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## REVIEW

# DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis

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**The heterocyclic amines (HCAs) are a family of mutagenic/carcinogenic compounds produced during the pyrolysis of creatine, amino acids and proteins. The major subclass of HCAs found in the human diet comprise the aminoimidazoazaarenes (AIAs) 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). All, except DiMeIQx, have been shown to be carcinogenic in animals. These compounds are present in cooked muscle meats at the p.p.b. level. Since the discovery of the HCAs in the late 1970s, many studies have examined the DNA adducts of these compounds. This review compiles the literature on AIA–DNA adducts including their identification and characterization, pathways of formation, mutagenesis *in vitro* and *in vivo*, and their association with carcinogenesis in animal models. It is now known that metabolic activation leading to the formation of DNA adducts is critical for mutagenicity and carcinogenicity of these compounds. All of the AIAs studied adduct to the guanine base, the major adduct being formed at the C8 position. Two AIAs, IQ and MeIQx, also form minor adducts at the $N^2$ position of guanine. A growing body of literature has reported on the mutation spectra induced by AIA–guanine adducts. Studies of animal tumors induced by AIAs have begun to relate AIA–DNA adduct-induced mutagenic events with the mutations found in critical genes associated with oncogenesis. Several studies have demonstrated the feasibility of chemoprevention of AIA tumorigenesis. Only a few studies have reported on the detection of AIA–DNA adducts in human tissues; difficulties persist in the routine detection of AIA–DNA adducts in humans for the purpose of biomonitoring of exposure to AIAs. The AIAs are nevertheless regarded as possible human carcinogens, and future research on AIA–DNA adducts is likely to help address the role of AIAs in human cancer.**

**Abbreviations:** AIA, aminoimidazoazaarene; AMS, accelerator mass spectrometry; BcPHDE, benzo[*c*]phenanthrene diol epoxide; CHL, chlorophyllin; CLA, conjugated linoleic acid; DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; dG-C8–IQ, *N*-(deoxyguanosin-8-yl)–IQ; dG-C8–MeIQ, *N*-(deoxyguanosin-8-yl)–MeIQ; dG-C8–MeIQx, *N*-(deoxyguanosin-8-yl)–MeIQx; dG-$N^2$–MeIQx, 5′-(deoxyguanosin-$N^2$-yl)–MeIQx; dG-C8-4,8–DiMeIQx, *N*-(deoxyguanosin-8-yl)-4,8–DiMeIQx; dG-C8–PhIP, *N*-(deoxyguanosin-8-yl)–PhIP; dG-$N^2$–IQ, 5′-(deoxyguanosin-$N^2$-yl)–IQ; GC–MS, gas chromatography–mass spectrometry; HCA, heterocyclic amine; I3C, indole-3-carbinol; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NAT, *N*-acetyltransferase; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; WBC, white blood cell.

## Introduction

Twenty years ago the discovery that cooked fish and beef showed highly mutagenic activity, as detected by the Ames/*Salmonella* test system (1,2), began an intensive search for the mutagens present in these foods. A number of studies showed that these mutagens were formed during the pyrolysis of amino acids and proteins, and during the cooking of a variety of muscle meats (1–6). The novel mutagens were identified as heterocyclic amines (HCAs). The major subclass of HCAs found in cooked meats was identified as the aminoimidazoazaarenes (AIAs), which includes those compounds with a quinoline, quinoxaline or pyridine moiety.

Studies to date indicate that the AIAs are present at the p.p.b. (ng/g) range in meats cooked by ordinary household methods (3–7). The formation of these compounds in meats depends largely on cooking temperature and duration (3,4,8,9). Chemical modeling has shown that several of the AIAs are produced by the reaction of creatinine and free amino acids at normal cooking temperatures (reviewed in refs 4,10). These compounds are distinct from the polycyclic aromatic hydrocarbons derived from the pyrolysis of fat in meat, which occurs, for example, during barbecuing. In addition to their mutagenic activity in *in vitro* assays, 10 HCAs bioassayed for carcinogenicity to date have all been shown to be carcinogenic in rats and/or mice (11,12); one of the AIAs, IQ, has also been shown to be carcinogenic in monkeys (13). The carcinogenicity of the AIAs most ubiquitously present in cooked beef (14) is summarized in Table I.

The HCAs, like the majority of chemical mutagens/carcinogens, form DNA adducts. It is generally accepted that DNA adducts of chemical carcinogens play a role in carcinogenesis; however, the relationship between DNA adducts and carcinogenesis is not fully understood (31). Since the identification of HCAs in cooked meats, considerable progress has been made in characterizing HCA–DNA adducts and clarifying pathways of metabolism necessary for DNA adduct formation. In addition, many investigations have attempted to relate HCA–DNA adduct formation to mutagenic and carcinogenic activity. In light of the considerable expansion of HCA research over the recent years, and the importance of increasing our understanding of the influence of DNA adducts in carcinogenesis, the current review examines HCA–DNA adducts and their possible role in carcinogenesis. Specific topics addressed in this review include (i) the characterization and identification of HCA–DNA adducts; (ii) the relationship between HCA–DNA adducts and mutagenic events; and (iii) the formation of DNA adducts in animal models as it relates to carcinogenesis. We have focused this review on the

**Table I.** Carcinogenicity of heterocyclic amines

| Compound | Strain/species | Target organs | Reference |
|---|---|---|---|
| IQ | F344 rat | Liver, small and large intestine, Zymbal gland, clitoral gland, skin | 15 |
| | Sprague–Dawley rat | Liver, mammary gland, Zymbal gland | 16 |
| | $CDF_1$ mouse | Liver, forestomach, lung | 17 |
| | $B6C3F_1$ mouse | Liver | 18 |
| | Monkey | Liver | 13 |
| MeIQ | F344 rat | Large intestine, Zymbal gland, skin, oral cavity, mammary gland | 19 |
| | $CDF_1$ mouse | Liver, forestomach | 20 |
| MeIQx | F344 rat | Liver, Zymbal gland, clitoral gland, skin | 21,22 |
| | $CDF_1$ mouse | Liver, lung, hematopoietic system | 23 |
| | $B6C3F_1$ mouse | Liver | 18 |
| PhIP | F344 rat | Large intestine, prostate, lymphoid tissue (males), mammary gland, large intestine (females) | 24,25,26 24,27 |
| | Sprague–Dawley rat | Mammary gland | 28 |
| | CD rat | Mammary gland | 29 |
| | $CDF_1$ mouse | Lymphoid tissue | 30 |
| | $B6C3F_1$ mouse | Liver | 18 |

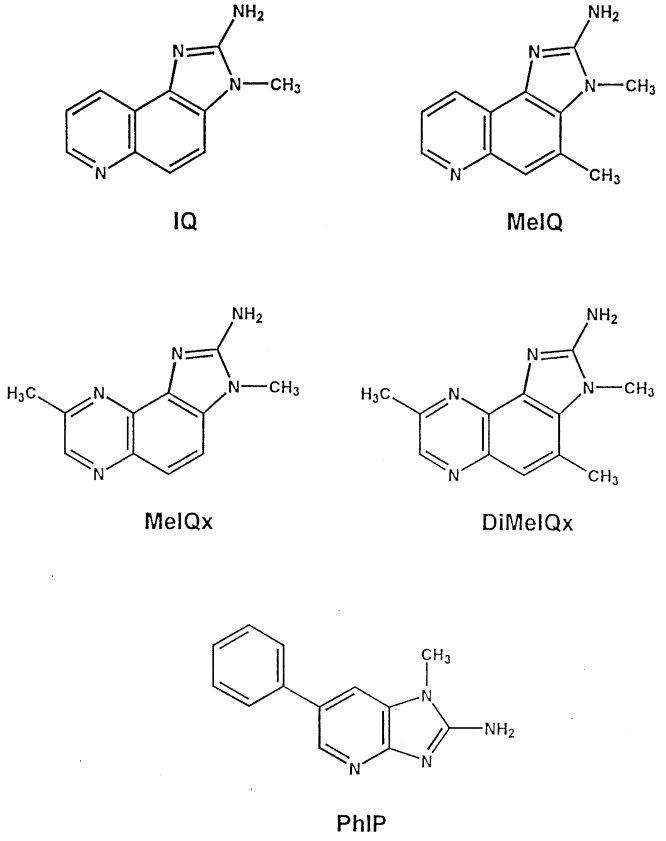


**Fig. 1.** Chemical structures of several of the AIA-type HCAs.

AIA-type HCAs (Figure 1), which are most consistently found in cooked meats and most prevalent in the human diet (14).

## Metabolic activation and AIA–DNA adduct formation

The AIAs are promutagens/pro-carcinogens that require metabolic activation for DNA adduct formation (32–71). The major pathway of AIA activation involves phase I hepatic cytochrome P450-mediated *N*-hydroxylation followed by phase II esterification of the *N*-hydroxylamines to reactive ester derivatives that covalently modify DNA. Numerous studies using inducers and inhibitors of cytochromes P450, antibodies to cytochromes P450, purified cytochromes P450 and recombinant enzymes have identified CYP1A2 as having high specificity and catalytic activity for AIA *N*-hydroxylation (33–67). Other cytochromes P450, including CYP1A1, CYP3A4, CYP2C9/10, CYP2A3 and CYP1B1, also carry out AIA *N*-hydroxylation, but these cytochromes generally show a lower capacity toward *N*-hydroxylation than CYP1A2 (46,48–54,57,59,61–63,68–71).

The *N*-hydroxylamine metabolites of the AIAs react relatively poorly with nucleic acids and require a second metabolic activation step to highly reactive ester derivatives to facilitate AIA–DNA adduct formation. To date, four mammalian phase II cytosolic enzymes have been implicated in the metabolic activation of the *N*-hydroxy-AIAs: *N*-acetyltransferase (NAT), sulfotransferase, prolyl tRNA synthetase and phosphorylase (35,36,57,58,67,72–81). These enzymes produce *N*-acetoxy, *N*-sulfonyloxy, *N*-prolyloxy and *N*-phosphatyl ester derivatives, respectively, at the exocyclic amino group (Figure 2). Many studies have now demonstrated that AIA–DNA adduct formation is greatly enhanced by phase II esterification of the *N*-hydroxylamine derivatives (35,36,67,72,74–82). For example, in an *in vitro* assay, NAT-mediated metabolism of *N*-hydroxy–IQ to *N*-acetoxy–IQ produced at least a 30-fold increase in IQ–DNA adduct levels (72). The four phase II activation pathways appear to give rise to the same AIA–DNA adduct(s) (76).

Several studies support the concept that the levels of AIA–DNA adducts formed in various tissues *in vivo* will largely depend on the balance between metabolic activation and detoxification (79,83–90). The liver is the major site of AIA metabolism *in vivo*, and hepatic phase I activation capacity, determined in large part by hepatic CYP1A2 levels, has a major impact on hepatic and as well as extrahepatic AIA–DNA adduct levels. For example, studies in monkeys and rats indicate that AIA–DNA adduct formation in liver and extrahepatic tissues is influenced by hepatic cytochrome P450-mediated AIA *N*-hydroxylation (79,83,84). The low level of MeIQx–DNA adducts in tissues of cynomolgus monkeys given MeIQx correlates with a low capacity for hepatic cytochrome P450-mediated *N*-hydroxylation of MeIQx in this species (63,83,84). In rats given PhIP, PhIP–DNA adducts are detected in colon despite the apparent lack of phase I activation of PhIP in colon (79). Recently, a breakdown product of *N*-acetoxy–PhIP was found in blood of rats given PhIP (79), which supports the idea that reactive metabolites, produced

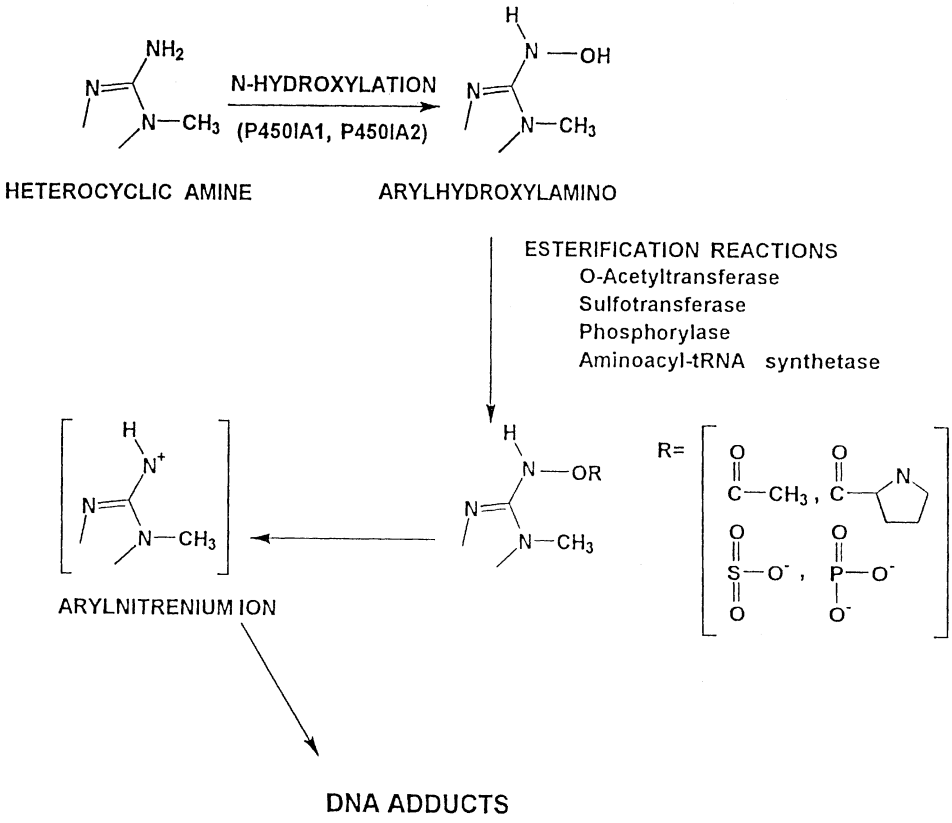


**Fig. 2.** Bioactivation of heterocyclic amines. Pathways of AIA metabolic activation leading to DNA adduct formation.

largely by hepatic metabolism, circulate via the bloodstream contributing to PhIP–DNA adducts in extrahepatic tissues such as colon (79) and mammary gland (90). In light of studies showing detectable AIA phase I metabolic activation by microsomes or cultured cells from several extrahepatic tissues, such as the lung, kidney, mammary gland and possibly pancreas (91–99), it is also possible that *in situ N*-hydroxylation will contribute to the formation of AIA–DNA adducts in several extrahepatic sites. In addition, DNA adducts in extrahepatic tissues might also arise from the metabolic activation of AIAs via prostaglandin H-synthetase (100–103). Recently the peroxygenase activity of cytochrome P450 has been shown to activate IQ to a reactive metabolite that binds to 2′-deoxyguanosine (104,105). The contribution of this pathway to tissue AIA–DNA adduct levels *in vivo*, however, is not yet known.

Extrahepatic phase II activity may also influence extrahepatic DNA adduct formation by contributing to the further metabolic activation or detoxification of the *N*-hydroxylamines or *N*-acetoxy metabolites derived from the circulation or generated *in situ* (79,88,90,106,107). In contrast to the comparatively low cytochrome P450-mediated activation capacity in extrahepatic tissue versus liver, phase II esterification activity in extrahepatic tissues is often similar to, or higher than, the activity found in liver (76,79,90). For example, in female rats, cytosolic NAT activation of *N*-hydroxy–PhIP was reported to be 16-fold higher in the mammary gland than in the liver (90). This difference in NAT activity may, in part, contribute to the 10-fold higher PhIP–DNA adduct levels in mammary gland than in liver in these animals (90,108).

The esters of *N*-hydroxy–AIAs generated by phase II metabolism are transient metabolites that react with nucleophilic sites in DNA. The ester moieties serve as leaving groups giving rise to putative electrophilic arylnitrenium ion intermediates (Figure 2) considered for many years to be involved in arylamine DNA adduct formation (109). Although to a much lesser extent, arylnitrenium ions may also be generated directly from the *N*-hydroxylamine metabolite following the protonation of the *N*-hydroxylamino group (109,110). This reaction mechanism explains the DNA adduction of certain *N*-hydroxy–AIAs, such as *N*-hydroxy–IQ, in the absence of esterification (110). The arylnitrenium ion is generally considered to be the ultimate carcinogenic form of the AIAs responsible for the formation of AIA–DNA adducts (109–115). The reactivity of the arylnitrenium ions and their carbenium ion resonance forms with particular nucleophilic sites on DNA give rise to specific DNA adducts, and with the AIAs, adducts have been found at the C8 and $N^2$ positions of guanine (110,116).

## Identification of AIA–DNA adducts

Several studies have examined AIA–DNA adduct formation by various methods, including the $^{32}$P-post-labeling assay (110–143). The structures of the various AIA–DNA adducts identified to date are shown in Figure 3. All form a major adduct with the C8 atom of guanine and, for IQ and MeIQx, a minor adduct with the $N^2$ atom of guanine has also been identified (110,116–122,128,129,134–138). *N*-acetoxy derivatives of several AIAs have also been shown to react with other bases *in vitro* (120,129,138), but such reactions appear to be of no quantitative importance *in vivo*. Studies that identified AIA adducts *in vitro* and *in vivo* are summarized in Table II.

Although each AIA forms one major guanine adduct, $^{32}$P-post-labeling analysis (130) has shown that most AIAs yield

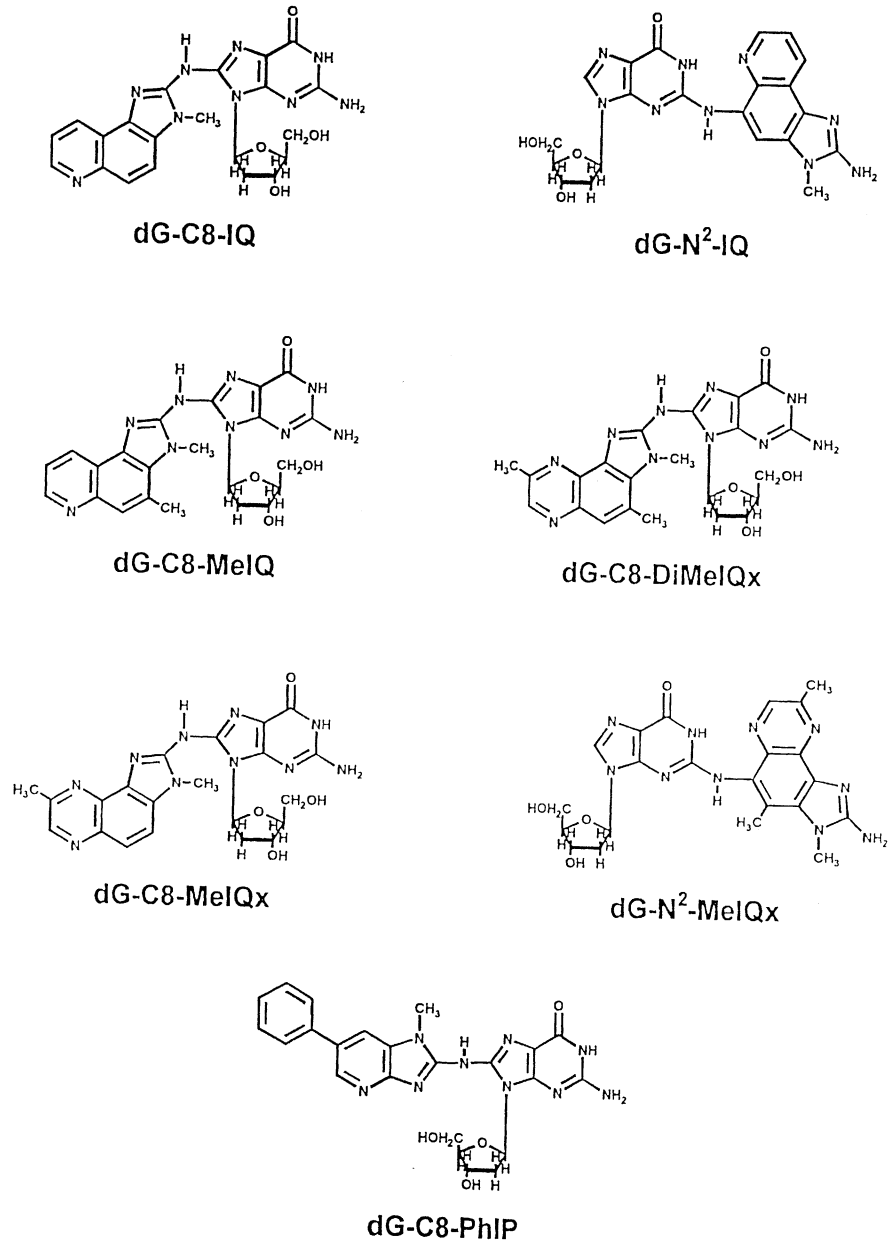


**Fig. 3.** Structures of the AIA–DNA adducts characterized to date. dG-C8–IQ, *N*-(deoxyguanosin-8-yl)–IQ; dG-N$^2$–IQ, 5′-(deoxyguanosin-*N*$^2$-yl)–IQ; dG-C8–MeIQ, *N*-(deoxyguanosin-8-yl)–MeIQ; dG-C8-4,8–DiMeIQx, *N*-(deoxyguanosin-8-yl)-4,8–DiMeIQx; dG-C8–MeIQx, *N*-(deoxyguanosin-8-yl)–MeIQx; dG-*N*$^2$–MeIQx, 5′-(deoxyguanosin-*N*$^2$-yl)–MeIQx; dG-C8–PhIP, *N*-(deoxyguanosin-8-yl)–PhIP.

multiple DNA adduct spots, especially in DNA from animals treated with AIAs (117,118,120–129,131–133,139–143). Several studies have indicated, however, that incomplete digestion of DNA to 3′-monophosphonucleotides prior to 5′ $^{32}$P-labeling may result in radiolabeled dimers and/or higher oligomers each containing the major adduct. Additional digestion procedures using nuclease P1 alone or with phosphodiesterase has been shown to reduce the multiple adducts observed with several HCAs to essentially one major adduct (128,129,142,143).

While the $^{32}$P-labeled adduct patterns of a particular AIA may differ greatly among various laboratories, the major identified DNA adduct for each AIA is similar in all species examined, including bacteria (*Salmonella*), rats, mice and monkeys (117–119,120–122,128,129,134–137,140). Examination of reaction products formed *in vitro* between *N*-acetoxy–PhIP and calf thymus DNA by fluorescence spectroscopy indicated the presence of four adduct fluorophores with different conformations (144). The data suggested heterogeneous formation of PhIP–DNA adducts in intact DNA but it could not be distinguished whether PhIP formed multiple adducts or if a given adduct existed in multiple conformations.

## Genotoxicity of HCA–DNA adducts in vitro

### *Mutagenicity in bacterial assays*

All the AIAs, with the exception of PhIP, are extremely potent mutagens in the Ames assay with GC frame-shift sensitive tester strains *Salmonella* TA98 and TA1538 (145,146). Mutagenicity of the AIAs in these bacterial strains is in the order of MeIQ > IQ > DiMeIQx ⩾ MeIQx > PhIP (3). Many studies in bacterial assays support the role of AIA–DNA

**Table II.** References for studies reporting the identification and detection of AIA–DNA adducts

| Adduct | Characterization[a] | Detection *in vitro*[b] | Detection *in vivo*[c] |
|---|---|---|---|
| dG-C8–IQ | 110,116 | 110,116 | 117–122 |
| dG-$N^2$–IQ | 116 | 116,119 | 119,121,122 |
| dG-C8–MeIQ | 128 | – | 128 |
| dG-C8–MeIQx | 116,129 | 116,129 | 116,120,129 |
| dG-$N^2$–MeIQx | 116 | 116 | – |
| dG-C8–DiMeIQx | 134 | 134 | 134,135 |
| dG-C8–PhIP | 136–138 | 136–138 | 136,137 |

[a]Adduct characterization is routinely carried out by nuclear magnetic resonance, mass and ultraviolet spectroscopy.
[b]AIA adducts are frequently produced *in vitro* by reacting *N*-acetoxy–AIA with calf thymus DNA or with deoxyguanosine. Prior to characterization, AIA adducts are purified from enzymatically hydrolyzed DNA or from the deoxyguanosine reaction mixture by high performance liquid chromatography (HPLC).
[c]AIA–DNA adducts have been detected *in vivo* by $^{32}$P-post-labeling or HPLC analysis of DNA from animals treated with AIAs.

adduct formation in mutagenesis (3,4,145–156). For example, in studying the relationship between PhIP–DNA adduct levels and mutagenicity, Malfatti *et al*. (149) determined that ~25 adducts are required for one mutational event in *Salmonella* TA98. In addition, Kerdar *et al*. (148) showed that there was a linear relationship between DNA binding and mutagenicity among several AIAs: the more potent the mutagen, the higher the level of adducts in bacterial DNA. Therefore, the difference in mutagenicity among the AIAs is largely attributable to quantitative differences in DNA adduct levels rather than to qualitative differences between AIA adducts.

In *Salmonella* strain TA1538, IQ, MeIQ and PhIP caused a single GC deletion in a run of GCs in the *hisD* gene, which is consistent with the known preference for AIA–DNA adduct formation at guanine (146,157). In addition, in the base substitution *Salmonella* strain TA100, PhIP induced predominantly GC→TA transversion; error-prone bypass of the dG-C8–PhIP adduct appeared to be associated with the induction of this mutation (156). Consistent with the lack of formation of adenine adducts, AIAs were poor mutagens in *Salmonella* strains specific for AT frameshift changes (3). AIA mutagenicity studies in other bacterial genes such as *lacZ*, *lacZa* and *lacI* of *Escherichia coli* further showed that mutations occur primarily at G:C pairs; however, the types of guanine mutations (base substitution or frameshift) were different among the various assays (158–160).

*Genotoxicity and mutagenicity in mammalian cells*

The genotoxicity of the AIAs in cultured mammalian cells has been assessed by multiple end points including mutagenesis, chromosomal aberrations, sister-chromatid exchange, DNA repair synthesis and DNA strand breaks (34,55,161–181). Whereas AIAs are potent frameshift mutagens in *Salmonella* bacterial reversion assays, in mammalian cell mutagenicity assays, AIA–DNA adducts induce primarily base substitution mutations at guanine (175–177,180). PhIP- and/or IQ-induced mutations have been examined in the *hprt*, *dhfr* and *aprt* genes of Chinese hamster ovary cells, and in the *hprt* gene of human lymphoblastoid cells (175–177,180). For both compounds, G→T transversion mutations in guanine nucleotides adjacent to either guanine or adenine nucleotides occurred most frequently. In the *supF* shuttle vector mutagenicity assay, ~25% of the mutations induced by PhIP in repair-proficient mammalian cells were at guanine in the sequence 5′-GCAGA-3′ (174). In the *dhfr* gene of Chinese hamster ovary cells treated with PhIP, recurring guanine mutations were also observed at CAGG, and one of 20 characterized mutants harbored a mutation in the sequence CAGA (175). AIA-induced frameshift mutations found in mammalian cells also largely involved a deletion of a guanine base adjacent to guanine and/or adenine (175,176,180). Yadollahi-Farsani *et al*. (180) reported finding a –1 frameshift mutation (guanine deletion) in a 5′-GGGA-3′ sequence in the *hprt* gene of Chinese hamster ovary cells exposed to PhIP that accounted for four of seven frameshift mutations observed in their study.

The role of AIA–DNA adducts in mutagenesis was explored in the *supF* shuttle vector replicated in mammalian cells (161,174). Mutation frequency increased linearly with IQ and PhIP–DNA adduct levels in the vector. $^{32}$P-post-labeling analysis showed that IQ and PhIP formed adducts only at guanine nucleotides in the *supF* plasmid, with the C8–guanine adduct being the major lesion (174). Using a polymerase arrest assay, these guanine adducts were shown to be distributed non-randomly in the *supF* gene with the concentration of adducts being different at different guanine sites. Although mutations were found at sites where IQ– and PhIP–guanine adducts formed, adduct level did not correlate with the likelihood of a mutation at a particular site. Therefore, the frequency of a mutation at a particular guanine nucleotide in the *supF* gene appeared to be attributable to other factors besides adduct levels. A similar observation was made in studies concerning MeIQx–DNA adducts in the *E.coli lacZa* gene (160).

In summary, the *in vitro* mutagenicity studies to date point to preferred sites for AIA–DNA adduct-driven mutations in mammalian genes. In support for the role of AIA adducts in G→T transversion mutations, a site-specific mutagenesis study recently reported that the C8-guanine adduct of PhIP induced this mutation (182). It is anticipated that the results from *in vitro* mutagenicity studies will provide insight into the role of AIA–DNA adducts in mutations found in genes associated with AIA-induced carcinogenesis.

## Genotoxicity and mutagenicity of AIA–DNA adducts in vivo

Cytogenetic assays, including chromosome aberrations, micronucleated normochromatic erythrocytes and sister chromatid exchanges of bone marrow and peripheral blood of rodents dosed with AIAs, indicate that the AIAs and, by extension, AIA–DNA adducts, appear to be only weakly clastogenic *in vivo* (183–187). In contrast, AIAs have been shown to induce base substitution and/or deletion mutations *in vivo* in the *lacI* or *lacZ* mutational reporter gene of transgenic mice (187–193), the colonic crypt cells of mice (194–196), *Dlb-1*-specific locus test in mouse small intestine (191,197,198) and the animal-mediated microbial assay system (199).

In *lacI* and *lacZ* transgenic mice, mutant frequency appears to depend on the dose and the duration of exposure to AIAs suggesting that the higher the level of adducts is and the more persistent the adducts are, the greater is the mutagenic effect in these mice (187,190,191). The role of persistent AIA adducts in mutagenesis *in vivo* is illustrated by the following example. In the *lacZ* gene in liver of the Muta™Mouse, the mutant frequency was 15- to 20-fold higher after exposure to MeIQx for 30 weeks than after just 10 days (189,193). This large increase in mutant frequency with chronic exposure to MeIQx occurred as the concentration of MeIQx–DNA adducts in liver

increased only 2-fold. Therefore, the likelihood of a mutagenic event increases with prolonged exposure to AIA–DNA adducts *in vivo*, apparently as ample time is provided for the fixation of mutations during cellular replication.

Tissue-specific factors, such as the rate of cell proliferation and cell death, play an important role in the mutagenicity of AIA–DNA adducts *in vivo*. Mutations in the *lacI* and *lacZ* genes of transgenic mice have also been shown to vary among tissue sites (187,188,190,191). In addition, the frequency of certain types of mutations was also different between organs indicating that multiple tissue-related factors potentially influence the mutagenic spectra induced by AIA–DNA adducts. In *lacI* transgenic mice on a diet of 0.03% MeIQ for up to 12 weeks, mutant frequency was highest in the colon, followed by the bone marrow, liver and forestomach; however, there was no increase in mutant frequency in heart, a tissue showing little proliferation (187). Over-expression of the *c-myc* oncogene has also been shown to increase the frequency of AIA-induced mutations in the *lacZ* reporter gene in liver of c-*myc*/*lacZ* double transgenic mice (189,193). The increased rate of hepatocellular proliferation associated with c-*myc* overexpression (200) may partly explain the higher mutant frequency in c-*myc* transgenic mice.

Whether *lacZ* or *lacI* reporter genes are general predictors of sites of chemical-induced carcinogenesis is still uncertain (190). Nevertheless, a recent study suggested that the mutational characteristics of individual AIAs in the *lacI* reporter gene of transgenic mice may represent the mutations induced by these AIAs in oncogenes and tumor suppressor genes involved in carcinogenesis. For example, PhIP was shown to induce a single-base deletion in the *lacI* gene in colon of transgenic mice that was characteristic of the single-base deletions found in the *Apc* gene in PhIP-induced rat colon tumors (192). The possible role of AIA–DNA adducts in carcinogenesis is discussed more fully below.

## Implications of HCA–DNA adduct formation in animal models

### *Carcinogenesis: DNA adducts and target organ specificity*

All of the AIAs demonstrated to be carcinogenic in rats and mice, including IQ, MeIQx, PhIP and MeIQ, form tissue DNA adducts in these species (79,89,90,118,121–141,201–212). In the liver, a target tissue for carcinogenesis by many AIAs (Table I), animal studies are consistent with the notion that AIA–DNA adduct levels are associated with carcinogenesis. Generally, the AIAs that induce hepatocellular carcinoma produce relatively high DNA adduct levels in the liver. IQ, but not MeIQx, is a potent hepatocarcinogen in cynomolgus monkeys (13,201,202). Accordingly, hepatic adduct levels in monkeys are ~50- to 100-fold higher with IQ than with MeIQx (83,120, 201). Both IQ and MeIQx are hepatocarcinogens in $CDF_1$ mice and Fischer-344 rats and both compounds produce relatively high hepatic DNA adduct levels (125,132,133,203–207). In contrast, PhIP, a compound that is not a hepatocarcinogen in adult mice or rats, forms relatively low hepatic DNA adduct levels in comparison with extrahepatic tissues, including the target organs mammary gland, prostate and colon (25,79,108,205,207,208).

Studies with MeIQx in rats are also consistent with the role of AIA–DNA adducts in hepatocarcinogenesis (22,132,133). MeIQx–DNA adduct levels in liver increased in a linear dose–response manner in rats fed various concentrations of MeIQx up to 400 p.p.m. (132,133). At 100, 200 and 400 p.p.m. MeIQx, the incidence of hepatocellular carcinoma was 0, 45 and 94%, respectively (22). These findings raise the possibility that a threshold dose of MeIQx, and perhaps a minimum level of MeIQx adducts, are needed for the induction of hepatocarcinogenesis. However, other factors associated with carcinogen exposure, such as tumor promotion, are also expected to play a role in hepatocarcinogenesis. It is noteworthy that MeIQx exposure is associated with the formation of 8-hydroxyguanine (213), a major species of oxidative DNA damage, which may also contribute to the carcinogenic effects of this compound.

Although the results from AIA–DNA adduct and carcinogenicity studies in liver support the role of AIA–DNA adducts in carcinogenesis, the finding that non-target tissues have relatively high AIA–DNA adduct levels belie a simple relationship between adducts and cancer (79,125,139,203–211). IQ–DNA adduct levels in Fischer-344 rats were reported to be highest in the liver, followed, in order, by the lungs, kidneys, stomach, colon, white blood cells (WBCs) and small intestine (205). Of these, only the liver, small intestine and colon are target organs in a carcinogenicity bioassay in Fischer-344 rats. The lungs, kidneys and stomach do not succumb to the carcinogenic effects of IQ, although adduct levels in these organs are higher than those in target tissues, including the colon and small intestine. As elevated AIA–DNA adduct levels are not observed in all target organs, it is possible that the threshold for the initiation of carcinogenesis by these adducts is unique among different tissues.

Neither the persistence of total AIA–DNA adducts nor the formation and persistence of specific AIA adducts appear to correlate with target organ specificity (121,122,133). In rats and monkeys, dG-C8–IQ, the principal IQ–DNA adduct formed *in vivo*, is removed more rapidly than dG-$N^2$–IQ in slowly dividing tissues, such as liver and kidney (121,122). In monkeys that develop hepatocellular carcinoma after chronic feeding with IQ, dG-$N^2$–IQ is the predominant adduct found in liver (122). However, this adduct is also the predominant adduct found in non-target tissues in monkeys, including kidney, pancreas and heart.

In summary, it is apparent that without metabolic activation and DNA adduct formation, AIAs would not be carcinogenic in animal models. However, since AIA–DNA adducts are found in both target and non-target tissues for AIA carcinogenesis, the relationship between AIA–DNA adduct formation and tumorigenesis is not a simple matter of cause and effect. In the subsequent section, the possible link between AIA–DNA adducts and carcinogenesis is further examined in a review of studies on mutations in specific oncogenes in AIA-induced tumors.

### *DNA adducts and oncogene activation in AIA-induced tumors*

Specific mutations are critical for the activation of oncogenes and inactivation of tumor suppressor genes associated with carcinogenesis (214–216). Several studies have examined AIA-induced tumors for mutations in genes including p53, Ki-*ras* and Ha-*ras*, *Apc* and B-catenin (217–230). Among the mutations detected in these genes, guanine base mutations occurred with the highest frequency, suggestive of the involvement of AIA–DNA adducts in these genetic alterations. For example, mutations in either Ha-*ras* and Ki-*ras* were found in nine of nine Zymbal gland tumors induced by IQ (224). Eight of these mutations occurred at guanine bases in

codons 13 of H-*ras* or 12 and 13 of Ki-*ras*. Seven mutations were transversion mutations, characteristic of IQ-induced mutations *in vitro* (174). Only one IQ-induced Zymbal gland tumor carried an adenine mutation in codon 61 of Ha-*ras*.

Recently, colon tumors induced by IQ and PhIP were shown to harbor either mutations in *Apc* or B-catenin, providing evidence for a role of B-catenin/*Apc* pathways in the development of AIA-induced colon tumors (230). In addition, Kakiuchi *et al*. (229) showed that four of eight PhIP-induced colon tumors in F344 rats carried a 5′-GGGA-3′ to 5′-GGA-3′ frameshift mutation in the *Apc* gene. This mutation was also the most common of the frameshift mutations found in the *hprt* gene of Chinese hamster ovary cells exposed to PhIP (180). Interestingly, a guanine deletion in this and similar sequence contexts was observed in other *in vitro* mutagenicity assays with PhIP in the *hprt* gene of human lymphoblastoid cells, in the *supF* shuttle vector system and in the *dhfr* gene of Chinese hamster ovary cells (174–176), as well as in the *lacI* transgenic mouse model after PhIP treatment (192). These studies provide supporting evidence for a mutational fingerprint for PhIP–DNA adduct formation that involves a –1 frameshift hotspot in a run of guanine nucleotides adjacent to an adenine. The finding that this same mutation occurs in the *Apc* gene in rat colon tumors induced by PhIP (229,231) supports the importance of this specific PhIP–DNA adduct induced mutation in colon carcinogenesis. It also raises the possibility that this specific mutation in the *Apc* gene in human colorectal tumors may serve as a fingerprint for PhIP exposure and a means to assess the role of PhIP as an etiological factor in human colorectal cancer (180,214,229).

*DNA adducts and cardiotoxicity*

In both monkeys and rats fed IQ and PhIP, high levels of carcinogen–DNA adducts have been observed in the heart (126,139,209,232). Ultrastructural studies on the myocardium of monkeys undergoing carcinogenicity bioassay with IQ revealed myocyte degeneration with mitochondrial abnormalities (233). Similar changes were also observed in rats given IQ or PhIP (234,235). Both PhIP and IQ formed adducts in mitochondrial DNA (236), a factor that may contribute to mitochondrial damage. However, the role of adducts in AIA cardiotoxicity is not fully known. In isolated cardiomyocytes, *N*-acetylcysteine was shown to protect against *N*-hydroxy–IQ- and *N*-hydroxy–PhIP-induced cardiotoxicity, but the protective effect of *N*-acetylcysteine in isolated cardiomyocytes was not associated with a reduction in DNA adduct levels (237). It is possible that the reactive metabolites of IQ and PhIP may have other toxic effects on cardiomyocytes besides DNA adduct formation that are mitigated by *N*-acetylcysteine. Superoxide radicals have been suggested to be produced from AIA metabolism (238); whether these radicals play a role in the cardiotoxic properties of AIAs requires further investigation.

## Inhibition of AIA–DNA adduct formation and chemoprevention of AIA-induced tumors

A number of cancer chemopreventive agents act by inhibiting the initiation phase of chemical carcinogenesis. One of the approaches in monitoring such inhibitory effects is by evaluating carcinogen–DNA adduct formation, and relating the extent of inhibition of this process to antimutagenic/anticarcinogenic effects of the agent. A variety of dietary components inhibit the mutagenicity of HCAs in the Ames *Salmonella* assay. These include hemin, chlorophyllin (CHL), retinol, fatty acids (arachidonic acid, oleic acid, linoleic acid, eicosapentaenoic acid, docosahexaenoic acid), flavonoids (morin, myricetin, quercetin, anthraflavic acid), polyphenols (ellagic acid, green tea polyphenols) and tryptamine (reviewed in refs 239,240). Human urine may also contain substances that inhibit HCA-induced bacterial mutagenicity (241); whether these substances are of dietary origin is not known.

In *in vivo* studies in rodents, a number of agents have been shown to inhibit AIA–DNA adduct formation in various organs, usually including the target organ (Table III) (242–256). The dietary agents studied most widely include CHL, conjugated linoleic acid (CLA) and indole-3-carbinol (I3C) (242–247). CHL is thought to act by non-covalent complexing with the AIA so that less is available for activation (242–245); it may also inhibit CYP1A2 (246). The mechanism by which CLA inhibits AIA–DNA adduct formation is not fully understood (248–250) whereas I3C appears to enhance AIA detoxification (247,254,255).

Whereas the chemopreventive properties of the inhibitors listed in Table III are known from studies in other model systems, the relationship between inhibition of AIA–DNA adducts and inhibition of AIA tumorigenesis has only been established for CHL as the inhibitor and IQ as the carcinogen (242,244,257). Phenethyl isothiocyanate, however, a well known experimental chemopreventive agent with other chemical carcinogens, did not inhibit PhIP–DNA adduct formation in liver and colon of male Swiss Albino mice (258).

With one exception (256), the dietary concentrations of the inhibitors listed in Table III were not toxic to the animals. In certain organs, or with certain protocols, however, both CHL and I3C may act as tumor promoters (257,259–261). Further studies are therefore needed on the mechanism of action of these agents and on their chemopreventive effects in AIA-induced tumorigenesis. In several studies, the relationship between inhibition of AIA–DNA adduct formation and inhibition of aberrant crypt foci formation, a putative pre-cancerous lesion in the colon, has already been established (247,249,254,255). In addition, several studies have been conducted on chemoprevention of AIA-induced tumors. Dietary CHL inhibited the multiplicity, but not the incidence, of PhIP-induced mammary adenocarcinomas in female F344 rats (262). The addition of lyophilized cultures of *Bifidobacterium longum* to the diet (0.5%) inhibits IQ-induced tumors in F344 rats (263). Dietary calcium inhibits PhIP-induced aberrant crypt foci formation in male F344 rats on low fat diets (264), whereas dietary oltipraz inhibits PhIP-induced lymphomas in male F344 rats (26). A number of antioxidants have been shown to possess anticarcinogenic properties against AIAs (265–267) but, with the exception of the studies on green and black tea (254), effects on DNA adduct formation were not evaluated in these studies.

## AIA–DNA adducts and human cancer risk

Carcinogen–DNA adducts are regarded as biomarkers of potential mutagenic events and of cancer risk (31,268). Measurement of AIA–DNA adducts in tissues from humans eating customary diets, however, has proven to be difficult. For the purpose of biomonitoring and risk assessment, a satisfactory method to routinely measure AIA–DNA adducts in people has not yet been developed.

Several laboratories have attempted to measure AIA–DNA adducts in tissues or WBCs from humans eating customary

**Table III.** *In vivo* inhibition of AIA–DNA adduct formation in rodents

| Inhibitor[a] | AIA[b] | Species (gender[c]) | Organ | Reference |
|---|---|---|---|---|
| CHL | IQ | F344 rat (m) | Liver, small intestine, colon | 242–244 |
| CHL | PhIP | F344 rat (m) | Cecum, liver, heart | 247 |
| CLA | IQ | $CDF_1$ mouse (m,f) | Liver, lungs, colon, kidneys | 248 |
| CLA | IQ | F344 rat (m) | Colon | 249 |
| CLA | PhIP | F344 rat (f) | Liver, mammary gland | 250 |
| Menhaden oil | IQ | F344 rat (m) | Liver, lungs, stomach, kidneys | 251 |
| Menhaden oil | IQ | $CDF_1$ mouse (m) | Stomach, cecum, colon, kidneys | 252 |
| ω-3 Fatty acids | PhIP | $CDF_1$ mouse (m) | Liver, stomach, small intestine, cecum, spleen | 253 |
| I3C | PhIP | F344 rat (m) | Colon, cecum, heart, spleen | 247 |
| I3C | IQ | F344 rat (m) | Colon | 254,255 |
| I3C | PhIP | F344 rat (f) | Liver, colon, mammary gland | 208 |
| 4-Ipomeanol | IQ | $CDF_1$ mouse (m) | Liver, lungs, stomach | 256 |
| Green tea | IQ | F344 rat (m) | Liver | 254 |
| Black tea | IQ | F344 rat (m) | Liver | 254 |

[a]The inhibitor typically was provided in the diet (most of the time powdered AIN-76A diet). Green tea and black tea were provided as the sole source of drinking water.
[b]The AIA was provided in the diet, or given i.p. or p.o. (single dose or multiple doses).
[c]m, male; f, female.

diets. One study reported the detection of PhIP–DNA adducts in surgical samples of human colon by $^{32}$P-post-labeling and gas chromatography–mass spectrometry (GC–MS) (210). Out of a total of 24 individual human tissue samples, including pancreas ($n$ = 12), colon mucosa ($n$ = 6) and urinary bladder epithelium ($n$ = 6), only two of the colon samples had detectable DNA adducts (210). In another study (269), a total of 38 human tissues (colon, rectum, liver, kidney, pancreas, lung, heart), obtained from surgery or autopsy, were examined for the presence of dG-C8–MeIQx by $^{32}$P-post-labeling. Only three specimens (one each of colon, rectum and kidney) were found to have detectable levels (1.8–14 adducts/$10^{10}$ nucleotides).

Human WBCs are an easily accessible source of DNA for DNA adduct studies and potentially for biomonitoring of AIA exposure. However, in humans consuming meals with well-done meat, WBC AIA–DNA adducts are non-detectable by the $^{32}$P-post-labeling method (P.T.Strickland and H.A.J.Schut, unpublished data). Studies in rats receiving daily doses of PhIP approximating the human daily intake (0.1 μg/kg) have shown that at this dose, or at doses that are even 10- or 100-fold higher, PhIP–DNA adducts are also non-detectable in tissues or WBCs when analyzed by $^{32}$P-post-labeling (211). Therefore, for the purpose of evaluating the potentially genotoxic dose of AIA derived from the diet in humans, the likelihood of detecting AIA–DNA adducts in WBCs using established methods is low.

Until recently, one of the most sensitive methods for detecting DNA adducts was $^{32}$P-post-labeling (270). This method has been applied to the detection of DNA adducts in situations where exposure to genotoxins is expected to be high, such as in smokers or in workers in certain occupational settings (268). The recent advent of the use of accelerator mass spectrometry (AMS) has made it possible to measure DNA adducts at levels as low as 1–10 adducts/$10^{12}$ nucleotides (271–276), i.e. several orders of magnitude lower than the most sensitive version of the $^{32}$P-post-labeling assay (270). Using AMS it has been possible to detect AIA–DNA adducts in tissues after dosing animals with amounts equivalent to human dietary exposures (273–275). Recently both MeIQx– and PhIP–DNA adducts have been detected in colon of humans given dietary equivalent levels of these AIAs labeled with $^{14}$C (K.W.Turteltaub, personal communication). These studies indicate that at doses derived from the human diet, AIAs are potentially genotoxic and carcinogenic at specific target sites. In combination with the existing epidemiological evidence linking consumption of well-cooked meat with colorectal cancer risk (14,277–284), the results from AMS may eventually help to determine if human colorectal cancer risk is associated with AIA–DNA adduct formation.

## Perspectives

There is considerable evidence to indicate that man is exposed to AIAs through the diet and is a species susceptible to the carcinogenic effects of AIAs (40,45,46,65,285–296). The unresolved question, however, is whether humans are exposed to levels of AIAs sufficient to play a significant role in human cancer incidence. The overall estimates of cancer risk from HCAs, calculated based on feeding studies in rodents (Table I), appear, so far, to be relatively small and insufficient to associate their presence directly with human cancer (14,297,298). However, there is increasing evidence to support that the risk of colorectal cancer and other cancers is higher in a subset of individuals who have particularly high intakes of well-done cooked meats likely to be rich in AIAs (279–284,299). Although there is wide interindividual variation in the capacity of human tissues to activate AIAs (45,83,293,300), in the case of colorectal cancer, risk appears to be especially elevated when high exposure is coupled with a strong capacity to metabolically activate the AIAs (283,284) which in turn implies the possibility of higher AIA–DNA adduct levels. The data summarized in this review support the notion that AIA–DNA adducts play a fundamental role in susceptibility to AIA carcinogenesis. As shown in animal studies, AIA–DNA adduct levels in target tissues are generally predictive of tumor incidence. Development of novel methods to detect AIA–DNA adducts in human tissues or the use of biomarkers of AIA–DNA adduct mediated genotoxic events is likely to provide further insight into the role of AIA–DNA adducts in carcinogenesis in humans.

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