Human N-Acetylation of Benzidine: Role of NAT1 and NAT2

Terry V. Zenser, Vijaya M. Lakshmi, Timothy D. Rustan, Mark A. Doll, Anne C. Deitz, Bernard B. Davis, and David W. Hein

Veterans Affairs Medical Center and Department of Biochemistry and Division of Geriatric Medicine, St. Louis University School of Medicine, St. Louis, Missouri 63125-4199

ABSTRACT

These studies were designed to assess metabolism of benzidine and N-acetylbenzidine by N-acetyltransferase (NAT) NAT1 and NAT2. Metabolism was assessed using human recombinant NAT1 and NAT2 and human liver slices. For benzidine and N-acetylbenzidine, Km and Vmax values were greater for NAT1 than for NAT2. The clearance ratios (NAT1/NAT2) for benzidine and N-acetylbenzidine were 54 and 535, respectively, suggesting that N-acetylbenzidine is a preferred substrate for NAT1. The much higher NAT1 and NAT2 Km values for N-acetylbenzidine (1380 ± 90 and 471 ± 23 μM, respectively) compared to benzidine (254 ± 38 and 333 ± 1.5 μM, respectively) appear to favor benzidine metabolism over N-acetylbenzidine for low exposures. Determination of these kinetic parameters over a 20-fold range of acetyl-CoA concentrations demonstrated that NAT1 and NAT2 catalyzed N-acetylation of benzidine by a binary ping-pong mechanism. In vitro enzymatic data were correlated to intact liver tissue metabolism using human liver slices. Samples incubated with either [3H]benzidine or [3H]N-acetylbenzidine had a similar ratio of N-acetylated benzenes (N-acetylbenzidine + N,N'-diacetylbenzidine/benzidine) and produced amounts of N-acetylbenzidine > benzidine > N,N'-diacetylbenzidine. With [3H]benzidine, p-aminobenzoic acid, a NAT1-specific substrate, increased the amount of benzidine and decreased the amount of N-acetylbenzidine produced, resulting in decreased ratio of acetylated products. This is consistent with benzidine being a NAT1 substrate. N-Acetylation of benzidine or N-acetylbenzidine by human liver slices did not correlate with the NAT2 genotype. However, a higher acetylation ratio was observed in human liver slices possessing the NAT1*10 compared to the NAT1*4 allele. Thus, a combination of human recombinant NAT and liver slice experiments has demonstrated that benzidine and N-acetylbenzidine are both preferred substrates for NAT1. These results also suggest that NAT1 may exhibit a polymorphic expression in human liver.

INTRODUCTION

NAT2 is a cytosolic enzyme that catalyzes the transfer of acetate to the nitrogen or oxygen atoms of aromatic amines, hydrazines, and N-hydroxylamines. This biotransformation can have important toxicological and carcinogenic consequences (1–3). Two NAT isozymes, NAT1 and NAT2, have been reported that differ significantly in their intrinsic stabilities and acceptor substrate selectivities (4, 5). In several species, including humans, acetylator polymorphism was demonstrated, resulting in rapid, intermediate, and slow acetylator phenotypes (6). This polymorphism is attributed to allelic variants in the coding region of the NAT2 gene locus (6, 7). The slow acetylator phenotype occurs at frequencies between 10 and 90% in various ethnic populations (3). Variant human NAT2s express a range of NAT and O-acetyltransferase activities and intrinsic stabilities (8, 9). Although NAT1 has been considered monomorphic (4, 5), a polymorphic distribution was demonstrated recently (10–12). Discrete NAT1 structural variants exist, with a prominent change in at least one of the variants (NAT1*10), which possesses an alteration of the consensus polyadenylation signal (10). In colon and bladder tissues, higher NAT1 activity was observed in individuals who inherited this NAT1*10 allele (rapid NAT1 genotype; Ref. 12).

NAT-catalyzed reactions can result in activation or detoxification of aromatic amines (13). Hepatic N-oxidation of aromatic amines is considered a necessary reaction for activation to occur (14, 15). The N-OH arylamine product is excreted either free or glucuronidated, and accumulates in urine within the lumen of the bladder. Acidic urine can hydrolyze the glucuronide to its N-OH arylamine that forms bladder DNA adducts (16). Because acetylated aromatic amines are difficult to oxidize, N-acetylation is considered a detoxification step (2). Slow acetylators would be expected to generate higher levels of N-OH products than rapid acetylators. This is consistent with the slow acylator phenotype being associated with an increased formation of macromolecular adducts by 4-aminobiphenyl, a monoarylamine (17–19), and with a higher incidence of bladder cancer (20, 21). Although N-OH arylamines at acidic pH form nitrenium ions that react with DNA, their O-acetylation to N-acetoxy esters facilitates DNA adduct formation (22). These esters are unstable and can be transformed through heterolytic fission into nitrenium ions (15). Individuals possessing the NAT1*10 allele were found to have higher levels of both bladder NAT1 activity and aromatic amine-DNA adducts (23). Carcinogen DNA adducts have been related to the subsequent development of cancer in humans (24–26). Individuals with the highest DNA adducts inherited slow NAT2 and rapid NAT1 genotypes (23). This genotype combination results in possible synergism between a hepatic inactivation pathway and an activation pathway in the bladder. Thus, NATs appear to play an important yet complex role in the etiology of bladder cancer.

Benzidine is an arylamine associated with the development of bladder cancer in occupationally exposed humans (27, 28). Workers exposed to high levels of benzidine have as much as a 100-fold increased risk for bladder cancer (29). Both NAT1 and NAT2 N-acetylate benzidine and N-acetylbenzidine (Fig. 1; Ref. 30). Although N-acetylation is considered a detoxification step in aromatic amine metabolism, studies in rodents suggest that N-acetylbenzidine is an active metabolite, whereas N,N'-diacetylbenzidine is a detoxified metabolite (31, 32). Therefore, the fate of N-acetylated arylamines, such as benzidine, and aryldiazenamines, such as 4-aminobiphenyl, may be different. In contrast to 4-aminobiphenyl, monoaetylation of benzidine yields a product (N-acetylbenzidine) that is still susceptible to N′ oxidation and N′ glucuronidation (33–35). Whereas acylator phenotype is considered a potential biomarker for bladder cancer in individuals with mixed exposure to arylmonoaamines and aryldiazenamines (36), this may not be the case for urinary bladder cancer related solely to benzidine exposure. A case-control study carried out among surviving workers revealed that slow acetylators did not have an increased risk of bladder cancer (37). This observation is supported by other studies in which workers exposed to benzidine contained a
N-acetylbenzidine might help explain the apparent lack of effect of the products. The primers used for amplification of human NAT1 were HSAT1 (5'-TTAGGAATTACGAGGATATATCTAGAATC-3') and HSNAT1D (5'-Cggccagttgctggtcgtgatgatgcataattgacagagttg-3'). The primers include engineered EcoR1 and SphI restriction sites for cloning into a prokaryotic expression vector. Representative clones were always obtained from at least two independent PCRs. NAT1 and NAT2 PCR products were gel purified, digested with EcoRI and SphI, ligated to similarly cut UC19, and transformed into Escherichia coli strain DH5α competent cells (Life Technologies, Inc., Gaithersburg, MD). Because Taq DNA polymerase lacks an editing function, the ligation was carried out at 16°C overnight. The ligation mixture was transformed to Escherichia coli DH5α competent cells (Life Technologies, Inc., Gaithersburg, MD). Reverse transcription reactions were carried out at 55°C, and 1.5 mm at 72°C. This was followed by 30 mm at 72°C to ensure complete extension. The primers and procedures used for PCR amplification of human NAT2 were as reported previously (8).

After amplification, NAT1 and NAT2 PCR products were gel purified, digested with EcoRI and SphI, ligated to similarly cut UC19, and transformed into Escherichia coli strain DH5α competent cells (Life Technologies, Inc., Gaithersburg, MD). Reverse transcription reactions were carried out at 55°C, and 1.5 mm at 72°C. This was followed by 30 mm at 72°C to ensure complete extension. The primers and procedures used for PCR amplification of human NAT2 were as reported previously (8).

After amplification, NAT1 and NAT2 PCR products were gel purified, digested with EcoRI and SphI, ligated to similarly cut UC19, and transformed into Escherichia coli strain DH5α competent cells (Life Technologies, Inc., Gaithersburg, MD). Reverse transcription reactions were carried out at 55°C, and 1.5 mm at 72°C. This was followed by 30 mm at 72°C to ensure complete extension. The primers and procedures used for PCR amplification of human NAT2 were as reported previously (8).

**Western Immunoblot Analysis.** Bacterial lysates (50 μg) containing NAT1 or NAT2 were mixed with SDS-polyacrylamide gel sample buffer containing 5% (final) N,N'-diacetylbenzidine concentration was determined by fluorometric detection with a Bio-Rad HPLC apparatus with a DMSO column. Reaction times (20 μl) were injected onto a Whatman 215 X 4.6-mm EQC C18 column and eluted at 2 ml/min with 20 mM sodium phosphate buffer (pH 6.8) and methanol (B). The gradient consisted of 65% A and 35% B for 2 min, followed by a linear gradient to 45% A and 55% B for 10 min. This was maintained for an additional 2 min. Benzidine, N-acetylbenzidine, and N,N'-diacetylbenzidine concentrations were determined using A254 nm peak areas versus calibration curves. Michaelis-Menten kinetic constants for the N-acetylation of benzidine and N-acetylbenzidine were obtained from proportionally weighted Lineweaver-Burk plots as described previously (42).

**Protein Determinations.** Protein concentrations of bacterial lysates were determined by a Bio-Rad dye-binding method (43). Enzyme velocities were then normalized relative to the quantity (arbitrary units) of immunoreactive NAT2 protein detected by Western immunoblot analysis as described above.

**NAT2 Genotype Determinations.** DNA was extracted from human liver tissues and used to determine NAT1 and NAT2 genotypes. NAT2 genotypes were determined by a recently described PCR-RFLP method (44) that distinguishes between the 23 human NAT2 alleles identified to date.

**NAT1 Genotype Determinations.** NAT1 genotype was assessed by a PCR-RFLP method designed to distinguish between the NAT1*3, *4, *5, *10, *11, *14, and *15 alleles that have been identified in human populations (46). Briefly, NAT1 was amplified by PCR using 0.5 μg of genomic DNA in a 100-μl reaction containing 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1.5 mm MgCl2, 0.2 mm of each deoxynucleotide triphosphate, 1 μg of each primer, and 2.5 U of Taq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT). The mixture was subjected to 30 cycles (1.5 min at 94°C, 1.5 min at 55°C, and 1.5 min at 72°C). This was followed by 30 min at 72°C to ensure complete extension. The primers and procedures used for PCR amplification of human NAT2 were as reported previously (8).

**Expression of Recombinant Human NAT1 and NAT2.** JM105 bacteria harboring the human NAT1 or NAT2 plasmids were grown overnight in Luria-Bertani medium containing 100 μg/ml ampicillin at 37°C. Fresh Luria-Bertani ampicillin broth was used only once. NAT1 and NAT2 plasmids were re-expressed in Escherichia coli strain DH5α, and transformed into a prokaryotic expression vector. Representative clones were always obtained from at least two independent PCRs. NAT1 and NAT2 PCR clones were sequenced by a modified double-stranded deoxyxystar chain termination method (41) using Sequenase (United States Biochemical Corp., Cleveland, OH). Once identified, NAT1 and 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 E. coli strain JM105.

**Table 1. Michaelis-Menten kinetic constants for N-acetylation of benzidine and N-acetylbenzidine by human recombinant NAT1 and NAT2**

<table>
<thead>
<tr>
<th>Arylamine substrate</th>
<th>Apparent kinetic constant</th>
<th>NAT1</th>
<th>NAT2</th>
<th>NAT1/ NAT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzidine</td>
<td>Vmax (mmol/min)</td>
<td>4414 ± 389</td>
<td>10.5 ± 0.3</td>
<td>420</td>
</tr>
<tr>
<td>Kcat (μM)</td>
<td></td>
<td>254 ± 38</td>
<td>33.3 ± 1.5</td>
<td>7.6</td>
</tr>
<tr>
<td>Clearance (Vmax/Kcat)</td>
<td></td>
<td>17.4</td>
<td>0.32</td>
<td>54</td>
</tr>
<tr>
<td>N-Acetylbenzidine</td>
<td>Vmax (mmol/min)</td>
<td>1476 ± 82</td>
<td>1.17 ± 0.04</td>
<td>1262</td>
</tr>
<tr>
<td>Kcat (μM)</td>
<td></td>
<td>1380 ± 90</td>
<td>471 ± 23</td>
<td>2.9</td>
</tr>
<tr>
<td>Clearance (Vmax/Kcat)</td>
<td></td>
<td>1.07</td>
<td>0.002</td>
<td>535</td>
</tr>
</tbody>
</table>

* The initial AcCoA concentration was 300 μM. N-Acetyltransferase activities were normalized relative to the quantity (in arbitrary units) of immunoreactive protein determined by Western blot with polyclonal antihuman NAT2 antiserum.
mutation sites. Therefore, each of these alleles was distinguished by PCR-RFLP analysis (46) using nested PCR. The NAT1 PCR product was used as the template in five subsequent PCR amplifications along with primers engineered to contain a partial restriction site. The presence or absence of the mutation in the template determined whether or not the restriction site was completed and thus recognized by the restriction enzyme.

**Human Liver Slice Incubations.** To assess intact liver metabolism of benzidine, tissues were sliced with a Stadie-Riggs microtome, weighed, and placed in 20-mI plastic scintillation vials. Vials contained 1 ml of α-modified Eagle’s medium with 14 μM [3H]benzidine or [3H]N-acetylbenzidine and were gassed with 5% CO₂ and 95% O₂ at 37°C (34, 47, 48). All incubation conditions were not performed with some subjects because of the limited amount of human sample available. After 1 h, the samples were regassed. After the incubation, 1 ml methanol was added. Samples were homogenized and centrifuged, and supernatants were frozen at —70°C. Metabolism was assessed by a Beckman HPLC apparatus consisting of a 5-μm, 4.6 × 150-mm C₁₈ ultrasphere column attached to a guard column. Metabolites were separated using the following solvent system: for 25% methanol, 75% 0.02 M potassium phosphate buffer (pH 6.8), 0—2 mm; for 25—45% methanol, 2—17 mm; and for 45—85% methanol, 17—25 mm; flow rate was 1 ml/min. Radioactivity in HPLC eluents was measured using a FLO-ONE radioanalytical detector. Data were expressed as nmol/g wet tissue weight.

DNA was prepared from the tissue pellet obtained after homogenization and centrifugation. The pellet was resuspended in 50 mM Tris buffer (pH 7.4) containing 10 mM EDTA, proteinase K (1 mg/ml), and 1% SDS, and incubated for 90 min at 50°C. Samples were extracted with phenol and then chloroform:isoamyl alcohol (24:1; Ref. 49). The aqueous fraction was adjusted to a final concentration of 0.25 M NaCl, cold ethanol added, and nucleic acid precipitated at −20°C overnight. Pellets were washed with 70% ethanol and dissolved in H₂O. Purity of DNA was determined by absorbance at 260 and 280 nm.

**RESULTS**

**Michaelis-Menten Constants for Benzidine and N-Acetylbenzidine.** For benzidine (Table 1), both Km and Vₘₐₓ values for NAT1 were significantly higher than corresponding values for NAT2 (Figs. 2A and 3A). With clearance values for NAT1 and NAT2 of 17.4 and 0.32, respectively, the clearance ratio (NAT1/NAT2) was 54. For N-acetylbenzidine (Table 1), the clearance ratio of 535 was about 10-fold greater than that observed for benzidine. Both N-acetylbenzidine Km and Vₘₐₓ values for NAT1 were significantly higher than corresponding values for NAT2 (Figs. 2B and 3B). Kinetic parameters appear to favor benzidine metabolism over N-acetylbenzidine for each NAT. For NAT1, the clearance value for benzidine is 16-fold greater than that for N-acetylbenzidine. For NAT2, the clearance value for benzidine is 160-fold greater than that for N-acetylbenzidine.

**Michaelis-Menten Constants for Benzidine as a Function of AcCoA Concentrations.** Previous studies with human liver indicated that rapid and slow N-acetylation occurs by a binary ping-pong mechanism (50). This was assessed for benzidine with various concentrations of AcCoA (Table 2). As the concentration of AcCoA increased, the Km and Vₘₐₓ values for benzidine increased for both recombinant human NAT1 and NAT2. Proportional incremental increases in Km and Vₘₐₓ were very similar, such that the apparent clearance of NAT1 over the six concentrations of AcCoA tested was...
The benzidine concentrations ranged from 2 to 2000 μM. N-Acetyltransferase activities were normalized relative to quantity (in arbitrary units) of immunoreactive protein determined by Western blot with polyclonal antihuman NAT2 antiserum. Following densitometry, NAT1 immunoreactive protein was 88.1% of NAT2.

Human Liver Slice Metabolism of Benzidine. To relate in vitro enzymatic data to the intact liver cell, human liver slice incubations were performed. The NAT1 and NAT2 genotype and phenotype are listed for each sample in Table 3. As shown in Fig. 4, slices incubated with [3H]benzidine exhibited substantial conversion to N-acetylbendine, but not N,N'-diacetylbenzidine. This was observed with all the human liver slices irrespective of the expected acetylator phenotype (Table 4). The ratio of acetylated to nonacetylated products (N-acetylbenzidine) was lowest for human liver slices incubations with [3H]N-acetylbenzidine. The average acetylation ratio for each sample whether slices were incubated with [3H]benzidine or [3H]N-acetylbenzidine was proportional to the presumptive NAT1 phenotype (Table 5). Samples 1 and 6 have a wild-type presumptive NAT1 phenotype and have the lowest acetylation ratio. This is consistent with NAT1 of benzidine primarily via NAT1.

DISCUSSION

This is the first study to determine kinetic constants for benzidine and N-acetylbenzidine metabolism by NATs. These kinetic values are interpreted more readily when viewed in the context of data obtained with other aromatic amines. PABA and p-aminosalicylic acid are preferred human recombinant NAT1 substrates, whereas sulfamethazine and procainamide are substrates for human recombinant NAT2 (4). NAT1 : NAT2 clearance ratios obtained for recombinant human NAT1 and NAT2-catalyzed N-acetylation of p-aminosalicylic acid and sulfamethazine were 130,523 and 0.27, respectively. In the same study, both human NAT1 and NAT2 N-acetylated 4-aminobiphenyl with a NAT1 : NAT2 clearance ratio of 24 (13), which represents an intermediate ratio, but more similar to that obtained for sulfamethazine than p-aminosalicylic acid. In this context, the NAT1 : NAT2 clearance ratio of 54 (Table 1) obtained for benzidine in the present study is also intermediate between NAT1- and NAT2-selective substrates. The 10-fold higher NAT1 : NAT2 clearance ratio observed with N-acetylbenzidine (535) would favor NAT1. The much higher affinity (lower \( K_m \)) of both NAT1 and NAT2 for benzidine compared to N-acetylbenzidine suggests preferential N-acetylation of benzidine at low exposure levels.

The \( V_{\text{max}} \) and \( K_m \) values for benzidine increased for both recombinant human NAT1 and NAT2 as the concentration of AcCoA increased. This is consistent with recombinant human NAT metabolism occurring by a binary ping-pong mechanism. This proposes that AcCoA first binds to NAT, transferring its acetyl function to the

### Table 2

<table>
<thead>
<tr>
<th>AcCoA (μM)</th>
<th>Apparent ( V_{\text{max}} ) (nmol/min/μg)</th>
<th>Apparent ( K_m ) (μM)</th>
<th>Apparent clearance (( V_{\text{max}}/K_m ))</th>
<th>Apparent ( V_{\text{max}} ) (nmol/min/μg)</th>
<th>Apparent ( K_m ) (μM)</th>
<th>Apparent clearance (( V_{\text{max}}/K_m ))</th>
<th>Clearance ratio (NAT1/NAT2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.6</td>
<td>317 ± 7</td>
<td>16.7 ± 0.7</td>
<td>19.0</td>
<td>0.914 ± 0.014</td>
<td>2.45 ± 0.32</td>
<td>0.37</td>
<td>51</td>
</tr>
<tr>
<td>31.3</td>
<td>620 ± 25</td>
<td>25.6 ± 2.2</td>
<td>24.2</td>
<td>1.81 ± 0.02</td>
<td>4.64 ± 0.32</td>
<td>0.39</td>
<td>62</td>
</tr>
<tr>
<td>62.5</td>
<td>1287 ± 59</td>
<td>58.3 ± 4.3</td>
<td>22.1</td>
<td>3.51 ± 0.04</td>
<td>8.64 ± 0.37</td>
<td>0.41</td>
<td>54</td>
</tr>
<tr>
<td>125</td>
<td>2302 ± 109</td>
<td>108 ± 7</td>
<td>21.3</td>
<td>6.78 ± 0.09</td>
<td>16.5 ± 0.6</td>
<td>0.41</td>
<td>52</td>
</tr>
<tr>
<td>250</td>
<td>4285 ± 173</td>
<td>209 ± 11</td>
<td>20.5</td>
<td>12.8 ± 0.22</td>
<td>32.4 ± 1.2</td>
<td>0.40</td>
<td>51</td>
</tr>
<tr>
<td>300</td>
<td>4414 ± 389</td>
<td>254 ± 38</td>
<td>17.4</td>
<td>10.5 ± 0.3</td>
<td>33.3 ± 1.5</td>
<td>0.32</td>
<td>54</td>
</tr>
</tbody>
</table>

The benzidine concentrations ranged from 2 to 2000 μM. N-Acetyltransferase activities were normalized relative to quantity (in arbitrary units) of immunoreactive protein determined by Western blot with polyclonal antihuman NAT2 antiserum. Following densitometry, NAT1 immunoreactive protein was 88.1% of NAT2.

20.8 ± 1.0. The apparent clearance of NAT2 over this AcCoA concentration range was 0.38 ± 0.01. A NAT1/NAT2 clearance ratio of 54 ± 1.7 further illustrates the consistent effect of AcCoA observed over a 20-fold concentration range. These results suggest recombinant human NAT1- and NAT2-catalyzed N-acetylation of benzidine by a binary ping-pong mechanism.

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>NAT2 genotype</th>
<th>NAT2 phenotype</th>
<th>NAT1 genotype</th>
<th>NAT1 phenotype</th>
<th>N-Acetylbenzidine + N,N'-Diacetylbenzidine/Benzidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NAT2<em>4/NAT2</em>4</td>
<td>Rapid</td>
<td>NAT1<em>4/NAT1</em>4</td>
<td>Wild-type</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>NAT2<em>4/NAT2</em>5A</td>
<td>Intermediate</td>
<td>NAT1<em>10/NAT1</em>4</td>
<td>Rapid</td>
<td>4.1</td>
</tr>
<tr>
<td>4</td>
<td>NAT2<em>5B/NAT2</em>5B</td>
<td>Slow</td>
<td>NAT1<em>4/NAT1</em>10</td>
<td>Rapid</td>
<td>8.7</td>
</tr>
<tr>
<td>5</td>
<td>NAT2<em>6A/NAT2</em>6A</td>
<td>Slow</td>
<td>NAT1<em>4/NAT1</em>10</td>
<td>Rapid</td>
<td>4.3</td>
</tr>
<tr>
<td>6</td>
<td>NAT2<em>5B/NAT2</em>5B</td>
<td>Slow</td>
<td>NAT1<em>4/NAT1</em>4</td>
<td>Wild-type</td>
<td>2.2</td>
</tr>
</tbody>
</table>

a Sample numbers correspond to those in Table 4.

b The ratio of acetylated benzidine products represents the average value obtained from corresponding sample incubations with [3H]benzidine and [3H]N-acetylbenzidine in Table 4. For NAT2, the average acetylation ratio for the slow and rapid/intermediate phenotypes is 5.1 ± 1.9 and 2.7 ± 0.8, respectively. For NAT1, the average acetylation ratio for the wild-type and rapid phenotypes is 1.8 ± 0.4 and 4.9 ± 1.3, respectively.
### Table 4. N-Acetylation of benzidine and N-acetylbenzidine by human liver slices

<table>
<thead>
<tr>
<th>Sample</th>
<th>NAT2 phenotype/incubation conditions</th>
<th>NAT1 phenotype</th>
<th>Benzidine (nmol/g wet tissue weight)</th>
<th>N-Acetylbenzidine (nmol/g wet tissue weight)</th>
<th>N,N'-Diacyetylbenzidine (nmol/g wet tissue weight)</th>
<th>N-Acetylbenzidine + N,N'-Diacyetylbenzidine (nmol/g wet tissue weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rapid</td>
<td>Wild-type</td>
<td>50</td>
<td>69</td>
<td>0</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>[3H]Benzidine</td>
<td></td>
<td>84</td>
<td>29</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate</td>
<td>Rapid</td>
<td>19</td>
<td>33</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>[3H]Benzidine with PABA</td>
<td></td>
<td>37</td>
<td>15</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>Intermediate</td>
<td>Rapid</td>
<td>11</td>
<td>32</td>
<td>1.5</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>[3H]Benzidinec</td>
<td></td>
<td>9</td>
<td>36</td>
<td>0.7</td>
<td>4.1</td>
</tr>
<tr>
<td>4</td>
<td>Slow</td>
<td>Rapid</td>
<td>16</td>
<td>30</td>
<td>0.8</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>[3H]Benzidine with PABA</td>
<td></td>
<td>10</td>
<td>37</td>
<td>4.2</td>
<td>4.1</td>
</tr>
<tr>
<td>5</td>
<td>Slow</td>
<td>Rapid</td>
<td>12</td>
<td>39</td>
<td>1.9</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>[3H]Benzidine with PABA</td>
<td></td>
<td>6.3</td>
<td>31</td>
<td>0.9</td>
<td>5.1</td>
</tr>
<tr>
<td>6</td>
<td>Slow</td>
<td>Wild-type</td>
<td>40</td>
<td>82</td>
<td>3</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>[3H]Benzidine with PABA</td>
<td></td>
<td>69</td>
<td>18</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>[3H]N-Acetylbenzidine</td>
<td></td>
<td>35</td>
<td>71</td>
<td>5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Notes:<br>1. Slices from different subjects were incubated with 0.014 mM [3H]benzidine or [3H]N-acetylbenzidine for 2 h at 37°C. Where indicated, 0.25 mM PABA was added. Metabolites were identified by HPLC. Values represent the average of duplicate incubations from one subject.<br>2. Deduced NAT2 acetylator phenotype based on recombinant human NAT2 expression systems (8, 9).
N-ACETYLATION OF BENZIDINE

Fig. 5. HPLC of a human liver slice incubation with [3H]benzidine and 0.25 mM PABA. BZ-NG, benzidine N-glucuronide; ABZ-NG, N-acetylbenzidine N'-glucuronide; BZ, benzidine; ABZ, N-acetylbenzidine; DABZ, N,N'-diacetylbenzidine.

lators and 97% in rapid acetylators, the importance of this small difference is uncertain. Furthermore, N’-(3’-monophosphodeox-yguanosine-8-yl)-N-acetylbenzidine content in exfoliated bladder cells did not correlate with NAT2 activity (38). These results support those from a study of workers exposed to benzidine in which the slow acetylators did not have an increased risk to bladder cancer (37). Thus, whereas 4-aminobiphenyl appears to function as a NAT2 substrate in vivo, this does not appear to be the case for benzidine.

N-Acetylation of 4-aminobiphenyl and benzidine can have different outcomes. N-Acetyl-4-aminobiphenyl undergoes much less N-oxidation than 4-aminobiphenyl. The N-(deoxyguanosine-8-yl)-4-aminobiphenyl adduct observed in bladder cells is attributed to O-acetylation of N-OH-4-aminobiphenyl and does not involve N-OH-N-acetyl-4-aminobiphenyl (58). In contrast, N-acetylbenzidine is relatively reactive and is metabolized by rat liver microsomes at a 50-fold greater rate than N,N'-diacetylbenzidine (33). The major DNA adduct observed after benzidine exposure in rodents (31, 32) and humans (38) is acetylated [N’-(deoxyguanosine-8-yl)-N-acetylbenzidine] and is presumed to be formed by O-acetylation of N'-hydroxy-N-acetylbenzidine. Thus, in contrast to 4-aminobiphenyl, N-acetylation appears to be an important part of the benzidine activation process.

In the intact rat (55), perfused rat liver (56), and rat liver slices (39, 57), N,N'-diacetylbenzidine appears to be the major product with little or no N-acetylbenzidine observed. In contrast, in human liver slices, N-acetylbenzidine is the major product with little N,N'-diacetylbenzidine formed (Table 4; Ref. 39). Workers exposed to benzidine exhibit N-acetylbenzidine as the predominant urinary metabolite with little N,N'-diacetylbenzidine observed (38, 59). The lower affinity of both human NAT1 and NAT2 for N-acetylbenzidine (high Ka) reflects this important species difference. Because N-acetylbenzidine is an active metabolite and N,N'-diacetylbenzidine inactive, the tendency for humans to accumulate N-acetylbenzidine may increase their risk of developing bladder cancer from benzidine exposures.

The complexity and multiple interactions that influence overall metabolism of benzidine are illustrated by the liver slice experiments. In addition to acetylation, glucuronidation occurs and is a competing pathway. Whether incubations were initiated with either [3H]benzidine or [3H]N-acetylbenzidine, the acetylation ratios within each sample were similar (Table 4), suggesting an equilibrium that favors N-acetylbenzidine accumulation. A substantial amount of deacetylation appears to occur and is illustrated by dramatic increases in N,N'-diacetylbenzidine observed in human liver slices incubated with paraoxon, a deacetylase inhibitor (39). This demonstrates N-acetylbenzidine acetylation with rapid deacetylation preventing the diacetylated product from accumulating.

In summary, N-acetylation plays an important role in benzidine carcinogenesis. Kinetic parameters for human recombinant NAT1 and NAT2 metabolism of benzidine and N-acetylbenzidine were determined. Whereas kinetic data suggest preferential metabolism of N-acetylbenzidine by NAT1, results with benzidine were less conclusive. Human liver slice data suggest that benzidine is a preferred substrate for NAT1. Thus, low NAT2 activity (slow acetylation phenotype) should not be expected to affect benzidine acetylation, adduct formation, or incidence of bladder cancer. These conclusions are supported by recent human studies (37–39). These results also suggest that human liver may exhibit polymorphic expression of NAT1.

ACKNOWLEDGMENTS
The authors thank Cindee Rettke and Priscilla DeHaven for excellent technical assistance.

REFERENCES
N-ACETYLATION OF BENZIDINE


Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 1996 American Association for Cancer Research.
Human N-Acetylation of Benzidine: Role of NAT1 and NAT2


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/17/3941

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.