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PRINCIPAL INVESTIGATOR: Lori Millner

CONTRACTING ORGANIZATION: University of Louisville Research Foundation  
Louisville, KY 40292

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## Introduction

Human arylamine *N*-acetyltransferases 1 (NAT1) is a phase II cytosolic enzyme responsible for the biotransformation of many arylamine compounds including pharmaceuticals and environmental carcinogens. A common environmental carcinogen found in cigarette smoke is an aromatic amine, 4-aminobiphenyl (ABP). Arylamines such as ABP can either be inactivated via *N*-acetylation or activated via *O*-acetylation by NAT1. ABP can be *N*-acetylated and then excreted from the body. However, if ABP is first hydroxylated by cytochrome p450 1A1 (CYP1A1), the hydroxyl-ABP then can be further activated by NAT1-catalyzed *O*-acetylation resulting in *N*-acetoxy-ABP. This compound is very unstable and spontaneously degrades to form a nitrenium ion that can react with DNA to produce bulky adducts. If these adducts are not repaired, mutagenesis can occur and result in cancer initiation.

The only known endogenous NAT1 substrate is p-aminobenzoylglutamate (PABG), a catabolite of folate. NAT1 has been associated with various birth defects that may be related to deficiencies in folate metabolism. NAT1 polymorphisms and maternal smoking have been associated with increased incidence of oral clefts, spina bifida and increased limb deficiency defects. NAT1 polymorphisms have also been associated with increased risk for breast, pancreatic, prostate, urinary bladder and colorectal cancers non-Hodgkin lymphoma, mammary cell growth and breast cancer survival.

NAT1\*4 is referred to as the referent allele because it was the most common allele in the population in which it was first identified. To date, 26 human NAT1 alleles have been identified (<http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-gene-nomenclature/>). Although the effects of NAT1 polymorphisms on catalytic activity have been studied, the results are ambiguous. Within single NAT1 genotypes, conflicting phenotypes have been reported, and the relationship between phenotype and genotype remains poorly understood. Genotype as well as other factors are likely affecting phenotype, therefore it is important to understand transcriptional and translational control of NAT1.

The NAT1 gene spans 53 kb and contains nine exons (Figure 1a). Several NAT1 transcripts have been identified containing various combinations of 5'-untranslated region (UTR) exons and are known to originate from two distinct promoters, NATa and NATb. NATb, the major promoter, is located 11.8 kb upstream of the open reading frame (ORF). NATb promotes transcription of Type II transcripts and the major transcript, Type IIA, has been detected in all tissues studied to date. An alternative promoter, NATa, originates 51.5 kb upstream of the NAT1 ORF and promotes transcription of Type I transcripts expressed primarily in kidney, lung, liver, and trachea (Barker et al. 2006).

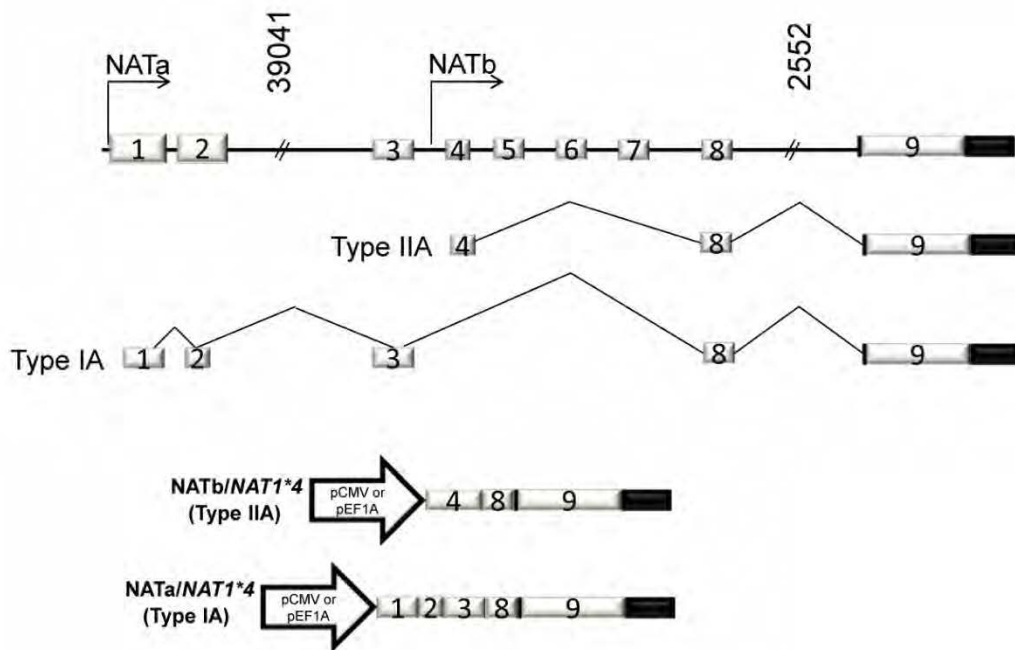
This project has examined the role of NAT1 polymorphisms including SNPs in the coding region and in the 3'-UTR. Polymorphisms examined in this study include T1088A and C1095A (NAT1\*10), T1571C, A1642C, ΔCT1647, C1716T (NAT1\*10B) and G560A (NAT1\*14B). In addition to SNP effects, this study has also examined effects of the two major 5'-UTRs (NATb and NATa) on NAT1 enzymatic activity, mRNA level and stability, protein expression, ABP-induced DNA adduct formation, and ABP-induced mutants.

## Body

**Objective 1: To create pcDNA5/FRT vector constructs that possess the human *NAT* alleles including *NATa* and *NATb* 5'-UTR exons, the coding region, and 885 nucleotides of the 3'-UTR (with 6 potential polyadenylation signals). In addition to the reference *NAT1\*4*, alleles possessing individual or combinations of genetic polymorphisms present in *NAT1\*10*, *NAT1\*11*, and *NAT1\*14* will be constructed.**

Completed and reported in Annual Report 2009.

Figure 1. (a) Genomic organization of *NAT1* gene; (b) Type IIA and Type IA *NAT1* RNA (c) and representative *NATb/NAT1\*4* and *NATa/NAT1\*4* constructs.



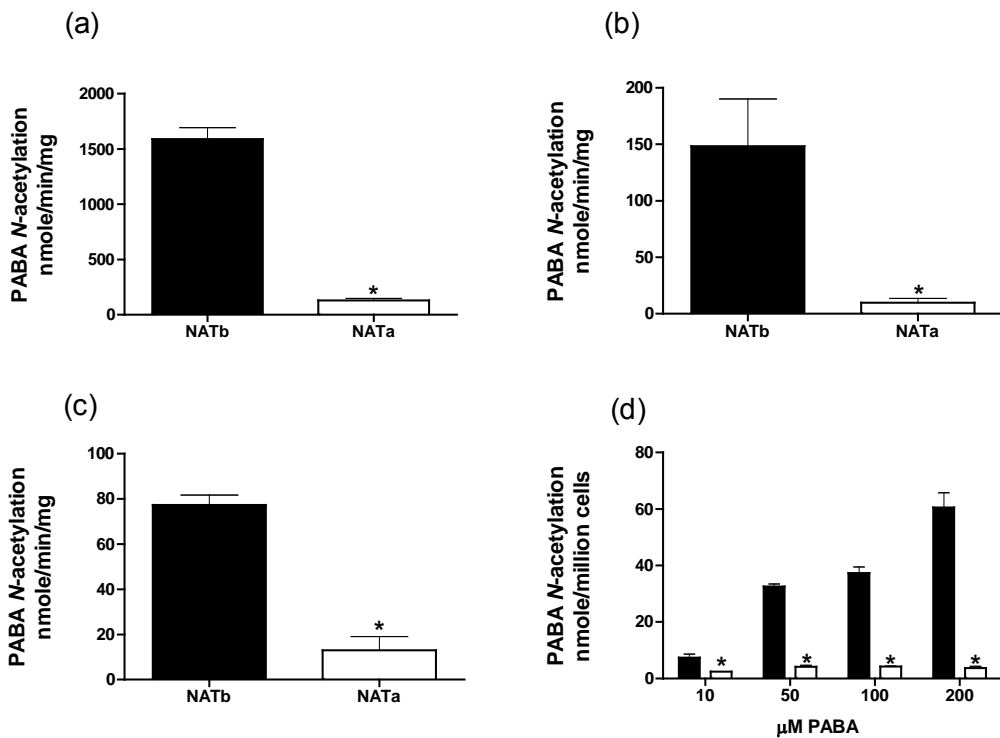
**Objective II. Nucleotide excision repair deficient Chinese hamster ovary cells expressing human CYP1A1 will be transfected with pcDNA5/FRT vectors containing human NAT1 constructs. The functional effects of genetic polymorphisms in NAT1\*10, NAT1\*11 and NAT1\*14 will be compared to the reference allele NAT1\*4 in transient transfection experiments. Functional assays will include determinations of N- and O-acetylation catalytic activities (HPLC assays), mRNA levels (Taqman assays) and protein (Western blot assays).**

**Methods**

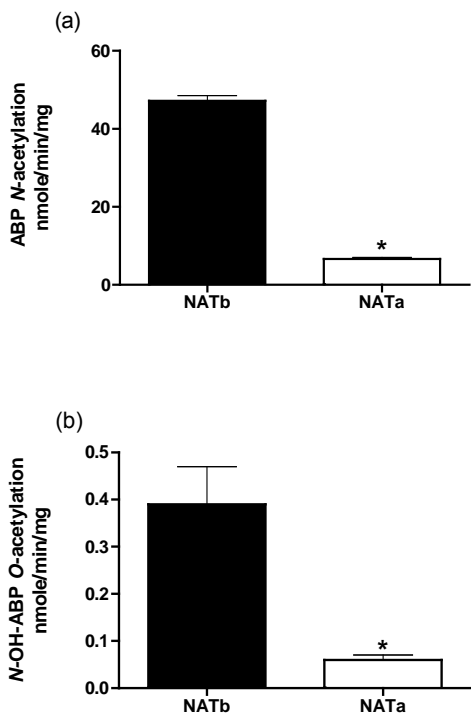
*In situ* N-acetyltransferase activity was studied in a whole cell assay using media spiked with differing concentrations of PABA (10 – 300 μM). The cells were incubated at 37°C and media was collected after 5 hours, 1/10 volume of 1M acetic acid was added, and the mixture was centrifuged at 13,000xg for 10 minutes. The supernatant was injected into the reverse phase HPLC column and N-acetyl-PABA was separated and quantitated as described above. All other methods for Objective II were reported in the annual report 2009.

**Results**

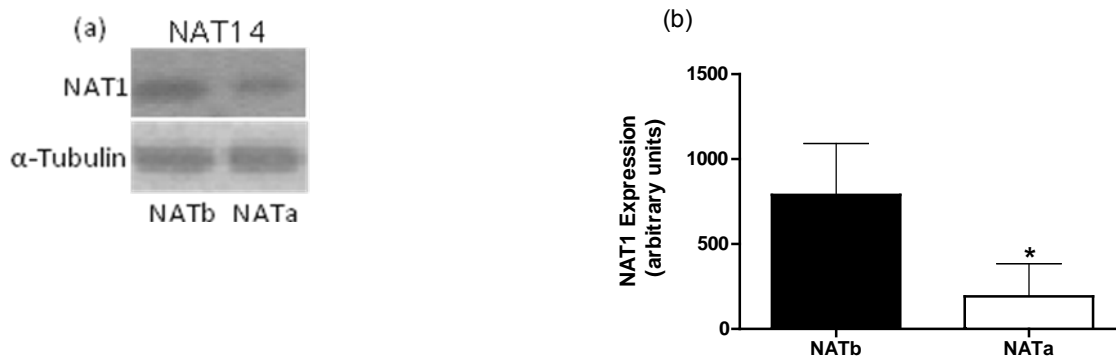
**FIG. 2.** N-acetylation of PABA in UV5/1A1 cells expressing CYP1A1 and NATb/NAT1\*4 (solid bars) or NATa/NAT1\*4 (open bars). (a) PABA N-acetylation activity following transient transfection with pcDNA5/FRT; (b) PABA NAT1 catalytic activity following stable transfection with pcDNA5/FRT of 3 different clones of each NATb/NAT1\*4 and NATa/NAT1\*4; (c) PABA N-acetylation activity following transient transfection with pEF1/V5-His; (d) PABA N-acetylation *in situ* following stable transfection of pcDNA5/FRT. Each bar represents mean ± S.E.M. for three transient transfections (a, c), 3 separate collections of 3 clones (b) or 3 separate collections of 1 clone (d). Asterisks (\*) represent a significant difference (p<0.05) (a, b, d) or (p<0.0001) (c) following a student's t-test.



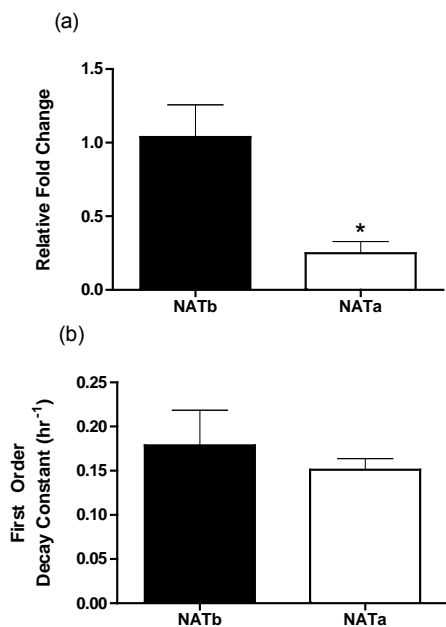
**FIG. 3.** (a) *N*-acetylation of ABP and (b) *O*-acetylation of *N*-hydroxy-ABP in UV5/1A1 cells stably expressing *CYP1A1* and either NATb/*NAT1*\*4 (solid bars) or NATa/*NAT1*\*4 (open bars) in pcDNA5/FRT. Each bar represents mean  $\pm$  S.E.M. for three separate collections. Asterisks (\*) represent a significant difference ( $p < 0.0001$ ) (a) or ( $p < 0.05$ ) (b) following a student's t-test.



**FIG. 4.** NAT1 protein expression in UV5/1A1 cells stably expressing *CYP1A1* and NATb/*NAT1*\*4 (solid bars) or NATa/*NAT1*\*4 (open bars) in pcDNA5/FRT. (a) Representative western blot of 20  $\mu$ g of total protein loaded; (b) Arbitrary intensity units of densitometric analysis performed on three independent Western blots. Asterisks (\*) represent a significant difference ( $p < 0.05$ ) following a student's t-test.



**Figure 5.** (a) NAT1 mRNA expression levels; (b) mRNA stability in UV5/1A1 cells stably expressing *CYP1A1* and NATb/NAT1\*4 (solid bars) or NATa/NAT1\*4 (open bars) in pcDNA5/FRT. Each bar represents mean  $\pm$  S.E.M. for (a) three or (b) nine determinations. Asterisks (\*) represent a significant difference ( $p < 0.05$ ) following a student's t-test.



**Figure 6.** (a) P-aminobenzoic acid (PABA), a NAT1 specific substrate was used to determine NAT1 enzymatic activity performed on lysate collected from stably transfected cells. Activities are expressed in nmoles acetylated PABA /min/mg of protein. Each bar represents mean ( $n=2$ )  $\pm$  SEM. In stably transfected cells, NATb/NAT1\*10 and NATb/NAT1\*10B resulted in significantly ( $p < 0.05$  and  $p < 0.0001$ , respectively) higher PABA N-acetylation activity than NATb/NAT1\*4. (b) 4-aminobiphenyl (ABP) was used to determine NAT1 enzymatic activities performed on lysate from stably transfected cells. Significantly more ( $p < 0.05$ ) activity was detected for NATb/NAT1\*10 and NATb/NAT1\*10B than with NATb/NAT1\*4. Each bar represents mean ( $n=3$ )  $\pm$  SEM. (c) *N*-OH-ABP was used to determine NAT1 O-acetylation activity. No significant differences were observed between NATb/NAT1\*4, NATb/NAT1\*10 or NATb/NAT1\*10B. Each bar represents mean ( $n=3$ )  $\pm$  SEM. All significance testing was done using one-way ANOVA followed by a Bonferroni post test.

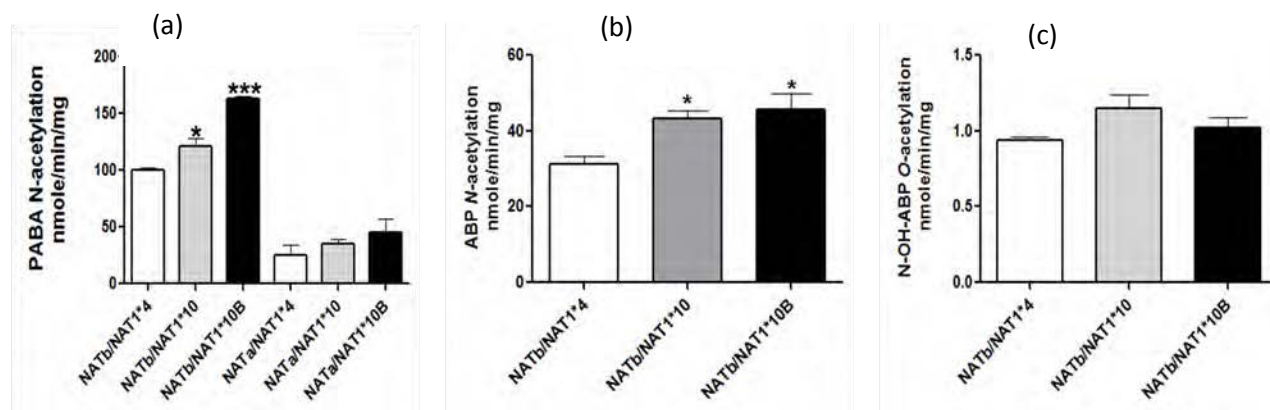
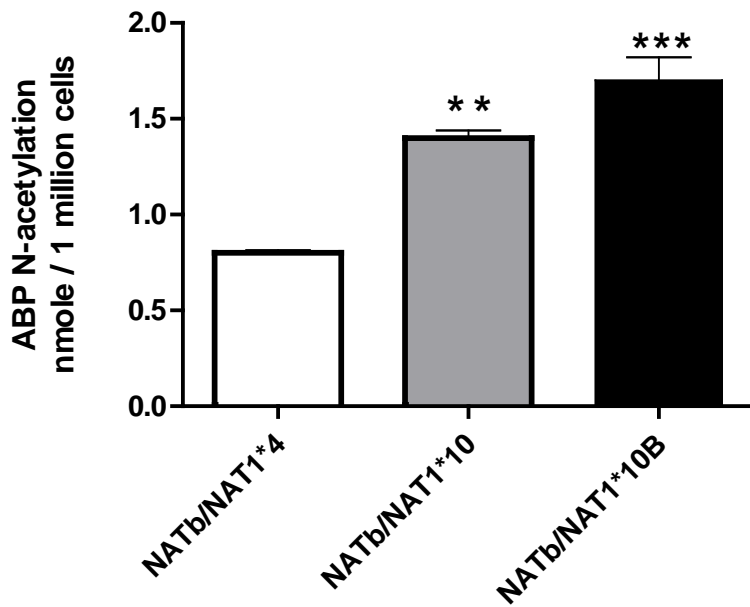
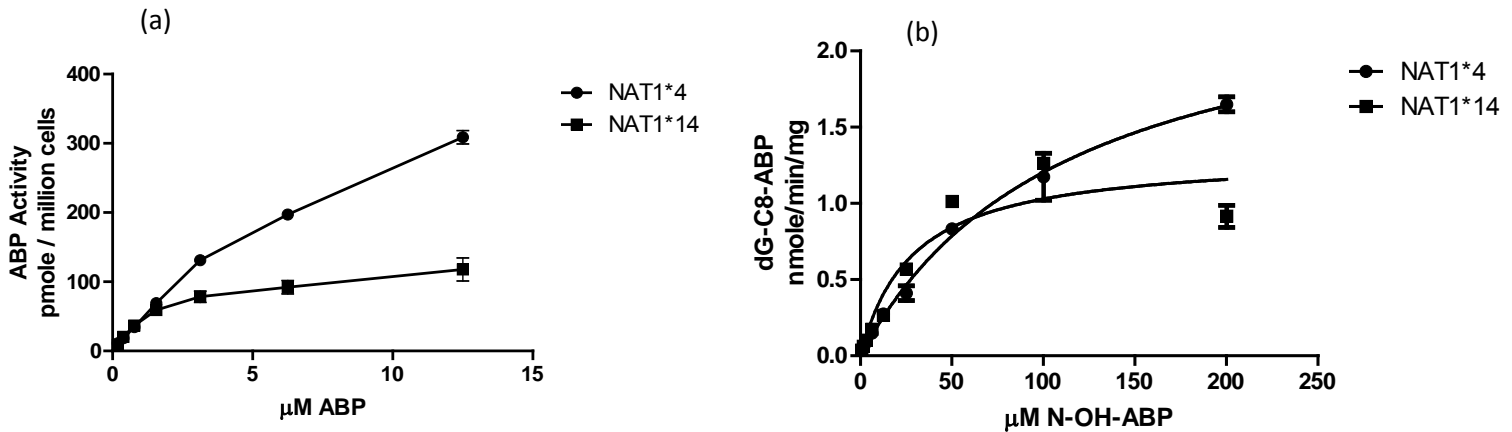


Figure 7



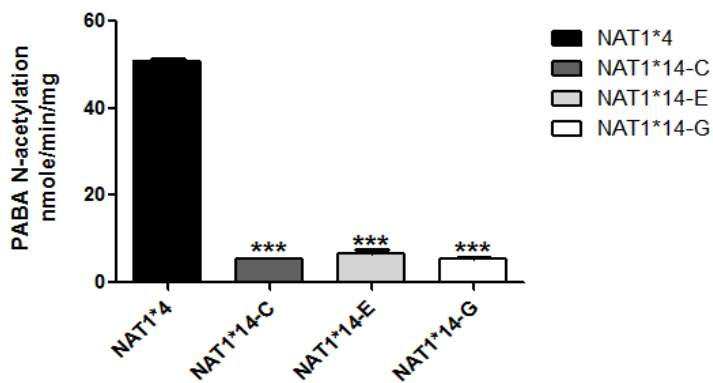
**Figure 7.** *N*-acetylation of ABP *in situ*. *N*-acetylated ABP was quantitated from media containing 100  $\mu$ M ABP incubated with whole cells for 3 hours. Values of ABP *N*-acetylation are reported in nmole / 1 million cells. Each bar represents mean ( $n=3$ )  $\pm$  SEM for one clone. Significantly more ABP *N*-acetylation was observed in cells transfected with NATb/NAT1\*10 and NATb/NAT1\*10B than in cells transfected with NATb/NAT1\*4 following analysis with one-way ANOVA and a Bonferroni post test.

Figure 8.



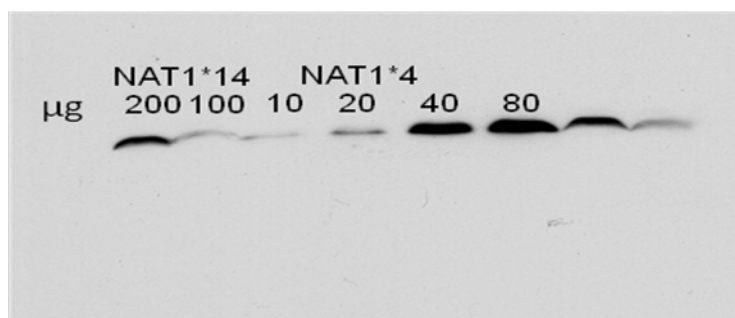
**Figure 8.** (a) *In situ* ABP *N*-acetylation. NAT1\*14 represents 3 separate clones and error bars represent mean ( $n=3$  experiments)  $\pm$  SEM. Values are reported as pmole of acetyl-ABP / 1 million cells. At concentration greater than 5  $\mu$ M ABP, NAT1\*4 *N*-acetylation activity was significantly higher than NAT1\*14 activity. However at concentrations below 5  $\mu$ M ABP, there was no significant difference following a student's *t*-test. (b) *In vitro* N-OH-ABP *O*-acetylation performed on cell lysate. NAT1\*14 represents 3 separate clones and error bars represent mean ( $n=3$  experiments)  $\pm$  SEM. Values are reported as dG-C8-ABP nmole/min/mg. At 200  $\mu$ M N-OH-ABP, NAT1\*4 results in significantly higher *O*-acetylation than NAT1\*14. However at concentrations below 100  $\mu$ M ABP, there was no significant difference following a student's *t*-test.

**Figure 9.**



**Figure 9.** PABA *N*-acetylation activity. PABA was used to determine NAT1 enzymatic activity performed on lysate collected from stably transfected cells. Activities are expressed in nmoles acetylated PABA /min/mg of protein. Each bar represents mean (n=3) ± SEM. Three clones were tested for NAT1\*14, they are referred to as NAT1\*14-C, NAT1\*14-E, and NAT1\*14-G. Each NAT1\*14 clone resulted in significantly ( $p < 0.0001$ ) lower activity than the referent allele, NAT1\*4 following analysis with one-way ANOVA.

**Figure 10.**



**Figure 10.** NAT1 protein expression in UV5/1A1 cells stably expressing *CYP1A1* and NATb/NAT1\*4 and NATb/NAT1\*14-E in pcDNA5/FRT. Representative western blot of 20 μg of total protein loaded. Approximately 6-fold less NAT1 protein was detected in cells transfected with NAT1\*14-E than cells with NAT1\*4.

III. Where functional effects are observed in the comparison of *NAT1\*10*, *NAT1\*11*, and *NAT1\*14* with the reference allele, *NAT1\*4*, stable CHO cell transfectants with these alleles will be constructed and exposed to various aromatic and heterocyclic amine carcinogens to test their effects on levels of covalent DNA adducts (liquid chromatography-mass spectrometry assays) and mutagenicity (*HPRT* mutants).

## Methods

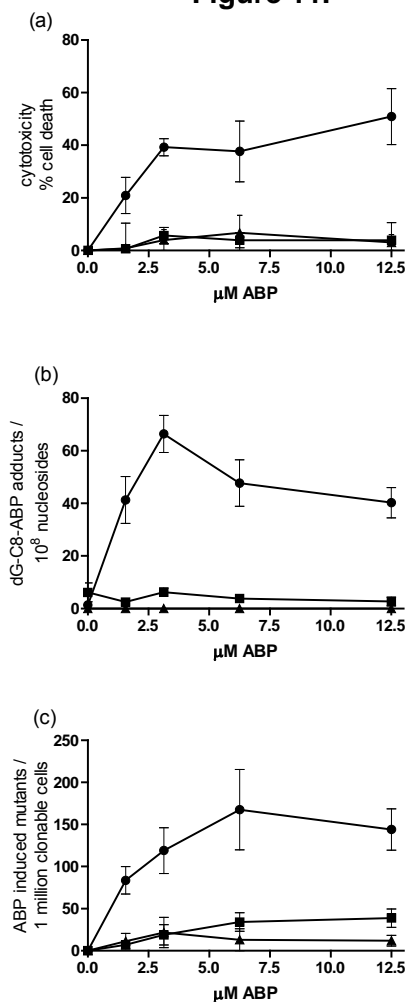
**DNA Isolation and dG-C8-ABP quantitation.** DNA was isolated and dG-C8-ABP adducts were quantitated with modifications to a previously described method (Metry et al., 2007). Cells grown to approximately 80% confluency in 15 cm dishes were incubated in complete  $\alpha$ -MEM media containing 1.56, 3.13, 6.25, 12.5  $\mu$ M ABP or vehicle alone (0.5% DMSO) at 37°C. The cells were collected following 24 h of treatment, centrifuged for 5 min at 13,000xg, and the pellet was resuspended in 2 volumes of homogenization buffer (20 mM sodium phosphate pH 7.4, 1 mM EDTA), 0.1 volumes of 10% SDS and 0.1 volume of 20 mg/mL Proteinase K and allowed to incubate overnight at 37°C. The DNA was extracted using phenol/chloroform:isoamyl alcohol and precipitated with isopropanol. The pellet was dried and resuspended in 500  $\mu$ L of DNA adduct buffer (5 mM Tris pH 7.4, 1 mM CaCl<sub>2</sub>, 1 mM ZnCl<sub>2</sub>, and 10 mM MgCl<sub>2</sub>). The DNA was quantitated by spectrophotometry at A<sub>260</sub>. Five hundred pg of internal standard (dG-C8-ABP-d5, Toronto Research Chemicals, North York, Ontario, Canada) was added to 30  $\mu$ g of sample DNA, treated with 10 units *DNase I* (US Biological, Swampscott, MA) for 1 h at 37°C followed by treatment with 10 units nuclease P1 (Sigma) for 6 h. The reactions were then treated with 10 units of alkaline phosphatase (Sigma) overnight at 37°C. The samples were then loaded onto PepClean C-18 Spin Columns (Thermo Fisher Scientific), washed with 10% acetonitrile (ACN), eluted with 50% ACN by centrifugation at 2000xg and dried. The samples were reconstituted with 25  $\mu$ L 5% ACN in 2.5 mM NH<sub>4</sub>HCO<sub>3</sub> just before analysis and 10  $\mu$ L of the sample was analyzed by Accela LC System (Thermo Scientific, San Jose, CA) coupled with a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA). Samples were loaded onto a 30  $\times$  1mm  $\times$  1.9  $\mu$ m Hypersil GOLD column (Thermo Scientific, San Jose, CA) and eluted with a 12.5 minute binary solvent gradient (Solvent A: 5% acetonitrile/0.1% formic acid and Solvent B: 95% acetonitrile/0.1% formic acid) at 50  $\mu$ L/min. The gradient started from 5% Solvent B, increased linearly to 75% Solvent B in 10 minutes, and then remained at 75% B for 2.5 minutes. The eluates were ionized by electrospray ionization and dG-C8-ABP and dG-C8-ABP-d5 were detected with linear ion trap and 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 and mutagenesis.** Assays for cell cytotoxicity and mutagenesis were carried as previously described (Wu et al., 1997) with slight modifications. Cells were grown in HAT medium (30 mM hypoxanthine, 0.1 mM aminopterin, and 30 mM thymidine) for 12 doublings. Cells ( $1 \times 10^6$ ) were plated, allowed to grow for 24 h and were then treated with 1.56,

3.13, 6.25 or 12.5  $\mu\text{M}$  ABP (Sigma) or vehicle alone (0.5% DMSO) in media. After 48 hours, cells were plated to determine survival and mutagenic response to ABP. To determine cloning efficiency following each dose of ABP, 100 cells were plated in triplicate in 6 well-plates and allowed to grow for 7 days in non-selective media. Colonies were counted and expressed as percent of vehicle control. To determine mutagenic response following ABP exposure,  $5 \times 10^5$  cells were plated and sub-cultured for 7 days and then seeded with  $1 \times 10^5$  cells/100 x 20 mm dish (10 replicates) in complete  $\alpha$ MEM containing 40 mM 6-thioguanine (Sigma). Mutant *hprt* cells were allowed to grow for 7 days and colonies were counted to determine ABP-induced mutants and corrected by cloning efficiency.

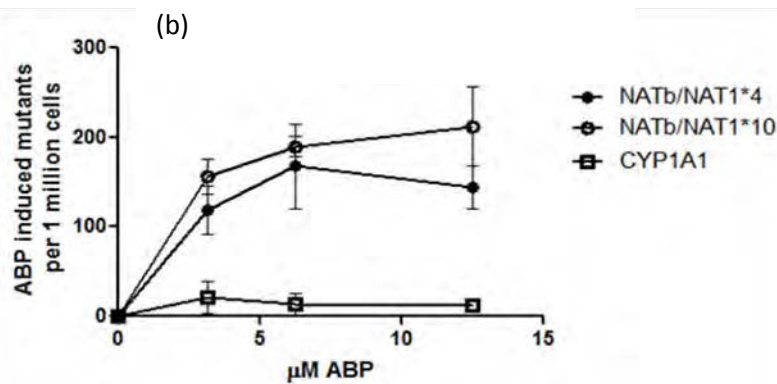
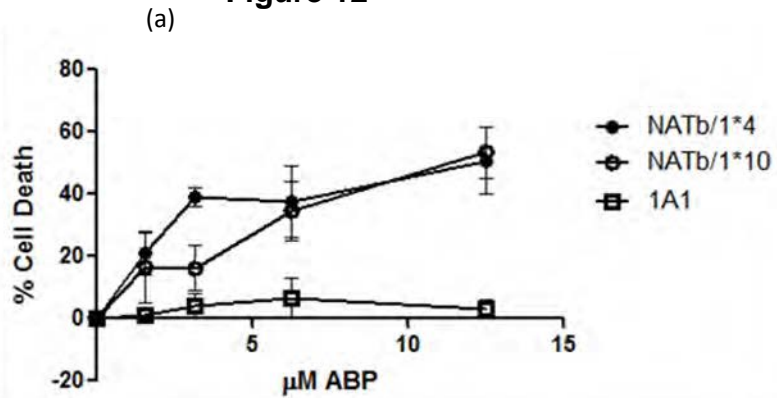
## Results

**Figure 11.**



**Figure 11.** ABP-induced cytotoxicity, mutagenesis, and DNA adduct formation in CHO cells stably expressing *CYP1A1* only (triangles) and *NATb/NAT1\*4* (circles) or *NATa/NAT1\*4* (squares) in pcDNA5/FRT. Each data point represents mean  $\pm$  S.E.M. for three determinations. (a) ABP-induced cytotoxicity; (b) ABP-induced dG-C8-ABP adducts/ $10^8$  nucleosides; (c) ABP-induced *hprt* mutant levels.

**Figure 12**



**Figure 12.** (a) ABP-induced cytotoxicity in NATb/NAT1\*4, NATb/NAT1\*10 and 1A1 (transfected with CYP1A1 only) stably transfected cells. Cytotoxicity was measured by plating 40,000 cells following exposure to ABP and is expressed as % cell death. Each error bar represents mean cell survival (n=3) ± SEM. No difference in cytotoxicity was observed between NATb/NAT1\*4 and NATb/NAT1\*10. Figure 10b. ABP-induced mutants at the *hprt* locus. Values are reported as mutants per 1 million cells. Each error bar represents mean (3 experiments) ± SEM. ABP-induced mutants following 3.13, 6.25, 12.5 μM ABP exposures (CYP1A1<NATb/NAT1\*4<NATb/NAT1\*10) differed significantly (p<0.05) following one-way ANOVA.

## Key Research Outcomes

- All cells transfected with NATb constructs (NATb/NAT1\*4, NATb/NAT1\*10, NATb/NAT1\*10B, NATb/NAT1\*14) resulted in higher N- and O- enzymatic activity than cells transfected with NATa constructs (NATa/NAT1\*4, NATa/NAT1\*10, NATa/NAT1\*10B, NATa/NAT1\*14).
- Cells transfected with NATa/NAT1\*4 resulted in significantly less ABP-induced cytotoxicity, DNA adduct formation and mutagenesis than cells transfected with NATb/NAT1\*4.
- Cells transfected with NAT1\*10 resulted in higher PABA and ABP N-acetylation activity than cells transfected with NAT1\*4.
- Three NAT1\*14 clones were examined and each clone resulted in lower protein expression and lower N- and O- acetylation activity than NAT1\*4 at higher concentrations. However, at lower concentrations, no difference was observed between NAT1\*14 and NAT1\*4.

## Reportable Outcomes

### Manuscripts

1. NATb/NAT1\*4 promotes greater arylamine *N*-acetyltransferase 1 mediated DNA adducts and mutations than NATa/NAT1\*4 following exposure to 4-aminobiphenyl. Lori M. Millner, Mark A. Doll, Jian Cai, J. Christopher States, and David W. Hein. Toxicological Sciences (submitted and under review).

### Published Abstracts

1. Lori M. Millner, Mark A. Doll, J. Christopher States and David W. Hein. Differences in Arylamine-induced Mutagenesis Associated with N-acetyltransferase 1 Alternative mRNA Isoforms. Abstracts Midwest DNA Repair Symposium, University of Louisville, Louisville, KY, May 15, 2010.
2. Lori M. Millner, Mark A. Doll, J. Christopher States and David W. Hein. Functional Effects of NAT1\*14 Polymorphism in a NATb mRNA Construct. 5<sup>th</sup> International Workshop on Arylamine N-acetyltransferases, Université Paris Diderot, Paris France, September 2, 2010.
3. Lori M. Millner, Mark A. Doll, J. Christopher States and David W. Hein. Differences in Arylamine-Induced Mutagenesis with N-acetyltransferase 1 Alternative mRNA Isoforms. 5<sup>th</sup> International Workshop on Arylamine N-acetyltransferases, Université Paris Diderot, Paris France, September 2, 2010.
4. Lori M. Millner, Mark A. Doll, J. Christopher States and David W. Hein. Effects of *N*-acetyltransferase 1 (NAT1\*10) polymorphisms in NATb and NATa derived mRNA constructs on DNA adducts and mutations from 4-aminobiphenyl. Society of Toxicology

## Conclusions

### **NATb/NAT1\*4 versus NATa/NAT1\*4**

Significantly more NAT1 activity, protein, mRNA, ABP-induced cytotoxicity, DNA adducts and mutagenesis were detected in cells stably transfected with NATb/NAT1\*4 than in cells transfected with NATa/NAT1\*4 ( $p < 0.05$ ). DNA adduct and mutant levels following exposure to ABP are biological endpoints that are very relevant to cancer risk. Because DNA adduct and mutant levels were significantly higher in cells transfected with NATb/NAT1\*4 than cells transfected with NATa/NAT1\*4, cancer risk may be altered in tissues that express both NATb and NATa transcripts following exposure to arylamine carcinogens due to altered gene expression. These results suggest that differential regulation occurs in the NAT1 5'-UTR which could contribute to overexpression in disease states, causing increased mutagenesis, enhanced tumor growth, and decreased chemotherapeutic sensitivity. This study focused only on the referent allele, NAT1\*4, but future studies should investigate 5'-UTR control with other NAT1 alleles to determine if any effect occurs between combinations of 5'-UTRs and alleles.

### **NAT1\*10 versus NAT1\*4**

NAT1\*10 is putatively considered to be a rapid acetylator, however there are many conflicting results about the acetylator phenotype of NAT1\*10. One study reported significantly higher enzyme activity in urinary bladder and colon tissue for individuals heterozygous for NAT1\*10/\*4 compared to those individuals homozygous for NAT1\*4 (Bell et al., 1995). Similar findings were reported in colorectal tissue. In contrast, another study employing recombinantly expressed alleles reported no difference between NAT1\*10 and NAT1\*4 activities (de Leon et al., 2000).

This study reports on *in vitro* and *in situ* N-acetylation experiments which show that NAT1\*10 and NAT1\*10B may be rapid acetylators compared to the referent, NAT1\*4. ABP-induced mutagenesis also show increased mutants in cells transfected with NAT1\*10 than in cells transfected with NAT1\*4. NAT1\*10 has been of high interest because of its association with many different cancers and because it has a very high allelic frequency in many different populations. Our constructs are a useful tool in the study of NAT1\*10 and may help elucidate the molecular mechanism causing NAT1\*10 to be associated with increased acetylation activity and increased cancer risk.

### **NAT1\*14 versus NAT1\*4**

Previous studies have shown that NAT1\*14 is associated with decreased protein expression and decreased acetylation activity (Fretland et al., 2001). These studies confirm that NAT1\*14 results in decreased protein expression when compared to NAT1\*4. Our studies also report that at higher concentrations, NAT1\*14 is associated with decreased acetylation capacity. However, at concentrations more biologically relevant (0-5  $\mu$ M for ABP and 0-100  $\mu$ M for N-OH-ABP), there is no difference observed between NAT1\*14 and NAT1\*4. This could indicate that a person with NAT1\*14 may not be at altered cancer risk following exposure to arylamine carcinogens.

## References

Bell, D.A., Badawi, A.F., Lang, N.P., Ilett, K.F., Kadlubar, F.F., and Hirvonen, A. (1995). Polymorphism in the N-acetyltransferase 1 (NAT1) polyadenylation signal: association of NAT1\*10 allele with higher N-acetylation activity in bladder and colon tissue. *Cancer Res* 55, 5226-5229.

de Leon, J.H., Vatsis, K.P., and Weber, W.W. (2000). Characterization of naturally occurring and recombinant human N-acetyltransferase variants encoded by NAT1. *Mol Pharmacol* 58, 288-299.

Fretland, A.J., Doll, M.A., Leff, M.A., and Hein, D.W. (2001). Functional characterization of nucleotide polymorphisms in the coding region of N-acetyltransferase 1. *Pharmacogenetics* 11, 511-520.