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DNA adduct formation and mutation induction by aristolochic acid in rat kidney and liver

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Abstract

Aristolochic acid (AA) is a potent nephrotoxin and carcinogen and is the causative factor for Chinese herb nephropathy. AA has been associated with the development of urothelial cancer in humans, and kidney and forestomach tumors in rodents. To investigate the molecular mechanisms responsible for the tumorigenicity of AA, we determined the DNA adduct formation and mutagenicity of AA in the liver (nontarget tissue) and kidney (target tissue) of Big Blue rats. Groups of six male rats were gavaged with 0, 0.1, 1.0 and 10.0 mg AA/kg body weight five times/week for 3 months. The rats were sacrificed 1 day after the final treatment, and the livers and kidneys were isolated. DNA adduct formation was analyzed by ³²P-postlabeling and mutant frequency (MF) was determined using the λ Select-*cII* Mutation Detection System. Three major adducts (7-[deoxyadenosin-*N*⁶-yl]-aristolactam I, 7-[deoxyadenosin-*N*⁶-yl]-aristolactam II and 7-[deoxyguanosin-*N*²-yl]-aristolactam I) were identified. There were strong linear dose-responses for AA-induced DNA adducts in treated rats, ranging from 25 to 1967 adducts/10⁸ nucleotides in liver and 95–4598 adducts/10⁸ nucleotides in kidney. A similar trend of dose-responses for mutation induction also was found, the MFs ranging from 37 to 666 $\times 10^{-6}$ in liver compared with the MFs of 78–1319 $\times 10^{-6}$ that we previously reported for the kidneys of AA-treated rats. Overall, kidneys had at least two-fold higher levels of DNA adducts and MF than livers. Sequence analysis of the *cII* mutants revealed that there was a statistically significant difference between the mutation spectra in both kidney and liver of AA-treated and control rats, but there was no significant difference between the mutation spectra in AA-treated livers and kidneys. A:T $\rightarrow$ T:A transversion was the predominant mutation in AA-treated rats; whereas G:C $\rightarrow$ A:T transition was the main type of mutation in control rats. These results indicate that the AA treatment that eventually results in kidney tumors in rats also results in significant increases in DNA adduct formation and *cII*MF in kidney. Although the same treatment does not produce tumors in rat liver, it does induce DNA adducts and mutations in this tissue, albeit at lower levels than in kidney.

Keywords

Aristolochic acid; DNA adduct; Mutagenicity; Transgenic rat

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1. Introduction

Herbal drugs derived from *Aristolochia* species have been used for medicinal purposes since antiquity. Aristolochic acid (AA) is a family of structurally related nitrophenanthrene carboxylic acids, mainly consisting of aristolochic acid I (AAI) and aristolochic acid II (AAII), and is the active component of the extracts used in these herbal medicines. Following the observation that AA is mutagenic and carcinogenic, the sale of pharmaceuticals containing AA was banned in many European (e.g. Germany, UK) and Asian countries (e.g. Japan), Australia, and Canada.

Long-term oral treatment of mice and rats with AA results 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 [1]. In mice, AA treatment results in squamous cell carcinoma of the forestomach, adenocarcinoma of the glandular stomach, kidney adenomas, lung carcinomas, and uterine haemangiomas [2]. AA is also mutagenic in bacterial [3,4] and mammalian [5,6] short-term tests.

In the early 1990s, a rapidly progressive interstitial nephropathy, originally called Chinese herb nephropathy (CHN) and now known as aristolochic acid nephropathy (AAN [7]), was reported in Belgian patients who used Chinese herbs containing AA as part of a weight-loss program [8,9]. Similar cases subsequently were observed world-wide [10,11]. Soon thereafter, AA-associated urothelial cancer was reported in CHN/AAN patients [12–14], and specific AA-derived DNA adducts, identified as 7-(deoxyadenosin- N^6 -yl)-aristolactam I (dA-AAI), were found in the kidney, ureter, bladder, liver, lung, and spleen of the patients [15–17]. These findings provide strong evidence linking the use of herbal products containing AA with cancer development. Due to the potential for serious public health risk, the U.S. Food and Drug Administration issued a Consumer Advisory in April 2001 warning consumers against using dietary supplements and other botanical products containing AA and requesting a recall of these products [18].

It is hypothesized that AA is bioactivated by cytochrome P450s and subsequently reacts with cellular proteins and DNA, leading to multiple forms of toxicity, including gene mutation and tumor induction. Previously we found that the mutant frequency (MF) in the kidneys of Big Blue rats treated with different doses of AA was positively correlated with the tumor incidence previously reported for the same treatments [19]. The available data indicate that AA is activated in the liver, kidney, and other tissues, but only targets the kidney and forestomach for tumor induction in rodents. In the present study, we used transgenic Big Blue rats to compare DNA adduct and mutation induction in kidney (target tissue) and liver (nontarget tissue) to determine their relationships with tumorigenicity.

2. Materials and methods

2.1. Chemical and animals

Aristolochic acid (AA) was purchased from Sigma (St. Louis, MO). The AA content of the test agent was 96% (40% AAI and 56% AAI). Male Big Blue transgenic rats were obtained

from Taconic Laboratories (Germantown, NY) through purchase from Stratagene (La Jolla, CA). All animal procedures followed the recommendations of the NCTR Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and sacrifice of the rats.

2.2. Treatments

The treatment schedule was based on the previous carcinogenesis study [1]. Six-week-old Big Blue rats were treated with AA as its sodium salt at concentrations of 0.1, 1.0, and 10.0 mg/kg body weight by gavage five times/week for 12 weeks. Vehicle control rats were gavaged with 0.9% sodium chloride using the same schedule as for the AA-treated rats. Six rats from each treatment group were sacrificed 1 day after the last treatment. The livers and kidneys were isolated, frozen quickly in liquid nitrogen, and stored at -80°C .

2.3. DNA adduct analysis by ^{32}P -postlabeling

Liver and kidney DNAs were isolated by a standard phenol extraction method. DNA adducts were determined by the ^{32}P -postlabeling procedure described recently [20], with minor modifications. DNA samples (4 μg) were digested with micrococcal nuclease (240 mU; Sigma, Poole, UK) and calf spleen phosphodiesterase (60 mU; Calbiochem, Nottingham, UK) in digestion buffer containing 20 mM sodium succinate and 10 mM calcium chloride (pH 6.0) for 3 h at 37°C in a total volume of 10 μl . For nuclease P1 enrichment, the digests were incubated with 4 μg nuclease P1 (MP Biomedicals, London, UK) in 3 μl of a buffer containing 0.8 M sodium acetate (pH 5.0) and 2 mM zinc chloride for 30 min at 37°C . The reaction was terminated by the addition of 3 μl Tris base (427 mM). DNA digests were then ^{32}P -labeled by adding 4 μl of a mixture consisting of 400 mM bicine (pH 9.5), 200 mM magnesium chloride, 300 mM DTT, 10 mM spermidine, 50 μCi [γ - ^{32}P]ATP (approximately 7000 Ci/mmol; MP Biomedicals), and 6 U T4 polynucleotide kinase (USB, Cleveland, OH), and incubated for 30 min at 37°C . Resolution of ^{32}P -labeled adducts was by thin layer chromatography (TLC) on polyethyleneimine-cellulose (PEI-cellulose) sheets (10 cm $\times$ 20 cm; Macherey-Nagel, Düren, Germany). TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, IL), and DNA adduct levels were calculated from the adduct cpm, the specific activity of [γ - ^{32}P]ATP, and the amount of DNA (pmol of DNA-P) used. Results are expressed as DNA adducts per 10^8 normal nucleotides. Enzymatic preparation of AA–DNA adduct reference compounds was performed as described previously [21].

2.4. cII mutation assay

High-molecular-weight genomic DNA was extracted from rat livers and kidneys using the RecoverEase DNA Isolation Kit (Stratagene) and stored at 4°C until DNA packaging was performed. The packaging of the phage, plating the packaged DNA samples, and determination of MF were carried out following the manufacturer's instructions for the Select-*cII* Mutation Detection System for Big Blue Rodents (Stratagene). Briefly, the shuttle vector containing the *cII* target gene was rescued from total genomic DNA with phage packaging extract (Transpack; Stratagene), and the resulting phage plated on *Escherichia coli* host strain G1250. To determine the total titer of packaged phages, G1250 bacteria were mixed with 1:3000 dilutions of phage, plated on TB1 plates, and incubated overnight at 37°C (nonselective conditions). For mutant selection, the packaged phages were mixed with

G1250, plated on TB1 plates, and incubated at 24 °C for about 42 h (conditions for *cII* selection). Under the selective conditions, phages with wild-type *cII* genes undergo lysogenization and become part of the developing bacterial lawn, whereas phages with mutated *cII* genes undergo lytic growth and give rise to plaques. When incubated at 37 °C, phages with wild-type *cII* genes also undergo a lytic cycle, resulting in plaque formation. Assays were repeated until a minimum of 2×10^5 plaque-forming units (pfus) from each sample were examined for mutation. The *cII*MF is defined as the total number of mutant plaques (determined at 24 °C) divided by the total number of plaques screened (determined at 37 °C).

2.5. Sequence analysis of the *cII* mutants

cII mutant plaques from liver and kidney were chosen at random from different animals and replated at low density to verify the mutant phenotype. Single, well-isolated plaques were transferred from these plates to a microcentrifuge tube containing 100 µl of sterile distilled water. The tube was heated at 100 °C for 5 min and centrifuged at $12,000 \times g$ for 3 min. *cII* target DNA released by this procedure was amplified by PCR using primers 5'-AAAAAGGGCATCAAATTAACC-3' (upstream) and 5'-CCGAAGTTGAGTATTTTGTGCTG-3' (downstream). For PCR amplification, 10 µl of the supernatant was added to 10 µl of a PCR Master Mix (Promega, Madison, WI) and the primers. The final concentrations of the reagents were 1× Taq polymerase reaction buffer, 0.2 µmol of each primer, 200 µM of each dNTP, 1.5 mM MgCl₂, and 0.25 U of Taq DNA polymerase. The PCR reaction was performed using a PCR System 9700 (Applied Biosystems, Foster City, CA), with the following cycling parameters: 3 min denaturation at 95 °C; followed by 35 cycles of 30 s at 95 °C, 1 min at 60 °C, and 1 min at 72 °C; with a final extension of 10 min at 72 °C. The PCR products were isolated using a PCR purification kit (Qiagen, Chatsworth, CA). The *cII* mutant DNA was sequenced with a CEQ Dye Terminator Cycle Sequencing Kit and a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The primer for *cII* mutation sequencing was the upstream primer used for the PCR.

2.6. Statistical analyses

Analyses were performed using SigmaStat 2.03 (SPSS, Chicago, IL). All DNA adduct and MF data are expressed as the mean ± standard deviation (S.D.) from six rats per group. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey test. Since the variance increased with the magnitude of the MF, these data were log-transformed before conducting the analysis. Mutational spectra were compared using the computer program written by Cariello et al. [22] for the Monte Carlo analysis developed by Adams and Skopek [23].

3. Results

3.1. Growth of rats treated with AA

AA was administered to male Big Blue transgenic rats 5 days a week for 12 weeks, according to the protocol of Mengs [1]. The relative mean body weights of the rats over the 12-week study are shown in Fig. 1. The relative body weight was calculated as the gain in

body weight during the experiment relative to the weight when the treatments started. Mean body weights of all AA-dosed groups were less than those of the vehicle controls throughout the study. By the end of the study, rats treated with 0.1, 1.0, and 10.0 mg/kg AA weighed 5%, 7%, and 15% less than the controls. The high-dose group of rats exhibited little weight gain during weeks 8–12 of the treatment.

3.2. AA-induced DNA adducts in liver and kidney

DNA adducts formed in livers and kidneys were analyzed by ^{32}P -postlabeling. AA-treated livers and kidneys had the same pattern of DNA adducts, whereas no AA–DNA adduct spots were found in control rats. Fig. 2 shows representative images of autoradiographic profiles generated by the ^{32}P -postlabeling analysis. Three major AA–DNA adducts were detected, dA–AAI, 7-(deoxyguanosin- N^2 -yl)-aristolactam I (dG–AAI), and 7-(deoxyadenosin- N^6 -yl)-aristolactam II (dA–AAII). These three adducts had similar levels in liver, while kidneys had more dG–AAI and dA–AAII than dA–AAI (Table 1). Levels of total AA–DNA adducts were 25, 199, and 1967 per 10^8 nucleotides for the livers, and 95, 705, and 4598 per 10^8 nucleotides for the kidneys of rats exposed to 0.1, 1.0, and 10.0 mg/kg of AA, respectively. Kidneys from treated rats averaged two- to four-fold more DNA adducts than livers. Total DNA adduct formation for both the livers and kidneys from AA-treated rats increased in a linear dose-dependent manner (Table 2), and statistically significant differences ($P < 0.001$) were observed between the two tissues for the three AA concentrations.

3.3. AA-induced MFs in the liver *cII* gene

DNA from each liver was packaged two to four times either to confirm the MF or to obtain a minimum of 2×10^5 pfus for mutant detection. The liver MFs for the control male Big Blue rats ranged from 21 to 39×10^{-6} , with an average of $28 \pm 6 \times 10^{-6}$, which is similar to the MF determined for female control rats [24]. The results from the *cII* mutation assay indicated that there was a dose-dependent increase in MF for the livers of AA-treated rats (Table 2). The liver MFs for rats treated with 0.1, 1.0, and 10.0 mg/kg AA were $37 \pm 8 \times 10^{-6}$, $113 \pm 16 \times 10^{-6}$, and $666 \pm 173 \times 10^{-6}$, respectively. The MFs for the middle- and high-dose groups were significantly higher than the MFs for control and low-dose groups ($P < 0.001$). There were also significant differences between the MFs of the high- and middle-dose groups ($P < 0.001$). Liver *cII* MFs were about half the MFs previously determined for the kidneys in these same rats (Table 2). We also plotted the AA-induced *cII* MFs per 10^6 cells as a function of total AA–DNA adducts per 10^8 nucleotides for liver and kidney (data not shown). Over the dose range studied, the relationship between the two endpoints was linear for both liver and kidney.

3.4. Mutation spectra in the liver *cII* gene

In our previous study [19], we found that there was a significant difference between the mutation spectra in kidneys of the AA-treated and control rats, and that A:T $\rightarrow$ T:A transversion was the predominant mutation for AA-treated rat kidneys. In contrast, we reported that G:C $\rightarrow$ A:T transition was the main type of mutation for control rat kidneys [19]. In the present study AA-induced mutations in the liver *cII* gene were evaluated by DNA sequence analysis of 137 mutants isolated from six rats treated with 10.0 mg/kg AA. Mutations that were found more than once among the mutants isolated from a single animal

were assumed to be siblings and to represent only one independent mutation. Accordingly, a total of 125 independent mutations were identified from the livers of AA-treated rats (Table 3). The overall pattern of mutations in the liver of AA-treated rats differed significantly ($P < 0.0001$) from the mutation spectrum previously determined for control rats [24]. A:T → T:A transversion (54%) was the major type of mutation in the liver of rats treated with AA. In addition, two tandem base substitutions (4%), AT → TA and AA → TT, were observed among the mutations from the AA-treated rat livers. By contrast, the mutation spectrum for control rat liver was dominated by G:C → A:T transition and frameshifts [24]. In comparison with the mutation spectra determined for the kidneys [19], there were no significant differences between either control livers and kidneys or between the AA-treated livers and kidneys.

4. Discussion

Reactive metabolites or intermediates, formed through the bioactivation of herbal constituents, are associated with herbal toxicity, mutagenicity, and carcinogenicity. As nitrophenanthrene alkaloids, both AAI and AAI 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 [25–27]. Several studies have established that AA is strongly nephrotoxic [28–30], genotoxic [31,32], and carcinogenic in humans and rodents [1,2,14,33]. AAI was found to be more toxic than AAI, and other structural analogues either have less overall toxicity or no toxicity [34]. When rabbits were injected intraperitoneally with 0.1 mg/kg of AA 5 days per week for 17–21 months, 25% developed severe hypocellular interstitial fibrosis, urothelial dysplasia, and tumors of the urinary tract [35]. Rats given daily doses of 10 mg/kg AA for 35 days developed papillary urothelial carcinoma by day 105 [36].

It was of particular interest to compare AA-induced DNA adducts and mutations in rat liver and kidney, because AA can be activated in both the kidney and liver [37–41] but the major target tissues for tumor induction in rodents are kidney and forestomach. The treatment schedule in the present study was based on the previous carcinogenesis study of Mengs [1], in which rats treated orally with 0.1, 1.0, and 10.0 mg AA/kg body weight for 3 months developed a high incidence of tumors (25%, 85%, and 100%, respectively), with 72%, 28%, and 17% of the animals treated with the 10.0 mg/kg dose having tumors of the forestomach, kidney and urinary tract, respectively. We used the same doses for treating Big Blue transgenic rats 5 days per week for 3 months by gavage, and determined DNA adducts and the *cHMFs* in liver and kidney. We identified three DNA adducts (dA-AAI, dA-AAII, and dG-AAI) in the liver and kidney of treated rats (Fig. 2). 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 (Table 1). Kidney had at least two-fold more AA-induced DNA adducts and two-fold higher MF than liver. The kidney to liver adduct concentration ratio was consistent with the adduct ratio in rats injected subcutaneously with 7 mg/kg of AA for 35 days [20]. Dose-dependent increases in MF and DNA adducts were observed for both liver and kidney (Table 2), suggesting that the mutagenic effects of AA are associated with the formation of AA–DNA adducts.

AA-DNA adducts have been found in the tissues of both exposed humans and rodents. The persistence of DNA adducts has been investigated in several rat organs after a single oral dose of pure AAI [42,43]. Both dG-AAI and dA-AAI adducts rapidly decrease during the first 2 weeks; afterwards, dG-AAI adducts continued to disappear, whereas dA-AAI levels remain practically unchanged between 4 and 36 weeks. Similar patterns of adduct reduction were observed in target tissues (forestomach and kidney) and nontarget tissues (liver and lung). Because the rates of reduction for dG-AAI adducts and dA-AAI adducts were different, the removal kinetics do not appear to be solely a function of cell turnover. It was suggested that persistent dA-AAI adducts may occupy specific genomic sites that are not amenable to repair and that these adducts may be converted into mutations [43]. It is tempting to speculate that nucleotide excision repair (NER) pathways may be involved in the removal of dG-AAI adducts whereas dA-AAI adducts can resist the repair process. However, a possible role of NER in the repair of individual AA-DNA adducts awaits further investigation.

It also has been reported that AAI forms high levels of DNA adducts in both target-organ forestomach and nontarget glandular stomach, with lower levels of adducts being detected in liver, kidney, and urinary bladder DNA [26]. The similarity of adduct patterns produced by AAI in all organs in this previous study indicates that the same reactive species are present in both the target and nontarget tissues. This study also evaluated the adducts formed by treatment with AAI and found that the levels of AAI adducts were generally lower than for AAI. AAI treatment, however, produced relatively higher amounts of adducts in the DNA of kidney than did AAI [26]. In AAN patients, the major adduct identified is dA-AAI [16], which persists in tissues for an extended period of time [14,43].

Differences in the plant extract AAs, the administrated doses, and the duration of treatment might result in different profiles of DNA adducts. The levels of DNA adducts are most likely the result of a balance between their formation and their loss through either DNA-repair processes or apoptosis [14], and their accumulation and persistence in different tissues may influence the ultimate mutagenicity and carcinogenicity of AA in different tissues. Kohara et al. [6] reported that treating Muta mice with 15 mg/kg AA per week for 4 weeks induced relatively high *cH*MFs in the target organs (663, 467, and 870×10^{-6} for forestomach, kidney, and bladder, respectively) and only small increases in *cH*MFs in nontarget tissues (e.g. 39×10^{-6} for liver). We observed much higher MFs in both liver and kidney (666 and 1319×10^{-6}) after administration of 10 mg/kg for 3 months. Although AA has similar carcinogenic potency in rats and mice [1,2], the different treatment regimens and different animal species may account for the differences in the MFs measured by Kohara et al. [6] and detected in our study.

The overall pattern of mutations induced by AA in liver was similar to that in kidney [19]. There were no significant differences between the mutation spectra for liver controls and kidney controls, and between AA-treated livers and kidneys. In contrast to the G:C → A:T transitions that dominated the mutation spectrum in control livers [24], the main type of mutation induced in liver by AA was A:T → T:A transversion (54%), which is also the predominant mutation detected in the kidney, bladder, and forestomach of AA-treated Muta mice [6]. These analyses suggest that the major mutagenic pathway for AA is reaction with

deoxyadenine, resulting in A → T transversion. Both adenine adducts formed by AA (dA-AAI and dA-AAII) have greater miscoding potential than the guanine adducts [44,45]. Translesional bypass of dA-AAI and dA-AAII adducts indicate a mutagenic potential resulting from dAMP incorporation by DNA polymerase [44], suggesting that an A → T transversion mutation would be the mutagenic consequence. Although nothing is known about the mutagenic potential of dA-AAI adducts *versus* dA-AAII adducts *in vivo*, their relative persistence and initial DNA binding may contribute to the overall MF.

Mutations also have been detected in the *p53* and *ras* genes of tissues from humans and rodents exposed to AA [16,30,46], and in AA-exposed primary mouse cells [47]. Since a high frequency of A → T transversion in codon 61 of the Ha-*ras* oncogene has been found in tumors from rodents treated with AAI, AA-DNA adducts at adenine residues might also be critical lesions in the carcinogenic process [46,48,49]. Mutations in protooncogenes, tumor suppressor genes, and genes that function in the maintenance of genomic stability are thought to be involved in the conversion of normal somatic cells to cancer cells [50,51]. The detection of mutations in the *p53* gene of AAN patients [16] and in the H-*ras* oncogene of tumors from AA-treated rodents [46,48] is consistent with a molecular mechanism whereby the mutagenicity of AA is a causal factor in the induction of urothelial cancer.

A large body of evidence suggests that AA-induced DNA adduct formation, followed by cellular proliferation and fixation of mutations, is responsible for cancer development in treated animals [10]. AA-DNA adducts in AAN patients, however, 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 [16,17,52]. In addition, DNA adduct levels in the liver of one patient were ninefold lower than the kidney [52], but in two cases DNA adduct levels in the liver were similar to those observed in the urinary tract [16,17]. It is not clear why AA has exhibited no liver tumors in humans or rodents. Although AA-induced DNA damage and *cHMF* measured in liver in this study were about half those in kidney (Table 2), the liver MFs were much higher than the liver *cHMF*s produced by riddelliine and comfrey, two botanical carcinogens that induce liver tumors in rats [24,53].

Debelle et al. [20] reported that injection of rats with AA for 35 days induces typical renal lesions, consisting of tubular atrophy, lymphocytic infiltration, and interstitial fibrosis, while there are no significant abnormalities in the liver. The possible reason for this targeting of toxicity to the kidney could be the ability of proximal tubules to transport and concentrate AA and their metabolites, resulting in renal toxicity. The cellular mechanisms of AA toxicity have been delineated in an established proximal tubule cell model [54]. In this model, transient exposure to AA significantly decreases expression of megalin, forms specific DNA adducts identical to those found in kidneys from AAN patients, and permanently inhibits tubular protein reabsorption. Similar observations recently have been made using a rat model of AAN [20,55]. Therefore, AA-DNA adducts in urothelial cells might impair physiological processes leading to proximal tubule dysfunction; such a mechanism might explain the seeming tissue specificity of the AAN-associated oncogenesis regardless of the widespread occurrence of AA-DNA adducts. On the other hand, a single nonnecrogenic dose of AA (10 mg/kg body weight, i.p. injection) to rats given 18 h after two-thirds partial hepatectomy initiated liver cell carcinogenesis (formation of hepatic foci and nodules) [56]. This suggests

that the lack of carcinogenicity of AA in the liver could be because AA is not sufficiently necrogenic to produce compensatory liver cell proliferation and fix mutations at the doses employed for carcinogenicity assays in rodents. However, we found that treatment with 10 mg/kg AA for 3 months, a treatment that does not result in live tumors [1], led to relatively high liver MFs, suggesting that factors other than DNA damage and mutation are necessary for tumor induction. We are conducting a microarray analysis of liver and kidney gene expression in rats exposed to AA to explore the possible mechanisms involved.

In summary, we have found that rats chronically treated with carcinogenic doses of AA had dose-responsive increases in total DNA adducts and *cII*MF in both liver and kidney. Sequence analysis of the *cII* mutants revealed that there were significant differences between the mutation spectra in livers and kidneys of AA-treated animals and their concurrent controls, whereas there was no significant difference between the mutation spectra in AA-treated livers and kidneys. While the levels of DNA adducts and MF detected in the kidney of AA-treated rats generally correlated with the tumor specificity of the treatment, relatively high levels of both DNA adducts and *cII* mutants were detected in the nontarget liver.

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Fig. 1.

Relative mean body weight of Big Blue rats treated with AA for 12 weeks. The rat relative body weight was calculated as a ratio of the body weight of rats during the 12-week dosing period to the body weight of 6-week-old rats at the initiation of the treatments. The data represent the means of groups of six rats. (▲) Vehicle control; (■) 0.1 mg/kg of AA; (◆) 1.0 mg/kg of AA; (●) 10.0 mg/kg of AA.

Fig. 2.

Representative autoradiographic profiles of AA-DNA adducts detected by the nuclease P1 enrichment version of the ^{32}P -postlabeling assay. Electronic autoradiography was performed for 5–10 min. Origins in the bottom left corners were cut off before exposure. (A) Liver control; (B) AA-treated liver; (C) kidney control; (D) AA-treated kidney; spot (1) dA-AAI; spot (2) dG-AAI; spot (3) dA-AAII.

Table 1

DNA adduct formation in liver and kidney of rats treated with different doses of aristolochic acid

Tissue	DNA adduct	Aristolochic acid (mg/kg)		
		0.1	1.0	10.0
Liver	dA-AAI	9.1 ± 2.8 ^a	49.4 ± 19.8	684.1 ± 198.3
	dG-AAI	8.2 ± 3.4	84.5 ± 34.5	720.9 ± 165.3
	dA-AAII	7.7 ± 2.1	65.4 ± 27.8	561.8 ± 124.5
Kidney	dA-AAI	9.2 ± 1.0	53.5 ± 13.5	911.4 ± 223.6
	dG-AAI	35.6 ± 7.2	266.5 ± 55.9	1676.6 ± 470.4
	da-AAII	49.1 ± 13.7	384.6 ± 87.0	2010.3 ± 496.8

^aThe values are the mean adducts ± S.D. per 10⁸ nucleotides for each group of six rats.

Table 2

Total DNA adducts and *cII* mutant frequency in liver and kidney of rats treated with different doses of aristolochic acid

Aristolochic acid (mg/kg)	Total DNA adducts ($\times 10^{-8}$ nucleotides) ^{a,b}		Mutant frequency ($\times 10^{-6}$) ^{a,c}	
	Liver	Kidney	Liver	Kidney
0	0	0	28 $\pm$ 6	29 $\pm$ 6
0.1	25 $\pm$ 8	95 $\pm$ 21	37 $\pm$ 8	78 $\pm$ 21
1.0	199 $\pm$ 75	705 $\pm$ 153	113 $\pm$ 16	242 $\pm$ 104
10.0	1967 $\pm$ 468	4598 $\pm$ 148	666 $\pm$ 173	1319 $\pm$ 360

^aThe data represent the mean $\pm$ S.D. per for each group of six rats.

^bThe data for total DNA adducts are taken from Table 1.

^cThe data for kidney mutant frequency are from [19].

Table 3

Summary of independent mutations in the *cII* gene of liver and kidney from aristolochic acid-treated and control Big Blue rats

Type of mutation	Control		Aristolochic acid			
	Liver ^a		Liver ^b		Kidney ^c	
	Number	%	Number	%	Number	%
G:C → C:G	2	4	6	5	6	7
G:C → A:T	30	55	20	16	13	16
G:C → T:A	5	9	9	7	3	4
A:T → T:A	3	5	68	54	42	50
A:T → C:G	3	5	1	1	4	5
A:T → G:C	3	5	11	9	14	17
Frameshift	8	15	3	2	1	1
Tandem base substitution	0	0	2	2	0	0
Complex mutation	1	2	5	4	0	0
Total mutants screened	55	100	125	100	83	100

^aControl data are from our previous results [24].

^bSpectrum for AA-treated livers was significantly different from controls ($P < 0.0001$).

^cKidney data are from our previous results [19]. There was no significant difference between AA-induced mutation spectra in liver and kidney ($P = 0.106$).