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Monocyclic aromatic amines as potential human carcinogens: old is new again

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Alkylanilines are a group of chemicals whose ubiquitous presence in the environment is a result of the multitude of sources from which they originate. Exposure assessments indicate that most individuals experience lifelong exposure to these compounds. Many alkylanilines have biological activity similar to that of the carcinogenic multi-ring aromatic amines. This review provides an overview of human exposure and biological effects. It also describes recent investigations into the biochemical mechanisms of action that lead to the assessment that they are most probably more complex than those of the more extensively investigated multi-ring aromatic amines. Not only is nitrenium ion chemistry implicated in DNA damage by alkylanilines but also reactions involving quinone imines and perhaps reactive oxygen species. Recent results described here indicate that alkylanilines can be potent genotoxins for cultured mammalian cells when activated by exogenous or endogenous phase I and phase II xenobiotic-metabolizing enzymes. The nature of specific DNA damage products responsible for mutagenicity remains to be identified but evidence to date supports mechanisms of activation through obligatory *N*-hydroxylation as well as subsequent conjugation by sulfation and/or acetylation. A fuller understanding of the mechanisms of alkylaniline genotoxicity is expected to provide important insights into the environmental and genetic origins of one or more human cancers and may reveal a substantial role for this group of compounds as potential human chemical carcinogens.

Introduction

Aromatic amines are a class of organic compounds that include many members that are carcinogenic, both experimentally in the research setting and experientially in human life. The carcinogenesis literature documents innumerable studies demonstrating that administration of a considerable variety of aromatic amines to experimental animals of different species induces cancers in those animals (1). The epidemiological literature leaves little doubt that a specific few aromatic amines are the cause of bladder cancer in occupationally exposed persons and there is a convincing argument to be made that exposure to aromatic amines via tobacco smoke is a major, if not predominant, factor in causing bladder cancer in smokers (2).

The biochemical mechanisms by which aromatic amines might induce cancers have been investigated extensively and are now thought to be reasonably well understood (3–5). Human population studies that have incorporated measures of metabolic genotype and phenotype tend to support the biochemical mechanisms inferred from experimental studies (6). It appears that, for this class of chemical carcinogens, the linkage between the experimental setting and the human condition is as strong as any. Yet, there remains a conundrum: outside the occupational setting there appears to be far too little exposure to any of the known human bladder carcinogens to account for the observed incidence rates.

Various possibilities may be considered for resolving this conundrum. The models used for extrapolating from high to low dose may underestimate potency at human exposure levels. There may be environmental exposures to known compounds that are largely cryptic or there may be amines in the environment that have not yet been identified. Unanticipated synergies among multiple exposures may occur. Other possibilities no doubts exist. Because of their environmental prevalence, one relatively unexplored group of aromatic amines, the alkylanilines, may be significantly involved in one or more of these explanations. In this paper, we will review much of the research on alkylanilines that addresses the question of why they could be playing an important role in human cancer.

Abbreviations: 4-ABP, 4-aminobiphenyl; CHO, Chinese hamster ovary; 2,6-DMA, 2,6-dimethylaniline; 3,5-DMA, 3,5-dimethylaniline; 3-EA, 3-ethylaniline; NAT, *N*-acetyltransferase; NER, nucleotide excision repair; ROS, reactive oxygen species; TGHQ, 2,3,5-*tris*-(glutathion-*S*-yl)hydroquinone.

Environmental prevalence

In considering the importance of any specific chemical carcinogen or class of carcinogens to human disease incidence, weight must be given to the extent to which the compounds are distributed in the environment. Alkylanilines, as a class, appear to be distributed widely, making exposure to them nearly ubiquitous. Early efforts at exposure assessment were predicated on the fact that alkylanilines are present in tobacco smoke (7–9). Accordingly, indoor spaces contaminated with tobacco smoke exhibited higher levels of alkylanilines than uncontaminated spaces (8,10). Outdoor air was generally lower with exceptions that may be attributable to industrial sources (10). Exposure assessment based on hemoglobin adduct levels also indicates that tobacco smoke is a significant, if not predominant, source for many alkylanilines (11,12). In both studies, 2,6-dimethylaniline (2,6-DMA) was an exception, being actually higher in non-smokers than smokers in a study conducted in Italy (11). Much higher levels (7- to 8-fold) of 3,5-dimethylaniline (3,5-DMA) were also observed in non-smokers in the Italian population. This finding attains greater significance in light of a recent investigation in Canada (13) that found levels of 3,5-DMA in indoor and outdoor air that are two to three orders of magnitude higher than those reported elsewhere.

Exposure assessment through hemoglobin adduct analysis has now been extended to include non-tobacco sources in the USA (12), Italy (11), Germany (14) and most recently by us in China (unpublished results). In all of these studies, there have been virtually no subjects that do not have hemoglobin adducts of all of the compounds investigated. Results tend to be comparable: while there may be a considerable range of values among individual subjects, mean values tend to fall within a range of $<$10-fold. The inescapable conclusion from these results is that environmental prevalence of many alkylanilines is extensive and that exposure to them is not confined to limited population subgroups such as the occupationally exposed.

Figure 1 illustrates most of the important routes by which alkylanilines come to be present in the environment. It is based on the comprehensive information about production and possible releases into the environment of specific alkylanilines that can be found in the

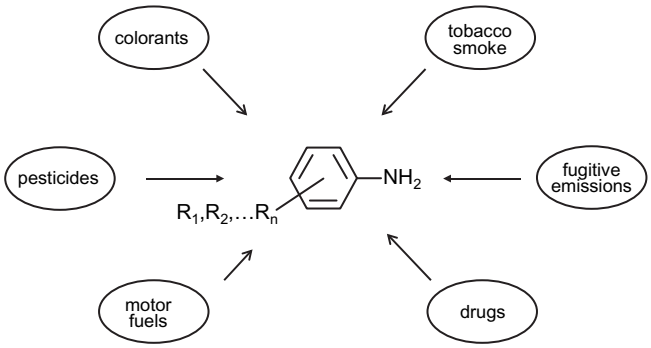

Fig. 1. Environmental sources of alkylanilines.

Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB) as well as the references cited above.

Evidence for carcinogenicity in a population-based study

In 2004, we published results from a population-based study of bladder cancer conducted in Los Angeles county in which chemical markers of exposure to a specific set of alkylanilines were included (12). This was the first, and is still the only, population-based bladder cancer study of any aromatic amines having the basic aniline ring structure. In the absence of additional studies with similar findings, the results are not definitive, but considering the power of the study, we were strongly motivated to pursue additional mechanistic and *in vitro* toxicology studies in an effort to provide biological plausibility for the epidemiological findings.

The Los Angeles study examined exposure to nine different alkylanilines, including all three ethyl and all six dimethyl isomers. Statistical analysis found that three of the nine—2,6-DMA, 3,5-DMA and 3-ethylaniline (3-EA)—were independently associated with risk for development of bladder cancer. Others were also associated with risk but not independently as there were strong correlations among the various anilines as well as with 4-aminobiphenyl exposure. Importantly, no meaningful differences were found when analysis was restricted to lifelong non-smokers, implying that smoking was not a confounder in this study.

Evidence for carcinogenicity from occupational exposure

There exist only very limited data from occupational studies that bear directly on the human carcinogenicity of monocyclic amines and none specifically related to the alkylanilines discussed here. The closest analog that has been evaluated comprehensively is *o*-toluidine. In a study of workers exposed to aniline and *o*-toluidine, it was found that levels of exposure to these chemicals were associated with a statistically significant increase in incidence of bladder cancer (15). The initial report did not distinguish between aniline and *o*-toluidine exposure but argued that the latter was more important. The study was challenged (16,17) on the grounds that prior exposure of the same workers to more potent chemicals could not be ruled out or could ongoing exposure to 4-aminobiphenyl (4-ABP). The issue of prior exposure was rebutted (18,19) and the issue of ongoing 4-ABP exposure was addressed through a follow-up study in which current exposure to aniline, *o*-toluidine and 4-ABP was assessed through biomonitoring of hemoglobin adducts (20). Biomonitoring clearly revealed that 4-ABP exposure was independent of workplace exposure status and, as observed in numerous other studies, was mostly associated with cigarette consumption. Thus, the most probable finding of this retrospective cohort study is that *o*-toluidine is indeed a human bladder carcinogen. A follow-up study at the same chemical plant reported in 2004 (21), long after potential co-exposures to other chemicals is thought to have ceased, revealed a continuing excess of bladder cancer among exposed workers.

Experimental carcinogenicity

A comprehensive review of the carcinogenicity of alkylanilines is outside the scope of this paper mainly because so many of the results, while suggestive of carcinogenic potential, are largely inconclusive. Furthermore, the National Toxicology Program has recently conducted a review of the literature as part of its process for nominating several alkylanilines for future study. The review can be found in the nominating document at http://ntp.niehs.nih.gov/ntp/noms/Support_Docs/Alkylanilines060809.pdf. The document includes genotoxicity data as well and therefore covers an expanded set of 14 compounds. Here, we will focus on the two compounds for which the International Agency for Cancer has found sufficient evidence to categorize them as either carcinogenic to humans (*o*-toluidine; Group 1) or possibly carcinogenic to humans (2,6-DMA; Group 2B).

As described by the International Agency for Cancer, '*ortho*-toluidine was tested for carcinogenicity as its hydrochloride salt in two experiments in mice and in three experiments in rats and as the free base in one limited experiment in hamsters. After oral administration to mice, it induced an increased incidence of haemangiomas and haemangiosarcomas and hepatocellular carcinomas or adenomas. In rats, oral administration of *ortho*-toluidine increased the incidence of tumours in multiple organs, including fibromas, sarcomas, mesotheliomas, mammary fibroadenomas and transitional cell carcinomas of the urinary bladder'. Based on these results, the International Agency for Cancer has concluded that there is sufficient evidence for the carcinogenicity of *o*-toluidine in experimental animals. The lack of organ specificity casts some doubt on the significance of this conclusion but the occurrence of transitional cell carcinomas of the urinary bladder is notable, as this is typical of many aromatic amine carcinogens.

In contrast to *o*-toluidine, 2,6-DMA is an organ-specific carcinogen, inducing carcinomas and papillary adenomas of the nasal cavity in male and female rats in a dose-dependent manner (22). Subsequent studies strengthen the bioassay findings. When [^{14}C]-2,6-DMA was given to rats, DNA in the ethmoid turbinate as well as in liver became radiolabeled (23). In contrast to hepatic DNA, labeling of the ethmoid turbinate DNA was only observable after pretreatment with unlabeled 2,6-DMA, indicating that the amine induced an enzyme present in the nasal cavity that is responsible for its activation and that the same enzyme is a minor constituent in liver. Recent studies in our laboratories support this inference: when various sources of P450s were screened for their ability to convert 2,6-DMA to the corresponding hydroxylamine, rat liver microsomes exhibited undetectable activity, whereas human liver microsomal activity was readily apparent (24; unpublished results). Of five recombinant human P450s tested (1A1, 1A2, 1B1, 2A6 and 2E1), only 2A6 exhibited *N*-hydroxylation activity. Notably, one member of the 2A family, 2A3, appears to be expressed preferentially in the rat nasal cavity (25).

Chemical reactivity: comparison of alkylanilines with multi-ring carcinogenic amines

A paradigmatic model for aromatic amine genotoxicity has evolved over the last forty years from the seminal work of James and Elizabeth Miller and their coworkers that is described in detail in several later publications (5,26,27). In this model, aromatic amines (and amides) first undergo P450-catalyzed oxidation at the nitrogen atom. The resulting *N*-hydroxylamine has the potential to undergo *N–O* bond heterolysis to produce a reactive nitrenium ion that reacts with nucleobases in DNA to induce genotoxicity. Heterolysis is promoted in most cases by the intermediate step of phase II conjugation of the hydroxyl group but may also be catalyzed by protonation.

In view of the apparent genotoxicity or carcinogenicity of at least some aniline derivatives, several groups have undertaken investigations to determine whether the nitrenium ion mechanism of DNA damage

is operative for single-ring aromatic amines. Certainly, DNA adducts of the same basic structure as formed by multi-ring amines can be synthesized via nitrenium ions. Aniline, the simplest aromatic amine, forms the dG–C8 adduct when it is present in the reaction in the activated form, *N*-acetoxyaniline (28). Marques *et al.* first reported the synthesis of dG–C8 adducts of *o*-, *m*- and *p*-toluidine, 2,3-dimethylaniline and 2,4-dimethylaniline by reaction of the corresponding *N*-(acyloxy)arylamines with dG, dG nucleotides and DNA, where the acyl group was either acetyl or pivaloyl (29). They later reported dG adducts of all the isomeric methyl, ethyl and dimethylanilines using the same synthetic methods (30). dG adducts of *p*-chloroaniline and *p*-anisidine have also been described as products of synthesis via nitrenium ion chemistry (31). Jones *et al.* (32) later expanded the range of substituted anilines that form dG adducts through their acyloxy derivatives to include 2-chloroaniline. The structures of these and the other adducts described below are shown in Figure 2.

A more detailed study of 2,6-DMA revealed that not only does this aniline form C8 adducts with dG but also its acyloxy derivative reacts with DNA to form two other dG adducts (33). One occurs by forming a bond between the *para*-carbon and O^6 of the nucleobase, whereas the other occurs by attack at N^2. One dA adduct is also formed, again by attack of the *para*-carbon, in this case at N^6 of the nucleobase. In our own work, we found that 3,5-DMA, when converted to the acetoxy derivative, produces four distinct adducts in DNA (34). One was the expected dG adduct. The analagous dA–C8 adduct also occurred. In addition, there was the adduct formed by attack of the *para*-carbon at N^6 of adenine, as was reported for 2,6-DMA. Lastly, we found an adduct formed with dC through attack of the nitrogen atom at C5 of the nucleobase. Despite the novelty of two of the adducts, all were consistent with the intermediacy of a nitrenium ion.

Demonstration that the DNA adducts expected from nitrenium ion chemistry do occur when DNA is treated with appropriately activated alkylanilines may be considered a necessary condition to expect their occurrence *in vivo* following exposure to the parent amines. Following up on these demonstrations, several attempts have been made to detect the chemically synthesized adducts in treated animals. Jones *et al.* (32) developed a method for quantitative analysis of dG and dA adducts of a set of aromatic amines that included the three toluidines, 2,4-dimethylaniline and 2,6-DMA. The method was based on HPLC/MS/MS and could distinguish among different adduct structures. When hepatic DNA from rats given 0.5 mmol/kg of the compounds was analyzed, no adducts of any of the monocyclic amines were detected above the level of 3.2 adducts per 10^8 normal bases. A polycyclic aromatic amine, 4-aminobiphenyl, was included for comparison and it produced adducts at ~500 per 10^8 bases.

Adopting a different strategy (35), we treated wild-type C57/BL6 mice with [^{14}C]-labeled 2,6-DMA, 3,5-DMA and 3-EA and analyzed tissue DNA for isotopic labeling by accelerator mass spectrometry. All the compounds produced detectable labeling of DNA at levels that ranged up to more than one per 10^7 bases for 3,5-DMA in liver. In this experiment, the dose of alkylaniline was ~1 μmol/kg, which is 1/500th of the dose given in the experiment described above where no adducts were detectable. Only one alkylaniline, 2,6-DMA, is common to both studies. In our study, it also produced adducts at the level of one per 10^7 bases but only at 4 h post-dosing; at 24 h—the time point used in the other study—the level had declined to about one per 10^8 bases. While this level is below the limit of detection by the HPLC/MS/MS method, the difference in dose should be kept in mind when comparing the two studies.

Since the analytical method we used, accelerator mass spectrometry, was capable only of detecting isotope, it cannot be inferred that any of the adducts detected were structurally identical with any of the adducts produced by nitrenium ion reactions. To gain further insight into the nature of the bound isotope, we have been performing high-performance liquid chromatography analyses of enzymatic digests of the DNA isolated from tissues harvested from animals given [^{14}C]-labeled 3,5-DMA. The choice of 3,5-DMA-treated animals was governed by the availability of nitrenium adducts of

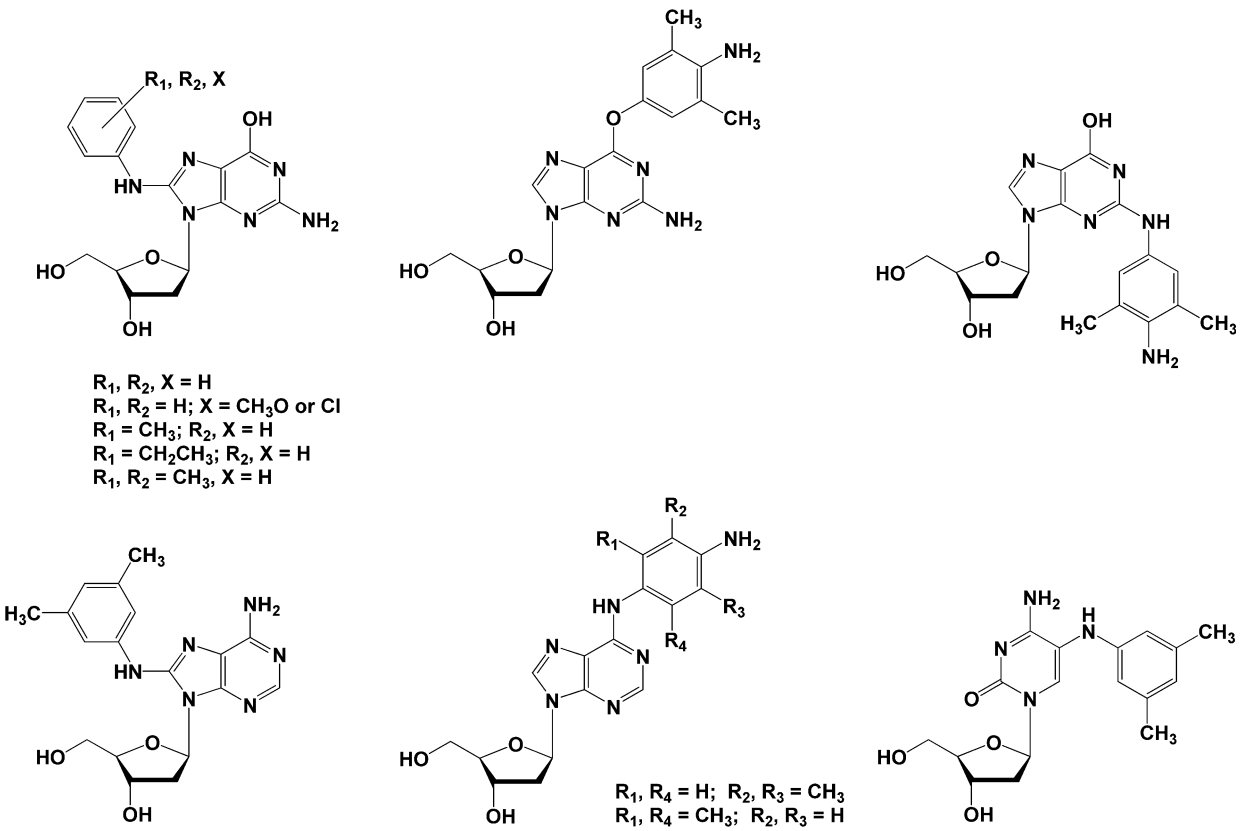

Fig. 2. Structures of the various alkylaniline DNA adducts that have been elucidated.

defined structure as described above (34). Digests of hepatic DNA were combined with a mixture of the four adduct standards at a level that produced well-defined UV-detectable peaks in the chromatogram and subjected to high-performance liquid chromatography analysis with fraction collection. Accelerator mass spectrometry analysis of the collected fractions was performed to generate a radiochromatogram that could be compared with the UV trace. Several such analyses have been conducted with inconsistent results in the radiochromatograms. In none of the analyses, though, have we observed co-chromatography of radiocarbon with UV peaks produced by the standards, suggesting that if any of the DNA adducts formed *in vivo* are identical to the standards they are present only as minor constituents.

Thus, far, little evidence has been developed to support a model for genotoxicity of alkylanilines that is comparable with the very successful model that characterizes multicyclic aromatic amine carcinogenicity. However, the available information is far from sufficient to rule the model out and the ability of nitrenium ions derived from monocyclic amines to form DNA adducts is reason to remain open minded. In the next section, then, we will show that *N*-hydroxylation, a critical metabolic step for activating many aromatic amines, is important also in monocyclic amine genotoxicity.

N-hydroxylation as a critical activation step

There is ample evidence that alkylanilines and other monocyclic amines are readily *N*-hydroxylated by mammalian metabolism. In perhaps, the most extensive investigation of the phenomenon to date, Sabbioni (36) tested 32 compounds in rats for their ability to bind to hemoglobin in a manner that is demonstrative of the generation of *N*-hydroxylamines *in vivo*. Only one of the 32 appeared to be resistant to metabolic transformation to a hemoglobin-binding hydroxylamine. Data from human studies are similarly indicative that alkylanilines are near-universally susceptible to metabolic *N*-hydroxylation (11). Direct evidence for production of an *N*-hydroxylamine *in vivo* in rats has also been presented by Kulkarni *et al.* (37), who demonstrated urinary excretion of *N*-hydroxy-*o*-toluidine following administration of the amine.

It has thus widely been assumed that generation of *N*-hydroxylamines *in vivo* is associated with genotoxicity but never demonstrated that *N*-hydroxylation is a critical step in leading to DNA adduct formation or damage. We recently undertook a multi-species multi-compound study to assess the role of *N*-hydroxylation (38). Using S9 from human, rat, dog and mouse liver, we determined the dependence of DNA adduct formation by three alkylanilines *in vitro* on the presence of NADPH. Results and experimental details are given in Table I. It is clear from the data that NADPH is a nearly absolute requirement for DNA adduct formation to occur. Whether the levels observed in the absence of NADPH represent true adduct formation is difficult to assess. Control experiments were conducted in which [^{14}C]alkylanilines were not added until immediately before terminating the incubation in order to assess non-covalent binding. Results from those experiments were comparable with results from experiments in which NADPH was omitted; thus, it is not apparent that any adduct formation occurred in the absence of NADPH.

Not all the ramifications implicit in the data given in Table I have been explored in detail but one feature in particular deserves further discussion. Little activation of 3-EA to DNA-binding metabolites was observed using S9 from three of the four species, mouse being the exception. This finding was discordant with results for *N*-hydroxylation, which indicated that 3-EA was generally *N*-hydroxylated at a rate several times greater than the rates observed with 3,5-DMA or 2,6-DMA (38). To explore this discrepancy further, DNA binding was measured at lower concentrations of alkylanilines using human S9. At 25–1000 nM, the same order of reactivity was observed as at 25 μM but the differences between 3-EA and the other two alkylanilines were much smaller, ranging from nearly identical to ~6-fold less (Figure 3). *N*-hydroxylation rates at the lower substrate concentrations have not been measured; so we can only speculate about the reasons for the change in relative DNA adduct formation. One possibility is that generation of the ultimate electrophilic species from 3-EA, whether directly from the *N*-hydroxylamine or by a different pathway, becomes kinetically favored at lower substrate concentration. Whatever the actual explanation, the results are equivocal with regard to the role of *N*-hydroxylamines in DNA adduct formation.

Further studies to attempt to demonstrate involvement of *N*-hydroxylamine derivatives in DNA damage involved manipulating *in vitro* activation systems with respect to phase II metabolism. Using the same three alkylanilines listed in Table I, we assessed DNA binding of radioisotope catalyzed by human liver S9 in the absence and presence of cofactors that promote *N*-acetyltransferase (NAT), sulfotransferase and glucuronyl transferase activities. Results are presented in Figure 4, along with experimental details.

Promotion of NAT activity did not have the same effect on all the compounds tested. It produced a striking negative effect on binding by 2,6-DMA, decreasing the adduct level by 78%. The effect on 3-EA binding was also negative but considerably smaller at 28%. In contrast, the effect with 3,5-DMA was to enhance adduct formation by 53%. Decreased binding under the conditions of the experiment is uninformative with respect to role of an *N*-hydroxylamine in DNA adduct formation because the non-oxidized amine is frequently a substrate for NAT. Enhancement, though, is a better indicator because *O*-acetylation of the hydroxylamine promotes its reactivity. Results appear to indicate a significant role for the *N*-hydroxylamine

Table I. Formation of DNA adducts by three [^{14}C]alkylanilines[a]

		3-EA	3,5-DMA	2,6-DMA
Human	Control	15 ± 6	36 ± 11	13 ± 1
	Experiment	15 ± 3	1392 ± 74	258 ± 187
Rat	Control	39 ± 12	5 ± 1	11 ± 1
	Experiment	30 ± 5	1101 ± 204	50 ± 39
Dog	Control	15 ± 8	21 ± 14	35 ± 21
	Experiment	22 ± 1	727 ± 37	180 ± 53
Mouse	Control	68 ± 16	77 ± 23	33 ± 2
	Experiment	162 ± 9	139 ± 35	146 ± 22

[a]Values are given as number of adducts per 10^8 normal nucleotides (±SD; $N = 4$) and were determined by accelerator mass spectrometry analysis of the amount of radioisotope bound to DNA after purification. Calf thymus DNA was incubated *in vitro* with 25 μM of each alkylaniline and hepatic S9 from each of four species with and without NADPH. Each 0.2 ml incubation also included 1 mg/ml DNA, 2 mg/ml S9, 50 mM pH 7.4 Tris buffer and an NADPH-regenerating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$ and 0.1 U glucose-6-phosphate dehydrogenase).

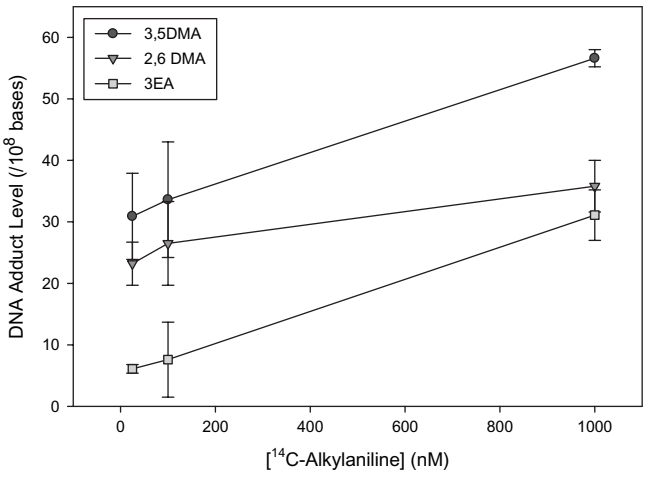

Fig. 3. Formation of DNA adducts catalyzed by human S9 at low doses. Experimental details given in footnote to Table I except that the alkylaniline concentrations used were 25, 100 and 1000 nM.

of 3,5-DMA in DNA binding (but, see comments on Bamberger rearrangement below).

Likewise, the promotion of DNA binding of all three alkylanilines by sulfotransferase activity points to a significant role for *N*-hydroxylation since it would be expected to produce sulfate esters, which, like acyl esters, are more reactive than the unconjugated hydroxylamines. Decreased binding as a result of promotion of glucuronyl transferase activity is largely uninformative, again because the non-oxidized amine can be a substrate.

Other mechanisms—*N*-hydroxylation as a first step to other genotoxins

Largely overlooked until recently is the potential existence of other chemical reaction pathways leading to DNA adducts or other DNA damage that do not directly involve electrophilic nitrenium ions. Phenols are prominent products of aromatic amine metabolism that may arise by direct oxidation of the aromatic ring or, as others have conjectured on theoretical grounds (39), from the radical cation that is the precursor to the *N*-hydroxylamine as shown in Figure 5. The phenols have generally been regarded as detoxification products. To the extent that the phenols are subsequently conjugated with sulfate, this is probably accurate because sulfates are highly polar water-soluble compounds. Moreover, with the acidic proton of the phenol no longer present, the aminophenol is highly resistant to oxidation. If the phenolic oxygen is not conjugated, the aminophenol can undergo spontaneous or peroxidase-catalyzed oxidation, as shown in Figure 5, to a quinone imine, which is highly reactive as an electrophile and can undergo redox cycling as well.

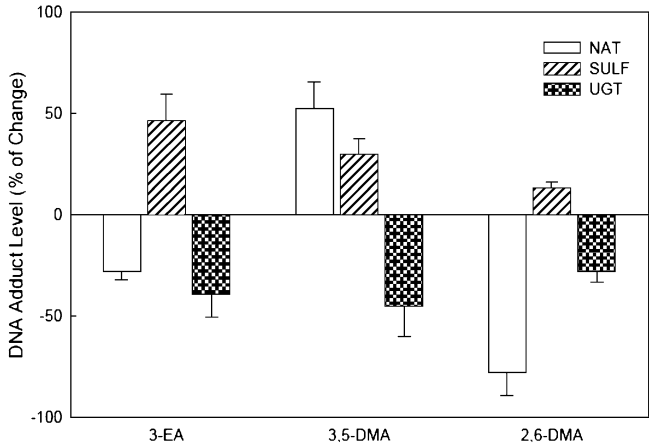

Fig. 4. Effect of Phase II conjugation activity on DNA adduct formation *in vitro*. NAT: All incubations were conducted in 0.2 ml 50 mM Tris buffer (pH 7.4) containing 1 mg/ml ct-DNA, 2 mg/ml human liver S9 fraction, NADPH-regenerating system and 1 μM of the respective [^{14}C]amine for 6 h at 37°C. Supplementation was as follows: NAT, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1.08 mg/ml acetyl-D,L-carnitine, 0.22 U/ml carnitine acetyltransferase and 0.1 mM acetyl coenzyme A; SULF, 0.1 mM PAPS and UGT, 8 mM $MgCl_2$, 25 μg/ml alamethicin and 2 mM UDPGA.

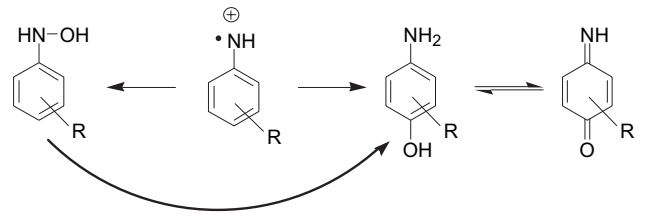

Fig. 5. Generation of aminophenols/quinone imines from *N*-hydroxylamines. The transformations shown here are alternative routes to aminophenols produced by direct ring oxidation by P450s.

Significantly, complicating any analysis of the role played by aminophenol products of metabolism is the fact that these compounds can also form by rearrangement of existing *N*-hydroxylamines. As a chemical reaction, this transformation is referred to as the Bamberger rearrangement and requires strong acid catalysis. It also proceeds in biological systems, where the rearrangement can be catalyzed by cytochrome P450 2E1, alcohol dehydrogenase and perhaps other enzymes as discussed or referenced in ref. (40). Thus, whenever an *N*-hydroxylamine is introduced into a biological system, the possibility that it is converted into an aminophenol before it undergoes any direct reaction with a biological target must be considered.

Direct evidence of genotoxicity of quinone imines has been reported (41) from studies that measured sister chromatid exchanges in cultured human lymphocytes. Of particular note is that Hill *et al.* included the *p*-quinone imine derived from 2,6-DMA in the study and found that it induced sister chromatid exchanges at doses as low as 0.1 μM. Most of the evidence, though, for biological activity of quinone imines or aminophenols comes from studies of other toxic effects.

Circumstantial evidence for a possible role of quinone imines in alkylaniline cytotoxicity comes from studies of acetaminophen linking the transcription factors Nrf2 and Keap1 and toxicity. Oxidation of acetaminophen to *N*-acetyl-*p*-benzoquinone imine is thought to be largely responsible for the hepatoxicity of this widely used analgesic. Nrf2-null mutant mice consequently exhibit increased sensitivity to acetaminophen hepatoxicity (42,43). More recently, it has also been demonstrated that Keap1-knockout mice acquire resistance against acetaminophen hepatoxicity through activation of Nrf2-antioxidant-responsive element pathways (44). Similarly, it has also been shown that acetaminophen can initiate nuclear translocation of Nrf2 *in vivo* in mice (45) and that it is then functionally active.

Aminophenols are better known as nephrotoxins than genotoxins but their mechanistic actions may be similar in that they are electrophilic reactants. The chemical reaction pathways shown in Figure 6 are presented as an illustration of current hypotheses to explain the nephrotoxicity and nephrocarcinogenicity of aminophenol/quinone imines as well as the closely related hydroquinone/quinones. At present, available data support a mechanism in which oxidation and glutathione conjugation occur in the liver. *Mono-*, *bis-* and *tris*-glutathionyl adducts are exported and taken up by the kidney where conversion to mercapturic acids occurs to a variable extent. Lastly, the thioether conjugates become bound to proteins through the same Michael reaction that produces the glutathione adducts or through addition–elimination reactions in which the protein displaces one of the thioether moieties. Examples of each of these steps have been reported (46–48), including the formation of multiple glutathione adducts of aminophenol (49) as well as the aminophenol metabolite of 3-(*N*-phenylamino)propane-1,2-diol considered responsible for toxic oil syndrome (50). Further studies (51,52) have probed the molecular biological effects of 2,3,5-*tris*-(glutathion-*S*-yl)hydroquinone (TGHQ) as well as effects on cell signaling and mutagenesis (53–55). Other important findings discussed in Shao *et al.* (56) indicate that *p*-aminophenol nephrotoxicity *in vivo* is mediated by extrarenal bioactivation comprised of both oxidation and glutathione conjugation. A full mechanistic understanding of the toxicity of aminophenols has not yet been developed, but the evidence at this stage points to a central role for quinone imines. Whether they also can react directly with DNA to produce genotoxic outcomes remains an open question.

Studies on the effects of TGHQ on cell signaling are of additional interest because they suggest yet another mechanism through which these species may be capable of producing toxic responses: generation of reactive oxygen species (ROS). TGHQ was reported to induce mutation spectra in the supF gene replicated in human AD293 cells and bacterial MBL50 cells that were similar to those induced by hydroxyl radical (53), one of several ROS. TGHQ reportedly modulates 12-*O*-tetradecanoyl phorbol 13-acetate responsive element and nuclear factor-kappaB-binding activity in an ROS-dependent fashion (54) and exhibits other ROS-inducible events such as posttranslational modification of Bcl-2 and subcellular localization of Bax, culminating in the release of cytochrome c and caspase activation (55). Whether

similar effects could be elicited by glutathione conjugates of quinone imines remain unexplored but considering the very close similarity between quinone imines and quinones it seems probably that generation of ROS by the former occurs *in vivo*.

Evidence of alkylaniline genotoxicity: recent *in vitro* studies in mammalian cells

Genotoxicity of alkylanilines has received very limited investigation to date, and the fragmented available database supports only limited conclusions. Combined evidence produced in cultured hepatocytes and cell-free systems, some of which has been discussed above, show that both phase 1 (*N*-hydroxylation) and phase 2 metabolism (mainly sulfation or acetylation) are required for activation of the alkylanilines to DNA-binding forms. Proposed metabolic pathways leading to the formation of genotoxic DNA damage products of 2,6-DMA are summarized in Figure 7. The proposed pathways provide the rationale underlying our experimental approaches to characterize mechanisms of mutagenesis and cytotoxicity of this class of chemicals.

Most previous investigations have focused on 2,6-DMA, in view of its demonstrable carcinogenic activity for the nasal cavity of rats. Information published to date can be briefly summarized as follows. In the *Salmonella typhimurium* assay, 2,3-, 2,4-, 2,5- and 3,4-dimethylanilines were weakly mutagenic and caused DNA damage in mammalian V79 cells (57). In the same assay procedure, 2,6-DMA was neither mutagenic in multiple bacterial strains in the presence or absence of exogenous activating systems nor was it active in a DNA damage and repair assay in *Escherichia coli*. It induced mutations in mouse L5178Y lymphoma cells and sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without exogenous metabolic activation (58,59). No genotoxic effect in *E.coli* K12 was observed in a host-mediated assay after feeding of 2,6-DMA to mice, nor was feeding or injecting it effective in a sex-linked recessive lethal assay in *Drosophila melanogaster* (58). No increase of micronuclei was detected in the bone marrow of male mice after oral doses of up to 375 mg/kg/day (60) and it did not induce unscheduled DNA synthesis in rat hepatocytes (61).

N-hydroxy-2,6-DMA, the proposed product of metabolic activation of 2,6-DMA *in vivo*, was mutagenic in *S.typhimurium* TA100 (62) and

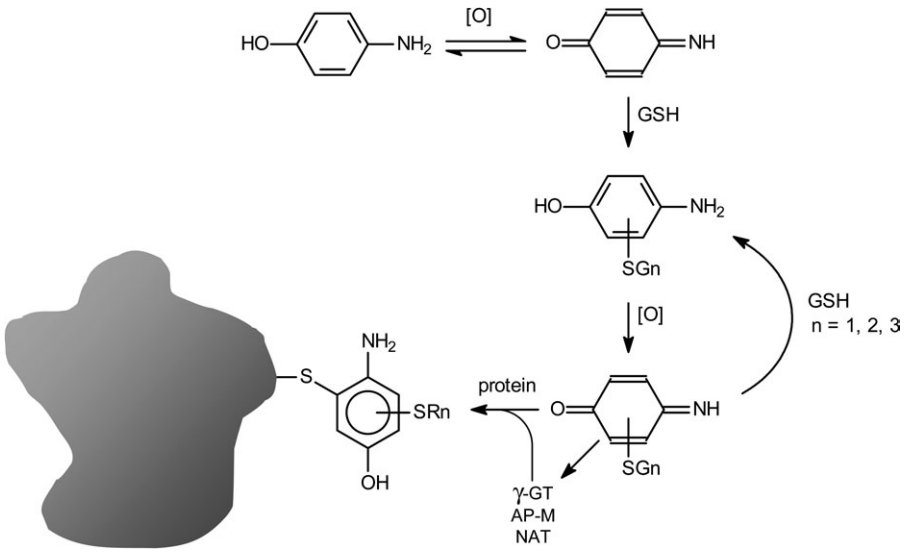

Fig. 6. Formation of protein adducts by aminophenols. A fundamental question posed by this article is whether similar reactions occur between aminophenols and DNA.

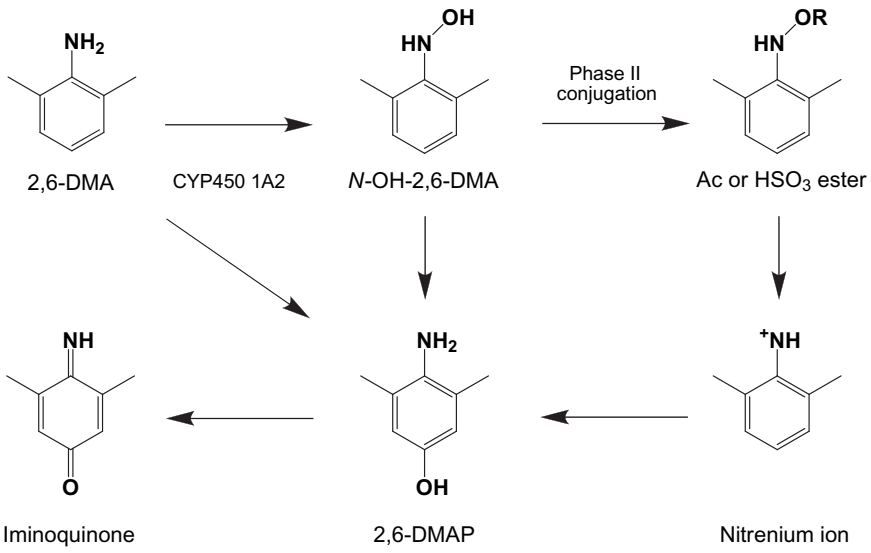

Fig. 7. Proposed metabolic pathways leading to formation of genotoxic DNA damage products of 2,6-DMA. Modified from Gan *et al.* (24).

was the most mutagenic member of a series of *N*-hydroxylated aniline derivatives in the absence of metabolic activation (30,63). *N*-hydroxy-3,5-DMA was mutagenic in the *HPRT* gene of human lymphoblastoid TK6 cells (see below; 64).

Because of the diversity of exposure conditions, target genomes and host cells in which mutagenic events produced by carcinogenic aromatic amines have been studied, extrapolation of observations from one system to another entails substantial uncertainty. The spectra of DNA lesions created also differ qualitatively and quantitatively, and the modified bases identified in any given experiment probably represent only a subset of lesions actually present. Thus, it is difficult to formulate valid generalizations regarding mutagenic efficiency of specific DNA lesions or mechanisms through which they are created. In view of the widespread human exposure to alkylanilines and other evidence summarized in the preceding sections, we have undertaken investigations to expand the database concerning their genotoxicity in mammalian cell systems. We are systematically investigating mutagenic responses and mutation spectra induced by metabolites of alkylanilines that have received little previous study. Evaluations are being done in well-defined genetic targets and mutagenesis models exposed under controlled conditions with the ultimate objective of identifying DNA alterations responsible for specific mutational events. To date, our studies of cytotoxicity have focused exclusively on cell survival, assessed by dye exclusion 24 h after exposure to test compounds.

We have focused on three members of this group, namely 3-EA, 2,6-DMA and 3,5-DMA, and their *N*-hydroxy and aminophenol metabolites synthesized as described above. Initial studies conducted in human lymphoblastoid TK6 cells revealed that 3-EA and *N*-hydoxy-3-EA did not cause reduced viability and were not mutagenic to cells exposed to millimolar concentrations and they have not been studied further. In contrast, 2,6- and 3,5-DMA both induced cell death, but exhibited considerable variation in potency, in which the aminophenol metabolites were considerably more potent than the corresponding *N*-hydroxylamines. Also, each metabolite of 3,5-DMA was more toxic than its 2,6-DMA counterpart. When these compounds were tested for *HPRT* mutagenicity, both the *N*-hydroxy and aminophenol of 3,5-DMA were clearly mutagenic but these derivatives of 2,6-DMA were not active over the same range of dose levels (64).

We have also studied cytotoxicity and mutagenesis induced by the alkylanilines and their major metabolites in AS52 cells, which were derived from *HPRT*$^-$ CHO cells, and contain a functional single copy of the *E.coli gpt* gene in an SV40-pBR322 plasmid stably integrated into their genomic DNA (65–68). The *gpt* gene encodes the purine salvage pathway enzyme xanthine-guanine phophoribosyl transferase, analagous to mammalian *HPRT*, and thus provides the basis for 6-thioguanine selection of mutants. The system is highly suited for determining mutational spectra induced by chemical mutagens of different types. Results to date show that 2,6-DMA and 3,5-DMA when activated by a human liver S9 preparation were both cytotoxic and mutagenic, showing linear dose-response relationships in both parameters at exposure levels between 10 μM and 1 mM. Their *N*-hydroxy derivatives were also cytotoxic and mutagenic, but had higher potency, showing comparable dose-related responses at exposure levels between 10 and 100 μM. Similarly, their aminophenols were both cytotoxic and mutagenic and were substantially more potent, causing nearly 100% cell killing and significant mutagenicity at levels below 10 μM. Mutation spectra induced by these treatments are currently under investigation.

We are also characterizing alkylaniline genotoxicity in cell systems designed to provide further information about their metabolic activation. Felton *et al.* (69,70) developed CHO-derived cell lines in which both phase I and phase II enzymes were introduced and have shown these cells to be highly sensitive to mutagenesis by the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. CHO AA8 cells served as the original source of the UV5 line, which lacks nucleotide excision repair (NER) due to an induced defect in the *XPD* (*ERCC2*) gene (70,71). Transfection of UV5 cells with mouse *P450 1A2* complementary DNA established the UV5P3 line (72), which was subsequently transfected with human aryl sulfotransferase (*HAST1*) or *NAT2* from a human liver complementary DNA library (70,73), producing CHO cells that are NER deficient (or proficient) and express mouse P4501A2 plus human aryl sulfotransferase or NAT genes. Based on their heterozygosity at the *aprt* locus, they can be used for mutational analysis based on selection with 8-azaadenine.

Cells were treated with the parent alkylanilines 2,6-DMA and 3,5-DMA at exposure levels of 50 μM–1 mM or with *N*-hydroxy or aminophenol metabolites at 5–500 μM. Cell viability was determined 24 h after treatment by trypan blue exclusion and mutant fraction by 8-azaadenine selection. Major findings of studies to date can be summarized as follows. The influence of NER status was investigated by comparison of responses of AA8 CHO cells, which are NER proficient, and UV5 cells, which are NER deficient. Neither cell line expresses significant levels of phase I nor phase II enzymes. In the absence of human liver S9, the parent alkylanilines were cytotoxic, but not mutagenic, at exposure levels of 100 μM–1 mM, as expected. Interestingly, cytotoxicity was enhanced by NER deficiency, suggesting that DNA damage by products that were not mutagenic in the assay used contributed to the toxicity of the compounds. The *N*-hydroxy derivatives of 2,6-DMA and 3,5-DMA were also toxic, causing dose-related loss of viability at doses between 100 μM and 1 mM; toxic potency was substantially increased by NER deficiency, resulting in similar loss of viability at levels of 10–200 μM. These derivatives were not mutagenic at doses up to 1 mM. Responses to the aminophenol derivatives were virtually identical to those of the *N*-hydroxylated forms, i.e. they were potent inducers of cytotoxicity, which was enhanced by NER deficiency, but not mutagenic under these conditions.

The influence of NER status was also studied by several other comparisons. In two cell lines both expressing CYP1A2 and NAT2, but one deficient in NER, 2,6-DMA and 3,5-DMA were cytotoxic and mutagenic at dose levels between 100 μM and 1 mM. Their potency in inducing both responses was greatly enhanced in the NER-deficient cells. In two cell lines both deficient in NER and one expressing CYP1A2 and NAT2, 2,6-DMA and 3,5-DMA were cytotoxic at dose levels of 100 μM –1 mM but were mutagenic only in cells expressing the metabolizing enzymes, as expected. Very similar results were obtained in a comparison of cells expressing CYP1A2 and aryl sulfotransferase. The relative contributions of aryl sulfotransferase and NAT2 to the induction of cytotoxicity and mutagenicity were compared in NER-deficient cells expressing CYP1A2 and either of the phase II enzymes. Aryl sulfotransferase proved to be more effective in potentiating cytotoxicity but not mutagenicity of both 2,6-DMA and 3,5-DMA.

Thus, results to date indicate that the alkylanilines are potent genotoxins when activated by exogenous or endogenous phase I and phase II xenobiotic-metabolizing enzymes. The nature of specific DNA damage products responsible for mutagenicity remains to be identified, although evidence to date supports the suggestion that activation through *N*-hydroxylation and subsequent conjugation by sulfation and/or acetylation are important contributors. It is also evident that NER is an important determinant of the magnitude of mutagenic responses. Interestingly, repair deficiency also enhances cytotoxicity under conditions in which mutagenicity is not detected, suggesting that DNA damage other than proposed DNA adducts may also be important in determining the observed responses.

NER of DNA is essential for maintenance of genomic integrity and cell viability. Different types of DNA lesions have distinct consequences with respect to helix distortion as well as ability to suspend or obstruct DNA replication, block ongoing transcription or hamper the battery of genome repair systems and caretakers that continuously safeguard the genome (74). Depending on the type of DNA damage, cell death may be triggered via apoptosis or replicative senescence (cytotoxic or cytostatic lesions, e.g. double-strand breaks) or mutations may be induced by DNA adducts of various structural types. NER is primarily involved in repair of severely helix-distorting damage, such as bulky nucleotide adducts and intrastrand crosslinks that interfere with base pairing, thereby interfering with transcription and normal replication. Current evidence suggests that different DNA

adducts may induce very different effects on cell proliferation, cell death via necrosis or apoptosis, as well as mutagenicity, possibly attributable to activation of cellular responses such as p53 activation.

The importance of NER status and the nature of DNA adducts as determinants of cytotoxicity and mutagenicity is well illustrated by results of a comparison of cytotoxicity and mutagenicity of a series of carcinogens in normal diploid human fibroblasts and in cells deficient in one or more DNA repair processes (75). Carcinogens studied included UV radiation; reactive derivatives of structurally related aromatic amides; metabolites of benzo[*a*]pyrene; the alkylating agent MNNG and aflatoxin B_1 dichloride, a model for the reactive 2,3-epoxide of aflatoxin B_1. Exponentially growing cells were exposed to agents and assayed for mutations (induction of 6-thioguanine resistance) and cell killing (loss of colony-forming ability). Cells deficient in repair of particular DNA adducts or lesions proved more sensitive to the agent causing those lesions than did normally repairing cells. These results are similar to our observations on alkylanilines. Many of the carcinogens were compared for their mutagenic and/or cytotoxic effect not only as a function of dose administered but also as a function of the initial number of adducts or photoproducts induced in DNA and the number remaining at critical times posttreatment. The results demonstrated a high correlation between the number of DNA lesions remaining unexcised at the time the DNA was replicated and the frequency of mutations induced. These observations will need to be taken into account in interpreting results of our future studies, and extensive further investigations will be required for more complete elucidation of underlying mechanisms.

Summary

Alkylanilines comprise a class of chemicals that, while clearly not as carcinogenic as multi-ring aromatic amines, may hold far more significance for human health than has yet been appreciated. It appears that few individuals are spared lifelong exposure to many of these compounds. The universal nature of exposure results from an abundance of sources, some, like tobacco smoke that are well documented and others that are poorly understood but that may reflect the widespread use of alkylanilines as components of numerous pharmaceuticals and chemical commodities. Whether their potency is sufficient to create substantial attributable risk remains an unresolved question but there is certainly some evidence to that effect with regard to bladder cancer and several specific amines. Research on the mechanisms of action of alkylanilines has yet to produce a well-defined model like the model for multi-ring aromatic amines. The present lack of a model may reflect more than a lower level of interest; the mechanisms may actually be more complex ranging from nitrenium ion chemistry to reactions involving quinone imines and possibly encompassing the chemistry of ROS. The potential mechanistic complexity of alkylanilines relative to multi-ring aromatic amines is an especially important aspect of their genotoxicity. The carcinogenic multi-ring aromatic amines are deemed to target the urinary bladder in part because the ultimate electrophilic form, a nitrenium ion, is transported in a stable form to the bladder, where it becomes available through a change in acidity. If additional mechanisms that do not require a bladder-specific environment are characteristic of alkylanilines, then there is reason to expect that they may target additional organs such as the kidney, for which there are few candidate chemical carcinogens. A better understanding of the mechanisms of alkylaniline genotoxicity is probably to provide important insights into the environmental and genetic origins of one or more human cancers and may reveal a substantial role for this group of compounds as human chemical carcinogens.

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