mM pepstatin A). The resuspended cell pellet was subjected to three rounds of freezing at -80°C and thawing at 37°C and then was centrifuged at 15,000g for 10 min. In vitro assays using PABA or ABP were conducted, and acetylated products were separated using HPLC as previously described (Hein et al., 2006). Preliminary studies optimized reactions with respect to linearity of time and protein concentration. PABA and ABP kinetic constants were determined at a fixed concentration of 100 μ M CoASA. PABA kinetic constants were determined using varying PABA concentrations between 11.7 and 3000 μ M. ABP kinetic constants were determined using varying ABP concentrations between 11.7 and 3000 μ M. Reactions containing substrate, CoASAc, and enzyme were incubated at 37°C for 10 min. Reactions were terminated by the addition of one-tenth volume of 1M acetic acid and were centrifuged at 15,000g for 10 min. Measurements were adjusted according to baseline measurements using lysates of the UV5/IA1 cell line and were normalized by the amount of total protein. Protein concentrations were measured using the Bradford (1976) method (Bio-Rad Laboratories, Hercules, CA). $V_{\max}$, k_m , and k_{cat} were determined by fitting substrate concentration and velocity data to the hyperbolic Michaelis-Menten model. All calculations were determined using GraphPad Prism Software version 4 (GraphPad Software, Inc., San Diego, CA).

Determination of In Situ (Whole-Cell Assay) Kinetic Parameters of NAT1 4 and NAT1 14B. In situ kinetic parameters were determined with a whole-cell assay using media spiked with varying concentrations of PABA or

ABP. PABA kinetic constants were determined using varying PABA concentrations between 2.25 and 300 μ M. ABP kinetic constants were determined using varying ABP concentrations between 0.19 and 25 μ M. The cells were incubated at 37°C , and media was collected after 1 h (PABA) or 22 min (ABP), one-tenth volume of 1M acetic acid was added, and the mixture was centrifuged at 13,000g for 10 min. Values were normalized to the amount of cells present at the time of media removal. After media removal, cells were washed with 1 $\times$ PBS, trypsinized, and diluted in counting buffer (aqueous 1% sodium chloride). The number of cells was determined using a Z Series Coulter Counter (Beckman Coulter, Fullerton, CA). The supernatant was injected into the reverse-phase HPLC column, and *N*-acetyl-PABA and *N*-acetyl-ABP were separated and quantitated as described above. $V_{\max}$ and k_m were determined as described above.

Determination of In Vitro Kinetic Parameters of O-Acetylation for NAT1 4 and NAT1 14B. Cells and lysates were collected and prepared as described above. *N*-OH-ABP *O*-acetyltransferase assays were conducted, and product was separated from substrate using HPLC as described previously (Hein et al., 2006). Assays containing 50 μ g of total protein, *N*-OH-ABP, CoASAc, and 1 mg/ml deoxyguanosine (dG) were incubated at 37°C for 10 min. *N*-OH-ABP kinetic constants were determined at a fixed concentration of 100 μ M CoASAc and *N*-OH-ABP concentrations between 0.78 and 200 μ M. Reactions were stopped with the addition of 100 μ l of water-saturated ethyl acetate and were centrifuged at 13,000g for 10 min. The organic phase was removed, evaporated to dryness, redissolved in 100 μ l of 10% acetonitrile (ACN), and injected onto the HPLC. $V_{\max}$, k_m , and k_{cat} were determined as described above.

Measurement of NAT1 Protein. The amount of NAT1 produced in UV5/IA1 cells stably transfected with NAT1*4 or NAT1*14B was determined by Western blot. Cells and lysates were collected and prepared as described above. Varying amounts of lysate were mixed 1:1 with 5% β -mercaptoethanol in Laemmli buffer (Bio-Rad Laboratories), boiled for 5 min, and resolved by 12% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred by semidry electroblotting to polyvinylidene fluoride membranes. The membranes were probed with G5 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a monoclonal mouse anti-NAT1(1:200) antibody (Santa Cruz Biotechnology, Inc.), specific for an epitope mapping between amino acids 261 to 290 at the C terminus of NAT1. Because the substitution R187Q lies outside this region, the antigenic site was not affected. Membrane was then probed with horseradish peroxidase-conjugated secondary donkey anti-mouse IgG antibody (1:2000) (Santa Cruz Biotechnology, Inc.). SuperSignal West Pico Chemiluminescent Substrate was used for detection (Thermo Fisher Scientific). To determine a quantitative amount of NAT1 protein in lysate collected from cells stably transfected with NAT1*4 or NAT1*14B, a standard curve was obtained from loading 1 to 140 ng of purified NAT1 (Abnova, Taipei, Taiwan). Intensities of varying amounts of lysate (55, 28, and 14 μ g) from NAT1 4 and NAT1 14B were compared to intensities of the standard curve to determine the amount of NAT1 protein in the lysate. Kinetic properties of the NAT1 antibody binding of the purified protein and to NAT1 from sample lysate were assumed to be the same. Densitometric analysis was performed using Quantity One Software (Bio-Rad Laboratories).

DNA Isolation and dG-C8-ABP Quantitation. Cells were prepared as described above. DNA was isolated, and dG-C8-ABP adducts were quantitated as described previously (Millner et al., 2011). Stably transfected cells grown to approximately 80% confluence in 15-cm dishes were incubated in complete α -MEM containing 1.56, 3.13, 6.25, and 12.5 μ M ABP or vehicle alone (0.5% dimethyl sulfoxide) at 37°C. The cells were collected after 24 h of treatment and were centrifuged for 5 min at 260g. The pellet was resuspended in 2 volumes of homogenization buffer (20 mM sodium phosphate, pH 7.4, 1 mM EDTA), 0.1 volume of 10% SDS, and 0.1 volume of 20 mg/ml proteinase K and was allowed to incubate overnight at 37°C. The DNA was extracted using phenol/chloroform/isoamyl alcohol and was precipitated with isopropanol. The pellet was dried and resuspended in 500 μ l of DNA adduct buffer (5 mM Tris, pH 7.4, 1 mM CaCl_2 , 1 mM ZnCl_2 , and 10 mM MgCl_2). The DNA was quantitated by spectrophotometry at A_{260} . Five hundred picograms of internal standard (dG-C8-ABP-d5; Toronto Research Chemicals, Inc., North York, ON, Canada) was added to 30 μ g of sample DNA and was treated with 10 units of DNase I (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C followed by treatment with 10 units of nuclease P1 (Sigma-Aldrich) for 6 h. The reactions were then treated with 10 units of alkaline phosphatase (Sigma-Aldrich) overnight at 37°C. The samples were then loaded onto PepClean C-18 Spin Columns (Thermo Fisher Scientific), washed with 10% ACN, eluted with 50% ACN by centrifugation at 2000g, and dried. The samples were reconstituted with 25 μ l of 5% ACN in 2.5 mM NH_4HCO_3 just before analysis, and 10 μ l of the sample was analyzed by Accela LC System (Thermo Fisher Scientific) coupled with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Samples were loaded onto a 30 $\times$ 1 mm $\times$ 1.9 μ m Hypersil GOLD column (Thermo Fisher Scientific) and were eluted with a 12.5-min binary solvent gradient (solvent A, 5% ACN/0.1% formic acid, and solvent B, 95% ACN/0.1% formic acid) at 50 μ l/min. The gradient started from 5% solvent B, increased linearly to 75% solvent B in 10 min, and then remained at 75% solvent B for 2.5 min. The eluates were ionized by electrospray ionization, and dG-C8-ABP and dG-C8-ABP-d5 were detected with linear ion trap and were detected by multiple reaction monitoring using the transitions of m/z 435.2 to m/z 319.2 (dG-C8-ABP) and m/z 440.2 to m/z 324.2 (dG-C8-ABP-d5). Concentrations of dG-C8-ABP were calculated from peak areas of dG-C8-ABP and dG-C8-ABP-d5 with a calibration curve from synthetic dG-C8-ABP and dG-C8-ABP-d5.

Measurement of Cytotoxicity. Assays for cell cytotoxicity were carried out as described previously (Millner et al., 2011). Stably transfected cells expressing NAT1*4 and NAT1*14B were grown in HAT medium (30 μ M hypoxanthine, 0.1 μ M aminopterin, and 30 μ M thymidine) for 12 doublings. Cells (1×10^6) were plated, allowed to grow for 24 h, and were then treated with 1.56, 3.13, 6.25, or 12.5 μ M ABP (Sigma-Aldrich) or vehicle alone (0.5% dimethyl sulfoxide) in media. After 48 h, cells were plated to determine survival after exposure to ABP. To determine cloning efficiency after each dose of ABP, 100 cells were plated in triplicate in 6-well plates and were allowed to grow for 7 days in nonselective media. Colonies were counted and were expressed as percentage of vehicle control.

Results

Initial experiments performed in yeast (in situ) resulted in higher NAT1 14B N-acetylation at 10 μ M ($p < 0.001$) and 50 μ M ABP ($p < 0.05$) compared to NAT1 4. There was no difference in N-acetylation between NAT1 14B and NAT1 4 after exposure to 100 μ M ABP (Fig. 2). The results of subsequent experiments performed in CHO cells are described below.

Kinetic parameters of the referent, NAT1 4, and the variant, NAT1 14B, in vitro (per milligram of total protein in solution biochemistry) are shown in Table 2. The apparent k_m of NAT1 14B was higher for PABA ($p < 0.0001$) compared to NAT1 4, whereas the apparent k_m of NAT1 14B was lower for ABP ($p < 0.0001$) and *N*-OH-ABP ($p < 0.0001$) compared to NAT1 4. The apparent V_{max} of NAT1 14B was lower for PABA ($p < 0.0001$), ABP ($p < 0.0001$), and *N*-OH-ABP ($p < 0.0001$) compared to NAT1 4. The apparent V_{max}/k_m of NAT1 14B was lower for PABA ($p < 0.0001$), higher for *N*-OH-ABP ($p <$

FIG. 2. In situ ABP N-acetylation in yeast cultures recombinantly expressing NAT1*4 and NAT1*14B. Each bar illustrates mean $\pm$ S.E.M. for nanomole of acetylated ABP per million cells after three separate collections. Significant differences between NAT1*4- and NAT1*14B-expressing yeast cultures were determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.001$.

0.0001), and not significantly different for ABP ($p > 0.05$) compared to NAT1 4.

The kinetic parameters, apparent k_m and k_{cat} , were also determined in vitro (per milligram of NAT1 protein in solution biochemistry) for the referent, NAT1 4, and the variant, NAT1 14B (Table 2). The apparent k_{cat} of NAT1 14B was lower for PABA ($p < 0.0001$) but higher for *N*-OH-ABP ($p < 0.0001$) compared to NAT1 4. There was no significant difference in apparent k_{cat} for ABP between NAT1 14B and NAT1 4 ($p > 0.05$). The apparent k_{cat}/k_m of NAT1 14B was lower for PABA ($p < 0.0001$) but higher for ABP ($p < 0.05$) and *N*-OH-ABP ($p < 0.0001$) compared to NAT1 4.

Apparent k_m and V_{max} for PABA and ABP were also determined in situ (per million cells in a whole-cell-based assay) for the referent, NAT1 4, and the variant, NAT1 14B (Table 3). The apparent k_m of NAT1 14B was not significantly different for PABA ($p > 0.05$) but was significantly lower for ABP ($p < 0.0001$) compared to NAT1 4. The apparent V_{max} of NAT1 14B was lower for PABA ($p < 0.05$) and ABP ($p < 0.0001$) compared to NAT1 4. The apparent V_{max}/k_m of NAT1 14B was significantly less for PABA ($p < 0.05$) but was significantly higher for ABP ($p < 0.05$) compared to NAT1 4.

Expression of NAT1 14B and NAT1 4 was determined by Western blot (Fig. 3). The standard curve obtained by loading 1 to 140 ng of purified NAT1 protein (Abnova) was used to compare intensities of lysate from stably transfected cells. Fifty-five, 28, or 14 μ g of total protein lysate corresponded to 154, 77, and 38 ng of NAT1 4 protein and 38, 19, and 10 ng of NAT1 14B protein, respectively. Overall, NAT1 14B resulted in a 4-fold reduction in NAT1 protein compared to NAT1 4 ($p < 0.001$).

ABP-induced cytotoxicity was also determined in cells stably transfected with NAT1*4 or NAT1*14B (Fig. 4a). Significantly more ABP-induced cytotoxicity was observed in NAT1*14B-transfected cells after exposures to each ABP concentration. ABP-induced dG-C8-ABP adducts in cells stably transfected with NAT1*4 and NAT1*14B were determined (Fig. 4b). Significantly more dG-C8-ABP adducts were observed after exposures between 1.56 and 12.5 μ M ABP in cells transfected with NAT1*14B than in cells transfected with NAT1*4.

Discussion

Smokers possessing NAT1*14B have been associated with increased risk for lung cancer compared with individuals possessing NAT1*4 (Bouchardy et al., 1998). Previous studies have reported that NAT1*14B is associated with reduced N- and O-acetylation of various substrates including PABA, *p*-aminosalicylic acid, and various arylamine carcinogens (Hughes et al., 1998; Fretland et al., 2001, 2002;

TABLE 2

NAT1 4 and NAT1 14B kinetic constants determined in vitro (per milligram of total protein)

PABA, ABP, and *N*-OH-ABP constants were determined at a fixed concentration of 100 μ M CoASAc. Table values represent mean $\pm$ S.E.M. for three to six individual determinations. Differences were tested for significance by Student's *t* test.

Allele	Substrate	$k_{m(app)}$ μ M	$V_{max(app)}$ $nmoles \cdot min^{-1} \cdot mg^{-1}$	$V_{max}/k_{m(app)}$ $ml \cdot min^{-1} \cdot mg^{-1}$	$k_{cat(app)}$ min^{-1}	$k_{cat}/k_{m(app)}$ min^{-1}/μ M
<i>NAT1*4</i>	PABA	42.9 $\pm$ 3.3	116 $\pm$ 3	2.72 $\pm$ 0.21	2400 $\pm$ 57	56.5 $\pm$ 4.3
<i>NAT1*14B</i>	PABA	430 $\pm$ 1 ^a	18.5 $\pm$ 1.5 ^b	0.043 $\pm$ 0.002 ^b	1550 $\pm$ 97 ^b	3.61 $\pm$ 0.20 ^b
<i>NAT1*4</i>	ABP	273 $\pm$ 46	57.7 $\pm$ 5.8	0.218 $\pm$ 0.018	1200 $\pm$ 122	4.52 $\pm$ 0.38
<i>NAT1*14B</i>	ABP	65.6 $\pm$ 3.9 ^b	18.0 $\pm$ 4.3 ^b	0.280 $\pm$ 0.031	1760 $\pm$ 128	22.9 $\pm$ 0.23 ^c
<i>NAT1*4</i>	<i>N</i> -OH-ABP	141 $\pm$ 1.1	2.97 $\pm$ 0.19	0.0211 $\pm$ 0.0014	35.1 $\pm$ 68	0.250 $\pm$ 0.02
<i>NAT1*14B</i>	<i>N</i> -OH-ABP	46.8 $\pm$ 1.3 ^b	1.76 $\pm$ 0.03 ^b	0.038 $\pm$ 0.001 ^c	147 $\pm$ 7 ^a	3.15 $\pm$ 0.23 ^c

^a Significantly higher than NAT1 4 ($p < 0.0001$).

^b Significantly lower than NAT1 4 ($p < 0.0001$).

^c Significantly higher than NAT1 4 ($p < 0.05$).

Zhu and Hein, 2008). Recombinant NAT1 14B expression in yeast demonstrated lower *N*-acetylation, *O*-acetylation, and NAT1-specific protein levels and increased NAT1 proteasomal degradation (Fretland et al., 2001, 2002; Butcher et al., 2004). Likewise, NAT1 14B expressed in COS-1 cells also resulted in lower NAT1 *N*- and *O*-acetylation, lower NAT1 protein level, and lower PABA V_{max} , but higher PABA k_m compared to the referent, NAT1 4 (Zhu and Hein, 2008). Our kinetic constant determinations performed in CHO cells confirmed that NAT1 14B results in a lower apparent V_{max} (both in vitro and in situ) for PABA compared to the referent, NAT1 4. We also confirmed the higher PABA apparent k_m in NAT1 14B determined in vitro compared to NAT1 4. In addition to PABA acetylation, we also report on *N*- and *O*-acetylation of ABP and *N*-OH-ABP. ABP is a human urinary bladder carcinogen found as a contaminant in cigarette smoke, food dyes, paints, textile dyes, engine exhaust, and commercial hair dyes (Nauwelaers et al., 2011).

The arylamine substrate k_m of NAT1 is dependent on the CoASAc concentration because acetylation proceeds via a "Ping-Pong bi-bi" reaction (Weber and Hein, 1985). CoASAc concentrations have been measured in vivo in the low micromolar range (Reeves et al., 1988). The lowest concentration of CoASAc (100 μ M) was used, which allowed repeatable and accurate measurements of acetylated product. To better mimic NAT1-catalyzed acetylation in vivo, kinetic constants were determined in situ (when possible) allowing the concentration of CoASAc to be provided by the cell.

Studies performed in situ using NAT1 14B and NAT1 4 produced in yeast did not result in lowered NAT1 14B *N*-acetylation of ABP (Fig. 2) as previous studies had shown in vitro (Fretland et al., 2002). This result was surprising because previous studies had reported NAT1 14B activity and protein expression to be lower than NAT1 4. To further explore the NAT1 14B acetylation status, studies were conducted in stably transfected CHO cells.

Comparing apparent V_{max} (in vitro), the NAT1 14B apparent V_{max} was lower than the NAT1 4 for all substrates studied. The apparent

V_{max} describes the maximum enzyme velocity extrapolated to maximum substrate concentrations. The lower apparent V_{max} values for PABA, ABP, and *N*-OH-ABP indicate that at high substrate concentrations, NAT1 14B has a decreased ability to metabolize the substrate compared to NAT1 4. The apparent V_{max}/k_m , or intrinsic clearance, describes an enzyme's ability to metabolize a substrate at substrate concentrations well below the k_m and has also been shown to correlate well to human liver clearance (Northrop, 1999; Chen et al., 2011). Although there are limitations in using V_{max}/k_m as a comparator of two enzymes, we determined apparent V_{max} for comparison at high substrate concentrations and apparent V_{max}/k_m for comparison at low substrate concentrations (Eisenthal et al., 2007). For PABA, the NAT1 14B apparent V_{max}/k_m was lower than NAT1 4. In contrast, no significant difference was observed between NAT1 14B and NAT1 4 apparent V_{max}/k_m toward the *N*-acetylation of ABP. We were surprised to find that the NAT1 14B apparent V_{max}/k_m for the *O*-acetylation of *N*-OH-ABP was higher in NAT1*14B CHO cell lysate compared to NAT1*4 CHO cell lysate. This indicates that the status of NAT1 14B intrinsic clearance compared to NAT1 4 intrinsic clearance is substrate-dependent.

Transfection of NAT1*14B resulted in a $\sim$ 4-fold less NAT1 protein expression compared to NAT1*4. When the amount of NAT1 protein was used to calculate apparent k_{cat} (determined in vitro), the results suggested that the lower NAT1 14B apparent V_{max} for these substrates is due to a reduction in NAT1 protein, not a reduction in the acetylation rate of the NAT1 14B enzyme. For example, although the NAT1 14B apparent V_{max} for *N*-OH-ABP was lower than the NAT1 4, the NAT1 14B apparent k_{cat} for *N*-OH-ABP was higher than NAT1 4. This difference in V_{max} compared to k_{cat} indicates that the lowered NAT1 14B apparent V_{max} is caused by a reduction in protein expression. Butcher et al. (2004) reported that NAT1 14B and other NAT1 genetic variants associated with reduced enzymatic activity have reduced ability to be acetylated, which resulted in an unstable NAT1 protein. Therefore, NAT1 14B was reported to be less stable and have

TABLE 3

NAT1 4 and NAT1 14B kinetic constants determined in situ (per million cells)

Table values represent mean $\pm$ S.E.M. for three to six individual determinations. Differences were tested for significance by Student's *t* test.

Allele	Substrate	$k_{m(app)}$ μ M	$V_{max(app)}$ $nmoles \cdot min^{-1} \cdot million\ cells^{-1}$	$V_{max}/k_{m(app)}$ $nmoles \cdot min^{-1} \cdot million\ cells^{-1} \cdot \mu$ M ⁻¹
<i>NAT1*4</i>	PABA	95.5 $\pm$ 1.1	0.16 $\pm$ 0.01	1.71 $\pm$ 0.08
<i>NAT1*14B</i>	PABA	72.1 $\pm$ 11.1	0.101 $\pm$ 0.018 ^a	1.1 $\pm$ 0.09 ^a
<i>NAT1*4</i>	ABP	10.5 $\pm$ 0.6	0.024 $\pm$ 0.0007	2.4 $\pm$ 0.1
<i>NAT1*14B</i>	ABP	2.3 $\pm$ 0.2 ^b	0.0063 $\pm$ 0.0005 ^b	2.9 $\pm$ 0.1 ^c

^a Significantly lower than NAT1 4 ($p < 0.05$).

^b Significantly lower than NAT1 4 ($p < 0.0001$).

^c Significantly higher than NAT1 4 ($p < 0.05$).

increased proteasomal degradation compared with NAT1 4 (Butcher et al., 2004). Our study confirmed that NAT1 14B resulted in reduction of NAT1 protein.

Because determination of kinetic parameters is dependent upon CoASAc concentration, acetylation was measured in situ to allow the concentration of CoASAc to be provided by the cell. When comparing $V_{\max}$ (in situ), the NAT1 14B apparent $V_{\max}$ was lower than the NAT1 4 for PABA and ABP. When evaluated in situ, PABA NAT1 14B apparent $V_{\max}/k_m$, or intrinsic clearance, was lower compared to NAT1 4. In contrast, for ABP, the in situ NAT1 14B apparent $V_{\max}/k_m$ was higher compared to NAT1 4. Because kinetic parameters of *N*-OH-ABP could not be determined in situ, an in vitro determination was performed. As with ABP, the NAT1 14B apparent $V_{\max}/k_m$ for *N*-OH-ABP was higher compared to NAT1 4. These findings indicate that differences in apparent $V_{\max}/k_m$ between NAT1 14B and NAT1 4 are substrate-dependent. Risk for individuals possessing NAT1*14B is also likely exposure-dependent. Increased apparent $V_{\max}/k_m$ indicates that NAT1 14B has an increased ability to metabolize ABP and *N*-OH-ABP at low substrate concentrations compared to NAT1 4 (Northrop, 1999). Because low substrate concentrations are relevant in vivo, the higher NAT1 14B apparent $V_{\max}/k_m$ suggests that differences between NAT1 14B- and NAT1 4-catalyzed ABP acetylation should be observed in vivo. Therefore, risk for individuals possessing NAT1*14B is dependent on exposure type and can also be altered depending on exposure level.

NAT1 homology modeling predicted that the R187Q could affect NAT1 active site acetylation and enzymatic activity (Walraven et al., 2008). Because changes in binding of CoASAc and substrate specificity are probably altered as a result of the R187Q, it is not surprising that differences in intrinsic clearance between NAT1 14B and NAT1 4 were observed. We confirmed that R187Q modifies substrate affinity, albeit in opposite directions depending on substrate. Further epidemiological studies are necessary to determine which carcinogen exposures result in increased risk for individuals possessing NAT1*14B. Kinetic parameters have been previously reported for an NAT2 variant allele, NAT2*7B. (Hickman et al., 1995; Zang et al., 2007). Both articles reported differences in k_m for sulfamethazine between NAT2 4 and NAT2 7B. Zang et al. (2007) also found that

FIG. 3. Western blot to determine relative protein expression of NAT1 4 and NAT1 14B. a, representative Western blot. b, densitometric analysis of Western blot of 28 μ g of total protein loaded. Loading either 28 or 14 μ g of total protein lysate, NAT1 14B resulted in a ~4-fold less NAT1 protein than NAT1 4 (**, $p < 0.001$). Bars represent mean $\pm$ S.E.M. for three Western blots, and significance was determined by Student's *t* test.

FIG. 4. ABP-induced cytotoxicity (a) and dG-C8-ABP adducts (b) in cells stably transfected with NAT1*4 and NAT1*14B. Significantly more cytotoxicity was observed for NAT1 14B than NAT1 4 after all ABP exposures between 1.56 and 12.5 μ M. Significantly more adducts were observed after all exposures examined in cells expressing NAT1 14B than in cells expressing NAT1 4. Values were adjusted for baseline values of UV5/IAI cells. Bars represent mean $\pm$ S.E.M. for three determinations, and significance was determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

NAT2 7B *O*-acetylates *N*-OH-PhIP as effectively as NAT2 4 but does not *O*-acetylate *N*-OH-ABP as effectively as NAT2 4. Our study is the first report of exposure-dependent behavior for a variant of NAT1. Studies in our laboratory are currently underway to examine the interplay between NAT1 and NAT2 on ABP metabolism.

In addition to higher apparent $V_{\max}/k_m$ for NAT1 14B toward ABP and *N*-OH-ABP compared to NAT1 4, ABP-induced DNA adducts and cytotoxicity were higher for NAT1 14B compared to NAT1 4. Measurement of DNA adduct levels after exposure to ABP is a biological endpoint very relevant to cancer risk. Because NAT1 14B resulted in increased ABP-induced DNA adducts, our results suggest that individuals possessing the NAT1*14B allele likely have increased risk compared with those who are homozygous for NAT1*4 after low (environmental) dose exposure to ABP. NAT1 14B is not simply associated with "slow acetylation" but rather is substrate-dependent, because NAT1 14B exhibits lower *N*-acetylation catalytic efficiency of PABA but higher *N*- and *O*-acetylation catalytic efficiency, as well as DNA adducts after exposure to the human carcinogen ABP.

Authorship Contributions

Participated in research design: Millner, Doll, States, and Hein.

Conducted experiments: Millner, Doll, and Cai.

Performed data analysis: Millner, Cai, and Hein.

Wrote or contributed to the writing of the manuscript: Millner, Doll, Cai, States, and Hein.

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