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 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-Acetylbenzidine 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.

1 Abbreviations used are: HPLC, high-performance liquid chromatography; UDP, uridine diphosphate; 1/2, half-time.
detergents Emilgen 911 (lot 2379; gift from Kao Atlas Chemicals, Tokyo, Japan), Triton X-100, Lubrol PX, and CHAPS (all from Sigma Chemical Co., St. Louis, MO) were used to stimulate transferase activity, the activity was qualitatively similar to that observed in the absence of detergents with human > dog > rat. No glucuronidation of 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 Emilgen 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 estradiol, 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 $^3$H-benzidine. For dogs, an HPLC peak corresponding to the glucuronide conjugate of benzidine represented as much as 30% of the total radioactivity recovered (Babu et al., 1992). This glucuronide was also observed in human liver slices incubated with $^3$H-benzidine. In addition, a new acid-labile HPLC peak was observed in humans. This peak corresponded to that observed during microsomal incubations with $^3$H-N-acetylbenzidine and UDP-glucuronic acid and was designated the glucuronide conjugate of N-acetylbenzidine (Babu et al., 1993). While human liver produced a considerable amount of N-acetylbenzidine, little N,N'-diacetylbenzidine was detected. This was shown to be due to rapid deacetylation of N,N'-diacetylbenzidine to N-acetylbenzidine (Lakshmi et al., 1995). Rat liver rapidly acetylated benzidine to N-acetylbenzidine and N,N'-diacetylbenzidine, with the major product being N,N'-diacetylbenzidine (Babu et al., 1993; Lakshmi et al., 1995). No glucuronides were detected under these incubation conditions in rats.

A model was developed to interpret our results relative to species differences in benzidine-induced bladder cancer (Figure 1). This model considers the role of hepatic acetylation, deacetylation, and glucuronidation on benzidine metabolism in rats and humans. According to this model, rats rapidly acetylate benzidine to N,N'-diacetylbenzidine, a detoxified product, with little or no N-glucuronides detected. In humans, benzidine is acetylated to N-acetylbenzidine, with little N,N'-diacetylbenzidine detected. The lack of detectable N,N'-diacetylbenzidine is due, in part, to the immediate deacetylation of N,N'-diacyctlbenzidine to N-acetylbenzidine. Extensive N-glucuronidation of both benzidine and N-acetylbenzidine occurs in humans. Interestingly, the high level of glucuronidation in humans and, therefore, the expected high level of urinary glucuronides correlates with the development of bladder cancer. Humans develop bladder cancer while rats develop liver cancer (Haley, 1975; Radomski, 1979). Thus N-glucuronidation may help to explain species differences in benzidine-induced bladder cancer.

**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>
<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.
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-acetylbenzidine oxidation products yielded similar results (Babu et al., 1995). The O-glucuronides of the hydroxamic acids, N-hydroxy-N-acetylbenzidine and N-hydroxy-N,N'-diacetylbenzidine, were not acid-labile. The N-glucuronide of N'-hydroxy-N-acetylbenzidine was acid-labile, with a $t_{1/2}$ at pH 5.5 of 3.5 hours, compared with that for N-acetylbenzidine N'-glucuronide of 7.5 min. Thus the N-glucuronide of N-acetylbenzidine 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-acetylbenzidine present as free (unconjugated) compounds. Diet is an important determinant of urine pH (Remer and Manz, 1995). Most fruits and vegetables contribute to urine alkalization, 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 amines 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 phorbol ester (Danon et al., 1986; Wong et al., 1989; Zenser et al., 1990; Zenser et al., 1988).

**Table 2**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Benzidine N-Glucuronide $t_{1/2}$ (min)</th>
<th>N-Acetylbenzidine N'-Glucuronide $t_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer pH 5.3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>pH 6.3</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>104</td>
<td>140</td>
</tr>
<tr>
<td>pH 9.3</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Dog plasma pH 7.4</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Experiments were conducted at 37°C (Babu et al., 1993).

$^*$ greater than 80% remaining after 4 hr.

![Fig. 2. Model illustrating a mechanism for benzidine-induced bladder cancer in humans. Benzidine and N-acetylbenzidine (Ar-NH$_2$) can be either oxidized (O) or N-glucuronidated (UDPG) 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-acetylbenzidine are metabolically activated (i.e. prostaglandin H synthase) in bladder to bind DNA and initiate carcinogenesis.](image)

Thus bladder prostaglandin H synthase has the potential to activate benzidine and N-acetylbenzidine.

Human bladder cells have recently been shown to activate N-OH-4-acetylbaminophenyl peroxidatically to form specific DNA adducts (Hatcher and Swaminathan, 1995). Prostaglandin H synthase from ram seminal vesicles has been used to activate N-acetylbenzidine to form a specific DNA adduct, N'-{3-monophospho-deoxyguanosin-8-yl}–N'-acylbenzidine (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; Hefflich et al., 1986; Melchior et al., 1994; Talaska et al., 1987). Thus the peroxidatic activity of bladder is capable of activating N-acetylbenzidine 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-acetylbenzidine can be either oxidized or glucuronidated 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. These N-glucuronides are acid-labile and are converted to their carcinogenic aromatic amines in acidic urine. Prostaglandin H synthase represents a potential peroxidatic pathway for activation of aromatic amines by the bladder to form DNA adducts. These adducts may initiate carcinogenesis by producing mutations that become fixed in the genome and eventually contribute to tumor formation. In vivo support for this hypothesis is provided by studies demonstrating an inverse relationship between urinary pH and urine levels of free (unconjugated) benzidine and N-acetylbenzidine in workers exposed to benzidine (Rothman et al., 1997). An inverse relationship between urinary pH and bladder cell content of N'-{3'-
monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine was also observed.

References


