1996 ASPET N-Glucuronidation of Xenobiotics Symposium

N-GLUCURONIDATION OF BENZIDINE AND ITS METABOLITES

Role in Bladder Cancer

TERRY V. ZENGER, VIJAYA M. LAKSHMI, AND BERNARD B. DAVIS

VA Medical Center, and Department of Biochemistry and Division of Geriatric Medicine, St. Louis University School of Medicine

This paper is available online at http://www.dmd.org

ABSTRACT:

Workers exposed to high levels of benzidine have a 100-fold increased incidence of bladder cancer. This review evaluates the overall metabolism of benzidine to determine pathways important to initiation of bladder cancer. Upon incubation of benzidine with liver slices from rats, dogs, and humans, different proportions of this diamine were N-acetylated and N-glucuronidated. With dogs, a non-acetylator species, N-glucuronidation was the major pathway. In contrast, little glucuronidation was observed in rats with N,N'-diacetylbenzidine, the major metabolite of benzidine. Human liver slices demonstrated both extensive N-acetylation and N-glucuronidation. Differences between rats and humans were attributed to rapid deacetylation by human liver with N-acetylbenzidine rather than an accumulation of N,N'-diacetylbenzidine. N-Acetylbenezidine oxidative metabolism was also observed. The acid lability of glucuronide products of benzidine, N-acetylbenzidine, and oxidation products of N-acetylbenzidine metabolism was assessed. N-Glucuronides of benzidine, N-acetylbenzidine, and N'-hydroxy-N-acetylbenzidine were acid-labile, with the latter having a much longer half-time than the former two glucuronides. Because bladder epithelium contains relatively high levels of prostaglandin H synthase and not cytochrome P450, the peroxidative metabolism of N-acetylbenzidine was assessed. N'- (3'-Monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine was the only DNA adduct detected. This adduct is also the major adduct detected in bladder cells from workers exposed to benzidine. In urine from these workers, an inverse relationship between urine pH and levels of free (unconjugated) benzidine and N-acetylbenzidine was observed. A similar inverse relationship was observed for urine pH and levels of bladder cell N'- (3'-monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine. These results suggest multiple pathways (acetylation, glucuronidation, peroxidation) in multiple organs (liver, blood, kidney, bladder) are important in benzidine-induced bladder cancer.

Bladder cancer was one of the first cancers to be recognized as being caused by chemical exposure in the workplace. A pioneering observation by Rehn in 1895 noted a high frequency of bladder cancer among workers in the aniline dye industry (Rehn, 1895). The high incidence of bladder cancer among smokers and workers in dye, rubber, and chemical industries is associated with their exposure to aromatic amines (Case et al., 1954; Doll and Peto, 1981; Rehn, 1895). Bladder cancer represents approximately 7% of human malignancies and is the third most prevalent cancer type in men 60 years of age and older (Parker et al., 1996; Silverberg and Lubera, 1987).

Workers exposed to high levels of benzidine have as much as a 100-fold increased risk for bladder cancer (Bi et al., 1992). Hepatic N-oxidation of aromatic amines is considered a necessary reaction for activation to occur (Miller, 1970; Miller and Miller, 1977). Because acetylated aromatic amines are difficult to oxidize, N-acetylation is considered a detoxification step (Hein, 1988). Benzidine, an aromatic diamine, is acetylated to N-acetylbenzidine and N,N'-diacetylbenzidine. The latter is considered a detoxification product, while N-acetylbenzidine is oxidized to reactive intermediates that form DNA adducts (Frederick et al., 1985; Kennely et al., 1984; Lakshmi et al., 1997; Martin et al., 1982). Both benzidine and N-acetylbenzidine are preferred substrates for the type 1 N-acetyltransferase enzyme (Zenser et al., 1996).

Benzidine-induced bladder cancer has been difficult to study in animals. This chemical induces bladder cancer in dogs and predominately liver cancer in rats (Haley, 1975; Radomski, 1979). While dogs do develop bladder cancer, more than 5 years are required for tumors to develop, making this a difficult model for study. One distinguishing characteristic between these two species is that rats are acetylators, while dogs are non-acetylators (Lower and Bryan, 1973; Poirier et al., 1963). Thus acetylation does not appear to be necessary for bladder tumor formation. We have hypothesized that N-glucuronidation plays an important role in aromatic amine-induced bladder cancer and have used benzidine as a model compound in the following studies to evaluate this hypothesis.

N-Glucuronidation of Benzidine by Human, Dog, and Rat Liver Microsomes and Slices

Microsomes from humans, dogs, and rats produced a new HPLC$^1$ peak when UDP-glucuronic acid was incubated with 3-H-benzidine (Babu et al., 1993; Babu et al., 1994a; Babu et al., 1994b). An identical peak, which was acid-labile, was formed by all three species. Human microsomes incubated in the absence of detergents had the most UDP-glucuronosyltransferase activity for benzidine and N-acetylbenzidine (Table 1). Rats had the lowest activity. When the

---

$^1$ Abbreviations used are: HPLC, high-performance liquid chromatography; UDP, uridine diphosphate; $t_{1/2}$, half-time.
detoxification of benzidine and N,N'-diacetylbenzidine was observed, which is consistent with the lack of glucuronidation of arylamides (Babu et al., 1993).

Kinetic parameters of glucuronidation were evaluated with human liver microsomes. For benzidine, $K_m$ and $V_{max}$ values of 0.8 mM and 4.2 nmol/mg protein/min, respectively, were observed with Emulgen 911–treated microsomes (Babu et al., 1994a). The kinetics of N-acetylbenzidine glucuronidation were more complex with high- and low-affinity UDP-glucuronosyltransferases, such that the $K_m$ values were 0.36 and 1.1 mM, respectively, and $V_{max}$ values were 2.3 and 3.1 nmol/mg protein/min, respectively (Babu et al., 1994b). This is consistent with multiple transferases metabolizing N-acetylbenzidine.

To determine the specificity of the transferase reaction with benzidine and N-acetylbenzidine, a wide range of inhibitors (known substrates) were tested. Benzidine glucuronidation was inhibited in a dose-response manner by estriol, testosterone, and 4-aminobiphenyl. When maximum effective concentrations of each inhibitor were added with another inhibitor, nearly additive decreases in activity were observed (Babu et al., 1994). Similar results were also observed with N-acetylbenzidine (Babu et al., 1994). This is consistent with multiple transferases metabolizing benzidine and N-acetylbenzidine. Green et al. demonstrated benzidine glucuronidation by the human UGT1.4 enzyme and suggested that all gene products of the human UGT1 gene family glucuronidate primary amines (Green et al., 1995).

To correlate results with microsomes to those in intact tissues, liver slices from these three species were examined. In each experiment, slices were incubated with 0.014 mM 3H-benzidine. For dogs, an HPLC peak corresponding to the glucuronide conjugate of benzidine was observed during microsomal incubations with 3H-benzidine. Because of high deacetylation activity, little N,N'-diacetylbenzidine is detected. Extensive N-glucuronidation of both benzidine and N-acetylbenzidine occurs. Human urine contains N-glucuronides, which are susceptible to acidic urine hydrolysis to benzidine and N-acetylbenzidine.

Identification of N-Glucuronides of Benzidine and N-Acetylbenzidine by Mass Spectrometry

Confirmation of the chemical structure of our compounds was determined by mass spectrometry. Using positive-ion Thermospray mass spectrometry for analysis of the glucuronide of benzidine, a molecular ion at $m/z$ 361 was observed, with the base peak at $m/z$ 185 for the protonated aglycone (Babu et al., 1992). Ions at $m/z$ 257 and 227 represented cleavage across the pyran ring. These results are consistent with the compound being benzidine N-glucuronide. For the glucuronide conjugate of N-acetylbenzidine, electron ionization mass spectrometry of the trimethylsilyl derivative was performed by direct probe in the positive ion mode (Babu et al., 1993). The molecular ion was at $m/z$ 762, structures of the major ions were verified by high-resolution mass measurements, and the compound was identified as N-acetylbenzidine N-glucuronide.

**pH Stability of N-Glucuronides**

The pH stability of benzidine and N-acetylbenzidine N-glucuronides was of interest because pH might play an important role in the effects of these compounds. After 4 or 5 min at pH 5.3 and 37°C, half of these glucuronides are hydrolyzed to their parent amine (Table 2) (Babu et al., 1993; Babu et al., 1992). These glucuronides are quantitatively hydrolyzed to their parent amine. At pH 7.4, the half-lives of benzidine and N-acetylbenzidine glucuronides are 104 and 140 min, respectively. The half-lives of both glucuronides are greatly extended at neutral pH by plasma with $>80\%$ remaining after 4 hr. Plasma is known to bind glucuronides (Dutton, 1980) and is thought to contribute to the observed increases in half-lives. Thus N-glucuronidation provides a mechanism for hepatic excretion, transport by plasma, filtration by the kidney, and accumulation in urine. Because of their

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>$^3$H-Benzidine</th>
<th>$^3$H-N-Acetylbenzidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>62</td>
<td>55</td>
</tr>
<tr>
<td>Dog</td>
<td>47</td>
<td>11</td>
</tr>
<tr>
<td>Rat</td>
<td>13</td>
<td>8</td>
</tr>
</tbody>
</table>

Values represent averages of at least duplicate experiments.

---

**FIG. 1.** Model illustrating hepatic acetylation, deacetylation, and glucuronidation of benzidine (BZ) in rat and human. Rat N-acetyltransferase (NAT) rapidly acetylates benzidine to N,N'-diacetylbenzidine (DABZ), with little deacetylase (DAT) activity and little N-glucuronidation detected. In humans, benzidine is acetylated to N-acetylbenzidine (ABZ) and N,N'-diacetylbenzidine. Because of high deacetylase activity, little N,N'-diacetylbenzidine is detected. Extensive N-glucuronidation of both benzidine and N-acetylbenzidine occurs. Human urine contains N-glucuronides, which are susceptible to acidic urine hydrolysis to benzidine and N-acetylbenzidine.
acid lability, $N$-glucuronides could be hydrolyzed by acidic urine to their corresponding carcinogenic amines in the lumen of the bladder.

These results prompt the following questions: Is this acid lability exhibited by other aromatic amine $N$-glucuronides, and how does the acid lability of an aromatic amine $N$-glucuronide compare with that of its N-OH analogue? The glucuronides of 4-aminobiphenyl and N-OH-4-aminobiphenyl were both acid-labile with $t_{1/2}$ values of 10.5 and 32 min, respectively, at pH 5.5 (Babu et al., 1996). In contrast, the $O$-glucuronide of N-OH-N-acyl-4-aminobiphenyl was not acid-labile, with $t_{1/2}$ values at pH 5.5 and 7.4 of 55 and 68 min, respectively. Thus other aromatic amine $N$-glucuronides are also acid-labile and may have a shorter half-life than their corresponding $N$-OH $N$-glucuronides. O-Glucuronides are not acid-labile.

The $N$- and $O$-glucuronides of N-acyetylbenzidine oxidation products yielded similar results (Babu et al., 1995). The $O$-glucuronides of the hydroxamic acids, $N$-hydroxy-N-acyetylbenzidine and $N$-hydroxy-$N,N'$-diacetylbenzidine, were not acid-labile. The $N$-glucuronide of $N'$-hydroxy-N-acyetylbenzidine was acid-labile, with a $t_{1/2}$ at pH 5.5 of 3.5 hours, compared with that for $N$-acyetylbenzidine $N'$-glucuronide of 7.5 min. Thus the $N$-glucuronide of N-acyetylbenzidine is much more likely to be involved in acidic urine--catalyzed hydrolysis than is its $N$-OH metabolite.

Experiments were designed to determine whether the acid lability demonstrated in these in vitro experiments was applicable in vivo (Rothman et al., 1997). Urine samples were obtained from workers in India manufacturing benzidine or benzidine-based dyes and compared with those from workers at a construction company. Post-workshift urine pH was inversely correlated with the proportions of benzidine and $N$-acyetylbenzidine present as free (unconjugated) compounds. Diet is an important determinant of urine pH (Remer and Manz, 1995). Most fruits and vegetables contribute to urine alkalinization, while meat, cheese, fish, and grain products contribute to urine acidification. These results would suggest that urine pH may be a new risk factor in bladder cancer.

**Bladder Prostaglandin H Synthase Activation of Arylamines**

Does the bladder metabolize primary arylamines derived from acidic urine hydrolysis? For urinary arylamines to initiate bladder cancer, they must be activated by the bladder. Bladder epithelium contains substantial amounts of prostaglandin H synthase but little cytochrome P450 activity (Wise et al., 1984). Prostaglandin H synthase activates a variety of aromatic amine carcinogens, because of its peroxidatic activity, to bind protein, t-RNA, and DNA (Flammang et al., 1989; Wise et al., 1984). Prostaglandin synthesis by cultured human and dog urothelial cells increases in response to bradykinin, calcium ionophore, arachidonic acid, and phosphol ester (Danon et al., 1986; Wong et al., 1989; Zenser et al., 1990; Zenser et al., 1988). Thus bladder prostaglandin H synthase has the potential to activate benzidine and N-acyetylbenzidine.

Human bladder cells have recently been shown to activate N-OH-4-aminobiphenyl peroxidatically to form specific DNA adducts (Hatcher and Swaminathan, 1995). Prostaglandin H synthase from ram seminal vesicles has been used to activate N-acyetylbenzidine to form a specific DNA adduct, $N'$-(3'-monophospho-deoxyguanosin-8-yl)-N'-acyetylbenzidine (Lakshmi et al., 1998). This adduct was demonstrated to be the major adduct observed in exfoliated urothelial cells from workers exposed to benzidine (Rothman et al., 1996). In addition, after controlling for internal dose, individuals with urine pH values $<$6 had tenfold higher levels of this adduct than did subjects with urine pH values $>$7 (Rothman et al., 1997). The adduct has been shown to cause genotoxic lesions, resulting in mutations in various bacterial and mammalian test systems in vitro and in oncogenes of tumors (Beland et al., 1983; Fox et al., 1990; Heflich et al., 1986; Melchior et al., 1994; Talaska et al., 1987). Thus the peroxidatic activity of bladder is capable of activating N-acyetylbenzidine to form DNA adducts with the potential to participate in initiation of tumor formation.

Our results are summarized in Figure 2 and illustrate a mechanism for benzidine-induced bladder cancer. According to this model, benzidine and N-acyetylbenzidine can be either oxidized (O) or $N$-glucuronidated (UDP) in liver. $N$-Glucuronides are transported by the blood and filtered by the kidneys, with their resulting accumulation in urine within the lumen of the bladder. Acidic urine hydrolyzes these $N$-glucuronides to their parent amines. Benzidine and N-acyetylbenzidine are metabolically activated (i.e. prostaglandin H synthase) in bladder to bind DNA and initiate carcinogenesis.

![Fig. 2. Model illustrating a mechanism for benzidine-induced bladder cancer in humans. Benzidine and N-acyetylbenzidine (Ar-NH$_2$) can be either oxidized (O) or N-glucuronidated (UDP) in liver. $N$-Glucuronides are transported by the blood and filtered by the kidneys, with their resulting accumulation in urine within the lumen of the bladder. Acidic urine hydrolyzes these $N$-glucuronides to their parent amines. Benzidine and N-acyetylbenzidine are metabolically activated (i.e. prostaglandin H synthase) in bladder to bind DNA and initiate carcinogenesis.](image-url)
monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine was also observed.

References


