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Role of Human *N*-Acetyltransferase 2 Genetic Polymorphism on Aromatic Amine Carcinogen-induced DNA Damage and Mutagenicity in a Chinese Hamster Ovary Cell Mutation Assay

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Abstract

Carcinogenic aromatic amines such as 4-aminobiphenyl (ABP) and 2-aminofluorene (AF) require metabolic activation to form electrophilic intermediates that mutate DNA leading to carcinogenesis. Bioactivation of these carcinogens includes *N*-hydroxylation catalyzed by CYP1A2 followed by *O*-acetylation catalyzed by arylamine *N*-acetyltransferase 2 (NAT2). To better understand the role of NAT2 genetic polymorphism in ABP- and AF-induced mutagenesis and DNA damage, nucleotide excision repair-deficient (UV5) Chinese hamster ovary (CHO) cells were stably transfected with human *CYP1A2* and either *NAT2*4* (rapid acetylator) or *NAT2*5B* (slow acetylator) alleles. ABP and AF both caused significantly ($p < 0.001$) greater mutagenesis measured at the *hypoxanthine phosphoribosyl transferase* (*hprt*) locus in the UV5/*CYP1A2*/*NAT2*4* acetylator cell line compared to the UV5, UV5/*CYP1A2*, and UV5/*CYP1A2*/*NAT2*5B* cell lines. ABP- and AF-induced *hprt* mutant cDNAs were sequenced and over 80% of the single-base substitutions were at G:C base pairs. DNA damage also was quantified by γ H2AX in-cell western assays and by identification and quantification of the two predominant DNA adducts, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) and *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) by liquid chromatography-mass spectrometry. DNA damage and adduct levels were dose-dependent, correlated highly with levels of *hprt* mutants, and were significantly ($p < 0.0001$) greater in the UV5/*CYP1A2*/*NAT2*4* rapid acetylator cell line following treatment with ABP or AF as compared to all other cell lines. Our findings provide further clarity on the importance of *O*-acetylation in CHO mutagenesis assays for aromatic amines. They provide evidence that NAT2 genetic polymorphism modifies aromatic amine-induced DNA damage and mutagenesis that should be considered in human risk assessments following aromatic amine exposures.

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Statement of Author Contributions

KJB and DWH designed the study.

KJB, RASG, and MAD collected the data.

KJB, RASG, and DWH analyzed the data.

KJB and DWH prepared the manuscript draft with important intellectual input from the other co-authors, all of whom approved the final manuscript.

Keywords

N-acetyltransferase 2; genetic polymorphism; 4-aminobiphenyl; 2-aminofluorene

INTRODUCTION

As recently reviewed [Wang et al., 2019], more than one in eight known or suspected human carcinogens is an aromatic amine, or a chemical that can convert into one, making them a major class of human carcinogens. Two aromatic amines, 4-aminobiphenyl (ABP) and 2-aminofluorene (AF) are frequently the subject of laboratory research investigations. ABP is a known human carcinogen present in tobacco smoke and dye products whereas *N*-acetylaminofluorene (a metabolite of AF) is reasonably anticipated to be a human carcinogen [National Toxicology Program, 2016].

Aromatic amine-induced DNA adduct formation and mutagenesis require metabolic activation, and the primary pathway includes *N*-hydroxylation catalyzed by hepatic CYP1A2 [Schut and Snyderwine, 1999]. The *N*-hydroxy metabolites formed are weakly reactive but are more likely to undergo further activation catalyzed by phase II enzymes [Snyderwine et al., 2002]. Arylamine *N*-acetyltransferase 2 (NAT2) activates *N*-hydroxy metabolites by *O*-acetylation to *N*-acetoxy metabolites which break down to electrophilic intermediates that form DNA adducts [Snyderwine, 2002]. In contrast, NAT2-catalyzed *N*-acetylation of aromatic amines competes with *N*-hydroxylation and in this role is considered a detoxification pathway.

ABP- and AF-induced DNA-adducts primarily form at the C8 position of deoxyguanosine [Heflich and Neft, 1994; Chen et al., 2018]. Bulky DNA adducts such as these are recognized by the nucleotide excision repair (NER) pathway. Since DNA damage and mutagenesis are a function of both environmental exposure and carcinogen metabolism, they are informative biomarkers to investigate genetic variation in carcinogen metabolism. *N*-hydroxy-ABP mutagenesis at the *hprt* and *TK* loci, and dG-C8-ABP DNA adduct formation have been investigated in human lymphoblastoid cells [Ricicki et al., 2005]. The metabolic activation of AF has been investigated in V79 Chinese hamster cells expressing human *CYP1A2* and *NAT2* alleles [Scheuenpflug et al., 2005], as well as other strains of CHO cells, by laboratories investigating both mutagenesis and DNA adduct formation [Heflich et al., 1988; Nairn et al., 1988].

Since aromatic amines require metabolic activation to exert their carcinogenic effects, genetic polymorphisms in carcinogen-metabolizing enzymes such as NAT2 may modify cancer risk following exposure [Hein, 2002]. Humans exhibit genetic polymorphism in *NAT2* resulting in rapid and slow acetylator phenotypes. The *NAT2**4 allele is associated with rapid acetylator phenotype whereas the *NAT2**5B allele is associated with slow acetylator phenotype. Higher levels of ABP-DNA adducts have been reported in human breast [Ambrosone et al., 2007] from rapid versus slow NAT2 acetylators, whereas ABP-hemoglobin adducts are higher in slow acetylators [Bartsch et al., 1990]. Epidemiological studies suggest a role for NAT2 genetic polymorphism in susceptibility to various cancers

[Hein et al, 2000]. However, laboratory-based experiments are required to support the biological plausibility and the conclusions inferred from these epidemiological studies.

Our laboratory has reported on previous investigations in which DNA repair-deficient CHO cells transfected with *CYP1A1* or *CYP1A2* and *NAT2*4* or *NAT2*5B* were incubated with heterocyclic amine carcinogens such as 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), 2-amino-3-methylimidazo [4,5-f] quinoline (IQ), and 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx). The results differed with the heterocyclic amine carcinogen tested, as rapid acetylator *NAT2*4* did not affect levels of mutagenesis and DNA adduct formation following incubation with PhIP [Metry et al., 2007] whereas both mutagenesis and DNA adduct formation were markedly higher in CHO cells possessing rapid acetylator *NAT2*4* following incubation with IQ or MeIQx [Metry et al., 2010]. The conclusion that *NAT2* is necessary for the DNA damage and mutagenesis induced by MeIQx and IQ but not PhIP was recently confirmed in V79 Chinese hamster cells [Chevereau et al., 2017]. Our laboratory also reported higher levels of ABP-induced *hprt* mutants and DNA adducts in DNA repair-deficient CHO cells transfected with *CYP1A1* and *NAT2*4* [Bendaly et al., 2009]. However, a study in V79 Chinese hamster cells with heterologous co-expression of human *CYP1A2* and *NAT2*4* found lower levels of AF-induced mutants in cells transfected with both *CYP1A2* and *NAT2*4* compared to transfection with *CYP1A2* alone [Scheuenpflug et al., 2005] suggesting that the findings might differ with aromatic amine (ABP vs. AF) or cytochrome P450 (*CYP1A1* vs. *CYP1A2*).

To better understand the role of *CYP1A2* and *NAT2* in ABP- and AF- induced mutagenesis and DNA damage, NER-deficient CHO cells were stably transfected with human *CYP1A2* and either *NAT2*4* (rapid acetylator) or *NAT2*5B* (slow acetylator) alleles. ABP- and AF-induced mutagenesis was measured at the hypoxanthine phosphoribosyl transferase (*hprt*) locus. DNA damage was quantified by γ H2AX in-cell western assays and by identification and quantification of the two predominant DNA adducts, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) and *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF).

MATERIALS AND METHODS

Construction of transformed UV5-CHO cell lines

The UV5-CHO cell line, a NER-deficient derivative of the AA8 line [Thompson et al., 1980], was obtained from the ATCC (Catalog number: CRL-1865). Since UV5-CHO lacks NER due to a mutation in the XPD (ERCC2) gene [Weber et al., 1988], it is hypersensitive to bulky adduct mutagens and belongs to the excision repair cross complementation group 2. All cells were grown in alpha-modified minimal essential medium (Cambrex) without L-glutamine, ribosides, and deoxyribosides, supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, 100 units/ml streptomycin (Cambrex), and 2 mM L-glutamine (Cambrex) at 37 °C in 5% CO₂. Media were supplemented with appropriate selective agents to maintain stable transfectants.

Construction and characterization of the UV5-CHO cell lines stably transfected with *CYP1A2* only (UV5/*CYP1A2*), *CYP1A2* plus *NAT2*4* (rapid acetylator) (UV5/*CYP1A2*/

NAT2*4), and *CYP1A2* plus *NAT2*5B* (slow acetylator) (UV5/*CYP1A2*/*NAT2*5B*) alleles has been described in detail previously [Metry et al., 2007].

***N*-Acetyltransferase assays**

N-acetyltransferase activities for ABP and AF were measured by separation of parent and their *N*-acetylated metabolite via high performance liquid chromatography (HPLC) as previously described [Leff et al., 1999; Fretland et al., 2002]. In order to isolate cell cytosols, 1×10^6 cells were seeded into 10 cm culture dishes and incubated for 48 hr. The cells were trypsinized and collected. After washing once with PBS, the cells were disrupted by three cycles of freezing and thawing in lysis buffer, and centrifuged for 10 min at $10,000 \times g$. Lysis buffer consisted of 20 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, 100 μ M phenylmethylsulphonylfluoride (PMSF), 1 μ M pepstatin A, and 1 μ g/ml aprotinin. Supernatants were collected and used immediately or stored at -80°C until used. Cell lysates were incubated with 300 μ M ABP or AF and 1 mM acetyl-coenzyme A at 37°C for 10 min. Reactions were terminated by the addition of 1 M perchloric acid. Arylamine and *N*-acetylated products were separated with a mobile phase of 20 mM sodium perchlorate (pH 2.5) and acetonitrile at a ratio of 91:9. Protein was quantified using the Bio-Rad protein assay kit (Bio-Rad).

Cell survival and *hprt* mutations

Assays for cell survival and mutagenesis were modified slightly from methods previously described [Wu et al., 1997; Metry et al., 2007]. Briefly, cells were grown for 12 doublings, with selective agents in complete HAT medium (30 μ M hypoxanthine, 0.1 μ M aminopterin, and 30 μ M thymidine). Cells were plated at a density of 5×10^5 cells/T-25 flask and incubated for 24 hr, after which media were changed and the cells treated for 48 hr with various concentrations of AF or ABP dissolved in DMSO or vehicle control (0.5% DMSO). Survival was determined by a colony forming assay and expressed as percent of vehicle control. The remaining cells were replated and subcultured for 7 days of growth. Then, cells were plated for cloning efficiency in complete media and for *hprt* mutations in complete medium containing 40 μ M 6-thioguanine (6-TG) (Sigma, St. Louis, MO). Dishes were seeded with 1×10^5 cells/100 mm dish (10 replicates) and incubated for 7 days in 6-TG media; cloning efficiency dishes were seeded with 100 cells/well/6-well plate in triplicate and incubated for 6 days.

Sequencing of *hprt* mutants

Sequencing of *hprt* mutants following incubation with ABP or AF was carried out as previously described [Metry et al., 2010]. Briefly, one viable colony was picked from each 6-TG containing culture dish of 2.0 μ M ABP-treated or 1.0 μ M AF-treated UV5/*CYP1A2*/*NAT2*4* cells and propagated until one confluent 10-cm dish was obtained. The cells were harvested and pelleted. RNA was extracted immediately using the RNeasy Mini Kit (Qiagen) or pellets were snap-frozen in liquid nitrogen and stored at -80°C for later use. RNA (1 μ g) was reverse transcribed with oligo(dT) primer from the SuperScript III First-Strand Synthesis System (Invitrogen) as described by the manufacturer. The *hprt* coding region was amplified and the PCR product was purified and sequenced as previously described [Metry et al., 2010].

Identification and quantification of DNA adducts

Cells grown in 15-cm plates were treated with ABP or AF as described above for the cytotoxicity and mutagenesis assays. Cells were harvested after 48 hr of treatment, centrifuged, and the pellet resuspended in 500 μ l of 50 mM Tris-HCl, 10 mM EDTA, pH 8.0. DNA samples (200 μ g) from ABP- or AF- treated cells were digested and prepared for LC-MS-MS analysis as previously described [Metry et al., 2007]. Samples used for quantitative analysis of *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP; Toronto Research Chemicals) were spiked with one ng deuterated internal standard dG-C8-ABP-d5 (Toronto Research Chemicals; >97% purity by NMR spectroscopic analysis, 97.5% pure by mass spectral analysis) before any sample treatment. *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) was generously provided by Dr. Fred Beland from the National Center for Toxicological Research, Jefferson, Arkansas. Samples used for quantitative analysis of dG-C8-AF were spiked with one ng of dG-C8-ABP (97.5% pure by mass spectral analysis) as the type C internal standard. HPLC conditions and MS analysis parameters were set as previously described [Metry et al., 2007], with a few modifications. Electrospray ionization was set at 2.5 kV, cone voltages were 25 V and the collision energy was 25 V for both dG-C8-ABP and dG-C8-AF. Argon was used as the collision gas and set at 2.0×10^{-4} mBar. Collision energy was 20 V set to minimize fragmentation and to cleave the glycosidic bond, producing one major fragment, which was $[(M-116) + H]^+$ for each adduct.

The dG-C8-ABP adduct was monitored using the multiple reaction monitoring (MRM) transition from m/z 435 to 319 m/z and the deuterated internal standard (dG-C8-ABP-d5) was monitored using the transition from m/z 440 to m/z 324 as previously described [Neale et al., 2008]. Similarly, the dG-C8-AF adduct was monitored using the MRM transition from m/z 446 to m/z 330 with the internal standard (dG-C8-ABP) using the transition from m/z 435 to m/z 319.

Quantification of DNA damage

DNA damage was assessed by a γ H2AX in-cell western staining protocol as previously described [Audebert et al., 2010]. Cells were grown with selective agents in 10-cm plates. Then 1×10^5 cells were plated into black/clear bottom 96-well plates (Corning, Corning, NY, USA) and allowed to attach overnight. The next morning media was removed (cell debris and non-adherent cells were washed away and removed) and attached cells washed with PBS and replaced with fresh pre-warmed media containing 0.01–100 μ M ABP or AF. Cells were incubated for up to 24 hr, after which media were removed and γ H2AX in-cell western staining protocol was performed as follows; cells were fixed to the plate using 3.7% formaldehyde and incubating at room temperature for 20 min. Then, the cells were permeabilized by washing five times with 0.1% Triton X-100 in TBS. After permeabilization, the cells were blocked using FISH Gelatin Blocking Agent (Biotium, Fremont, CA, USA) diluted in TBS for 90 min at room temperature with constant agitation. Primary antibody anti-phospho-histone H2AX (Millipore-Sigma, Burlington, MA, USA) was diluted to 2 μ g/mL and added to the cells and then incubated overnight at 4°C. The next morning cells were washed with 0.1% Tween 20 in TBS for 5 min, five times. Secondary antibody IRDye® 800CW goat anti-mouse IgG (H+L) (LI-COR, Lincoln, NE, USA) was used at a 1:1000 dilution and DNA dye RedDot™ 2 diluted to 1X (Biotium, Fremont, CA,

USA) to normalize for DNA content. Cells were incubated with this combination for 60 min and washed again with the Tween 20 solution as previously described. DNA and the γ H2AX were simultaneously visualized using an Odyssey CLx imaging system (LI-COR, Lincoln, NE, USA) with the 680 nm fluorophore (red) and the 800 nm fluorophore (green). Relative fluorescence units from the scanning allowed a quantitative analysis. Relative fluorescent units for γ H2AX per cell (as determined by γ H2AX divided by DNA content) were divided by untreated cells to determine percent change in phosphorylation of H2AX levels relative to control.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (La Jolla, CA). Differences between measurements in the UV5, UV5/CYP1A2, UV5/CYP1A2/NAT2*5B and UV5/CYP1A2/NAT2*4 cell lines were analyzed for significance by two-way ANOVA followed by Tukey's multiple comparison tests. Correlations between levels of ABP- and AF-induced DNA adducts and mutants in the UV5, UV5/CYP1A2, UV5/CYP1A2/NAT2*5B and UV5/CYP1A2/NAT2*4 cell lines were analyzed by the Pearson correlation coefficient. Differences or correlations were considered significant if $p < 0.05$.

RESULTS

N-acetylation capacity in NAT2-transfected cells

ABP and AF *N*-acetyltransferase activity was measured and found to be greater in the UV5/CYP1A2/NAT2*4 rapid acetylator cell line than the UV5/CYP1A2/NAT2*5B slow acetylator cell line (Figure 1). ABP and AF *N*-acetyltransferase activity in the UV5 and UV5/CYP1A2 cell lines were below the level of detection (< 20 pmol/min/mg).

Aromatic amine-induced cytotoxicity and mutagenesis

Only the UV5/CYP1A2/NAT2*4 cells showed concentration-dependent cytotoxicity and *hprt* mutagenesis following incubation with ABP or AF (Figure 2). There were significantly more ABP- and AF-induced *hprt* mutants in the UV5/CYP1A2/NAT2*4 rapid acetylator cell line ($p < 0.001$) than in all other cell lines for all concentrations tested (Figure 2).

Mutant sequencing

Mutations in *hprt* cDNAs from 23 independent ABP-induced *hprt* mutants and 22 independent AF-induced mutants are described in Tables I and II, respectively. For ABP-induced *hprt* mutants, 74% of the mutants were single base substitutions, 88% of which were transversions. The remaining 26% were exon deletions presumably resulting from splice-site mutations. Half the deletion mutants had exon 5 deleted. Furthermore, 88% of the single base substitutions were at G:C base pairs, with 60% G:C $\rightarrow$ T:A, 7% G:C $\rightarrow$ A:T, and 33% G:C $\rightarrow$ C:G.

For AF-induced *hprt* mutants, 86% of the mutants were single base substitutions, 74% of which were transversions. (The remaining were exon deletions presumably caused by splice-site mutations). Two of the three deletion mutants had exon 8 deleted. Also, of the single-base substitutions, 84% were at GC base pairs, with 62.5% G:C $\rightarrow$ T:A, 12.5% G:C $\rightarrow$ A:T,

and 25% G:C → C:G. Mutants in untreated cells were not sequenced because the frequency of spontaneous mutants was less than 10% of either ABP- or AF-induced mutants.

In order to compare single-base substitutions induced by ABP and AF, we mapped these mutations in the *hprt* cDNA sequence (Figure 3). For comparison, we added heterocyclic amine-induced single-base substitutions in the *hprt* cDNA sequence previously described [Metry et al., 2010].

Identification and measurement of DNA adducts and DNA double-strand breaks

Under the low energy MRM mass spectrometry scanning conditions employed, one principal deoxyribonucleoside adduct was observed following incubation with ABP or AF. The adduct following incubation of ABP had a molecular mass of 435 reflecting dG-C8-ABP. The adduct following incubation with AF had a molecular mass of 447 reflecting dG-C8-AF.

dG-C8-ABP and dG-C8-AF adduct levels were dose-dependent and significantly ($p < 0.001$) greater in the UV5/CYP1A2/NAT2*4 rapid acetylator cell line than all other cell lines (Figure 4). As shown in Figure 5, DNA adduct levels correlated highly with induced-*hprt* mutants in all cell lines following treatment with ABP ($r^2 = 0.96$) or AF ($r^2 = 0.94$). γ H2AX-positive DNA double-strand breaks in the CHO cells transfected with CYP1A2 and NAT2 were dose-dependent following incubation with both ABP and AF (Figure 4). The DNA double-strand breaks were at background levels in the UV5 and UV5/CYP1A2 cells. DNA damage was significantly higher in the UV5/CYP1A2/NAT2*4 than the UV5/CYP1A2/NAT2*5B cells ($p < 0.0001$). As shown in Figure 5, levels of DNA double-strand breaks correlated highly with dG-C8-ABP adducts ($r^2 = 0.85$) following incubation with ABP and dG-C8-AF adducts ($r^2 = 0.94$) following incubation with AF.

DISCUSSION

Carcinogenic aromatic amines such as ABP and AF require metabolic activation to form electrophilic intermediates that mutate DNA leading to carcinogenesis. Bioactivation includes *N*-hydroxylation catalyzed by CYP1A2 followed by *O*-acetylation catalyzed by NAT2. NER-deficient CHO cells were stably transfected with human *CYP1A2* and either *NAT2*4* (rapid acetylator) or *NAT2*5B* (slow acetylator) alleles. ABP and AF both caused significantly ($p < 0.001$) greater mutagenesis measured at the *hprt* locus in the UV5/CYP1A2/NAT2*4 compared to the UV5, UV5/CYP1A2, and UV5/CYP1A2/NAT2*5B cell lines. Both ABP- and AF-induced *hprt* mutant cDNAs were sequenced and over 80% of the mutations were single-base missense or nonsense substitutions at G:C base pairs. dG-C8-ABP and dG-C8-AF adduct levels were dose-dependent and significantly greater in the rapid *NAT2*4* acetylator cell line following treatment with AF or ABP as compared to all other cell lines. DNA adduct levels correlated very highly with *hprt* mutagenesis for both ABP and AF and were in the following order: UV5 < UV5/CYP1A2 < UV5/CYP1A2/NAT2*5B < UV5/CYP1A2/NAT2*4. Similar findings have been reported following administration of heterocyclic amines MeIQx and IQ [Metry et al., 2010] but not PhIP [Metry et al., 2007]. More recently, it was confirmed that transfection of human NAT2 into V79 Chinese hamster cells increases mutagenicity of MeIQx and IQ, but not PhIP [Chevereau et al., 2017].

Consistent with the CHO cell data described above, dG-C8-MeIQx, but not dG-C8-PhIP adducts are higher in liver from rapid NAT2 acetylators than slow acetylators congenic rats following administration of MeIQx [Metry et al., 2009]. The role of *N*-acetyltransferase in levels of DNA adduct formation following administration of ABP and AF also has been investigated in animal models. Higher levels of DNA damage were observed following incubation with AF [McQueen et al., 1982] but not ABP [McQueen et al., 1983] in hepatocytes from rapid than slow acetylators rabbits. Levels of the major DNA adduct dG-C8-AF were higher in urinary bladders of rapid versus slow acetylators Syrian hamsters [Flammang et al., 1992] as well as in Syrian hamsters congenic for NAT2 [Feng et al., 1996]. Hepatic DNA adduct levels were higher in rapid than slow acetylators mice [Levy and Weber, 1989] and Syrian hamsters [Flammang et al., 1992] following administration of AF. In contrast, rapid and slow acetylators mice did not differ in levels of hepatic DNA adducts following administration of ABP [McQueen et al., 2003]. Hepatic dG-C8-ABP levels were significantly higher in neonatal double NAT knockout mice compared to wildtype mice administered ABP [Sugamori et al., 2012]. Hepatic levels of dG-C8-ABP were lower in adult mice than in neonatal mice and did not differ significantly in adult double NAT knockout compared to wildtype mice [Sugamori et al., 2012]. Perhaps these findings are influenced by the important role of sulfotransferases in the metabolic activation of ABP in mice [Li et al., 2018].

CHO cells have negligible endogenous phase I and II enzymes, and our present findings showed that both ABP and AF induced highest levels of mutagenesis and DNA adduct formation in the cell line expressing both human *CYP1A2* and rapid acetylators *NAT2*4*. A study in V79 Chinese hamster cells with heterologous co-expression of human *CYP1A2* and rapid or slow *NAT2* alleles found that AF induced mutants in the *CYP1A2* cell line with negligible mutants in the *CYP1A2/NAT2*4* cell line [Scheuenpflug et al., 2005]. These results differ from those obtained in our experiments in which we found highest levels in the UV5/*CYP1A2/NAT2*4* cell line. The reason(s) for this difference is unknown, but based on our results, it does not appear to be related to aromatic amine or cytochrome P450. While the results in V79 Chinese hamster cells [Scheuenpflug et al., 2005] are consistent with *N*-acetylation as a deactivation pathway for AF mutagenesis, our results with both AF and ABP suggest that rapid acetylators have increased mutations as compared to slow acetylators because *O*-acetylation is required to form DNA adducts leading to mutagenesis. Although we have investigated NAT2 only in combination with *CYP1A1* [Bendaly et al., 2009] and *CYP1A2*, it is likely that other cytochrome P450s activate aromatic amines depending upon the cell and tissue. For example, a recent report showed that *CYP2A13* expressed in the urinary bladder bioactivates ABP to form DNA adducts [Bellamri et al., 2019]. The ability of recombinant human NAT2 allozymes to catalyze metabolic activation of *N*-hydroxy-ABP and *N*-hydroxy-AF to DNA adducts via *O*-acetyltransferase is markedly lower when catalyzed by NAT2 5B as compared to the NAT2 4 [Hein et al., 1995; Hein, 2002]. Similar results towards the metabolic activity of *N*-hydroxy-ABP have been reported in COS-1 cells transfected with *NAT2*4* and *NAT2*5B* [Zang et al., 2007] and in rapid and slow acetylators cryopreserved human hepatocytes [Doll et al., 2010]. Previous studies in our laboratory quantified ABP-induced *hprt* mutations and DNA adducts in *NER*-deficient UV5 cells transfected with *CYP1A1* and *NAT2* [Bendaly et al., 2009]. ABP-induced *hprt* mutants and

DNA adduct levels were negligible in UV5 cells transfected with *CYP1A1* alone. However, whether co-transfected with *CYP1A1* [Bendaly et al., 2009] or *CYP1A2* (current study), ABP-induced *hprt* mutant and DNA adduct levels were higher following transfection with the *NAT2*5B* slow acetylator allele, and much higher following transfection with the *NAT2*4* rapid acetylator allele.

Our findings confirm previous conclusions [Heflich et al., 1988] that proposed a major difference in the metabolism of *N*-hydroxy arylamines in CHO versus bacterial mutagenesis assays to account for different sensitivities towards aromatic amines. The bacterial mutagenicity of AF is highly dependent upon bacterial *O*-acetylase [McCoy et al., 1982; 1983]. *N*-hydroxy-AF is about 10-fold more mutagenic in *Salmonella typhimurium* strain TA98 than it is in strain TA98/1,8-DNP6 [McCoy et al., 1983] which is a derivative deficient in endogenous *O*-acetylase activity [McCoy et al., 1982; 1983]. Transformation of TA98/1,8-DNP6 with human NAT increases sensitivity to AF-induced mutagenesis [Grant et al., 1992].

The levels of *hprt* mutants and DNA adducts correlated very highly following administration of either ABP or AF. The majority of induced mutations for both aromatic amines were missense/nonsense single-base substitutions, in which over 80% were at G:C base pairs. There were a few locations in the *hprt* cDNA where both aromatic amines had induced mutations such as within a string of G's at position 208-209, 482 and 550-551. Furthermore, when adding previously published heterocyclic amine-induced mutations [Metry et al., 2010], positions 134-135 (GpG), 208-209 (string of G's), 418-419 (GpG) and 589 were frequent mutation locations. It is also important to note that for both ABP and AF-induced *hprt* mutants, over 60% of the single-base substitutions at G:C base pairs were G to T transversions and about 30% were G to C transversions. These results are similar to our studies for PhIP, IQ, and MeIQx [Metry et al., 2010] and to previous studies with PhIP and IQ [Wu et al., 1997]. Also, in a study with transgenic Big Blue rats dosed with *N*-hydroxy-2-acetylaminofluorene, G:C to T:A transversions predominated [Chen et al., 2001].

In conclusion, CHO cells stably transfected with *CYP1A2* and *NAT2* exhibited dose-dependent increases in mutagenesis and DNA damage following incubation with ABP or AF. The levels of *hprt* mutants, dG-C8 adducts, and double-strand breaks correlated very highly for both ABP and AF. The levels were dose-dependent and consistently highest in the UV5/*CYP1A2*/*NAT2*4* rapid acetylator cell line. Our findings provide further clarity on the importance of *O*-acetylation in CHO mutagenesis assays for aromatic amines. Since more than one in eight known or suspected human carcinogens is an aromatic amine, or a chemical that can convert into one [Wang et al., 2019], mutagenesis assays for aromatic amines or chemicals that can be converted to aromatic amines should be conducted in CHO cells stably transfected with human *CYP1A2* and *NAT2*. Furthermore, the findings provide further evidence that the *NAT2* genetic polymorphism modifies aromatic amine-induced mutagenesis and DNA damage and should be considered in human risk assessments following aromatic amine exposures.

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Figure 1. ABP and AF NAT activity in UV5/CYP1A2/NAT2*5B or UV5/CYP1A2/NAT2*4 cells. Each bar represents Mean $\pm$ SEM for three independent experiments. **A:** The difference in ABP NAT activity between the CYP1A2/NAT2*5B and the CYP1A2/NAT2*4 CHO cell lines was significant ($p < 0.0001$). **B:** The difference in AF NAT activity between the CYP1A2/NAT2*5B and the CYP1A2/NAT2*4 CHO cell lines was significant ($p = 0.0003$).

Figure 2.

Cytotoxicity and *hprt* mutants. Percent survival measured by colony forming ability plotted for each of the four UV5 CHO cell lines following treatment with ABP (A) or AF (C). Data represent Mean $\pm$ SEM for two independent experiments. *hprt* mutant fractions following treatment with ABP (B) or AF (D). Data presented as Mean $\pm$ SEM for 4 independent experiments. Error bars not shown fall within the symbol. The UV5/CYP1A2/NAT2*4 cells had significantly more ($p < 0.001$) *hprt* mutants as compared to all other cell lines following treatment with ABP or AF at each concentration tested. The UV5/CYP1A2/NAT2*5B cells had significantly more *hprt* mutants than the UV5 and UV5/CYP1A2 cells following treatment with 2.0 μ M ($p < 0.001$) or 3.0 μ M ($p < 0.01$) AF.

Figure 3.

Location of ABP- and AF- induced base substitutions in the mutated *hprt* cDNAs. Highlighted regions designate deleted exons. Previously identified heterocyclic amine-induced mutations are also shown: B: ABP; F: AF; I: IQ; M: MeIQx; P: PhIP.

Figure 4.

DNA adduct formation and double-strand breaks indicated by gamma-H2AX staining. dG-C8-ABP (**A**) or dG-C8-AF (**B**) DNA adduct levels in CHO cells treated with ABP or AF, respectively. Gamma H2AX staining indicating DNA double-strand breaks in CHO cells following incubation with ABP (**C**) or AF (**D**), respectively. Data for UV5 cells (closed squares), UV5/CYP1A2 cells (open squares), UV5/CYP1A2/NAT2*5B cells (closed triangles) and UV5/CYP1A2/NAT2*4 cells (open triangles) represent Mean $\pm$ SEM for three independent experiments in each of the stably transfected CHO cell lines. dG-C8-ABP (**A**) and dG-C8-AF (**B**) DNA adduct levels were significantly greater in the UV5/CYP1A2/NAT2*4 compared to all other cell lines at each concentration tested ($p < 0.0001$). ****, Differences between the CHO cell lines were significant ($p < 0.0001$) as indicated.

Figure 5.

Relationships of *hprt* mutants and DNA double strand breaks to dG-C8-adduct levels. **A.** Correlation of ABP-induced *hprt* mutants to dG-C8-ABP adduct levels in all four cell lines ($r^2 = 0.96$). **B.** Correlation of ABP-induced DNA double strand breaks to dG-C8-ABP adduct levels in all four cell lines ($r^2 = 0.85$). **C.** Correlation of ABP-induced *hprt* mutants to dG-C8- AF adduct levels in all four cell lines ($r^2 = 0.94$). **D.** Correlation of AF-induced DNA double strand breaks to dG-C8- AF adduct levels in all four cell lines ($r^2 = 0.94$). Data from Figures 2 and 4 have been combined to produce the correlation plots.

Table I.ABP-induced *hprt** mutations in UV5/CYP1A2/NAT2*4 CHO cells

Missense/Nonsense Single-Base Substitutions					
Mutant #	Nucleotide	Exon	Type of Mutation [†]	Surrounding	Amino Acid
	Position			Sequence	Change
1	74	2	C:G > G:C	attcetaat	Pro > Arg
2	113	2	C:G > A:T	attcctcat	Pro > His
3	197	3	G:C > T:A	ctctgtgtg	Cys > Phe
3	208	3	G:C > T:A	aaggggggc	Gly > Trp
4	209	3	G:C > A:T	aaggggggc	Gly > Glu
5	209	3	G:C > T:A	aaggggggc	Gly > Val
6	236	3	T:A > G:C	ctgctggat	Leu > Arg
8	464	6	T:A > C:G	aacctcaaa	Leu > Pro
9	482	6	C:G > A:T	gttgcaagc	Ala > Glu
10	482	6	C:G > A:T	gttgcaagc	Ala > Glu
11	550	8	C:G > G:C	attcagac	Pro > Ala
12	550	8	C:G > G:C	attcagac	Pro > Ala
13	575	8	C:G > A:T	tatgccctt	Ala > Asp
14	582	8	C:G > G:C	cttgactat	Asp > Glu
15	617	9	G:C > T:A	atttgtgtc	Cys > Phe
16	628	9	G:C > T:A	agtgaact	Glu > Stop
17	648	9	C:G > G:C	aaatacaaa	Tyr > Stop
Deletions ^{††}					
18	319-384	4	Deletion	actgt aatgt	Frameshift
19	385-402	5	Deletion	gaaag gacat	Frameshift
20	385-402	5	Deletion	gaaag gacat	Frameshift
21	385-402	5	Deletion	gaaag gacat	Frameshift
22	486-532	7	Deletion	ctgct ttgct	Frameshift
23	533-609	8	Deletion	agact catat	Frameshift

* GenBank sequence accession #J00060.

[†] Mutated or inserted nucleotides are bolded.^{††} The actual single-base substitution in the splice-site region which led to the deletions cannot be determined with certainty because sequencing was performed on mutant cDNAs.

Table II.AF-induced *hprt** mutations in UV5/CYP1A2/NAT2*4 CHO cells

Missense/Nonsense Single-Base Substitutions					
Mutant #	Position	Exon	Type of Mutation [†]	Surrounding Sequence	Amino Acid change
1	97	2	G:C > T:A	ttg g aaaag	Glu > Stop
2	134	2	G:C > T:A	gacag g act	Arg > Met
3	134	2	G:C > T:A	gacag g act	Arg > Met
4	134	2	G:C > T:A	gacag g act	Arg > Met
5	166	3	G:C > A:T	aaagag a tg	Glu> Lys
6	209	3	G:C > T:A	aa g gggggc	Gly > Val
7	229	3	G:C > T:A	gct g acctg	Asp > Phe
8	292	3	G:C > C:G	gtagatt t t	Asp > His
9	419	6	G:C > T:A	act g gtaaa	Gly > Val
10	439	6	C:G > G:C	ctg e ttcc	Leu > Val
11	464	6	T:A > C:G	aac t caaa	Leu > Pro
12	482	6	C:G > A:T	gtt g caagc	Ala > Glu
13	496	7	A:T > G:C	gt g aaaagg	Lys > Glu
14	533	8	T:A > C:G	gactt t gtt	Phe > Ser
15	538	8	G:C > C:G	gtt g gattt	Gly > Arg
16	551	8	C:G > G:C	att c agac	Pro > Arg
17	589	8	G:C > T:A	aat g agtac	Glu > Stop
18	589	8	G:C > T:A	aat g agtac	Glu > Stop
19	599	8	G:C > A:T	ttcag g gat	Arg > Lys
Deletions ^{††}					
20	403-485	6	Deletion	ttgag aatga	Frameshift
21	532-609	8	Deletion	agact catat	Frameshift
22	533-609	8	Deletion	agact catat	Frameshift

* GenBank sequence accession [#J00060](#).[†] Mutated or inserted nucleotides are bolded.^{††} The actual single-base substitution in the splice-site region which led to the deletions cannot be determined with certainty because sequencing was performed on mutant cDNAs.