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# Genotoxicity of Aristolochic Acid: A Review

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(The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.)

## ABSTRACT

Aristolochic acid (AA), a mixture of aristolochic acid I (AAI) and aristolochic acid II (AAII), is present in *Aristolochiaceae* plants, many of which are used as herbal folk remedies. Plants containing AA, however, can be nephrotoxic, genotoxic and carcinogenic in humans. AA has been also associated with the development of tumors in mice and rats. Therefore, plant products containing AA have been banned in many countries. Because quantitative cancer risk assessment is based upon an understanding of the chemical's mode-of-action, it is necessary to determine whether the chemical is a mutagenic carcinogen. In this review, we present the available information concerning the genotoxicity of AA and discuss the possible mechanisms for mutation induction by AA. The evidences indicate that AA is mutagenic and this activity is mediated mainly by the formation of AA-DNA adducts. Not only does AA induce genetic damage and mutations in bacteria, mammalian cells, *Drosophila*, and rodents, but it is also demonstrated to induce mutations in the target tissues of the model animals and oncogenes from human. Many evidences from genotoxicity tests also indicate that AA is a clastogenic agent that breaks DNA and results in chromosome damage and chromosome mutations. These results indicate that AA is a mutagenic carcinogen.

Key words: aristolochic acid, genotoxicity, DNA adduct, clastogenicity, mutation, mutagenicity, oncogene

## INTRODUCTION

Aristolochic acid (AA, CAS No. 10190-99-5) is a mixture of aristolochic acid I (AAI, 3,4-methylenedioxy-8-methoxy-10-nitrophenanthrene-1-carboxylic acid, molecular weight 341.276, CAS No. 313-67-7) and aristolochic acid II (AAII, 3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid, molecular weight 311.250, CAS No. 475-80-9)[1] (Figure 1). AA is an active component of herbal drugs derived from *Aristolochiaceae* family of plants. These herbal drugs containing AA have been used for medicine purposes since antiquity worldwide, such as for treatment of snake bites, arthritis, gout, rheumatism, and festering wounds, as well as used in obstetrics[2-8].

Plants containing AA, however, can be nephrotoxic, genotoxic and carcinogenic in humans[7,9-19]. In the early 1992, cases of so-called Chinese herbs nephropathy (CHN), more appropriately replaced later by aristolochic acid nephropathy (AAN)[20] were reported in Belgium[21]. An outbreak of rapidly progressive renal fibrosis in Belgium involved at least 100 patients, mostly middle-aged women undergoing a weight-loss regimen that included use of a mixture of Chinese herbs containing *Aristolochia* species incorrectly labeled as *Stephania tetrandra*. About half of these AAN patients had renal replacement therapy[21-25]. Similar AAN cases subsequently were observed in many countries and repeatedly reported[26-34]. Soon thereafter, AA-associated urothelial cancer was reported, with near 50% incidence of upper urinary tract urothelial malignancy found in Belgian AAN patients[9,35,36]. Invasive urothelial carcinoma also were reported in patients without severe renal failure after exposure to Chinese herbal medicine containing AA[37,38]. AA is also associated to the etiology of Balkan endemic nephropathy-associated urothelial cancer[39]. Specific AA-derived DNA adducts were found in the kidney, ureter, bladder, liver, lung, and spleen of the AAN patients[40-42], providing strong evidence linking the use of herbal products containing AA with cancer development.

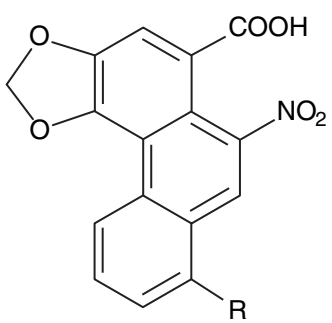


**Figure 1.** Chemical structural of aristolochic acids I ($R = OCH_3$) and II ($R = H$).

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Animal studies show that AA results in renal failure in rodents[11] and induces tumors in the kidney and other tissues of rabbits, rats and mice[16,17,19,43,44]. When rabbits were injected intraperitoneally with 0.1 mg/kg of AA 5 days per week for 17 to 21 months, 25% developed severe hypocellular interstitial fibrosis, urothelial dysplasia, and tumors of the urinary tract[44]. Long-term oral treatment of mice and rats with AA resulted in the time- and dose-dependent induction of tumors in multiple tissues. When AA was administered orally to rats for 3 months in doses ranging from 0.1 to 10.0 mg/kg, the animals developed squamous cell carcinomas in the forestomach and malignant tumors in the kidney and urinary tract[19]. Rats given daily doses of 10 mg/kg AA for 35 days developed papillary urothelial carcinoma by day 105[45]. In mice, AA treatment results in squamous cell carcinoma of the forestomach, adenocarcinoma of the glandular stomach, kidney adenomas, lung carcinomas, and uterine haemangiomas[17]. AA is found among the most potent 2% of the carcinogens in Carcinogenic Potency and Genotoxicity Databases[46].

Based on evidences from humans and animals, the International Agency for Research on Cancer (IARC) has classified herbal remedies containing plant species of the genus *Aristolochia* as human carcinogens (Group I)[47]. Several countries including United Kingdom, Canada,

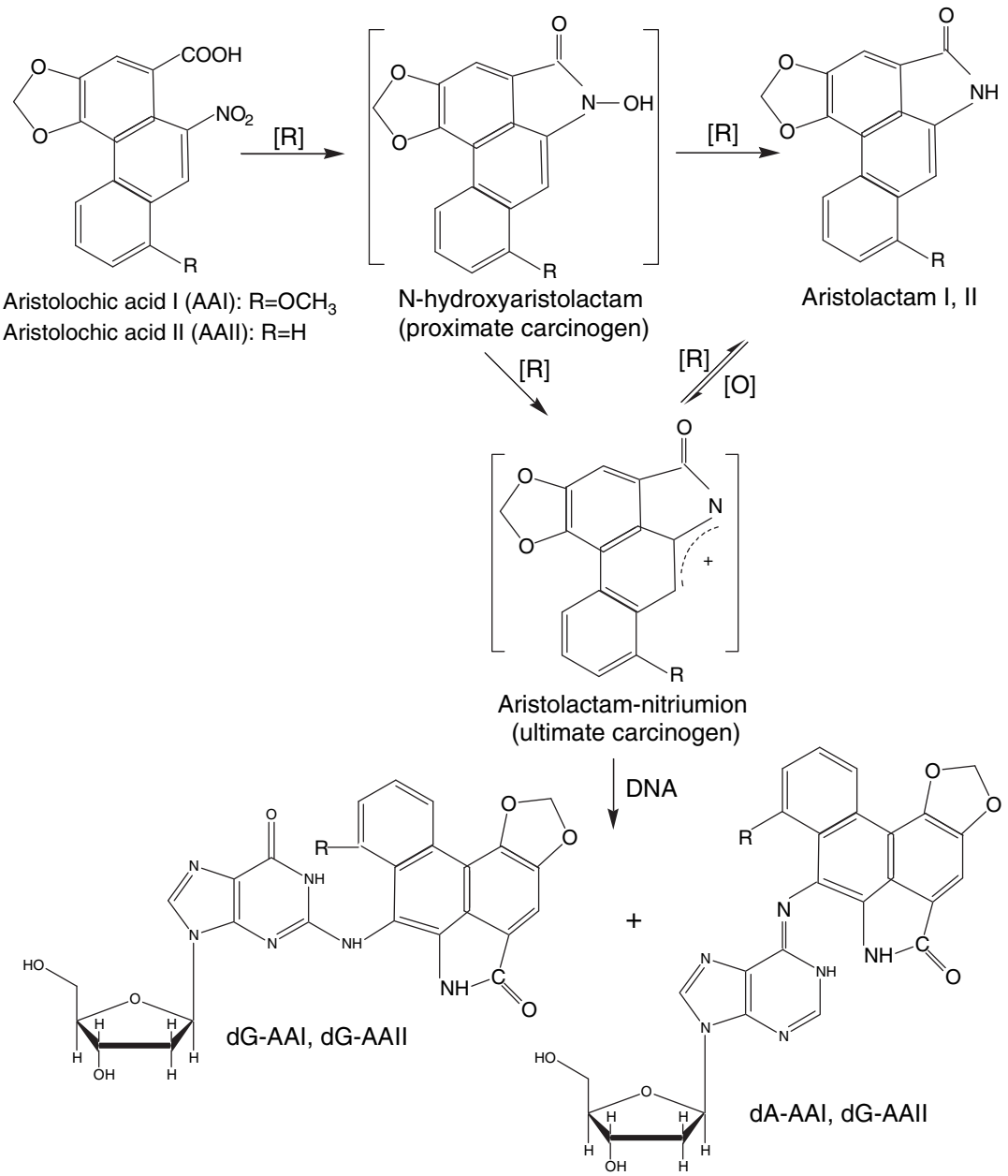


**Figure 2.** Metabolic activation of aristolochic acid (AA) and AA-DNA adduct formation. Data are from literatures(27,56-58,69). [R] means reduction and [O] represents oxidation.

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Australia, and Germany banned the use of herbs containing AA[48]. The U.S. Food and Drug Administration issued a Consumer Advisory in 2001 warning consumers against using dietary supplements and other botanical products containing AA and requesting a recall of these products and published a list of botanical products that contained AA[49].

Despite the actions of the FDA and other agencies, products containing AA have not been banned in the USA and many other countries. Many products containing or suspected to contain AA are still available in market or on web sites for sale for gastrointestinal symptoms, weight loss, cough, immune stimulation and other purposes[29,50-52]. The Dutch Food and Consumer Product Safety Authority investigated 190 Chinese traditional herbal preparations potentially containing AA between 2002 and 2006 in the Dutch market. AAI was found in 25 samples up to a concentration of 1,676 mg/kg. AAII was also found in 13 of these samples up to 444 mg/kg. These positive samples contained Mu Tong, Fang Ji, Tian Xian Teng or Xi Xin. In a worst-case scenario, use of a sample of Mu Tong with the highest AA content over a 7-day period would result in the same intake levels of AA that significantly raised the cancer risk in the Belgian AAN cases[51].

It has been reported that AA-initiated tumors are associated with activation of H-*ras* oncogene and inactivation of *p*53 tumor suppressor gene by AA-specific A:T → T:A transversion mutations[41,53,54], suggesting a genotoxic mechanism for mode of action of AA carcinogenesis. Genotoxicity of AA has been widely studied and the generated data demonstrate that AA is a potent mutagen. However, no review article specific to genotoxicity of AA has been published although AA and AAN have been extensively reviewed[27,28]. The purpose of this review is to present the knowledge regarding the genotoxicity of AA.

## DNA AND CHROMOSOME DAMAGE INDUCED BY ARISTOLOCHIC ACID

### I. *DNA Adducts Formed by AA*

Metabolism studies demonstrate that the major metabolites of AAI and AAII *in vitro* and *in vivo* are aristolactam I and II[55,56]. These metabolites can undergo reduction of the nitro group to form reactive cyclic nitrenium ions that are able to form covalent DNA adducts with the exocyclic amino groups of adenine and guanine[57-59] (Figure 2). Human P450 1A1, P450 1A2, nitroreductases, preoxidases and sulphotransferases were found capable of activating AA[60-68].

AA-DNA adducts have been induced in the tissues of both exposed humans and rodents[40-42,58,69,70], which are summarized in Table 1. Four major types of purine AA-DNA adducts have been detected by $^{32}$P-postlabelling assay[57-59]. They are 7-(deoxyadenosin-$N^6$-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-$N^2$-yl)aristolactam I (dG-AAI), 7-(deoxyadenosin-$N^6$-yl)aristolactam II (dA-AAII) and 7-(deoxyguanosin-$N^2$-yl)aristolactam II (dG-AAII). dA-AAI, dA-AAII, and dG-AAI were the major DNA adducts found in the forestomach, glandular stomach, liver, kidney and urinary bladder epithelium of rats treated with AA[58,70,71]. The three adducts were present at similar concentrations in liver, while more dG-AAI and dA-AAII adducts were detected than dA-AAI adducts in kidney. Kidney had at least 2-fold more AA-induced DNA adducts than liver[70]. In human, dA-AAI was the most abundant AA-DNA adduct found in urothelial tissue and other tissues of the AAN patients[9,42,72] because this AA-DNA adduct was the most persistent and the samples were examined a long period after exposure of AA. It has been reported that dA-AAI was detectable even 8 years after the Belgian AAN patients stopped taking the herbal slimming regimen[9]. The persistence of DNA adducts has been

**Table 1.** DNA adducts formatted by aristolochic acid

| Agent | Type of DNA adduct | Tissue | Reference |
|---|---|---|---|
| *Aristolochia fangchi* | dA-AAI, dA-AAII, dG-AAI | Human kidney, ureter, bladder, lung and spleen | (9, 40-42, 72) |
| AA | dA-AAI, dA-AAII, dG-AAI | Rat liver and kidney | (70, 71) |
| AAI | dA-AAI, dG-AAI, | Rat forestomach, glandular stomach, liver, kidney and urinary bladder epithelium | (58, 73) |
| AAII | dA-AAII | Rat liver, stomach, kidney and bladder | (58, 59) |
| AAI | dA-AAI, dG-AAI | Mouse liver, stomach, kidney, lung, spleen, intestine, and bladder | (75) |
| AAII | dA-AAII, dG-AAII | Mouse liver, stomach, kidney, lung, spleen, intestine, and bladder | (75) |

Abbreviation: aristolochic acid, AA; aristolochic acid I, AAI; aristolochic acid II, AAII; 7-(deoxyadenosin-$N^6$-yl)aristolactam I, dA-AAI; 7-(deoxyguanosin-$N^2$-yl)aristolactam I, dG-AAI; 7-(deoxyadenosin-$N^6$-yl)aristolactam II, dA-AAII; and 7-(deoxyguanosin-$N^2$-yl)aristolactam II, dG-AAII.

investigated in several rat organs after a single oral dose of pure AAI[72,73]. Both dG-AAI and dA-AAI adducts rapidly decrease during the first 2 weeks. While dG-AAI adducts continued to disappear, dA-AAI levels remain practically unchanged between 4 and 36 weeks in the target and non-target tissues. It was suggested that the persistent dA-AAI adducts may occupy specific genomic sites that are not amenable to repair and that these adducts may be converted into mutations more than other types of AA-DNA adducts due to its persistence[72] .

AAI was found more cytotoxic than AAII while other structural analogues either have less overall toxicity or no toxicity comparing to AAI and AAII[74]. However, it has also been suggested that AAI and AAII have similar genotoxic and carcinogenic potential[75]. To compare the genotoxicities of AAI and AAII, Shibutani and his colleagues treated mice with AAI and AAII, respectively. They found similar levels of DNA adducts derived from AAI and AAII in the target tissues, kidney and bladder, although the levels of the DNA adducts derived from AAI were significantly higher than those derived from AAII in non-target tissues, the liver, stomach, intestine and lung[75].

II. *DNA Strand Breakage*

Comet assay can be used to measure DNA fragments generated by DNA double strand breaks and single strand breaks. Three recent studies using comet assays demonstrate that AA can cause DNA damage via breaking the DNA (Table 2). In an *in vivo* comet assay with isolated nuclei from kidney cells, AA treatment significantly increased the DNA fragmentation in animals treated once with 20 or 40 mg AA/kg body weight by gavage in a 22–26 hour expression period[76]. In an *in vitro* comet assay using HepG2 cells, AA caused a significant induction of DNA breakage in a dose-dependent manner at concentrations 25–200 μM[77]. In the other *in vitro* study, Li *et al*. investigated the effect of AAI on DNA damage and cell cycle in porcine proximal tubular epithelial cell lines[78]. The cells were treated with AAI at the concentrations of 80, 320, and 1,280 ng/mL for 24 hr. DNA damage was examined by comet assay; and the cell cycle was assayed by flow cytometry. They found that AAI-induced DNA breakage prior to apoptosis and lysis in the treated cells in a dose-dependent manner and that the percentage of cells in the G2/M phase increased significantly. The authors suggested that AAI might cause DNA damage and cell cycle arrest through a wild-type p53-independent pathway prior to apoptosis or necrosis.

III. *Micronucleus Analysis*

Micronucleus induction by AA has been widely reported (Table 2). Mengs and Klein[79] measured genotoxic effects of AA using the micronucleus test on bone marrow cells. Male and female mice were given a single intravenous injection of 6, 20, or 60 mg/kg, respectively. Within 48 hr of administration, the males treated with 6 mg/kg or over and the females given 20 mg/kg or over showed statistically significant increases in the numbers of micronucleated polychromatic erythrocytes over the negative control. Kohara *et al*.[80] analyzed the clastogenicity of AA by evaluating the peripheral blood micronucleus after intragastric treatment of 15 mg AA/kg body weight per week for 4 weeks. However, no statistical difference in frequency of micronucleated reticulocytes was observed. This results was similar to a previous report for other strains of mice[81]. Different doses and

**Table 2.** Clastogenic effects of aristolochic acid

| Agent | Test system | Result | Reference |
|---|---|---|---|
| AA | *In vivo* comet assay on isolated kidney cells | Positive | (76) |
| AA | *In vitro* comet assay using HepG2 cells | Positive | (77) |
| AAI | *In vitro* comet assay using tubular epithelial cells | Positive | (78) |
| AA | Micronucleus test in mouse bone marrow | Positive | (79) |
| AA | Micronucleus test in mouse peripheral blood | Negative | (80, 81) |
| AA | Micronucleus test in CHO cells with or without S9 | Positive | (15) |
| AA | Micronucleus test in Hep-G2 cells with or without S9 | Positive | (77, 82) |
| AA | *In vitro* micronucleus test in human lymphocytes with or without S9 | Positive | (82) |
| AA | *In vitro* sister chromatid exchanges in human lymphocytes | Positive | (83) |
| AA | *In vitro* chromosome aberration in human lymphocytes | Positive | (83) |
| AA | Chromosomal aberration in CHO cells with or without S9 | Positive | (15) |
| AA | *In vivo* treatment and *in vitro* culture unscheduled DNA synthesis test in stomach pyloric mucosa of rats | Negative | (84) |
| AA | Chromosome loss in male germ cells of *Drosophila melanogaster* | Positive | (85) |

Abbreviation: aristolochic acid, AA; aristolochic acid I, AAI; aristolochic acid II, AAII.

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routes of administration were suggested for the different results from the mouse tests[80].

Several *in vitro* studies also showed that AA was a clastogenic agent in micronucleus assays. Significant increases in micronucleated binucleated cells were observed in CHO cells treated with AA at ≥ 25 μg/mL with or without S9[15]. Wu *et al*. studied induction of micronuclei by AA in the metabolically competent human hepatoma cell line HepG2 cells and found a significant increase of the micronuclei frequency in the range between 12.5 and 50 μM in the micronucleus test[77]. Also, AA caused a significant increase in the number of micronuclei in human lymphocytes in the presence and the absence of rat liver S9-mix and in HepG2[82].

IV. *Chromosome Damage*

Chromosomal damage caused by AA has been summarized in Table 2. Abel and Schimmer investigated the induction of structural chromosome aberrations and sister chromatid exchanges (SCEs) by AA in human lymphocytes[83]. Cells were treated with a range of 1 to 20 mg AA /mL for the throughout culture time or during the $G_0$ phase of the cell cycle. Both treatment conditions resulted in significant chromosome damage. The induction of chromosome gaps and breaks and SCEs was dose-dependent. The number of SCEs per metaphase was enhanced by a factor of 2 to 3.

The ability of AA to induce chromosomal aberrations was evaluated in CHO cells with or without S9[15]. The cells were treated with five concentrations of AA covering a range of 6.25–100 μg/mL. AA produced dose-dependent increases in the frequency of the structural chromosomal aberrations, with statistically significant increases observed at 25.0 and 50.0 μg/mL.

Unscheduled DNA synthesis in stomach pyloric mucosa of rats was examined in *in vitro* organ cultures after administration of AA *in vivo* and the test was negative[84]. AA was also tested with *Drosophila melanogaster* and the treatment of AA resulted in significant chromosome losses in male germ cells[85].

## MUTAGENICITY OF ARISTOLOCHIC ACID

The mutagenicity results of AA from testing in bacteria, mammalian cells, Drosophina, rodents and oncogenes have been summarized in Table 3.

I. *Mutagenicity in Bacteria*

AA was mutagenic to *Salmonella typhimurium*[14,15,55,60,86-88]. Although AA was a direct-acting mutagen in strains TA100, TA102, TA1537 and TM677, it was a week mutagen to TA98[15,87,88]. AAI was found not active in the nitroreductases-deficient strains TA98NR and TA100NR, indicating the necessity of nitroreduction for the bioactivation of AAI[86,87]. The mutagenicity of AA was strongly reduced, usually completely abolished, in the strains that were deficient in an endogenous nitroreductases[14,87]. Gotzl *et al*. studied mutagenicities of AAI and AAII in *S. typhimurium* tester strains that contained multicopy plasmids carrying the genes for the classical bacterial nitroreductase. They concluded from their study that only the nitro group is important for the mutagenicity of AA in *S. typhimurium*; AAII was more efficiently metabolized by endogenous nitroreductases than AAI; and the methoxy group is probably responsible for the lower activity of AAI, producing steric hindrance for binding of the genetically active intermediate to DNA or for binding of the substrate to the active site of the enzyme(s)[88].

AA, AAI and AAII were tested for genotoxicity, respectively, using *Escherichia coli* PQ37 genotoxicity assay (SOS chromotest) in the presence and in the absence of an exogenous metabolizing system. AA, AAI, and AAII, were genotoxic in the SOS chromotest in the absence of S9-mix, while AA and AAI showed genotoxic effects and AAII was marginal genotoxic effects in the presence of an exogenous metabolizing system[89].

II. *Gene Mutations in Mammalian Cells*

AA was mutagenic to mouse lymphoma L5178Y cells in both the presence and absence of S9 activation system. Mutations were induced by AA in mouse lymphoma cells at concentrations of 25 μg/mL and above. Percentage of small *Tk* mutant colonies increased with AA concentrations, indicating that AA might produce more chromosomal damages than point mutations at high cytotoxicity[15]. Also, both AAI and AAII were direct mutagens in the *Hprt* gene when evaluated with cultured Chinese hamster ovary cells[87].

Maier *et al*. evaluated the mutagenic potencies of AAI and AAII using a *Hprt* mutation assay in the subcutaneous connective tissue *in vitro*. The oxygen tension *in vitro* was adjusted to that found *in vivo*. The results showed that AAI was 19 times more mutagenic than AAII at this low oxygen tension, but only 4 times greater than AAII under standard culture conditions. The authors concluded that the genotoxicity of AA *in vivo* was mainly caused by AAI[90].

III. *Drosophina*

Mutagenicity of AA was measured with *Drosophila melanogaster*. AA induced sex-linked recessive lethal in male germ cells that measured the recombinogenic activity, and mutant single spots as well as twin spots that measured gene mutations. The results demonstrated that AA was both a point mutagen to *Drosophila* and a clastogen, inducing both gene mutations and recombinogenic activity leading to somatic recombination in mitotically active cells[85].

**Table 3.** Mutagenicity of aristolochic acid

| Agent | Test system | Result | Reference |
|---|---|---|---|
| AA | *Salmonella typhimurium* with or without S9 | Positive | (14, 15) |
| AAI | *Salmonella typhimurium* with or without S9 | Positive | (86, 87) |
| AAII | *Salmonella typhimurium* with or without S9 | Positive | (86, 87) |
| AA | *Escherichia coli* PQ37 genotoxicity assay with or without S9 | Positive | (89) |
| AAI | *Escherichia coli* PQ37 genotoxicity assay with or without S9 | Positive | (89) |
| AAII | *Escherichia coli* PQ37 genotoxicity assay with or without S9 | Positive | (89) |
| AAI | *In vitro Hprt* assay in CHO cells | Positive | (87) |
| AAII | *In vitro Hprt* assay in CHO cells | Positive | (87) |
| AA | Mouse lymphoma assay with or without S9 | Positive | (15) |
| AAI | *In vitro Hprt* assay in primary fibroblast-like rat cells | Positive | (90) |
| AAII | *In vitro Hprt* assay in primary fibroblast-like rat cells | Positive | (90) |
| AA | *Drosophila melanogaster* sex-linked recessive lethal in male germ cells and mutant single spots | Positive | (85) |
| AA | Transgenic mouse *lacZ* and *cII* assays | Positive in kidney, forestomach, bladder and colon; the major mutations are A:T → T:C | (80) |
| AA | *In vivo Hprt* assay in subcutaneous granuloma tissue | Positive | (91) |
| AAI | *In vivo Hprt* assay in subcutaneous granuloma tissue | Positive | (90) |
| AAII | *In vivo Hprt* assay in subcutaneous granuloma tissue | Positive | (90) |
| AA | Transgenic rat *cII* assay | Positive in liver and kidney; the major mutations are A:T → T:C | (70, 92) |
| *Aristolochia fangchi* | Mutations in the *p*53 genes in human urothelial tumors | The major mutations detected in the *p*53 gene are A:T → T:C | (41) |
| AAI | Human *p*53 DNA-binding domain mutation assay | The major mutations detected are A:T → T:C | (98, 99) |
| AA | Mutation in the *ras* genes in rat tumors | CAA → CTA mutations were found in c-Ha-*ras*, c-Ki-*ras*, c-N-*ras* genes | (53, 54) |
| AAI | Mutations in *ras* gene in mouse tumors | CAA → CTA mutations were found in c-Ha-*ras* gene | (54) |

Abbreviation: aristolochic acid, AA; aristolochic acid I, AAI; aristolochic acid II, AAII.

IV. *Mice*

Kohara and his colleagues[80] analyzed the mutant frequency (MF) and mutational spectra in the *lacZ* and *cII* genes in 10 target and non-target tissues of Muta mice intragastrically treated with 15 mg AA/kg body weight once a week for four weeks. They found that MFs in target tissues were significantly increased by AA over their concurrent controls in kidney, forestomach, and bladder (forestomach 33- and 15-fold; kidney 10- and 9-fold; bladder 16- and 31-fold, for the *lacZ* and *cII*, respectively) while the MFs in non-target organs, except the colon, showed only slight increases. Sequence analysis of *cII* mutants in target organs revealed that AA induced mainly A:T → T:A transversions whereas G:C → A:T transitions at CpG sites predominated among spontaneous mutations. The authors found that MFs induced by AA in *lacZ* and *cII* genes were correlated well with the carcinogenic data in mice[17] so they concluded that mutagenicity and carcinogenicity of AA were very selective to different organs.

V. *Rats*

Maier *et al*. measured the mutation induction in the *Hprt* gene of rats with the Granuloma Pouch Assay. AA was directly exposed to the subcutaneous granuloma tissue and induced a high MF in the *Hprt* gene at a relatively low cytotoxic level in the target cells. The mutagenicity of AA was even more potent than *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), a very strong mutagen, at equimolar doses. Further study using oral

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treatment of AA to rats also resulted in a dose-dependent mutation induction in the *Hprt* gene[91]. Maier *et al*. also compared the mutagenicities of AAI and AAII using the same method and found AAI induced 16 times more *Hprt* mutants than AAII[90].

Chen *et al*. studied the mutagenicity of AA in rat kidney using a protocol that resulted in tumors in order to compare the mutagenicity of AA with its carcinogenicity[92]. Groups of six male Big Blue transgenic rats were gavaged with 0, 0.1, 1.0 and 10.0 mg AA/kg body weight 5 times a week for 12 weeks. The treatment resulted in a significant increase in MF in the *cII* gene and a strong linear dose response. The *cII* MFs in rat kidney were $29 \times 10^{-6}$, $78 \times 10^{-6}$, $242 \times 10^{-6}$ and $1319 \times 10^{-6}$ in the control, low, medium and high dose treatment groups, respectively. The dose-response for MF was consistent with the previous carcinogenesis study[19], in which a similar treatment with AA resulted in 0%, 27%, 86% and 100% tumor incidences in rats. The correlation between induction of mutations and tumors by AA suggests that AA is a potent mutagenic carcinogen. Sequence analysis of the *cII* mutants from AA-treated rats revealed that A:T→T:A transversion was the predominant AA-induced mutation. If grouping into mutations occurring at A:T sites, a larger difference can be observed between the treated and control groups, 73% vs. 6%. These results support a mutagenic mechanism of action for tumor induction by AA, considering that the same type of mutations was also found in the *ras* gene in rat tumors resulted from AA treatment.

Mei *et al*. compared AA-induced DNA adducts and mutations in rat liver and kidney[70] (Figure 3). Both of the induction levels of DNA adducts and mutations are about 2 times lower in liver than in kidney. The levels of both DNA adducts and *cII* mutants detected in the non-target liver, however, were relative high. It is known that through AA can be activated in both the kidney and liver[61-64,68], tumor induction by AA only occurred in rat kidney but not in liver[19]. AA-DNA adducts in AAN patients have been observed in several organs in addition to the urinary tract, including the liver, but AAN-associated tumors thus-far have been observed only in urothelial tissue[37,41,42]. In addition, DNA adduct levels in the liver of one patient were 9-fold lower than the kidney[37], but in two cases DNA adducts levels in the liver were similar to those observed in the urinary tract[41,42]. It is not clear why AA has exhibited no liver tumors in humans or rodents. Although AA-induced DNA damage and *cll* MF measured in liver in this study were only about half of those in kidney, the induced MFs in liver were much higher than the liver *cII* MFs produced by riddelliine and comfrey, two botanical carcinogens that induce liver tumors in rats[93,94]. The overall pattern of mutations induced by AA in liver was similar to that in kidney[92]. The main type of mutations induced in liver by AA was also A:T → T:A transversion (54%), which is also the predominant mutation detected in the kidney, bladder, and forestomach of AA-treated Muta mice[80]. Therefore, the treatment that does not result in live tumors led to relatively high liver MFs, suggesting that factors other than DNA damage and mutation are necessary for tumor induction. A microarray analysis of liver and kidney gene expression in rats exposed to AA has been conducted and found that significant alteration of genes associated with defense response, apoptosis and immune response in kidney, but not in liver, may be responsible for the tissue-specific toxicity and carcinogenicity of AA[95]. It has also been suggested that sulpho conjugation in liver or other tissues can be exported into the circulation and uptaked into renal cells to cause renal and urothelial toxicity[60].

## VI. *Mutagenicity of Aristolochic Acid in Oncogenes*

Mutations in protooncogenes, tumor suppressor genes, and genes that function in the maintenance

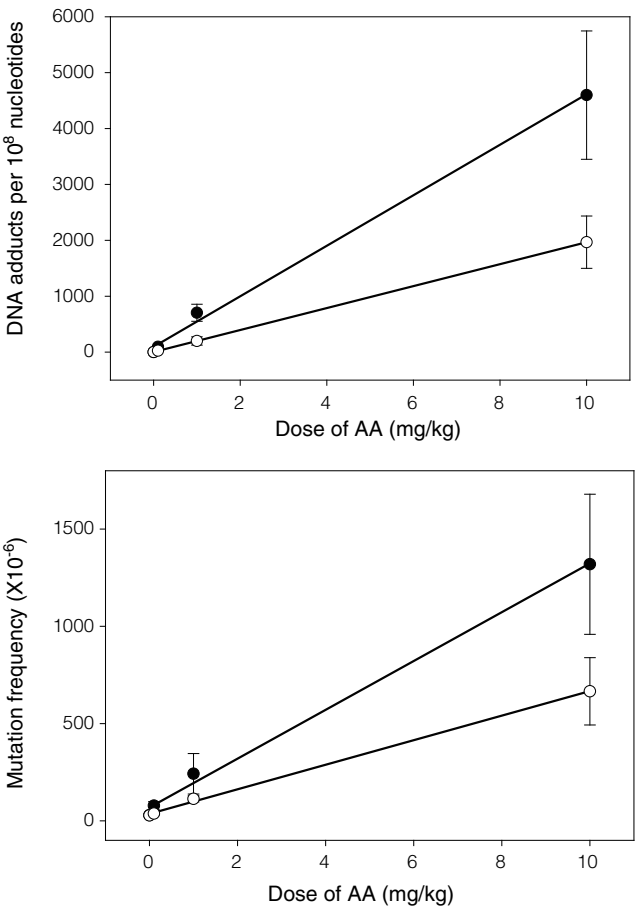


**Figure 3.** DNA adducts and mutant frequencies induced by aristolochic acid (AA). The open cycles indicate the results resulted from liver while the solid cycles represent the data from kidney. Up panel shows that DNA adducts increase with AA doses in both liver and kidney; down panel displays that the mutant frequencies in the *cII* gene enhance with doses in both liver and kidney. Data are from literatures[70,92].

of genomic stability are thought to be involved in the conversion of normal somatic cells to cancer cells[96,97]. Mutagenic signatures of carcinogens found in oncogenes can be used as finger prints to explore mechanisms of mode of actions because mutations in these genes are the critical steps for tumor initiation.

(I) *Mutations in the p53 Gene*

Lord *et al*. have analyzed AA-DNA adducts and mutations in the *p*53 gene, a tumor-suppressor gene, on the urothelial tumor DNA from a patient who had urothelial malignancy 6 years after presenting with AAN. The adduct detected was dA-AAI and the *p*53 mutation detected from the tumor DNA showed an AAG to TAG mutation in codon 139 (Lys → Stop) of exon 5[41].

Cosyns *et al*. assessed urothelial lesions and cellular expression of *p*53 for 9 kidneys and ureters removed during and/or after renal transplantation from 10 patients[36]. The study shows that the intake of Chinese herbs containing AA has a dramatic carcinogenic effect and the carcinogenesis is associated with the overexpression of *p*53, which suggests a role for mutations in the *p*53 gene.

Liu *et al*. used embryonic cells from Hupki (human *p*53 knock-in) mouse strain to generate human *p*53 DNA-binding domain (DBD) mutations experimentally. After primary Hupki cells were exposed to AAI, 5 of the 10 established cultures harbored *p*53 DBD mutations. Four out of the five mutations were A:T → T:A transversions on the nontranscribed strand[98].

To test the hypotheses on the origins of *p*53 mutations in human tumors, Feldmeyer *et al*. developed an assay using Hupki mouse embryonic fibroblasts (HUFs). They examined *p*53 mutations induced by AAI with these cells. Six immortalized cultures from 18 HUF primary cultures exposed to AAI harbored *p*53 mutations. The most frequently observed mutation was A:T → T:A transversion. One of the mutations was identical in position (codon 139) and base change (A:T → T:A on the nontranscribed strand) to the single *p*53 mutation that has thus far been characterized in a urothelial tumor of a nephropathy patient with documented AAI exposure[41]. Besides, of the seven *p*53 mutations identified thus far that immortalized spontaneously (no carcinogen treatment), none were A:T → T:A transversions; in addition, no A:T → T:A transversions were identified among the previously reported set of 18 mutations in HUF cell lines derived from B(a)P treatment[99], suggesting that A:T → T:A transversion is a mutagenic signature of AA exposure.

A recent study demonstrated that chronic dietary poisoning by AA was responsible for Endemic (Balkan) nephropathy and its associated urothelial cancer(100). AA DNA adducts were present in renal tissues and urothelial tumors of the patients. The AmpliChip *p*53 microarray was then used to sequence exons 2–11 of the *p*53 gene and 19 base substitutions in the *p*53 gene were found. A:T → T:A transversions dominated the *p*53 mutational spectrum in the tumors. Mutations at A:T pairs accounted for 89% of all *p*53 mutations, with 78% of these being A:T → T:A transversions[100].

(II) *Mutations in the ras Gene*

Schmeiser *et al*. reported that AA activated the *ras* genes in rat tumors at dA residues. They analyzed 35 various tumors from 18 male Wistar rats with long term oral administration of AAI. They detected an activated c-Ha-*ras* gene in 5 of 5 squamous cell carcinomas of the forestomach and all of them were A:T → T:A transversions at the second position of codon 61 of the c-Ha-*ras* gene (CAA to CTA). They also detected identical mutations in 93% (13 of 14) of forestomach tumors, in 100% (7 of 7) of ear duct tumors, and in the lung metastasis. Moreover, similar mutations were demonstrated at c-Ki-*ras* codon 61 in 1 of 7 ear duct tumors (CAA to CAT) and in 1 of 8 tumors of the small intestine (CAA to CTA) as well as at c-N-*ras* 61 (CAA to CTA) in a pancreatic metastasis. Additional analysis revealed a CAA to CTA transversion at codon 61 of the c-Ha-*ras* gene in 1 forestomach tumor as well as at codon 61 of the c-N-*ras* in 1 hyperplasia of the pancreas and in 1 lymphoma. The authors suggested that dA-AAI adducts were the critical lesions in the tumor initiation by AA[53].

Schmeiser *et al*. also examined the thin-tissue sections of rat tumors induced by AAI and of mouse tumors induced by AA for c-Ha-*ras* mutations in codon 61. Neoplastic and histologically normal tissues were separated and analyzed using the PCR and mutation detection by selective oligonucleotide hybridization. They found A:T → T:A transversions in DNA isolated from neoplastic tissues, but not in the adjacent normal tissues in both rats and mice[54].

## MECHANISM FOR MUTATION INDUCTION BY ARISTOLOCHIC ACID

It is clear that AA-induced DNA adduct formation results in fixation of mutations and the initiation of tumors. AA is first metabolized to aristolactam in tissues with specific enzymes like P450 1A1. The aristolactam then undergo reduction of the nitro group to form reactive cyclic nitrenium ions that are able to form covalent DNA adducts with the exocyclic amino groups of adenine and guanine.

Among AA-DNA adducts, dA-DNA adducts are more mutagenic for point mutation induction. If AA-DNA adducts are not repaired before DNA synthesis during cell proliferation, dAMP and dTMP are preferentially incorporated into the places opposite the adenine adducts, resulting A:T → T:A transversion, and A:T → A:T non-mutagenic events while dCMP was preferentially incorporated

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into the sites opposite guanine adducts, resulting in G:C → G:C non-mutagenic events[101,102]. Therefore, the four major types of DNA adducts preferentially produce A:T → T:A transversion. Both adenine adducts formed by AA (dA-AAI and dA-AAII) have greater miscoding potential than the guanine adducts[69,101]. Besides, dA-AAI adducts are persistent for very long time in tissues in experimental animals and in humans while dG-AA adducts are quickly removed by DNA repair. Therefore, dA-AAI has been suggested the most mutagenic DNA adducts for point mutation induction.

AA-DNA adducts also result in chromosomal damage and chromosomal mutations. AA-DNA adducts can induce single- or double-strand breaks of DNA. Repair of these DNA breaks can result in loss of heterozygosity, DNA deletions and insertions, chromosomal translocations and other types of chromosomal mutations. AA may also causes chromosomal damage via enhancing oxidative stress inside of cells. It was reported that a significant increase in the levels of NO and the formation of 8-OHdG in HepG2 cells[77].

## CONCLUSIONS

A large body of evidence suggests that AA-induced DNA adduct formation. Four major AA-DNA adducts, dA-AAI, dG-AAI, dA-AAII and dG-AAII, have been found in cell lines, in human urothelial tumors and in tissues of animals treated with AA. Among these AA-DNA adducts, dA-AAI adducts has been suggested the most mutagenic adduct.

AA is both a potent gene mutagen and a chromosomal mutagen. AA was positive in many different *Salmonella typhimurium* strains and induced mutations in the *Hprt* and *Tk* genes in cell lines. It induced mutations in the transgenic *cII* gene in many tissues of mice and rats, especially in the target tissues. The major type of mutation found in rodents are A:T → T:A transversions. Mutations have been detected in the *p*53 and *ras* genes of tissues from human tumors and rodents exposed to AA and in AA-exposed primary mouse cells. The major type of the mutations occurred at the oncogenes are also A:T → T:A transversions. Also, AA is a strong clastogenic agent that breaks DNA and results in chromosome damage and chromosome mutations. These kinds of genotoxic damage can cause genomic instability and loss of heterozygosity of genes that promote tumor development.

Consistence between mutations and DNA adducts generated from tissues of animals treated with AA and from the tumors of humans who exposed to AA suggest that gene and chromosomal mutations induced by AA, especially A:T → T:A transversions and chromosome aberrations, are the causal factors in the induction of urothelial cancer. Although it is still in the absence of direct evidence on mutagenicity of AA in human, mutagenicity data from studies *in vitro*, *in vivo* and in oncogenes provide sufficient evidences that mutations are responsible for the kidney-destructive fibrotic process and urothelial carcinogenesis.

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