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*-acetylarylamine 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 promotional effects of various aromatic amines from which different ratios of *N*-acetylarylamine DNA adducts to arylamine DNA adducts are formed in the rat liver. Our results show that formation of *N*-acetylarylamine 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*-acetylarylamine 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*-acetylarylamines 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 N^2) are formed by the first pathway, while nonacetylated arylamine adducts at C8 of deoxyguanosine and N^6 of deoxyadenosine 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*)].

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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-acetylaminofluorene (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 N^2 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 inactivate 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, mammalian 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*-acetylarylamine 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*-acetylarylamine 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-acetylaminobiphenyl in the rat liver and kidney (*20,21*)]. However, in other target organs, only arylamine adducts have been found, [e.g., from 4-aminobiphenyl 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-acetylaminobiphenyl 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-glutamyltrans-peptidase 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*-acetylarylamine 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*-acetylarylamine DNA adducts formed in this organ.

Blocking of DNA Replication by dG-C8-Acetylarylamine and dG-C8-Arylamine Adducts

Information on the effects of *N*-acetylarylamine 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 those adducts to block replication compared to 2-aminofluorene adducts. A lesser inhibition by the dG-C8-4-acetylaminobiphenyl (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-aminobiphenyl (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 (*11*). 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-acetylarylamine 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-FAABP, and dG-C8-AABP adducts always occured before a modified base, whereas replication of M13 DNA modified with dG-C8-AF, dG-C8-FABP, and dG-C8-ABP terminated before as well as opposite an adduct (*32,33*). These data suggest that all dG-C8-acetylarylamine adducts may block DNA replication equally effectively and that dG-C8-arylamine adducts are less effective. This difference seems to be determined mainly by the *N*-acetyl group rather than aromatic moiety.

Table 1. Blocking of replication of single-stranded ϕX174 DNA by dG-C8-*N*-acetylarylamine adducts and dG-C8-arylamine adducts.[a]

	Average number of adducts for a complete blockage
dG-C8-AAF[b]	1
dG-C8-FAABP[c]	1
dG-C8-AABP[c]	1
dG-C8-AF[d]	7
dG-C8-FABP[c]	2
dG-C8-ABP[c]	3

[a]For the methods of modification of ϕX174 DNA, see Van de Poll et al. (*32*). Different levels of modification were used with each adduct (0–25 adducts/phage molecule). ϕX174 phage was transfected into *E. coli* spheroplasts (AB 1157, repair proficient) and plated with *E. coli* host on soft agar to determine phage survival. [b]Data from Lutgering et al. (*9*). [c]Data from Van de Poll et al. (*32*). [d]Data from Lutgering et al. (*10*).

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

We have used *N*-hydroxy-2-acetylaminofluorene *N*-hydroxy-4′-fluoro-4-acetylaminobiphenyl, *N*-hydroxy-4-acetylaminobiphenyl, and in some experiments *N*-hydroxy-2-acetylaminophenanthrene.

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-acetylaminofluorene. This is not due to instability of the biphenyl adducts

Table 2. Formation of DNA adducts from *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF), *N*-hydroxy-4'-fluoro-4-acetylaminobiphenyl (N-OH-FAABP) and *N*-hydroxy-4-acetylaminobiphenyl (N-OH-AABP) in the rat liver.

		pmole/mg DNA			
	Dose,	Total,[a]	dG-C8 adduct		
	µmole/kg	before hydrolysis	Acetylated	Nonacetylated	Other
N-OH-AAF	30	81 ± 10[b]	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-acetylarylamines 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 Tates et al. (*44*). [a]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 64% for N-OH-AAF, N-OH-FAABP, and N-OH-AABP, respectively. [b]Results are expressed as mean ± SEM of four or five animals.

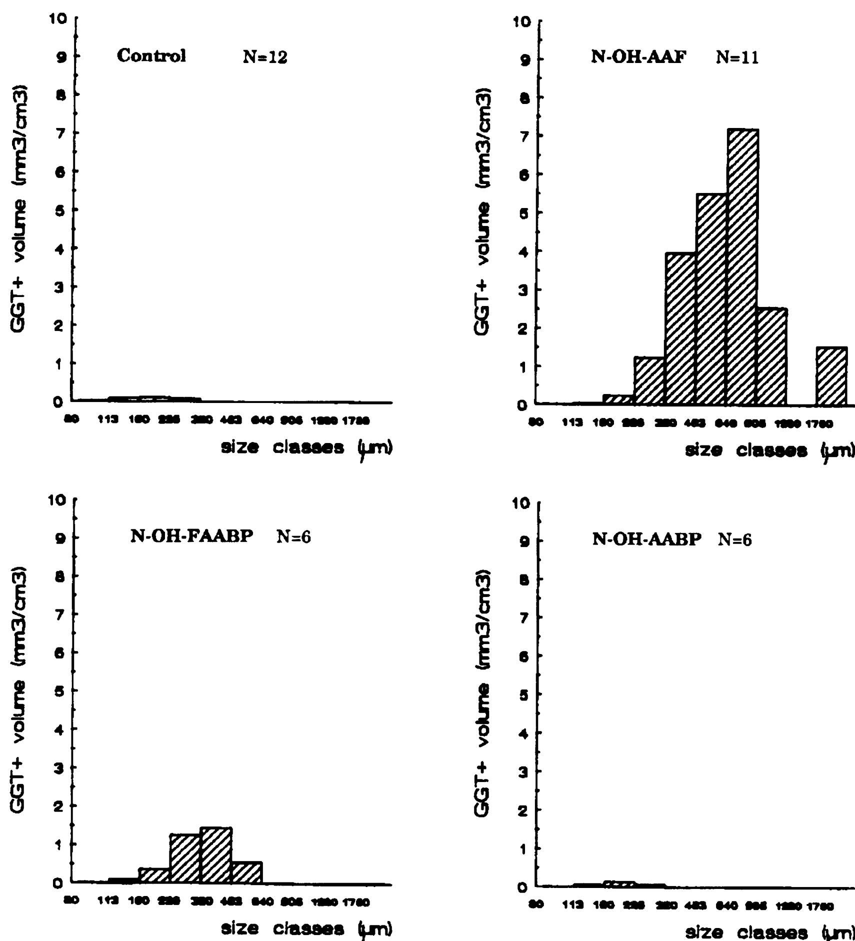

Figure 1. Promotional effects of *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF), *N*-hydroxy-4'-fluoro-4-acetylaminobiphenyl (N-OH-FAABP) and *N*-hydroxy-4-acetylaminobiphenyl (N-OH-AABP) in a modified Solt-Farber protocol. Male Wistar rats, 200 g, were initiated with diethylnitrosamine (200 mg/kg, ip). 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 γ-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 µmole/kg/injection; N-OH-FAABP and N-OH-AABP at doses of 120 µmole/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 diethylnitrosamine, 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 γ-glutamyltranspeptidase-positive foci of preneoplastic cells was determined. In the animals that had received *N*-hydroxy-2-acetylaminofluorene, a high number of large foci were found (Figure 1). *N*-Hydroxy-4'-fluoro-4-acetyl- aminobiphenyl, 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-acetylaminobiphenyl (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-acetylaminofluorene 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 (CCl_4) 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-acetylaminofluorene and (to a lesser degree) *N*-hydroxy-4'-fluoro-4-acetylaminobiphenyl, are hepatocarcinogenic.

Table 3. Induction of γ-glutamyltranspeptidase positive foci (GGT^+) by *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF), *N*-hydroxy-4′-fluoro-4-acetylaminobiphenyl (N-OH-FAABP), *N*-hydroxy-4-acetylaminobiphenyl (N-OH-AABP), and *N*-hydroxy-2-acetylaminophenanthrene (N-OH-AAP) in the rat liver.

	Dose, µmole/kg	Number of foci/cm^2	Volume, mm^3/cm^3
Controls	–	80 ± 27	1.0 ± 0.3
N-OH-AAF[a]	30	148 ± 30[b,c]	8.4 ± 3.0[c]
N-OH-FAABP[a]	120	197 ± 62[c]	8.7 ± 4.1[c]
N-OH-AABP[a]	120	200 ± 37[c]	12.0 ± 4.1[c]
N-OH-AAP	60	249 ± 90[d]	15.7 ± 8.6[c]

Results are expressed as means ± SEM of 10 to 13 animals per treatment group. Compounds were injected ip 24 hr after partial hepatectomy. Promotion was by administration of 2-aminofluorene in drinking water (0.92 mM) for 2 weeks in combination with a necrogenic dose of CCl_4. For further experimental details, see Van de Poll et al. (*60*). [a]Data are from Van de Poll et al. (*60*). [b]Significantly different from controls. [c]$p < 0.05$. [d]$p < 0.1$.

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-Acetylaminophenanthrene is not hepatocarcinogenic (*35*). Several hepatic promoters have been administered after administration of 2-acetylaminophenanthrene, but this did not result in hepatocarcinogenicity (*36,37*). In this study, we found that *N*-hydroxy-2-acetylaminophenanthrene 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-aminophenanthrene (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-acetylaminofluorene is a potent clastogen (Figure 2): a high frequency of micronuclei was found already at a dose of 25 µmole/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 µmoles/kg (*41*). At doses higher than 25 µmoles/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-acetylaminophenanthrene 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-

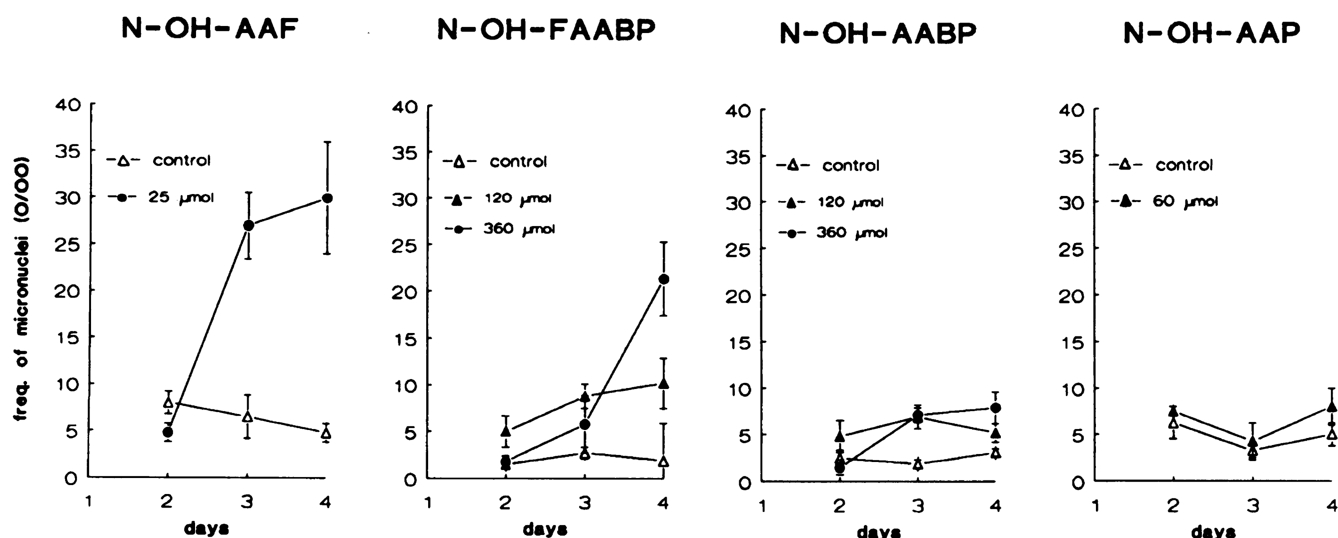

Figure 2. Frequency of micronuclei induced in rat liver *in vivo* by *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF), *N*-hydroxy-4′-fluoro-4-acetylaminobiphenyl (N-OH-FAABP), *N*-hydroxy-4-acetylaminobiphenyl (N-OH-AABP) and *N*-hydroxy-2-acetylaminophenanthrene (N-OH-AAP) injected 17 hr after partial hepatectomy. Hepatocytes were isolated 2, 3, and 4 days after injection. All values are mean ± SEM. Four to six animals were used for each time point.

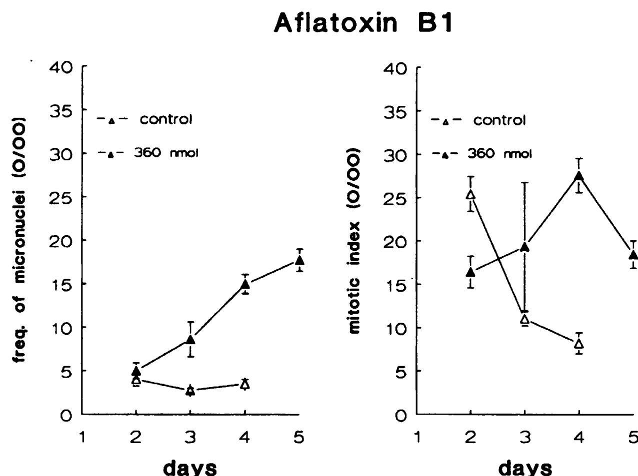

Figure 3. Frequency of micronuclei and mitotic index after administration of aflatoxin B1 (ip injection 17 hr after partial hepatectomy) in rat hepatocytes *in vivo*. Results are expressed as means ± SEM. Hepatocytes were isolated 2, 3, 4, and 5 days after injection. Four or five animals were used per time point. Data for controls are historic data from our laboratory (18–25 animals per time point).

acetylaminofluorene). This is consistent with our previous results, which showed a much reduced clastogenicity of *N*-hydroxy-2-acetylaminofluorene after inhibition of the fromation 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 B_1, 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, Tates 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 clastinogenic, 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 cyle 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 G_1-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 G_1 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 G_1.

[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.]

Preneoplastic cells proliferate during promotion with *N*-acetylarylamines. 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 G_1 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 *p*53 that result in escape from G_1 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*-acetylarylamine DNA adducts of various *N*-hydroxy acetylarylamines 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.

REFERENCES

1. Beland FA, Kadlubar FF. Metabolic activation and DNA-adducts of aromatic amines and nitroaromatic hydrocarbons. In: Handbook of Experimental Pharmacology, Vol 94/I (Cooper CS, Grover PL, eds). Berlin:Springer-Verlag, 1990; 267–325.
2. Daune MP, Fuchs RPP, Leng M. Carcinogenic and mutagenic *N*-substituted aryl compounds. Natl Cancer Inst Monograph 58:201–210 (1981).
3. Kriek E. Persistent binding of a new reaction product of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene with guanine in rat liver DNA *in vivo*. Cancer Res 32:2042–2048 (1972).
4. Westra JG, Kriek E, Hittenhaus H. Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA *in vivo*. Chem Biol Interact 15:149–164 (1976).
5. Beland FA, Dooley KL, Jackson CD. Persistence of DNA adducts in rat liver and kidney after multiple doses of the carcinogen *N*-hydroxy-2-acetylaminofluorene. Cancer Res 42:1348–1354 (1982).
6. Poirier MC, Hunt JM, True B, Laishes BA, Young JF, and Beland FA. Formation and removal of (guan-8-yl)DNA-2-acetylaminofluorene adducts in liver and kidney of male rats given dietary 2-acetylaminofluorene. Cancer Res 42:1317–1321 (1982).
7. Poirier MC, Fullerton NF, Patterson ED, Beland FA. DNA adduct formation and removal in hepatic chromatin fractions from rats chronically fed 2-acetylaminofluorene. Carcinogenesis 11:1343–1347 (1990).
8. Tamura N, King CM. Relationship between DNA distortion and the survival of arylamine-modified DNA-adducts in *E. coli.* Proc Am Assoc Cancer Res 31:626 (1990).
9. Lutgering JT, Retèl J, Loman H. Effects of adduct formation on the biological activity of single- and double-stranded ϕX174 DNA, modified by *N*-acetoxy-*N*-acetyl-2-aminofluorene. Biochim Biophys Acta 781:811–91 (1984).
10. Lutgering JT, Retèl J, Westra GJ, Welling MC, Loman H, Kriek E. By-pass of the major aminofluorene-DNA adduct during *in vivo* replication of single- and double-stranded ϕX174 DNA treated with *N*-hydroxy-2-aminofluorene. Carcinogenesis 6:1501–1506 (1985).
11. Tamura N, King CM. Comparative survival of aminobiphenyl- and aminofluorene-substituted plasmid DNA in *Escherichia coli* Uvr endonuclease deficient strains. Carcinogenesis 11:535–540 (1990).
12. Tang M-S, Lieberman WM, King CM. Uvr genes function differently in repair of acetylaminofluorene and aminofluorene DNA adducts. Nature 299:646–648 (1982).
13. Reid TM, Lee M-S, King CM. Mutagenesis by site-specific arylamine adducts in plasmid DNA: enhancing replication of the adducted strand alters mutation frequency. Biochemistry 29:6153–6161 (1990).
14. Koffel-Schwartz N, Maenhaut-Michel G, Fuchs RPP. Specific strand loss in *N*-2-acetylaminofluorene-modified DNA. J Mol Biol 193:651–659 (1987).
15. Moriya M, Takeshita M, Johnson F, Peden K, Will S, Grollman AP. Targeted mutations induced by a single acetylaminofluorene DNA aduct in cells and bacteria. Proc Natl Acad Sci USA 85:1586–1589 (1988).
16. Gupta PK, Pandrangi RG, Lee MS, King CM. Induction of mutations by *N*-acetoxy-*N*-acetyl-2-aminofluorene modified M13 viral DNA. Carcinogenesis 12:819–824 (1991).
17. Schaaper RM, Koffel-Schwartz N, Fuchs RPP. *N*-acetoxy-*N*-acetyl-2-aminofluorene-induced mutagenesis in the lacI gene of *Escherichia coli.* Carcinogenesis 11:1087–1095 (1990).
18. Beland FA, Dooley KL, Casciano DA. Rapid isolation of carcinogen-bound DNA and RNA by hydrosyapatite chromatography. J Chromatogr 174:177–186 (1979).
19. Meerman JHN, Mulder GJ. Prevention of the hepatotoxic action of *N*-hydroxy-2-acetylaminofluorene in the rat by inhibition of *N-O*-sulfation by pentachlorophenol. Life Sci 28:2361–2365 (1981).
20. Kriek E, Hengeveld GM. Reaction products of the carcinogen *N*-hydroxy-4-acetylamino-4′-fluorobiphenyl with DNA in liver and kidney of the rat. Chem Biol Interact 21:179–201 (1978).
21. Van de Poll MLM, Tijdens RB, Vondracek P, Bruins AP, Meijer DKF, Meerman JHN. The role of N-sulfation in the metabolic activation of *N*-hydroxy-4′-fluoro-4-acetylaminobiphenyl. Carcinogenesis 10:2285–2291 (1989).
22. Kadlubar FF, Beland FA, Beranek DT, Dooley KL, Heflich RH, Evans FE. Arylamine-DNA adduct formation in relation to urinary bladder carcinogenesis and *Salmonella typhimurium* mutagenesis. In: Environmental Mutagens and Carcinogens (Sugimura T, Kondo S, Takebe H, eds). New York:Liss, 1982; 385–396.
23. Kadlubar FF, Butler MA, Hayes BE, Beland FA, Guengerich FP. Role of microsomal cytochromes P-450 and prostaglandin H synthase in 4-aminobiphenyl-DNA adduct formation. In: Microsomes and Drug Oxidations (Miners J, Birkett DJ, Drew R, McManus M, eds). Taylor & Francis, 1988;370–379.
24. Beland FA, Beranek DT, Dooley KL, Heflich RH, Kadlubar FF. Arylamine-DNA adducts *in vitro* and *in vivo*: their role in bacterial mutagenesis and urinary bladder carcinogenesis. Environ Health Perspect 49:125–134 (1983).
25. Allaben WT, Weiss CC, Fullerton NF, Beland FA. Formation and persistence of DNA adducts from the carcinogen *N*-hydroxy-2-acetylaminofluorene in rat mammary gland *in vivo.* Carcinogenesis 4:1680–1686 (1985).
26. Westra JG, Flammang TJ, Fullerton NF, Beland FA, Weiss CC, Kadlubar FF. Formation of DNA adducts *in vivo* in rat liver and intestinal epithelium after administration of the carcinogen 3,2′-dimethyl-4-aminobiphenyl and its hydroxamic acid. Carcinogenesis 6:37–44 (1985).
27. DeBaun JR, Miller EC, Miller JA. *N*-hydroxy-2-acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and in protein-(methion-S-yl) binding in rat liver. Cancer Res 30:577–595 (1970).
28. Miller EC, Miller JA, Hartman HA. *N*-hydroxy-2-acetylaminofluorene: a metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. Cancer Res 21:815–824 (1961).
29. Gupta RC, Dighe NR. Formation and removal of DNA adducts in rat liver treated with *N*-hydroxy derivaties of 2-acetylaminofluorene, 4-acetylaminobiphenyl, and 2-acetylaminophenanthrene. Carcinogenesis 5:343–349 (1984).
30. Meerman JHN. The initiation of γ-glutamyltranspeptidase positive foci in the rat liver by *N*-hydroxy-2-acetylaminofluorene. The effect of the sulfation inhibitor pentachlorophenol. Carcinogenesis 6:893–897 (1985).

31. Kroese ED, Van de Poll MLM, Mulder GJ, Meerman JHN. The role of *N*-sulfation in the *N*-hydroxy-2-acetylaminofluorene mediated outgrowth of diethylnitrosamine-initiated hepatocytes to γ-glutamyltranspeptidase-positive foci in male rat liver. Carcinogenesis 9:1953–1958 (1988).
32. Van de Poll MLM, Lafleur MVM, Van Gog F, Vrieling H, Meerman JHN. *N*-Acetylated and decetylated 4′-fluoro-4-aminobiphenyl and 4-aminobiphenyl adducts differ in their ability to inhibit DNA replication of single-stranded M13 *in vitro* and of single-stranded ϕX174 in *Escherichia coli*. Carcinogenesis 13:751–758 (1992).
33. Moore PD, Rabkin SD, Strauss BS. Termnination of *in vivo* DNA synthesis at AAF adducts in the DNA. Nucleic Acids Res 8:4473–4484 (1980).
34. Van de Poll MLM, Venizelos V, Meerman JHN. Sulfation-dependent formation of *N*-acetylated and deacetylated DNA of *N*-hydroxy-4-acetylaminobiphenyl in male rat liver *in vivo* and in isolated hepatocytes. Carcinogenesis 11:1775–1781 (1990).
35. Miller JA, Sandin RB, Miller EC, Rusch HP. The carcinogenicity of compounds related to 2-acetylaminofluorene. II. Variations in the bridges and the 2-substituent. Cancer Res 15:188–199 (1955).
36. Scribner JD, Mottet NK. DDT acceleration of mammary gland tumors induced in the male Sprague-Dawley rat by 2-acetamidophenanthrene. Carcinogenesis 2:1235–1239 (1981).
37. Scribner JD, Woodworth B, Koponen G, Holmes EH. Use of 2-acetamidophenanthrene and 2-acetamidofluorene in investigations of mechanisms of hepatocarcinogenesis. Environ Health Perspect 49:81–86 (1983).
38. Gupta RC, Earley K, Fullerton NF, Beland FA. Formation and removal of DNA adducts in rats administered multiple doses of 2-acetylaminophenanthrene. Proc Am Assoc Cancer Res 26:86 (1985).
39. Evans HJ. Molecular mechanisms in the induction of chromosome aberrations. In: Progress in Genetic Toxicology (Scott P, Bridges BA, Sobels FH, eds). Amsterdam:Elsevier/North Holland, 1977;57–74.
40. Natarajan AT, Obe G, Van Zeeland AA, Palitti E, Meyers M, Verdegaal-Immerzaal, EAM. Molecular mechanisms involved in the production of chromosomal aberrations. II. Utilization of Neurospora endonuclease for the study of aberration production by X-rays in G1 and G2 stages of the cell cycle. Mutation Res 69:293–306 (1980).
41. Van de Poll MLM, Van der Hulst DAM, Tates AD, Mulder GJ, Meerman JHN. The role of specific DNA-adducts in the induction of micronuclei by *N*-hydroxy-2-acetylaminofluorene in rat liver *in vivo*. Carcinogenesis 10:717–722 (1989).
42. Ito N, Tatematsu M, Nakanishi K, Hasegawa R, Takano T, Imaida K, Ogiso T. The effects of various chemicals on the development of hyperplastic liver nodules in hepatectomized rats treated with *N*-nitrosodiethylamine or *N*-2-fluorenylacetamide. Gann 71:832–842 (1980).
43. Ito N, Tsuda H, Tatematsu M, Inoue T, Tagawa Y, Aoki T, Uwagawa S, Kagawa M, Ogiso T, Masui T, Imaida K, Fukushima S, Asamoto M. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione-S-transferase placental form positive foci in rats — an approach for a new medium-term bioassay system. Carcinogenesis 9:387–394 (1988).
44. Tates AD, Neuteboom J. Micronucleus test with hepatocytes to detect induction of clastogenic damage. In: Evaluation of Short Term Tests for Carcinogens. Report of the International Programme on Chemical Safety's Collaborative Study on *In Vivo* Assays, Vol I (Ashby J, de Serres FJ, Shelby MD, Margolin BH, Ishidate M, Berking GC, eds). Cambridge, England:Cambridge University Press, 1988;1400–1404.
45. Dock L, Scheu G, Jernstrom B, Martinez M, Torndal U, Eriksson L. Benzo[*a*]pyrene metabolism and induction of enzyme-altered foci in regenerating rat liver. Chem Biol Interact 67:243–253 (1988).
46. Van de Poll MLM. Clastogenicity and tumour promoting activity of carcinogenic arylamides in rat liver. Thesis. Leiden, The Netherlands:Leiden University, 1991; 143–160.
47. Gupta RC, Early K, Becker FF. Analysis of DNA adducts in putatative premalignant hepatic nodules and nontarget tissue of rats during 2-acetylaminofluorene carcinogenesis. Cancer Res 48:5270–5274 (1988).
48. Farber E. Clonal adaptation during carcinogenesis. Biochem Pharmacol 39:1837–1846 (1990).
49. Enomoto K, Farber E. Kinetics of phenotype modulation of remodeling of hyperplastic nodules during liver carcinogenesis. Cancer Res 42:2330–2335 (1982).
50. Tatematsu M, Takano T, Hasegawa R, Imaida K, Nakamowatori J, Ito N. A sequential quantitative study of the reversibility or irreversibility of liver hyperplastic nodules in rats exposed to hepatocarcinogens. Gann 70:843–855 (1980).
51. Saeter G, Schwarze PE, Nesland JM, Seglen PO. Acetylaminofluorene promotion of liver carcinogenesis by a non-cytotoxic mechanism. Carcinogenesis 9:581–587 (1988).
52. Titawech D, Hasegawa R, Kurata Y, Tatematsu M, Shibata MA, Thamavit W, Ito N. Dose-dependent effects of 2-acetylaminofluorene on hepatic foci development and cell proliferation in rats. Carcinogenesis 12:985–990 (1991).
53. Yerokun T, Norton TR, Hawell B, Ringer DP. Modulation of hepatic mRNA translation activity and specific expression of arylsulfotransferase IV during acetylaminofluorene-induced rat hepatocarcinogenesis. Cancer Res 51:504–509 (1991).
54. Kaderbhai MA, Bradshaw TK, Freedman RB. Alterations in the enzyme activity and polypeptide composition of rat hepatic endoplasmic reticulum during acute exposure to 2-acetylaminofluorene. Chem Biol Interact 39:279–299 (1982).
55. Kondo S, Carr BI, Takagi K, Huang TH, Chou YM, Yokoyama K, Itakura K. Expression of rat microsomal hydrolase gene during liver chemical carcinogenesis. Cancer Res 50:6222–6228 (1990).
56. Ringer DP, Kampschmidt K, King RL Jr, Jackson S, Kizer DE. Rapid decrease in *N*-hydroxy-2-acetylaminofluorene sulfotransferase activity of liver cytosols from rats fed carcinogen. Biochem Pharmacol 32:315–319 (1983).
57. Gilissen RAHJ, Meerman JHN. Bioactivation of the hepatocarcinogen *N*-hydroxy-2-acetylaminofluorene by sulfation in the rat liver changes during the cell cycle. Life Sci 51:1255–1260 (1992).
58. Lane DP. p53, Guardian of the genome. Nature 358:15–16 (1992).
59. Roberts E, Ahluwalia MB, Lee G, Chan C, Sorma DSR, Farber E. Resistance to hepatotoxins acquired by hepatocytes during liver regeneration. Cancer Res 43:28–44 (1983).
60. Van de Poll MLM, Van der Hulst DAM, Tates AD, Meerman JHN. Correlation between clastogenicity and promotion activity in liver carcinogenesis by *N*-hydroxy-2-acetylaminofluorene, *N*-hydroxy-4′-fluoro-4-acetylaminobiphenyl and *N*-hydroxy-4-acetylaminobiphenyl. Carcinogenesis 11:333–339 (1990).