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-acetylation 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; Kenneley 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 3H-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

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$^1$ Abbreviations used are: HPLC, high-performance liquid chromatography; UDP, uridine diphosphate; $t_{1/2}$, half-time.
detergents Emulgen 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 \( N,N' \)-diacetylbenezidine 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 \( N \)-acetylbenezidine 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 the \( V_{max} \) values were 2.3 and 3.1 nmol/mg protein/min, respectively (Babu et al., 1994b). This is consistent with multiple transferases metabolizing \( N \)-acetylbenezidine.

To determine the specificity of the transferase reaction with benzidine and \( N \)-acetylbenezidine, 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 to human liver microsomes (Babu et al., 1994a), 911–treated microsomes (Babu et al., 1994a), and 4.2 nmol/mg protein/min, respectively, were observed with Emulgen 911–treated microsomes (Babu et al., 1994a). The kinetics of \( N \)-acetylbenezidine 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 the \( V_{max} \) values were 2.3 and 3.1 nmol/mg protein/min, respectively (Babu et al., 1994b). This is consistent with multiple transferases metabolizing \( N \)-acetylbenezidine.

Identify the N-glucuronides of benzidine and N-acetylbenezidine 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 \( N \)-acetylbenezidine, 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 \( N \)-acetylbenezidine \( N \)-glucuronide.

PH Stability of N-Glucuronides

The pH stability of benzidine and \( N \)-acetylbenezidine \( N \)-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 \( N \)-acetylbenezidine 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
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-acyt-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 a benzidine manufacturing plant in India and from workers in a benzidine-based dye manufacturing 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.

**Table 2: Stability of benzidine and N-acyetylbenzidine N-glucuronides**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Benzidine N-Glucuronide</th>
<th>N-Acetylbenzidine N'-Glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</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</td>
<td>pH 7.4</td>
<td>*</td>
</tr>
</tbody>
</table>

Experiments were conducted at 37°C (Babu et al., 1993). * greater than 80% remaining after 4 hr.

**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 become fixed in the genome and eventually contribute to tumor formation. This model, based on bacterial and mammalian test systems in vivo 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), suggests that 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 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.

**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.

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

Human bladder cancer cells have recently been shown to activate N-OH-4-acytaminobiphenyl 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'-acetylbenzidine (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 vivo and in oncogenes of tumors. These adducts may initiate carcinogenesis by producing mutations that become fixed in the genome and eventually contribute to tumor formation.
monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine was also observed.

References


