Metabolic Activation Routes of Arylamines and Their Genotoxic Effects

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Two different types of DNA adducts are formed from many aromatic amines by bioactivation: N-acetylated and nonacetylated, arylamine DNA adducts. It has become clear from experiments using N-acetyl-2-aminofluorene and 2-aminofluorene adducts to C8 of deoxyguanosine that these two types of adducts may have different effects on DNA structure and DNA replication. We have determined blocking of DNA replication by various other N-acetylaniline and arylamine deoxyguanosine adducts. It was found that the N-acetyl group in general is required for blocking of DNA replication; the nature of the aromatic moiety seems to be of minor importance. Little information is available on the genotoxic effects of these adducts in mammalian cells in vivo. We have tried to get more insight in this by investigating the clastogenicity, the initiation of preneoplastic cells, and the promontional effects of various aromatic amines from which different ratios of N-acetylaniline DNA adducts to arylamine DNA adducts are formed in the rat liver. Our results show that formation of N-acetylaniline adducts to C8 of deoxyguanosine in the liver is correlated with clastogenicity and hepatic promoting effect. Initiation capacities, however, seem to be correlated with formation of nonacetylated, arylamine adducts. Mechanisms by which formation of N-acetylaniline DNA adducts may generate a promoting effect in the liver are discussed. — Environ Health Perspect 102(Suppl 6):153–159 (1994)

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Introduction

For aromatic amines, two major pathways of metabolic activation have been implicated in the generation of genotoxic metabolites in vivo (1). One is the formation of N-hydroxy-N-acetylanilines by subsequent N-acetylation and N-hydroxylation, followed by O-esterification; the other is formation of hydroxylamines. The hydroxylamines are reactive per se but further metabolism by O-esterification to more reactive metabolites is also involved. The two pathways lead to the formation of different types of DNA adducts: N-acetylated arylamine adducts at C8 of deoxyguanosine (and to a minor extent adducts at N7) are formed by the first pathway, while nonacetylated arylamine adducts at C8 of deoxyguanosine and N7 of deoxadenosine are formed by the second. With certain aromatic amines, other metabolic pathways may also be important (e.g., for benzidine and 4-aminobiphenyl DNA adducts may be formed by a prostaglandin H synthase-dependent route (1)).

Numerous studies have been conducted to determine the effects of the two types of adducts on DNA structure. In most of these studies, N-(deoxyguanosin-8-yl)-2-acetylanilinofluorene (dG-C8-AAF) and its nonacetylated analog, N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) were used as model adducts. Significant differences have been observed in the way these two types of adducts alter the conformation of DNA (2). dG-C8-AAF adducts either adopt a syn conformation and induce a major distortion of the DNA helix or induce a B to Z transition of DNA. In contrast, dG-C8-AF adducts seem to be incorporated in DNA without causing major distortions.

These conformational differences are probably responsible for the different rates of repair of the adducts. dG-C8-AAF adducts are repaired relatively rapidly in rat liver in vivo with a half-life of 7 days (3–5); dG-C8-AF adducts are repaired more slowly and accumulate during chronic feeding (5–7). The latter also applies to the minor N-acetylated adduct at N7 of deoxyguanosin, which has been taken to suggest that this adduct does not distort the DNA helix either. Structural differences seem also to be responsible for differences in replication of DNA strands modified with dG-C8-AAF or dG-C8-AF adducts. When incorporated in single-stranded phage DNA, dG-C8-AAF adducts very effectively block replication of this modified DNA after transfection in Escherichia coli. (8,9). Already one adduct per DNA molecule was sufficient to completely inactive single-stranded φX174 (9). This contrasts to the results obtained with dG-C8-AF adducts: on average seven adducts were needed to block the infectiveness of a φX174 molecule (10).

Also in double stranded DNA it was found that dG-C8-AAF adducts blocked replication more effectively than dG-C8-AF adducts. When randomly introduced in the plasmid pBR322, one to two adducts are needed to block replication of the plasmid in repair deficient E. coli strains, while 8 to 17 of the nonacetylated adducts are needed (11). Comparable results were obtained with modified double-stranded φX174 and M13mp9 DNA (8–10,12): dG-C8-AAF adducts always blocked replication more efficiently than dG-C8-AF adducts, both in wild-type and repair-deficient hosts. As a result of this efficient blockage, preferential use of unmodified strands was observed during replication of plasmids in which dG-C8-AAF adducts were introduced in one strand specifically (13,14).

The mutagenic effects of dG-C8-AAF and dG-C8-AF adducts have been studied extensively; they result in different types of mutations in various systems (15,16). It was suggested that many factors, such as type of DNA (single- or double-stranded, phage or plasmid, extrachromosomal or
not), host cell (bacteria, mamalian cells), repair, etc., may determine the mutation spectra of the two adducts (16,17).

Thus, there is much experimental evidence for a major effect of dG-C8-AAF adducts on the structure of DNA and subsequent blocking of DNA replication, while dG-C8-AF adducts have much less effect.

We have investigated if this might be a general feature of N-acetylated arylamine adducts by determining the effects of dG-C8-4'-fluoro-4-acetylaminobiphenyl and dG-C8-4'-acetylaminobiphenyl adducts and their nonacetylated analogs on blocking of DNA replication (see below). These experiments also indicated that for these adducts the presence of the N-acetyl moiety strongly enhances the blocking of DNA replication; the nature of the aromatic moiety seems to be of minor importance (see discussion in the next paragraph).

There is only limited information on the role of N-acetylaralmine versus arylamine DNA adducts for the process of chemical carcinogenesis. DNA adducts are found in many organs after administration of aromatic amines and derivatives, often also in nontarget organs. In target organs, N-acetylaralmine as well as arylamine adducts are found [e.g., from 2-acetylaminofluorene and N-hydroxy-2-acetylaminofluorene in the male rat liver (3,4,7,18,19), and from N-hydroxy-4'-fluoro-4-acetylamino- biphenyl in the rat liver and kidney (20,21)]. However, in other target organs, only arylamine adducts have been found, [e.g., from 4-aminoazobiphenyl in the dog bladder (22-24), N-hydroxy-2-acetylaminofluorene in the rat mammary gland (25), and 3,2'-dimethyl-4-aminobiphenyl in rat colon (26)]. These data suggest that formation of only arylamine adducts might be sufficient for carcinogenesis and that these adducts may bring about all the genetic changes needed for the induction of malignant tumors. Still, in various organs in which these adducts are formed at a high level, no tumors develop. For example, nonacetylated arylamine adducts are formed from N-hydroxy-2-acetylaminofluorene in the female rat liver, which is resistant to hepatocarcinogenesis by this compound (27,28), and from 4-acetylaminoazobiphenyl in the dog liver (22,24) and rat liver (29), which are not target sites.

We have shown previously that inhibition of the formation of dG-C8-AAF adducts did not change the initiation capacity (induction of foci of γ-glutamyltranspeptidase positive preneoplastic cells) of N-hydroxy-2-acetylaminofluorene in the rat liver (30). Because the amount of dG-C8-AF adducts was unaffected in these experiments, this suggests a role for these adducts in initiation. Inhibition of the formation of dG-C8-AAF adducts from N-hydroxy-2-acetylaminofluorene during a promotion experiment in the rat liver, however, greatly decreased the promoting effect of this compound (31). Therefore, these adducts may play a role in promotion.

We have further investigated the role of the different DNA adducts in the process of hepatocarcinogenesis by determining the initiation and promotional capacities of various analogs of N-OH-AAF from which different ratios of the two types of DNA adducts are formed in vivo.

To investigate if the N-acetylaralmine DNA adducts which block DNA replication quite efficiently in vitro and in bacteria, may also block replication in mammalian cells in vivo, we determined the clastogenicity of various analogs in rat liver and correlated this with the amounts of N-acetylaralmine DNA adducts formed in this organ.

**Blocking of DNA Replication by dG-C8-Acetylaralmine and dG-C8-Arylamine Adducts**

Information on the effects of N-acetylaralmine and arylamine DNA adducts on DNA replication has been obtained mainly from experiments with dG-C8-AAF and dG-C8-AF. Only a few studies have been performed with the analogous 4-aminobiphenyl adducts (8,11). These studies showed a difference in the ability of these adducts to block replication compared to 2-aminobiphenyl adducts. A lesser inhibition by the dG-C8-4-acetylaminoaralmine (dG-C8-AABP) adduct was observed when incorporated in double-stranded plasmid and phage DNA compared to dG-C8-AAF; its effect was similar to that of the nonacetylated adducts dG-C8-4-aminoaralmine (dG-C8-ABP) and dG-C8-AF. It has been suggested that the possibility of the dG-C8-AABP biphenyl adducts to adopt a nonplanar aromatic conformation may be responsible for the smaller effect of dG-C8-AABP (13). In single-stranded M13mp9 DNA; however, dG-C8-AABP adducts blocked replication much more than nonacetylated adducts (8), although still less effectively than dG-C8-AAF adducts.

We have found a comparable blocking by biphenyl and fluorene adducts in single stranded φX174 DNA (Table 1): on average, one dG-C8-acetylaralmine adduct was sufficient to inactivate φX174, irrespective of the nature of the aromatic moiety. Nonacetylated adducts, however, were less effective: on average two to seven adducts were needed for complete inactivation. In addition, termination of in vitro replication by DNA polymerase I (Klenow fragment) of single stranded M13mp9 DNA modified with dG-C8-AAF, dG-C8-AFBP, and dG-C8-AABP adducts always occurred before a modified base, whereas replication of M13 DNA modified with dG-C8-AF, dG-C8-AFBP, and dG-C8-ABP terminated before as well as opposite an adduct (32,33). These data suggest that all dG-C8-acetylaralmine adducts may block DNA replication equally effectively and that dG-C8-aralmine adducts are less effective. This difference seems to be determined mainly by the N-acetyl group rather than aromatic moiety.

**DNA Adduct Formation, Initiation, and Promotion by N-Hydroxy Acetylaralmines in the Rat Liver in Vivo**

We have used N-hydroxy-2-acetylaminoaralmine N-hydroxy-4'-fluoro-4-acetylaminoaralmine, N-hydroxy-4-acetylaminoaralmine, and in some experiments N-hydroxy-2-acetylaminoaralminephenanthrene. Comparable amounts of DNA adducts were formed, as determined by administration of the radiolabeled compounds (Table 2). Although not all adducts from the biphenyl derivatives could be identified, it is clear that far fewer N-acetylated adducts to the C8 of deoxyguanosine are formed from these compounds than from N-hydroxy-2-acetylaminoaralmine. This is not due to instability of the biphenyl adducts.
Table 2. Formation of DNA adducts from N-hydroxy-2-acetylanilinofluorene (N-OH-AAF), N-hydroxy-4'-fluoro-4-acetylanilinobiphenyl (N-OH-FAABP) and N-hydroxy-4-acetylanilinobiphenyl (N-OH-AABP) in the rat liver.

| Compound         | Dose, pmole/kg | Total, pmole/mg DNA | Acetylated | Nonacetylated | Other |
|------------------|---------------|---------------------|------------|---------------|-------|
|                  |               | before hydrolysis   |            |               |       |
| N-OH-AAF         | 30            | 81 + 10^5           | 23 ± 3     | 26 ± 4        | 6 ± 3 |
| N-OH-FAABP       | 120           | 58 ± 3              | 1.7 ± 0.1  | 6.2 ± 0.6     | 27 ± 2|
| N-OH-AABP        | 120           | 46 ± 8              | 1.0 ± 0.1  | 4.7 ± 1.9     | 19 ± 2|

Radiolabeled N-OH-acetylanilines were administered to male Wistar rats, 24 hr after PH. Livers were removed 5 hr later and DNA was isolated and hydrolyzed in trifluoroacetic acid. After removal of the acid, nonlabeled standard DNA adducts were added as UV markers and analyzed by HPLC. Quantitation of adducts was by determination of radioactivity coeluting with the unlabeled markers. Data from Van de Poll et al. (21,34) and Tate et al. (44). *Data for total adducts are based on covalent binding of radioactivity to DNA before hydrolysis and HPLC analysis. Recovery of radioactivity after HPLC analysis was 68, 60, and 94% for N-OH-AAF, N-OH-FAABP, and N-OH-AABP, respectively. Results are expressed as mean ± SEM of four or five animals.

Figure 1. Promotional effects of N-hydroxy-2-acetylanilinofluorene (N-OH-AAF), N-hydroxy-4'-fluoro-4-acetylanilinobiphenyl (N-OH-FAABP) and N-hydroxy-4-acetylanilinobiphenyl (N-OH-AABP) in a modified Solt-Farber protocol. Male Wistar rats, 200 g, were initiated with diethylnitosamine (250 mg/kg, ip). After 2 weeks they received three ip injections of the compounds (at days 18, 19, and 20), a partial hepatectomy at day 21, and finally a quarter of the dose at day 25. The liver was removed at day 28. Liver sections were stained for y-glutamyltranspeptidase activity (GGT), the number of GGT positive foci of cells was determined and the volume of GGT positive cells was calculated for each size class. N-OH-AAF was tested at doses of 40 pmole/kg/injection; N-OH-FAABP and N-OH-AABP at doses of 120 pmole/kg/injection. Controls received solvent during promotion. Data are from Van de Poll et al. (60). The number of animals per group (N) is indicated in the figure.

during the isolation and hydrolysis procedure of DNA because synthetic standards were stable. The unidentified adducts (Table 2) probably represent nonacetylated adducts because the majority of biphenyl DNA adducts formed in vivo are nonacetylated (20,21,29,34).

Promotional effects of several N-hydroxy acetylarylamines were determined in a modified Solt-Farber protocol. Rats were initiated with a high, necrogenic dose of diethylnitosamine, followed after 2 weeks by repeated ip injections of the compounds (four injections in 2 weeks). This was combined with partial hepatectomy (see legend, Figure 1). At the end of the experiments, the number of y-glutamyltranspeptidase-positive foci of preneoplastic cells was determined. In the animals that had received N-hydroxy-2-acetylanilinofluorene, a high number of large foci were found (Figure 1). N-Hydroxy-4'-fluoro-4-acetylaminobiphenyl, although administered at a 3-fold higher dose, was much less effective. No increased number of foci above control level was observed with N-hydroxy-4-acetylanilinobiphenyl (at the same dose as its 4-fluoro analog); it was completely ineffective as promoter. These results indicate that promotion by the various N-hydroxy acetylarylamines does not correlate with total covalent binding to DNA of these compounds. There seems to be, however, a correlation with the formation of arylamine adducts to C8 of deoxyguanosine (Table 2). This correlation probably is based on the formation of N-acetylated adducts to C8 of deoxyguanosine specifically, because our previous results showed a much decreased hepatic promotional effect of N-hydroxy-2-acetylanilinofluorene after inhibition of the formation of such adducts (31).

Initiation capacity of the N-hydroxy acetylarylamines in the rat liver was determined in a modified Solt-Farber protocol in which administration of 2-aminofluorene in the drinking water combined with a necrogenic dosis of carbon tetrachloride (CCl4) was used for promotion. The N-hydroxy acetylarylamines were administered after partial hepatectomy (during S-phase) because this makes it unlikely that differences in the rate of repair of the various DNA adducts may influence the outcome of the experiments to a great extent. All N-hydroxy acetylarylamines were good initiators (Table 3). However, only N-hydroxy-2-acetylanilinofluorene and (to a lesser degree) N-hydroxy-4'-fluoro-4-acetylanilinobiphenyl, are hepatocarcinogenic.
Comparison of the data on initiation with formation of N-acetylated adducts to the C8 of deoxyguanosine (Table 2) shows no correlation between initiation and formation of these adducts. It is not possible to correlate initiation with a specific type of other adduct because a large part of the biphenyl DNA adducts has not been identified.

2-Acetylanminophenanthrene is not hepatocarcinogenic (35). Several hepatic promoters have been administered after administration of 2-acetylanminophenanthrene, but this did not result in hepatocarcinogenicity (36,37). In this study, we found that N-hydroxy-2-acetylanminophenanthrene is a good initiator. This is probably due to the use of a stronger promotion stimulus in our study. Administration of N-hydroxy-2-acetylaminophenanthrene leads to the formation of only nonacetylated adducts in the liver (29). Two major deoxyguanosine adducts are formed; one of these was identified as N-(deoxyguanosin-8-yl)-2-aminophenan-threne (dG-C8-AP)(38).

Thus, the data on initiation by the various N-hydroxy acetylarylamines indicate that dG-C8-acetylarylamine adducts are most likely not involved in this.

Clastogenicity of N-Hydroxy Acetylarylamines in the Rat Liver in Vivo

The clastogenicity of various N-hydroxy acetylarylamines was studied by the induction of micronuclei in the rat liver. Micronuclei may arise from DNA adducts that block DNA replication during S-phase. The presence of such adducts may lead to gaps in the daughter strand opposite the adduct. Subsequently, this gap may be converted to a double strand break by the action of (repair) endonucleases that specifically attack single stranded regions (39,40). N-Hydroxy-2-acetylanminofluorene is a potent clastogen (Figure 2): a high frequency of micronuclei was found already at a dose of 25 μmol/kg. A clear delay in the partial hepatectomy-induced regenerative response was observed (41). Clastogenicity and delay in regeneration were also found at doses of 5 and 15 μmol/kg (41). At doses higher than 25 μmol/kg, regeneration was severely inhibited and clastogenicity, therefore, not expressed as micronuclei (results not shown).

The other N-hydroxy acetylarylamines were much less clastogenic; only N-hydroxy-4'-fluoro-4'-acetylaminobiphenyl induced a significant number of micronuclei at a dose equivalent to that in the initiation and promotion experiments (Figure 2). Delay in regeneration was observed only at day 2 after partial hepatectomy. At a 3-fold higher dose, a higher frequency of micronuclei was found and regeneration was still delayed at days 3 and 4. Both N-hydroxy-4-acetylaminobiphenyl and N-hydroxy-2-acetylanminophenanthrene induced very few micronuclei and caused no delay in regeneration.

Discussion

Our results indicate that formation of N-acetylarylamine adducts to C8 of deoxyguanosine may correlate with clastogenicity in the rat liver in vivo because only the compound that forms the most of these adducts is highly clastogenic (N-hydroxy-2-
acetylaminofluorene). This is consistent with our previous results, which showed a much reduced clastogenicity of N-hydroxy-2-acetylaminofluorene after inhibition of the formation of these adducts (41). Also, formation of N-acetylarylamine adducts to C8 of deoxyguanosine may be correlated with promoting activity. A correlation between clastogenicity and hepatic promoting activity has been found for other hepatocarcinogens and nonhepatocarcinogens: e.g., the very potent hepatocarcinogen aflatoxin B1, which is an extremely good promoter compared to 2-acetylaminofluorene (42,43), induced a similar frequency of micronuclei as N-hydroxy-2-acetylaminofluorene already at a 75 times lower dose (Figure 3). Regeneration of the liver, after the partial hepatectomy that was employed in these experiments, was severely delayed as is evident from the still high mitotic index 5 days after partial hepatectomy (Figure 3). In similar experiments, Bates et al. (44) have shown that the nonhepatocarcinogen benzo[a]pyrene is not clastogenic in the liver, although in combination with a promotion stimulus, it induces foci of preneoplastic cells in the regenerating liver (45) suggesting that it is nonhepatocarcinogenic because it lacks promotion effect. Other hepatocarcinogens and nonhepatocarcinogens (total of 23 compounds) have also been tested for clastogenicity in the liver micronucleus assay. In general, the hepatocarcinogens tested (13 compounds) all gave a positive response, whereas most of the nonhepatocarcinogens (8 compounds) did not or were very weak clastogens. Only two nonhepatocarcinogens were clearly clastogenic (46). Thus, clastogenicity may in general be related to hepatic promoting activity.

It is not clear how clastogenic damage may be involved mechanistically in promotion (if there is a causal relationship at all) because fewer clastogenic N-acetylarylamine DNA adducts are formed in preneoplastic cells compared to normal hepatocytes (47), and tumors are believed to develop from the preneoplastic cells. A possible explanation may be that reduced formation of clastogenic, mitoinhibitory DNA-adducts renders preneoplastic cells relatively resistant against the mitoinhibitory and toxic effects of hepatic promoters. Therefore, preneoplastic hepatocytes may proliferate faster than normal hepatocytes (clonal expansion model, 48), increasing the chance that a further conversion towards malignancy takes place in one of the cells of this expanded population.

This model requires that several conditions are met. For instance, a mitogenic signal must be generated, otherwise preneoplastic cells do not expand clonally because they are not autonomous in their growth (49,50). Regenerative proliferation does not seem to be important in this respect because promotion by N-hydroxy-2-acetylaminofluorene already can be achieved with non-cytotoxic doses (51,52). However, decreased hepatic functioning may play a role. Decreased protein synthesis and mRNA template function have been reported during promotion with this compound (53). The impaired hepatic functioning may be the trigger for a mitogenic response. Indeed, there are indications that normal hepatocytes enter the cell cycle because similar changes in enzyme activities have been found after administration of N-hydroxy-2-acetylaminofluorene that are also found after partial hepatectomy (54-57). Also, prolonged arrest of hepatocytes in the G1 phase of the cell cycle (see below) with a concomitant change in enzyme expression, may add to the generation of a mitogenic signal.

Recently, it was suggested that the tumor-suppressor protein p53 has an important role in blocking cells with damaged DNA in G1 to allow time for DNA repair (58). The protein accumulates in response to treatments that induce DNA damage (including clastogenic damage). Formation of N-acetylarylamine DNA adducts, that cause a major distortion of the DNA structure and are clastogenic may therefore lead to accumulation of p53 and arrest in G1.

[Recently, we found that there is indeed a large accumulation of p53 in the liver after administration of a nonhepatotoxic dose of N-hydroxy-2-acetylaminofluorene to male rats.]

Prenecoplasic cells proliferate during promotion with N-acetylamines. It is possible that this is due to lack of properly functioning suppressor protein p53. Once entered the cell cycle, they may remain resistant towards the mitoinhibitory and toxic effects of hepatic promoters because metabolic activation of these compounds is decreased during cell proliferation (57,59). Due to absence of G1 arrest in response to DNA damage, these cells are genetically instable and will accumulate mutations and be at high risk for malignant transformation. Of course, any other genetic defect of preneoplastic cells other than in p53 that result in escape from G1 arrest would have the same effect.

Our results also suggest that formation of nonacetylated arylamine adducts is related to initiation. This may explain why certain aromatic amines are sometimes not carcinogenic for an organ in which such
adducts are formed: initiation may have taken place, but promotion may be lacking. This may particularly apply to some organs with a low cell turnover (e.g., the liver). In other organs where promotion may be brought about by additional compounds (e.g., steroid hormones in the mammary gland), a high proliferation rate (colon, organs in the neonatal animal) or regenerative hyperplasia after cell damage (urine bladder), formation of only this type of adducts may be sufficient for carcinogenesis.

In conclusion, we have found a correlation between the formation of clastogenic, N-acetylaminofluorene DNA adducts of various N-hydroxy acetylaminofluorenes and hepatic promoting activity, whereas initiation seems to be correlated with formation of nonacetylated DNA adducts. At present, the exact mechanism by which formation of clastogenic DNA adducts may cause hepatic promotion is not yet clear.

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