Arylamine-DNA Adducts *In Vitro* and *In Vivo*: Their Role in Bacterial Mutagenesis and Urinary Bladder Carcinogenesis

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Hepatic *N*-oxidation, followed by *N*-glucuronidation, has been proposed as a route of metabolic activation for arylamine bladder carcinogens. It is postulated that the *N*-glucuronides are transported to the bladder lumen where they are hydrolyzed under slightly acidic conditions to release direct-acting carcinogenic and mutagenic *N*-hydroxyarylamines. In this study, 4-aminobiphenyl (ABP), 1-naphthylamine (1-NA), 2-naphthylamine (2-NA), 2-acetylaminofluorene (AAF), 4-nitrobenzidine (NBP), benzidine (BZ), and *N*-acetylbenzidine (ABZ) were administered to male beagle dogs (60 µ mole/kg), and the bladder epithelium DNA adducts were quantified at various times after treatment. At 24-48 hr after administration, the order of binding to bladder epithelium DNA was: ABP >> AAF > NBP ≈ 2-NA ≈ BZ ≈ ABZ >> 1-NA. The level of DNA modification by ABP remained constant for 7 days, whereas 2-NA and AAF residues decreased by 35% and 80%, respectively. The extent and relative persistence of total DNA binding correlated with the compounds' ability to induce bladder tumors in dogs. ABP, AAF, NBP, 2-NA and ABZ administration resulted in DNA binding sufficient for adduct analysis. Enzymatic hydrolysis of the DNA and examination of the adducts by high pressure liquid chromatography indicated that arylamine substitution at C8 of deoxyguanosine was the dominant product. Additional adducts were detected in animals treated with ABP, NBP, and 2-NA. Furthermore, the profiles of adducts obtained *in vivo* were remarkably similar to the profiles obtained when the *N*-hydroxy arylamine metabolites of these carcinogens were reacted with DNA *in vitro* at pH 5.0. To evaluate the mutagenic potential of these arylamine-DNA adducts, *Salmonella typhimurium* strains TA 1535 and TA 1538 were incubated with *N*-hydroxy-2-NA, *N*-hydroxy-2-aminofluorene (AF), *N*-hydroxy-ABP, and *N*-hydroxy-ABZ and the resulting DNA adducts and reversions were quantified. Arylamine-C8-deoxyguanosine substitution was correlated with frameshift reversions induced by these agents, with the lesions showing a relative order of mutagenic efficiency of ABZ > AAF > 2-NA > ABP. These data suggest that mutagenic *N*-hydroxyarylamines may be ultimate carcinogens for the bladder epithelium. Furthermore, if one assumes that a mutagenic lesion is important for tumor initiation, then C8-deoxyguanosine substitution by these compounds may be significant for urinary bladder carcinogenesis.

Introduction

In 1895, the German physician Rehn noted a relationship between workers exposed to aromatic amines and the incidence of bladder cancer (1). Although he named this disease "aniline cancer," subsequent studies indicated that the tumors were actually caused by 2-naphthylamine. During the intervening years, industrial exposure to other aromatic amines, such as benzidine and 4-aminobiphenyl, has also been associated with an increased risk to bladder cancer (2-4). Man is not the only species susceptible to bladder tumorigenesis following exposure to aromatic amines. Mice, rats, guinea pigs, hamsters, and rabbits are also sensitive; however, it is the dog that most closely parallels man's response to these chemicals (5, 6). From the studies of McDonald and Lund (6) and Scott and Boyd (7), it became apparent that an agent contained in the urine of dogs exposed to arylamines was responsible for tumor

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induction. Based on these data and others, various models for bladder tumor formation have been developed. One model suggests that in the liver there is a critical balance between N-acetylation followed by N-oxidation versus initial N-oxidation and subsequent N-glucuronidation (8-10). Species, such as the rat, which favor the former pathway, appear to preferentially develop liver tumors, while the bladder is the primary target site in animals for which the latter pathway predominates. As noted by Poirier et al. (11) and Lower and Bryan (12), dogs do not N-acetylate aromatic amines very well and, according to this model, should be quite susceptible to induction of bladder tumors. Humans, in contrast, show considerable genetic variability in their ability to N-acetylate amines (13), and this variation has been implicated in the etiology of aromatic amine-induced bladder tumors (9, 14).

According to the model, the N-glucuronidation of N-hydroxyarylamines serve to transport the carcinogen to the bladder (8, 10). These polar metabolites are quite stable at neutral pH; however, under slightly acidic conditions, they rapidly hydrolyze to the unconjugated N-hydroxyarylamines (8). The importance of urinary pH in the formation of these N-hydroxy metabolites has recently been demonstrated in rats treated with 2-naphthylamine (15). Rats normally have a urinary pH of 6.7 but by supplementation of their drinking water with either ammonium chloride or sodium bicarbonate, the pH of the urine was altered to 5.7 and 7.7, respectively. Although the total amount of urinary N-hydroxylated metabolites (free and N-glucuronidated) remained constant, as the pH became more acidic, the amount of free N-hydroxy-2-naphthylamine increased transformation frequency resulted when have acidic urine (8, 16), this acidity could facilitate the hydrolysis of N-hydroxy-N-glucuronides to N-hydroxyarylamines thus increasing their susceptibility to arylamine-induced bladder tumors.

The covalent interaction of carcinogens with DNA is considered to be an essential step in the initiation of tumors. N-Hydroxyarylamines readily react with DNA at slightly acidic pH (8, 17) and are directly mutagenic (18, 19) and carcinogenic (8). Furthermore, a recent study (20) demonstrated that an increased transformation frequency resulted when diploid human fibroblasts were incubated with either N-hydroxy-1-naphthylamine or N-hydroxy-2-naphthylamine at pH 5 compared to pH 7. Since the pH-dependent model predicts that N-hydroxyarylamines are the ultimate carcinogenic species in bladder tumor induction, the products formed in vitro from reacting N-hydroxyarylamines with DNA should correspond directly with the adducts formed in vivo in dogs administered the parent arylamines.

To investigate this concept, we have embarked upon studies to compare the adducts in both of these systems. In addition, we have attempted to assess the biological potential of individual DNA adducts by using a Salmonella typhimurium mutational assay.

Materials and Methods

The 4-aminobiphenyl-DNA adducts were prepared as previously described (21). The adducts were isolated by high pressure liquid chromatography and characterized by ¹H nuclear magnetic resonance and electron impact in-beam desorption mass spectroscopy (22) with Bruker WM 500 and Finnigan 4023 instruments, respectively. Before mass spectral analysis, the samples were treated with N-methyl-N-trimethylsilyl trifluoroacetamide:dimethylformamide (1:1) at 37°C for 30 min. Additional confirmation of adduct structure was provided from pH-dependent partitioning experiments (23) with 5% n-butanol in diethyl ether as the organic phase and 50 mM buffers of the appropriate pH.

Male beagle dogs (8-10 kg; Marshall Lab Animals, North Rose, NY) were treated PO with ¹H-aromatics in gelatin capsules (60 μ mole/kg, 170-1220 mCi/m mole; obtained from Robert Roth, Midwest Research Institute, Kansas City, MO) and were killed at selected intervals by Nembutal injection. Hepatic and bladder epithelium arylamine-modified DNA were isolated by solvent extraction and hydroxylapatite chromatography (24). In some instances, the DNA was further purified by cesium chloride centrifugation (25). Acetylaminofluorene-modified DNA was enzymatically digested with DNase I (Sigma), phosphodiesterase (Sigma) and alkaline phosphatase (Sigma; Ref. 26). Benzidine- and N-acetylbenzidine-modified DNA were hydrolyzed with DNase I, nuclease P1, (Calbiochem-Behring), and acid and alkaline phosphatase (Sigma) (27).

Mutagenicity experiments were conducted by use of Salmonella typhimurium strains TA 1535 and TA 1588 (obtained from B.N. Ames, University of California, Berkeley, CA) as previously described (21, 28).

Results and Discussion

In Vitro Reaction of N-Hydroxyarylamines with DNA

N-Hydroxyarylamines readily react with biological macromolecules. With nucleic acids, this process is acid-catalyzed with binding increasing approximately 5-fold for each unit decrease between pH 7 and 5 (8, 27, 29, 30). In contrast, protein binding does not appear to be pH-dependent, because incubations conducted between pH 5 and 7 show equivalent re-
Kriek and colleagues (29,30) have proposed that covalent linkage should occur between C8 of deoxyguanosine and the arylamine nitrogen of N-hydroxyaminofluorene. This assignment has recently been confirmed through the use of nuclear magnetic resonance spectroscopic techniques (26). In addition, Kriek and Westra have demonstrated that this C8-deoxyguanosine adduct is labile and will undergo hydrolysis at slightly alkaline pH to yield an imidazole ring-opened adduct (32). To date, the only other N-hydroxyarylamine that appears to give exclusively C8-deoxyguanosine substitution is N-hydroxy-N'-acetylbenzidine (27).

The adduct profiles obtained from reacting N-hydroxyarylamines with DNA in vitro at acidic pH are summarized in Figure 1. While N-hydroxy-2-aminofluorene and N-hydroxy-N'-acetylbenzidine gave exclusively C8-deoxyguanosine adducts, the other N-hydroxyarylamines yielded a mixture of products. With N-hydroxy-2-naphthylamine (30), C8-deoxyguanosine substitution also predominated; however, in this case it appeared to exist primarily in an imidazole ring-opened configuration. Two other minor N-hydroxy-2-naphthylamine adducts have been characterized as 1-deoxyguanosin-N²-yl)2-naphthylamine and 1-deoxyadenosin-N²-yl)2-naphthylamine which represented 30% and 15% of the total binding, respectively. The isomeric N-hydroxy derivative, N-hydroxy-1-naphthylamine, is unique among arylamines because two of the adducts that have been characterized are bound through O₈ of deoxyguanosine, with the major substitution (60%) occurring through the arylamine nitrogen and 30% of the binding through the ortho carbon (29). In a recent report (33), evidence for C8-deoxyguanosine substitution was presented, but we have been unable to confirm this observation.

When N-hydroxy-4-aminobiphenyl was reacted with DNA at pH 5, three DNA adducts resulted. We have previously identified the major product, which accounted for 80% of the total binding, as N-(deoxyguanosin-8-yl)4-aminobiphenyl (27). A second adduct, which represented 15% of the binding, was unambiguously characterized as a novel C8-deoxyadenosine product and a third adduct (5%) was tentatively assigned as an additional deoxyadenosine product. Additional studies have failed to confirm this latter structure; instead, the data strongly indicated that the minor adduct was N-(deoxyguanosin-N²-yl)4-aminobiphenyl. Following silylation, ion beam electron impact mass spectral analysis (22) gave a molecular ion at m/z 795 which was consistent with a pentamethylsilyldeoxyguanosine adduct. ¹H nuclear magnetic resonance spectra of this minor adduct indicated the presence of all nine aromatic protons associated with the biphenyl rings. Resonances were also detected that could be assigned to H₈ and H₁ of deoxyguanosine, plus two additional signals that were ascribed to a novel hydrazo linkage. Finally, the adduct had both acidic and basic pKₐ's, as determined by pH-dependent
partitioning experiments (29), which was consistent with the proposed deoxyguanosine substitution.

**In Vivo Arylamine–DNA Adducts**

Male beagle dogs were treated with [3H] carcinogens at a dose of 60 μmole/kg body weight and then were killed after 1, 2, or 7 days. Liver and bladder epithelium DNAs were isolated and enzymatically hydrolyzed to mononucleosides. The adducts were then identified through high pressure liquid chromatographic comparison to standards synthesized by reacting the respective N-hydroxyarylamine with DNA at acidic pH. In instances where there was sufficient radioactivity, the identity of the individual adducts was confirmed through application of the pH-dependent partitioning technique of Moore and Koreeda (23).

The results from the bladder epithelium DNA binding studies are shown in Figure 2a. The most extensive binding was detected with 4-aminobiphenyl, which correlated with it being the most potent bladder carcinogen investigated (34). The concentration of adducts was essentially constant at each time point with N-(deoxyguanosin-8-yl)-4-aminobiphenyl accounting for 76% of the total binding. In contrast to what was found when N-hydroxy-4-aminobiphenyl was reacted with DNA in vitro (Fig. 1), the next most abundant in vivo product was the N²-deoxyguanosine substituted adduct (15%). The remainder of the DNA binding (9%) was identified as being N-(deoxyadenosin-8-yl)-4-aminobiphenyl.

The nitrated analog, 4-nitrobiphenyl, gave a similar profile of adducts, although the extent of binding was only 5% of that observed with 4-aminobiphenyl. These data suggest that 4-nitrobiphenyl is, to a limited extent, metabolically activated through N-hydroxyarylamine formation. This reduction could occur in the liver, or alternatively, intestinal microbiota may reduce the nitro moiety to an amine and subsequent hepatic oxidation could then result in N-hydroxyarylamine formation. In support of this latter pathway, anaerobic intestinal bacteria have recently been implicated in the metabolic activation of 2,4-dinitrotoluene (35) and an in vitro culture of rat intestinal bacteria has been shown to reduce 1-nitropyrene to its corresponding amine (36). The much lower binding of 4-nitrobiphenyl compared to 4-aminobiphenyl correlated with its weak carcinogenicity in the dog bladder (34).

The carcinogenic amide, 2-acetylaminofluorene, also gave a much lower binding to bladder epithelium DNA than was observed with 4-aminobiphenyl. Only one adduct was detected and this was identical to the product obtained when N-hydroxy-2-aminofluorene was incubated with DNA. The formation of this nonacetylatted adduct is consistent with the ability of dog liver to deacetylate 2-acetylaminofluorene (9). As opposed to what was observed with the aminobiphenyl adducts, the concentration of N-(deoxyguanosin-8-yl)-2-aminofluorene decreased by 80% from 2 through 7 days. Whether this decrease is due to cell turnover as a result of cellular toxicity or DNA repair remains to be determined.

As previously reported, 2-naphthylamine caused the formation of three adducts at a total binding similar to that of 4-nitrobiphenyl (21, 37). The major DNA product was, again, a C8-substituted deoxyguano-
nosine which accounted for 48% of the total DNA adducts, but there were also substantial quantities (36%) of N-(deoxyadenosin-N'-yl)-2-naphthylamine. The remaining adduct detected was identical to the N²-deoxyguanosine product formed in vitro from reacting N-hydroxy-2-naphthylamine with DNA. Examination of the bladder epithelium DNA 7 days following treatment revealed a 33% decrease in activity. This reduction was due primarily to a loss of N-(deoxyadenosin-N'-yl)-2-naphthylamine. This suggests that bladder epithelial cells may be able to excise preferentially specific DNA adducts.

When bladder epithelium DNA from a dog treated with 1-naphthylamine was examined, binding could not be detected (37). The limit of sensitivity was approximately 1 adduct/10⁶ nucleotides. The failure to find binding corresponds to the lack of carcinogenicity for this compound (38). Radioactivity could be detected in bladder epithelium DNA of a dog treated with [³H]-benzidine; however, due to its low specific activity, benzidine binding was of an insufficient magnitude to perform adduct analysis. If it is assumed there is only one product formed (cf., liver and N-acetylbenzidine-treated dog), then based upon typical recoveries during enzymatic hydrolysis, the binding would not exceed 30 residues/10⁶ nucleotides, a level similar to 2-naphthylamine and 4-nitroso-biphenyl. Another dog was treated with N-acetylbenzidine, and in contrast to benzidine, a high level of radioactivity was associated with the bladder epithelial DNA. However, upon enzymatic hydrolysis, only 10% of the radioactivity partitioned into n-butanol, as compared to >70% for the other carcinogens studied. Examination of the n-butanol-soluble fraction revealed primarily one product which coeluted with N-(deoxyguanosin-8-yl)-N'-acetylbenzidine at a binding level that approximated that observed with the benzidine-treated dog. The radioactivity that did not partition into the n-butanol may represent protein-benzidine-DNA crosslinking as has been reported for N-acetoxy-2-acetylaminofluorene by Metzger and Werbin (39).

In addition to examining the bladder epithelium DNA, liver DNA from each dog was analyzed (Fig. 2b). Of the carcinogens administered, only 2-acetylaminofluorene has been reported to be hepatocarcinogenic in this species (40). The only hepatic DNA adduct identified following 2-acetylaminofluorene treatment was N-(deoxyguanosin-8-yl)-2-aminofluorene. Since N,O-acyltransferase has not been detected in dog liver (41), this suggests that the electrophilic species is N-hydroxy-2-aminofluorene. [We previously reported the presence of low levels of the acetylated adducts 3-(deoxyguanosin-N'-yl)-2-acetylaminofluorene and N-(deoxyguanosin-8-yl)-2-acetylsaminofluorene (21). However, pH-partitioning experiments indicate that the small quantities of radioactivity that coelute with these markers are not due to these adducts.] Qualitatively, the spectrum of adducts observed in hepatic DNA from each carcinogen was similar to that detected in the bladder epithelium. There were, however, quantitative differences. The binding of 2-acetylaminofluorene was seven times as high in the liver as in the bladder. 4-Aminobiphenyl gave similar adduct concentrations in both tissues, and 2-naphthylamine and 4-nitroso-biphenyl had more extensive adduct formation in the bladder. The benzidine- and N-acetylbenzidine-treated dogs had very high levels of radioactivity associated with hepatic DNA. However, after enzymatic hydrolysis, <1% of the radioactivity partitioned into n-butanol and the only adduct detected was N-deoxyguanosin-8-yl-N'-acetylbenzidine. Since both benzidine and N-acetylbenzidine give the same adduct and the same level of binding, this indicates that there is an equilibrium between acetylation and deacetylation with deacetylation greatly favored. Since individuals who have been exposed to benzidine-based azo dyes have been reported to excrete substantial quantities of N-acetylbenzidine (42), these data suggest either that the dog is a poor model or that it seriously underestimates the potential risk of benzidine exposure in man.

As was observed in the bladder, the DNA adducts obtained from 2-naphthylamine- and 2-acetylaminofluorene-treated dogs decreased in concentration from 2 through 7 days. With 2-acetylaminofluorene, there was an 80% loss of N-(deoxyguanosin-8-yl)-2-aminofluorene, while with 2-naphthylamine, both the C8-substituted deoxyguanosine and N²-substituted deoxyadenosine adducts decreased in concentration. Interestingly, the concentration of the 4-aminobiphenyl adducts remained constant with time and, although the binding of this arylamine to hepatic DNA was similar to the level observed with 2-acetylaminofluorene, 4-aminobiphenyl is not a hepatocarcinogen for the dog. This observation suggests that there may be fundamental differences in the biological activity of various arylamine DNA adducts.

Reversions and DNA Adducts Induced by N-Hydroxyarylamines in Salmonella typhimurium

To assess the relationship between arylamine-DNA adducts and mutation induction, the N-hydroxyarylamines were incubated with Salmonella typhimurium strains TA 1538 and TA 1535. After a 30-min exposure, aliquots were analyzed for both induced reversions and bacterial survival, while the
remainder of the bacteria were used for DNA-adduct determination.

Figure 3 illustrates mutation frequency and bacterial survival as a function of dose in strain TA

**FIGURE 3.** Revertants/10^5 surviving bacteria and percent survival of *Salmonella typhimurium* TA 1538 exposed to different concentrations of the N-hydroxyarylamines of 2-aminofluorene (2-AF), 4-aminobiphenyl (4-ABP), 2-naphthylamine (2-NA) and N-acetylbenzidine (ABZ).

1538. N-Hydroxy-2-aminofluorene was the most mutagenic and the second most cytotoxic (as indicated by percent survival) compound studied. N-Hydroxy-2-naphthylamine was the most toxic of these agents but was only weakly mutagenic, while N-hydroxy-N'-acetylbenzidine was quite mutagenic and only slightly cytotoxic. Thus, there does not appear to be an obvious relationship between cytotoxicity and reversions induced by these compounds in strain TA 1538.

The variation in mutagenicity between the N-hydroxyarylamines could reflect differences in the extent of reaction of the compounds with bacterial DNA, or, alternatively, it may indicate that some lesions were more efficient in inducing frameshift reversions than others. To differentiate between these possibilities, the bacterial DNA was isolated and the extent of binding and the adduct profile were established. Figure 4 illustrates revertants as a function of total binding. While N-hydroxy-N'-acetylbenzidine demonstrated intermediate mutage-

**FIGURE 4.** Comparison of the revertants/10^5 survivors versus arylamine residues/10^5 nucleotides for *Salmonella typhimurium* TA 1538 incubated with N-hydroxyarylamines. See Figure 3 for abbreviations used.
nicity when plotted as a function of dose, it was clearly the most mutagenic N-hydroxyarylamine in strain TA 1538 when expressed as a function of total binding. This was followed by N-hydroxy-2-aminofluorene and then N-hydroxy-2-naphthylamine and N-hydroxy-4-aminobiphenyl which showed approximately the same number of revertants per bound residue.

High pressure liquid chromatographic analysis of the enzymatic hydrolyzate of the bacterial DNA indicated that only C8-deoxyguanosine adducts were formed from N-hydroxy-N'-acetylbenzidine and N-hydroxy-2-aminofluorene (Fig. 5). This suggests that the frameshift reversions detected by strain TA 1538 are due to substitution at this position. The other two compounds gave the same mixture of adducts that was observed in vitro; however, N-hydroxy-2-naphthylamine gave substantially less C8-deoxyguanosine substitution and correspondingly more of the N7-deoxyguanosine adduct. When frameshift revertants are expressed as a function of C8-deoxyguanosine substitution, the following trends become evident (Fig. 6). The adduct resulting from N-hydroxy-N'-acetylbenzidine is clearly the most mutagenic lesion and causes approximately five times as many revertants per bound residue as N-deoxyguanosin-8-yl)-2-aminofluorene. Similarly, the C8 adduct formed from N-hydroxy-2-aminofluorene is much more efficient at inducing mutations than the analogous lesion from N-hydroxy-4-aminobiphenyl. The analysis of N-hydroxy-2-naphthylamine was complicated by its high toxicity and low mutagenicity in strain TA 1538. However, when revertants are expressed as a function of C8-deoxyguanosine substitution, it has a mutagenic efficiency similar to N-hydroxy-2-aminofluorene. It should be noted that if other adducts formed from N-hydroxy-2-naphthylamine or N-hydroxy-4-aminobiphenyl are also involved in frameshift reversion induction, then the mutagenic efficiency of the C8-deoxyguanosine adducts produced by these agents would be lower. The reason for the variations in mutation induction is not known but may be due to differences in the ability of the adducts to adopt a syn conformation about the glycosyl bond (43).

Similar incubations were conducted with strain TA 1535 which detects base-substitution reversions. In this strain, N-hydroxy-2-aminofluorene was the most toxic compound, but did not induce any reversions (Fig. 7). This observation reinforces the concept that, at least in Salmonella typhimurium, there is not a direct relationship between toxicity and mutagenicity. The only compound that induced reversions in strain TA 1535 was N-hydroxy-2-naphthylamine. The fact that N-hydroxy-2-aminofluorene and N-hydroxy-N'-acetylbenzidine were not muta-

**Figure 5.** Percent distribution of arylamine adducts in *Salmonella typhimurium* DNA from bacteria incubated with the N-hydroxyarylamine of N-acetylbenzidine, 2-aminofluorene, 4-aminobiphenyl and 2-naphthylamine. See Figure 3 for abbreviations used.

genic, suggests that C8-deoxyguanosine arylamine adducts do not result in base-substitution reversions. Comparison of the adducts formed from N-hydroxy-2-naphthylamine with the other N-hydroxyarylamines revealed two fundamental differences:
N-hydroxy-2-naphthylamine formed a N⁶-deoxyadenosine adduct and a ring-opened C8-deoxyguanosine DNA adduct. Further experimentation will be necessary to determine whether one or both of these particular lesions may be responsible for the base-substitution reversions.

Conclusions

A model has been proposed for arylamine-induced bladder carcinogenesis in which initial N-oxidation and N-glucuronidation occur in the liver. The conjugate is then transferred to the bladder where under slightly acid conditions it is hydrolyzed to yield an electrophilic N-hydroxyarylamine. To investigate this hypothesis, N-hydroxyarylamines were incubated with DNA at slightly acidic pH and the DNA adducts were identified. With the exception of N-hydroxy-1-naphthylamine, the predominant adduct from each N-hydroxyarylamine was substituted through C8 of deoxyguanosine. The same pattern of DNA adducts was observed in bladder epithelium DNA of dogs administered the parent arylamines and in Salmonella typhimurium TA 1538 incubated with N-hydroxyarylamines. Although a great difference in mutagenic efficiency was detected between the various C8-deoxyguanosine adducts, substitution at this position was associated with frameshift mutations in Salmonella typhimurium TA 1538. These data suggest that N-hydroxyarylamines are ultimate bladder carcinogens and that C8-deoxyguanosine substitution may represent an initiating lesion in bladder tumor formation.

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