Published in final edited form as:

Arch Toxicol. 2017 August ; 91(8): 2827–2835. doi:10.1007/s00204-017-1989-7.

Catalytic properties and heat stabilities of novel recombinant human N-acetyltransferase 2 allozymes support existence of genetic heterogeneity within the slow acetylator phenotype

David W. Hein and Mark A. Doll

Department of Pharmacology & Toxicology and James Graham Brown Cancer Center, University of Louisville School of Medicine, Louisville, Kentucky USA 40292

Abstract

Human N-acetyltransferase 2 (NAT2) catalyzes the N-acetylation of numerous aromatic amine drugs such as sulfamethazine (SMZ) and hydrazine drugs such as isoniazid (INH). NAT2 also catalyzes the N-acetylation of aromatic amine carcinogens such as 2-aminofluorene and the O- and N,O-acetylation of aromatic amine and heterocyclic amine metabolites. Genetic polymorphism in NAT2 modifies drug efficacy and toxicity as well as cancer risk. Acetyltransferase catalytic activities and heat stability associated with six novel NAT2 haplotypes (*NAT2*6C*, *NAT2*14C*, *NAT2*14D*, *NAT2*14E*, *NAT2*17* and *NAT2*18*) were compared with that of the reference *NAT2*4* haplotype following recombinant expression in *Escherichia coli*. N-acetyltransferase activities towards SMZ and INH were significantly ($p < 0.0001$) lower when catalyzed by the novel recombinant human NAT2 allozymes compared to NAT2 4. SMZ and INH N-acetyltransferase activities catalyzed by NAT2 14C and NAT2 14D were significantly lower ($p < 0.001$) than catalyzed by NAT2 6C and NAT2 14E. N-acetylation catalyzed by recombinant human NAT2 17 was over several hundred-fold lower than by recombinant NAT2 4 precluding measurement of its kinetic or heat inactivation constants. Similar results were observed for the O-acetylation of N-hydroxy-2-aminofluorene and N-hydroxy-2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine and the intramolecular N,O-acetylation of N-hydroxy-N-acetyl-2-aminofluorene. The apparent V_{max} of the novel recombinant NAT2 allozymes NAT2 6C, NAT2 14C, NAT2 14D, and NAT2 14E towards AF, 4-aminobiphenyl (ABP), and 3,2'-dimethyl-4-aminobiphenyl (DMABP) were each significantly ($p < 0.001$) lower while their apparent K_m values did not differ significantly ($p > 0.05$) from recombinant NAT2 4. The apparent V_{max} catalyzed by NAT2 14C and NAT2 14D were significantly lower ($p < 0.05$) than the apparent V_{max} catalyzed by NAT2 6C and NAT2 14E towards AF, ABP and DMABP. Heat inactivation rate constants for recombinant human NAT2 14C, 14D, 14E and 18 were significantly ($p < 0.05$) higher than NAT2 4. These results provide further evidence of genetic heterogeneity within the NAT2 slow acetylator phenotype.

Correspondence to: David W. Hein, Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, Kosair Charities CTR-Room 303, 505 South Hancock Street, Louisville, KY 40202 USA, Tel: (502) 852 6252; d.hein@louisville.edu.

Conflict of Interest None

Keywords

N-acetyltransferase 2; slow acetylator genotype; isoniazid; sulfamethazine; 4-aminobiphenyl; O-acetylation; N-acetylation; N,O-acetylation; cancer risk assessment

Introduction

Human N-acetyltransferase 2 (NAT2) catalyzes N-acetylation, O-acetylation, and N,O-acetylation of aromatic and heterocyclic amine drugs and carcinogens. A genetic polymorphism in NAT2 modifies the efficacy and/or toxicity following administration of numerous drugs (Weber and Hein, 1985; McDonagh et al, 2014). Human NAT2 also catalyzes the N-acetylation and O-acetylation of xenobiotics and carcinogens (Hein 1988). Genetic polymorphisms in *NAT2* have been shown to be associated with differential susceptibility to various cancers, particularly urinary bladder cancer (Golka et al., 2002; Garcia-Closas et al., 2005; Moore et al., 2011; Selinski et al., 2013). Associations also have been reported with colorectal adenoma (Moslehi et al., 2006) and polyps (Shin et al., 2008), particularly when combined with exposures to tobacco or dietary carcinogens and genetic polymorphisms in multiple enzymes that metabolize these carcinogens (Fu et al., 2012; 2013). As recently reviewed (Hein, in press), several studies have reported that associations of NAT2 genetic polymorphism with breast cancer (Deitz et al., 2000; Ambrosone et al., 2008) are strongest in smokers who are “very slow” acetylators (van del Hel et al., 2003; Baumgartner et al., 2009; Conlon et al., 2010).

Previous papers have discussed the possibility of genetic heterogeneity within the slow acetylator phenotype (Hein, 2002; 2009) and following evidence obtained from the in vivo metabolism of caffeine (Ruiz et al., 2012) a recent guest editorial (Selinski et al., 2015a) highlighted the importance of identifying and accounting for “ultra-slow” acetylators to enable more accurate and robust assessments of the effects of NAT2 genetic polymorphism on drug toxicity and cancer risk, and other health contexts such as cognitive function (Selinski et al., 2015b).

The first comprehensive review of *NAT2* alleles or haplotypes was published over 20 years ago (Vatsis et al., 1995) and recent updates have been reported (Walker et al., 2009; McDonagh et al., 2014). Individual single nucleotide polymorphisms (SNPs) in human *NAT2* haplotypes have been characterized following recombinant expression in yeast (Fretland et al., 2001). Human *NAT2* alleles with the single SNPs 191G>A, 341T>C, or 434A>C clearly resulted in reduced N-acetyltransferase activities, whereas recombinant expression of human alleles with the single SNPs 282C>T, 481C>T, 803A>G did not. Recombinant expression of 845A>C in yeast resulted in somewhat ambiguous findings, with slight but non-significant ($p>0.05$) reductions in N-acetyltransferase activity. Similarly, recombinant expression of human *NAT2* alleles in COS-1 cells with the single SNPs 191G>A and 341T>C clearly resulted in reduced N-acetyltransferase activities, whereas recombinant expression of human alleles with the single SNPs 282C>T, 481C>T, 803A>G did not (Zang et al., 2007). Recombinant expression of human *NAT2* alleles with the single SNPs 434A>C or 845A>C have not been reported in COS-1 cells.

Six novel *NAT2* haplotypes have previously been identified in human population studies (Lin et al., 1994; Martinez et al., 1995; Agundez et al., 1996). As summarized in Table 1, these six novel human *NAT2* haplotypes (possessing the single nucleotide polymorphisms in parenthesis) are *NAT2*6C* (282C>T; 590G>A; 803A>G), *NAT2*14C* (191G>A; 341T>C; 481C>T; 803A>G), *NAT2*14D* (191G>A; 282C>T; 590G>A), *NAT2*14E* (191G>A; 803A>G), *NAT2*17* (434A>C) and *NAT2*18* (845A>C). The importance of genetic heterogeneity within the slow acetylator phenotype has been emphasized particularly in cancer risk assessments (Hein 2009; Selinski et al, 2013; 2015a). Although the frequency of these novel *NAT2* haplotypes is relatively rare in the human populations studied (Lin et al., 1994; Martinez et al., 1995; Agundez et al., 1996), Golka and colleagues (2002) have described the importance of ethnic differences in the effects of slow *NAT2* acetylator phenotype on urinary bladder cancer susceptibility, which may be caused at least in part by the frequency of *NAT2* haplotypes among different ethnic groups. For example, the *NAT2*6C* haplotype was identified in a molecular epidemiological investigation of *NAT2* acetylator phenotype on gastric cancer among Koreans (Zhang et al., 2009). Failure to account for and understand relationships between *NAT2* genotype and phenotype leading to misclassification compromises molecular epidemiological investigations (Deitz et al., 2004).

To further explore a molecular basis for genetic heterogeneity within the slow acetylator phenotype, the N-, O-, and N,O-acetyltransferase activities and kinetic and heat inactivation rate constants of six novel human *NAT2* allozymes were compared with that of the reference human *NAT2* 4 allozyme following recombinant expression in *Escherichia coli*.

Materials and Methods

Source of human *NAT2* haplotypes

Human colon surgical samples were obtained from the National Disease Research Interchange (Philadelphia, PA) or the National Cancer Institute Cooperative Human Tissue Network. Colon tissues were snap frozen, placed at -70°C within 1 hr of removal, and shipped on dry ice. DNA was isolated from colon tissue following proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation.

PCR amplification, cloning and sequencing of *NAT2* haplotypes

DNA was isolated from colon tissue and the primers and procedures used for PCR amplification of human *NAT2* were as previously reported (Hein et al., 1994a). Following amplification, *NAT2* PCR products were gel-purified, digested with *EcoRI* and *SphI*, ligated to similarly cut pUC19, and transformed into *Escherichia coli* strain DH5 α competent cells (Gibco-BRL, Gaithersburg, MD). Since *Taq* DNA polymerase lacks an editing function, representative clones were always obtained from at least two independent PCRs. *NAT2* PCR clones were sequenced by a modified double-stranded dideoxy chain termination method (Sanger et al., 1977) using Sequenase (U.S. Biochemical, Cleveland, OH). Once identified, *NAT2* cloned alleles were digested with *EcoRI* and *HindIII* endonucleases to generate cohesive ends for ligation into *EcoRI/HindIII*-digested pKK223-3 prokaryotic expression vector, and transformed into competent *Escherichia coli* strain JM105 as described previously (Hein et al., 1994a).

Construction of NAT2 haplotypes

NAT2 haplotypes were generated by digestion of NAT2*4, *5B, *5C, *12B, or *14A alleles with appropriate endonucleases, followed by fragment isolation and religation as previously described (Hein et al., 1994a). Following religation, constructs of the NAT2 alleles were transformed into competent *Escherichia coli* JM105 cells.

Expression of recombinant human NAT2 4 and novel human NAT2 allozymes

JM105 bacteria harboring the human NAT2 plasmids were grown up overnight in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin (LB-Amp) at 37°C. Fresh LB-Amp broth were re-inoculated and NAT2-expression bacteria were grown to approximately 0.5 OD_{600nm}. Isopropyl β-D-thiogalactopyranoside (1 mM) was added to the broth for induction, and the cultures were grown for an additional 3 hr. The cells were harvested by centrifugation at 4000 × g for 10 min, then resuspended in 20 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA and DTT, 10 µM leupeptin and 100 µM phenylmethanesulfonyl fluoride to 5% of original culture volume. The cells were sonicated for 6 × 30 sec on ice with a probe sonicator. Lysates were centrifuged at 15,000 × g for 15 min to pellet bacterial debris. The supernatants were assayed for protein and acetyltransferase activities as described below.

N-Acetyltransferase assays

N-acetyltransferase assays towards sulfamethazine (SMZ) and isoniazid (INH) were conducted in duplicate reactions containing bacterial lysate (< 2 mg of protein/ml), 300 µM SMZ or INH, and 1 mM acetyl coenzyme A (AcCoA) at 37°C for time periods that activity is linear with time. Reactions were terminated by the addition of 1/10 volume of 1 M acetic acid. The reaction tubes were centrifuged to precipitate protein. The amount of acetyl-SMZ produced was determined following separation and quantitation by high performance liquid chromatography as described previously (Leff et al., 1999). The amount of acetyl-INH was determined following separation of INH and acetyl-INH using a 125 × 4 mm Lichrospher 100 RP-100 5µM C18 HPLC column eluted with an isocratic gradient of 86% 25 mM sodium phosphate, 10 mM heptane sulfonate pH 3.0, 14% acetonitrile. Acetyl-INH was quantitated by measuring the absorbance at 266 nm. Retention times for INH and acetyl-INH were 2.63 min and 2.93 min respectively.

N-OH-AF and N-OH-PhIP O-acetyltransferase assays

The AcCoA-dependent metabolic activation of [*ring*-³H] N-hydroxy-2-aminofluorene (N-OH-AF) and [*ring*-³H] N-hydroxy-2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (N-OH-PhIP) to products capable of forming DNA adducts was conducted as previously described (Hein et al., 1994b; 1995). The reaction mixture contained 20 mM sodium phosphate buffer (pH 7.4), 1 mM DTT, 1 mM EDTA, calf thymus DNA (1 mg/ml), and suitably diluted bacterial lysate. The initial [*ring*-³H] N-OH-AF or [*ring*-³H] N-OH-PhIP and AcCoA concentrations were 100 µM and 2 mM, respectively. Since N-hydroxyarylamines have been shown to bind directly to DNA at neutral pH, reaction controls without AcCoA were subtracted from the experimental determinations. Assays were carried out for 20 min and terminated by adding 1 ml of ice-cold water-saturated *n*-

butanol. The aqueous layer was extracted with phenol, and the DNA was precipitated and washed with 80% ethanol.

N-OH-AAF N,O-acetyltransferase assays

Assays for the metabolic activation of [*ring*-³H] N-hydroxy-N-acetyl-2-aminofluorene (N-OH-AAF) by N,O-acetylation were carried out as previously described (Hein et al., 1993). The assay is similar to the N-hydroxyarylamines O-acetyltransferase assays described above except that all assays were carried out in the absence of AcCoA and controls used heat-denatured enzymes.

Protein determinations

Protein concentrations in the lysates were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Enzyme velocities were then normalized relative to the quantity [arbitrary units (U)] of immunoreactive NAT2 protein detected by Western immunoblot (Hein et al., 1994a).

Determination of kinetic constants

N-acetyltransferase assays towards 2-aminofluorene (AF), 4-aminobiphenyl (ABP) and 3,2'-dimethyl-4-aminobiphenyl (DMABP) were carried out as previously described (Hein et al., 1994a) using a radiolabel assay that measures the formation of [³H]-2-acetylaminofluorene product and utilizes [³H]-acetate in a [³H]-AcCoA regenerating system. N-acetyltransferase assays were carried out for 20 min at 37°C in duplicate with AF substrate concentrations ranging from 0.5 – 500 µM in the presence of a fixed AcCoA concentration (1000 µM). N-acetyltransferase maximum velocities were calculated by linear regression of Eadie-Hofstee plots as previously described (Fretland et al., 1997).

Determination of heat inactivation rate constants

Heat inactivation assays were conducted at 50° C as previously described (Hein et al., 1994a). Briefly, bacterial lysates diluted to 1 mg/ml in homogenization buffer were incubated at 50°C for 5, 10, 20, 40, 60, or 80 min, transferred immediately to ice, and assayed for N-acetylation of AF. Results were normalized to activity of unincubated enzyme (time 0 = 100%) for the percentage of activity remaining. Heat inactivation rate constants were determined by fitting the data to a first-order decay curve using GraphPad Prism software.

Statistical analyses

Differences in catalytic activities, Michaelis-Menten kinetic constants, and heat inactivation rate constants among the recombinant NAT2 allozymes were tested for significance by one way analysis of variance followed by Tukey-Kramer multiple comparisons test.

Results

N-acetyltransferase activities

The recombinant human NAT2 allozymes catalyzed the N-acetylation of both SMZ and INH in the relative order NAT2 4 > NAT2 6C, NAT2 14E, NAT2 18 > NAT2 14C, NAT2 14D (Fig. 1). SMZ and INH N-acetyltransferase activities catalyzed by NAT2 14C and NAT2 14D were significantly lower ($p < 0.001$) than catalyzed by NAT2 6C and NAT2 14E. The N-acetyltransferase activities catalyzed by NAT2 17 were below the limit of detection towards SMZ (< 0.05 nmol/min/unit protein) and INH (< 0.5 nmol/min/unit protein).

O- and N,O-acetyltransferase activities

The recombinant human NAT2 allozymes catalyzed the O-acetylation of both N-OH-AF and N-OH-PhIP and the N,O-acetylation of N-OH-AAF in the relative order NAT2 4, NAT2 18 > NAT2 6C, NAT2 14E, > NAT2 14 C, NAT2 14D > NAT2 17 (Fig. 2). The O-acetylation of N-OH-PhIP catalyzed by NAT2 17 was below the limit of detection (< 3 pmol/min/mg DNA/U protein).

Michaelis-Menten kinetic constants for the N-acetylation of AF, ABP and DMABP

Apparent maximum velocities of the recombinant human NAT2 allozymes towards the N-acetylation of AF, ABP, and DMABP were in the rank order NAT2 4, NAT2 18 > NAT2 6C, NAT2 14E, > NAT2 14 C, NAT2 14D (Fig. 3). The apparent V_{max} catalyzed by NAT2 6C, NAT2 14C, NAT2 14D, NAT2 14E were significantly ($p < 0.001$) lower than catalyzed by NAT2 4 for AF, ABP and DMABP. The apparent V_{max} catalyzed by NAT2 14C and NAT2 14D were significantly lower ($p < 0.05$) than the apparent V_{max} catalyzed by NAT2 6C and NAT2 14E towards AF, ABP and DMABP. The N-acetyltransferase activities catalyzed by NAT2 17 were below the limit of detection which precluded determination of the apparent V_{max} . The apparent K_m towards AF, ABP and DMABP were similar among recombinant human NAT2 4 and the novel human NAT2 allozymes (Fig. 4).

Heat inactivation of recombinant human NAT2 allozymes

Intrinsic heat inactivation rate constants following incubations at 50°C were in the order NAT2 4 < NAT2 6C < NAT2 14C, NAT2 18 < NAT2 14E < NAT2 14D and each except NAT2 6C differed significantly ($p < 0.05$) from NAT2 4 (Fig. 5).

Discussion

Recombinant expression of human *NAT2* alleles or haplotypes have previously been shown to produce equivalent levels of NAT2 immunoreactive protein upon heterologous expression in *E. coli* (Hein et al., 1994a; Hickman et al., 1995; Grant et al., 1997), contrary to observations in liver cytosols where the slow acetylator phenotype has reduced NAT2 immunoreactive protein compared to rapid acetylators (Grant et al., 1990; Doll et al., 2010). Protein degradation pathways vary from single cell organisms such as bacteria to human cell lines (Wang et al, 2010) which likely accounts for the discrepancy. Although investigations of human *NAT2* genotypes have been conducted in cryopreserved human hepatocytes, the novel NAT2 haplotypes in the present study have not been characterized in cryopreserved

hepatocytes or any human tissues, due most likely to their very low frequency in human populations (Lin et al., 1994; Martinez et al., 1995; Agundez et al., 1996).

As summarized in Table 1, the recombinant human NAT2 allozymes catalyzed the N-acetylation of both SMZ and INH in the relative order NAT2 4 > NAT2 6C, NAT2 14E, NAT2 18 > NAT2 14C, NAT2 14D. Previous investigations following recombinant expression of human *NAT2* alleles possessing one SNP in yeast reported that G191A, A434C, and G590A each individually reduced N-acetyltransferase activities significantly towards the N-acetylation of SMZ and AF (Fretland et al., 2001). Following these recombinant expressions in yeast, the relative order of activities was NAT2 4 > G590A > G191A > A434C. Thus, the relative order of SMZ and INH N-acetylation among recombinant human NAT2 4 and the novel human NAT2 allozymes in the present study is consistent with the results reported previously in yeast (Fretland et al., 2001), with the greatest effect of A434C (NAT2 17), and a lower reduction (but still significant) for G191A (NAT2 14 C, 14D, 14E) and G590A (NAT2 6C). The N-acetyltransferase catalytic activities of the recombinant human NAT2 allozymes in the present study appear to reflect a greater effect from combinations of non-synonymous SNPs versus a single one. Thus the N-acetyltransferase activities catalyzed by NAT2 14C (possessing two non-synonymous SNPs resulting in R64Q and I114T) and NAT2 14D two non-synonymous SNPs resulting in R64Q and R197Q) were significantly ($p < 0.001$) lower than NAT2 6C (possessing one non-synonymous SNP resulting in R197Q) and NAT2 14E (possessing one non-synonymous SNP resulting in R64Q). The lowest activity associated with NAT2 17 (possessing one non-synonymous SNP resulting in Q145P) is consistent with the previous results reported in yeast which also showed the greatest reduction in activity associated with this SNP (Fretland et al., 2001).

The relative ability of human NAT2 4 and variant human NAT2 allozymes to catalyze O-acetylation and N,O-acetylation has previously been reported following recombinant expression in *E. Coli* (Ferguson et al., 1994; Hein et al., 1994b; 1995). The previous studies reported lower activities for NAT2 6A and 6B (which like NAT2 6C in the present study contain the signature non-synonymous SNP resulting in R197Q) and for NAT2 14A and 14B (which like NAT2 14C, 14D, and 14E in the present study contain the signature non-synonymous SNP resulting in R64Q) that were generally but not always reduced significantly. The current study provides much more comprehensive information for both O- and N,O-acetyltransferase activities clarifying the effects of both R64Q and R197Q on the metabolic activation of aromatic amine and heterocyclic amine carcinogens. Thus although both R64A and R197Q consistently reduced metabolic activation of N-OH-AF, N-OH-PhIP and N-OH-AAF catalyzed by recombinant human NAT2, the presence of multiple non-synonymous SNPs reduced activity more than one of these did alone, analogous to the results described for the N-acetylation of SMZ and INH described above.

The catalytic activity of NAT 18 (possessing one non-synonymous SNP resulting in K282T) yielded a modest reduction in N-acetyltransferase activity that reached significance towards the N-acetylation of some substrates (SMZ and INH) but not others (AF, ABP or DMABP). This is consistent with the very modest but non-significant effect of K282T following recombinant expression in yeast (Fretland et al., 2001). Similarly, NAT2 18 catalyzed the O-

acetylation of N-OH-AF and N-OH-PhIP as well as the N,O-acetylation of N-OH-AAF at rates equivalent to NAT2 4. Thus, it appears that the effects of K282T and therefore the phenotype of the NAT2 18 allozyme is substrate-dependent, as previously predicted (Walraven et al., 2008) and reported (Zang et al., 2007) for G286E, which also resides on the C-terminal tail of the NAT2 protein which can alter access to the active site (Hein, 2009).

Apparent maximum velocities of the recombinant human NAT2 allozymes towards the N-acetylation of AF, ABP, and DMABP were in the rank order NAT2 4, NAT2 18 > NAT2 6C, NAT2 14E, >NAT2 14C, NAT2 14D (Fig 3). The N-acetyltransferase V_{max} of the recombinant human NAT2 allozymes also a greater effect from combinations of non-synonymous SNPs versus a single one. Thus the N-acetyltransferase V_{max} catalyzed by NAT2 14C (possessing two non-synonymous SNPs resulting in R64Q and I114T) and NAT2 14D (possessing two non-synonymous SNPs resulting in R64Q and R197Q) were significantly ($p<0.05$) lower than NAT2 6C (possessing one non-synonymous SNP resulting in R197Q) and NAT2 14E (possessing one non-synonymous SNP resulting in R64Q). The N-acetyltransferase activities catalyzed by NAT2 17 were below the limit of detection which precluded determination of the apparent V_{max}. These results are consistent with the rank order for N-acetylation, O-acetylation, and N,O-acetylation described above. This result likely reflects the similar apparent affinities towards AF, ABP and DMABP among recombinant human NAT2 4 and the novel human NAT2 allozymes characterized in the present study.

Intrinsic heat inactivation rate constants following incubations at 50°C were in the order NAT2 4 < NAT2 6C < NAT2 14C, NAT2 18 < NAT2 14E < NAT2 14D and each except NAT2 6C differed significantly ($p<0.05$) from NAT2 4. These findings are very consistent with previous findings in which the G191A non-synonymous SNP resulting in R64Q yielded very large reductions in the thermostability of human NAT2 following recombinant expression in *E. Coli* (Ferguson et al., 1994; Hein et al., 1994a) and yeast (Fretland et al., 2001). Similarly, the effect of the A845C (K282T) resulting in reduction in the thermostability of NAT2 18 following recombinant expression in *E. Coli* in the present study is consistent with the effects following recombinant expression in yeast (Fretland et al., 2001). The more modest effect of the G590A (R197Q) on the thermostability of NAT2 6C following recombinant expression in *E. Coli* in the present study also is consistent with the effects following recombinant expression of the single non-synonymous SNP in *E. Coli* (Hein et al., 1994a) and yeast (Fretland et al., 2001).

In summary, the results of the present study support the molecular interactions in NAT2 catalytic activity and stability previously predicted for the novel NAT2 haplotypes (Walraven et al., 2008). Recent studies have described the importance of accounting for genetic heterogeneity in the slow NAT2 acetylator phenotype for drug efficacy/toxicity and carcinogen risk assessments (Hein, 2009; Selinski et al, 2013; 2015a). The results of the present study characterize the catalytic properties and heat stabilities of novel recombinant human NAT2 allozymes and provide important genotype/phenotype data to support further the existence of genetic heterogeneity within the NAT2 slow acetylator phenotype. Genetic heterogeneity in N-acetyltransferase 2 slow acetylator phenotype also has recently been reported in cryopreserved human hepatocytes (Doll and Hein, 2017). Investigations of N-

acetyltransferase catalytic activities associated with all *NAT2* haplotypes in cryopreserved human hepatocytes would strengthen the conclusions of the present study.

References

- Agundez JA, Olivera M, Martinez C, Ladero JM, Benitez J. Identification and prevalence study of 17 allelic variants of the human *NAT2* gene in a white population. *Pharmacogenetics*. 1996; 6(5):423–8. [PubMed: 8946474]
- Ambrosone CB, Kropp S, Yang J, Yao S, Shields PG, Chang-Claude J. Cigarette smoking, N-acetyltransferase 2 genotypes, and breast cancer risk: pooled analysis and meta-analysis. *Cancer Epidemiol Biomarkers Prev*. 2008; 17(1):15–26. [PubMed: 18187392]
- Baumgartner KB, Schlierf TJ, Yang D, Doll MA, Hein DW. N-acetyltransferase 2 genotype modification of active cigarette smoking on breast cancer risk among hispanic and non-hispanic white women. *Toxicol Sci*. 2009; 112(1):211–20. [PubMed: 19692670]
- Conlon MS, Johnson KC, Bewick MA, Lafrenie RM, Donner A. Smoking (active and passive), N-acetyltransferase 2, and risk of breast cancer. *Cancer Epidemiol*. 2010; 34(2):142–9. [PubMed: 20197243]
- Deitz AC, Rothman N, Rebbeck TR, et al. Impact of misclassification in genotype-exposure interaction studies: example of N-acetyltransferase 2 (*NAT2*), smoking, and bladder cancer. *Cancer Epidemiol Biomarkers Prev*. 2004; 13(9):1543–6. [PubMed: 15342459]
- Deitz AC, Zheng W, Leff MA, et al. N-Acetyltransferase-2 genetic polymorphism, well-done meat intake, and breast cancer risk among postmenopausal women. *Cancer Epidemiol Biomarkers Prev*. 2000; 9(9):905–10. [PubMed: 11008907]
- Doll MA, Hein DW. Genetic heterogeneity among slow acetylators N-acetyltransferase 2 phenotypes in cryopreserved human hepatocytes. *Arch Toxicol*. 2017; 91(7):2655–2661. [PubMed: 28516247]
- Doll MA, Zang Y, Moeller T, Hein DW. Codominant expression of N-acetylation and O-acetylation activities catalyzed by N-acetyltransferase 2 in human hepatocytes. *J Pharmacol Exp Ther*. 2010; 334(2):540–4. [PubMed: 20430842]
- Ferguson RJ, Doll MA, Rustan TD, Gray K, Hein DW. Cloning, expression, and functional-characterization of 2 mutant (*Nat2*(191) and *Nat2*(341/803)) and wild-type human polymorphic N-acetyltransferase (*Nat2*) alleles. *Drug Metabolism and Disposition*. 1994; 22(3):371–376. [PubMed: 7915226]
- Fretland AJ, Doll MA, Gray K, Feng Y, Hein DW. Cloning, sequencing, and recombinant expression of *NAT1*, *NAT2*, and *NAT3* derived from the C3H/HeJ (rapid) and A/HeJ (slow) acetylators inbred mouse: functional characterization of the activation and deactivation of aromatic amine carcinogens. *Toxicol Appl Pharmacol*. 1997; 142(2):360–6. [PubMed: 9070359]
- Fretland AJ, Leff MA, Doll MA, Hein DW. Functional characterization of human N-acetyltransferase 2 (*NAT2*) single nucleotide polymorphisms. *Pharmacogenetics*. 2001; 11(3):207–15. [PubMed: 11337936]
- Fu Z, Shrubsole MJ, Li G, et al. Using gene-environment interaction analyses to clarify the role of well-done meat and heterocyclic amine exposure in the etiology of colorectal polyps. *Am J Clin Nutr*. 2012; 96(5):1119–28. [PubMed: 23015320]
- Fu Z, Shrubsole MJ, Li G, et al. Interaction of cigarette smoking and carcinogen-metabolizing polymorphisms in the risk of colorectal polyps. *Carcinogenesis*. 2013; 34(4):779–86. [PubMed: 23299405]
- Garcia-Closas M, Malats N, Silverman D, et al. *NAT2* slow acetylation, *GSTM1* null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. *Lancet*. 2005; 366(9486):649–59. [PubMed: 16112301]
- Golka K, Prior V, Blaszkewicz M, Bolt HM. The enhanced bladder cancer susceptibility of *NAT2* slow acetylators towards aromatic amines: a review considering ethnic differences. *Toxicol Lett*. 2002; 128(1–3):229–41. [PubMed: 11869833]
- Grant DM, Hughes NC, Janezic SA, et al. Human acetyltransferase polymorphisms. *Mutat Res*. 1997; 376(1–2):61–70. [PubMed: 9202739]

- Grant DM, Morike K, Eichelbaum M, Meyer UA. Acetylation pharmacogenetics. The slow acetylator phenotype is caused by decreased or absent arylamine N-acetyltransferase in human liver. *J Clin Invest.* 1990; 85(3):968–72. [PubMed: 2312737]
- Hein DW. Acetylator genotype and arylamine-induced carcinogenesis. *Biochim Biophys Acta.* 1988; 948(1):37–66. [PubMed: 3293663]
- Hein DW. Molecular genetics and function of NAT1 and NAT2: role in aromatic amine metabolism and carcinogenesis. *Mutat Res.* 2002:506–507. 65–77.
- Hein DW. N-acetyltransferase SNPs: emerging concepts serve as a paradigm for understanding complexities of personalized medicine. *Expert Opin Drug Metab Toxicol.* 2009; 5(4):353–66. [PubMed: 19379125]
- Hein, DW. N-acetyltransferase 2 polymorphism and human urinary bladder and breast cancer risk. In: Sim, E., Laurieri, N., editors. *Arylamine N-Acetyltransferases in Health and Disease.* World Scientific Publishing; in press
- Hein DW, Doll MA, Nerland DE, Fretland AJ. Tissue distribution of N-acetyltransferase 1 and 2 catalyzing the N-acetylation of 4-aminobiphenyl and O-acetylation of N-hydroxy-4-aminobiphenyl in the congenic rapid and slow acetylator Syrian hamster. *Mol Carcinog.* 2006; 45(4):230–8. [PubMed: 16482518]
- Hein DW, Doll MA, Rustan TD, Ferguson RJ. Metabolic activation of N-hydroxyarylamines and N-hydroxyarylamides by 16 recombinant human NAT2 allozymes: effects of 7 specific NAT2 nucleic acid substitutions. *Cancer Res.* 1995; 55(16):3531–6. [PubMed: 7627960]
- Hein DW, Doll MA, Rustan TD, et al. Metabolic activation and deactivation of arylamine carcinogens by recombinant human NAT1 and polymorphic NAT2 acetyltransferases. *Carcinogenesis.* 1993; 14(8):1633–8. [PubMed: 8353847]
- Hein DW, Ferguson RJ, Doll MA, Rustan TD, Gray K. Molecular genetics of human polymorphic N-acetyltransferase: enzymatic analysis of 15 recombinant wild-type, mutant, and chimeric NAT2 allozymes. *Hum Mol Genet.* 1994a; 3(5):729–34. [PubMed: 8081359]
- Hein DW, Rustan TD, Ferguson RJ, Doll MA, Gray K. Metabolic activation of aromatic and heterocyclic N-hydroxyarylamines by wild-type and mutant recombinant human NAT1 and NAT2 acetyltransferases. *Arch Toxicol.* 1994b; 68(2):129–33. [PubMed: 8179482]
- Hickman D, Palamanda JR, Unadkat JD, Sim E. Enzyme kinetic properties of human recombinant arylamine N-acetyltransferase 2 allotypic variants expressed in *Escherichia coli*. *Biochem Pharmacol.* 1995; 50(5):697–703. [PubMed: 7669073]
- Leff MA, Epstein PN, Doll MA, et al. Prostate-specific human N-acetyltransferase 2 (NAT2) expression in the mouse. *J Pharmacol Exp Ther.* 1999; 290(1):182–7. [PubMed: 10381774]
- Lin HJ, Han CY, Lin BK, Hardy S. Ethnic distribution of slow acetylator mutations in the polymorphic N-acetyltransferase (NAT2) gene. *Pharmacogenetics.* 1994; 4(3):125–34. [PubMed: 7920692]
- Martinez C, Agundez JA, Olivera M, Martin R, Ladero JM, Benitez J. Lung cancer and mutations at the polymorphic NAT2 gene locus. *Pharmacogenetics.* 1995; 5(4):207–14. [PubMed: 8528267]
- McDonagh EM, Boukouvala S, Aklillu E, Hein DW, Altman RB, Klein TE. PharmGKB summary: very important pharmacogene information for N-acetyltransferase 2. *Pharmacogenet Genomics.* 2014; 24(8):409–25. [PubMed: 24892773]
- Moore LE, Baris DR, Figueroa JD, et al. GSTM1 null and NAT2 slow acetylation genotypes, smoking intensity and bladder cancer risk: results from the New England bladder cancer study and NAT2 meta-analysis. *Carcinogenesis.* 2011; 32(2):182–9. [PubMed: 21037224]
- Moslehi R, Chatterjee N, Church TR, et al. Cigarette smoking, N-acetyltransferase genes and the risk of advanced colorectal adenoma. *Pharmacogenomics.* 2006; 7(6):819–29. [PubMed: 16981843]
- Ruiz JD, Martinez C, Anderson K, et al. The differential effect of NAT2 variant alleles permits refinement in phenotype inference and identifies a very slow acetylation genotype. *PLoS One.* 2012; 7(9):e44629. [PubMed: 22970273]
- Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA.* 1977; 74(12):5463–7. [PubMed: 271968]
- Selinski S, Blaszkewicz M, Getzmann S, Golka K. N-Acetyltransferase 2: ultra-slow acetylators enter the stage. *Arch Toxicol.* 2015a; 89(12):2445–7. [PubMed: 26608182]

- Selinski S, Blaszkewicz M, Ickstadt K, Hengstler JG, Golka K. Refinement of the prediction of N-acetyltransferase 2 (NAT2) phenotypes with respect to enzyme activity and urinary bladder cancer risk. *Arch Toxicol.* 2013; 87(12):2129–39. [PubMed: 24221535]
- Selinski S, Getzmann S, Gajewski PD, et al. The ultra-slow NAT2*6A haplotype is associated with reduced higher cognitive functions in an elderly study group. *Arch Toxicol.* 2015b; 89(12):2291–303. [PubMed: 26615528]
- Shin A, Shrubsole MJ, Rice JM, et al. Meat intake, heterocyclic amine exposure, and metabolizing enzyme polymorphisms in relation to colorectal polyp risk. *Cancer Epidemiol Biomarkers Prev.* 2008; 17(2):320–9. [PubMed: 18268115]
- van der Hel OL, Peeters PHM, Hein DW, et al. NAT2 slow acetylation and GSTM1 null genotypes may increase postmenopausal breast cancer risk in long-term smoking women. *Pharmacogenetics.* 2003; 13(7):399–407. [PubMed: 12835615]
- Vatsis KP, Weber WW, Bell DA, et al. Nomenclature for N-acetyltransferases. *Pharmacogenetics.* 1995; 5(1):1–17. [PubMed: 7773298]
- Walker K, Ginsberg G, Hattis D, Johns DO, Guyton KZ, Sonawane B. Genetic polymorphism in N-Acetyltransferase (NAT): Population distribution of NAT1 and NAT2 activity. *Journal of toxicology and environmental health Part B, Critical reviews.* 2009; 12(5–6):440–72.
- Walraven JM, Zang Y, Trent JO, Hein DW. Structure/function evaluations of single nucleotide polymorphisms in human N-acetyltransferase 2. *Curr Drug Metab.* 2008; 9(6):471–86. [PubMed: 18680467]
- Wang T, Darwin KH, Li H. Binding-induced folding of prokaryotic ubiquitin-like protein on the Mycobacterium proteasomal ATPase targets substrates for degradation. *Nature structural & molecular biology.* 2010; 17(11):1352–7.
- Zang Y, Doll MA, Zhao S, States JC, Hein DW. Functional characterization of single-nucleotide polymorphisms and haplotypes of human N-acetyltransferase 2. *Carcinogenesis.* 2007; 28(8):1665–71. [PubMed: 17434923]
- Zhang YW, Eom SY, Kim YD, et al. Effects of dietary factors and the NAT2 acetylator status on gastric cancer in Koreans. *Int J Cancer.* 2009; 125(1):139–45. [PubMed: 19350634]

Fig. 1.

N-acetyltransferase activities towards SMZ (top) and INH (bottom) in nmol acetylated/min/unit protein catalyzed by recombinant human NAT2 allozymes. Each bar represents Mean $\pm$ S.E.M for three determinations. Rates of acetylation activity catalyzed by each of the novel recombinant human NAT2 allozymes (except NAT2 17 in which rates were non-detectable: ND) were significantly lower than catalyzed by NAT2 4 towards both SMZ and INH ($p < 0.0001$). SMZ and INH N-acetyltransferase activities catalyzed by NAT2 14C and NAT2 14D were significantly lower ($p < 0.001$) than catalyzed by NAT2 6C and NAT2 14E.

Fig. 2. O-acetyltransferase activities towards N-OH-AF (top) and N-OH-PhIP (middle), and N,O-acetyltransferase activity towards N-OH-AAF (bottom) in pmol acetylated/min/mg DNA/unit protein catalyzed by recombinant human NAT2 allozymes. Each bar represents Mean $\pm$ S.E.M for six (N-OH-AF) or three (N-OH-PhIP, N-OH-AAF) determinations. Rates of acetylation activity catalyzed by the novel recombinant human NAT2 allozymes were significantly lower than by NAT2 4 *($p < 0.05$); ** ($p < 0.01$), *** ($p < 0.001$).

Fig. 3. Apparent N-acetyltransferase catalytic Vmax in nmol acetylated/min/unit protein of recombinant NAT2 allozymes towards the N-acetylation of AF (top), ABP (middle) and DMABP (bottom). Each bar represents Mean $\pm$ S.E.M for three determinations. The apparent Vmax catalyzed by NAT2 6C, NAT2 14C, NAT2 14D, NAT2 14E were significantly ($p < 0.001$) lower than catalyzed by NAT2 4 for AF, ABP and DMABP. The apparent Vmax catalyzed by NAT2 14C and NAT2 14D were significantly lower ($p < 0.05$)

than the apparent $V_{\max}$ catalyzed by NAT2 6C and NAT2 14E towards AF, ABP and DMABP.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. Apparent K_m (μM) of recombinant NAT2 allozymes towards AF, ABP and DMABP. Each bar represents Mean $\pm$ S.E.M for three determinations. The apparent K_m of NAT2 4 towards AF or ABP did not differ significantly ($p > 0.05$) among the NAT2 allozymes. The apparent K_m of NAT2 4 towards DMABP differed significantly from NAT2 6C ($p < 0.05$), NAT2 14C ($p < 0.001$), and NAT2 14 D ($p < 0.01$).

Fig. 5.
Heat inactivation rate constants following incubations of human NAT2 allozymes at 50° C.
Intrinsic heat inactivation rate constants were in the order NAT2 4 < NAT2 6C < NAT2 14C,
NAT2 18 < NAT2 14E < NAT2 14D and each except NAT2 6C differed significantly
($p < 0.05$) from NAT2 4.

Table 1
Comparison of recombinant human NAT2 4 (reference) with novel recombinant human NAT2 allozymes

NAT2 Allele or Haplotype	Nucleotide Changes	RS Identifier	Amino Acid Change (s)	NAT2 heat stability relative to NAT2 4 ^a	NAT2 activity relative to NAT2 4 ^a	Amino Acid changes which reduce NAT2 activity ^b
NAT2*4 (Reference)	None		None	++++	++++	None
NAT2*6C	282C>T 590G>A 803A>G	1041983 1799930 1208	Y94Y R197Q K268R	+++	++	R197Q
NAT2*14C	191G>A 341T>C 481C>T 803A>G	1801279 1801280 1788829 1208	R64Q I114T L161L K268R	++	+	R64Q I114T
NAT2*14D	191G>A 282C>T 590G>A	1801279 1041983 1799930	R64Q Y94Y R197Q	+	+	R64Q R197Q
NAT2*14E	191G>A 803A>G	1801279 1208	R64Q K268R	+	++	R64Q
NAT2*17	434A>C	72554616	Q145P	Not determined due to low catalytic activity	+	Q145P
NAT2*18	845A>C	56054745	K282T	++	+++ (substrate-dependent)	K282T

^aRelative compared to NAT2 4 arbitrarily assigned +++++.

^bPredicted molecular interactions of these amino acid changes for human NAT2 catalytic activity and stability are described in Walraven et al., 2008.