Selective Toxicity of Aristolochic Acids I and II

Shinya Shibutani*, Huan Dong, Naomi Suzuki, Shiro Ueda,

Frederick Miller and Arthur P. Grollman

Laboratory of Chemical Biology, Department of Pharmacological Sciences, State University of New York, Stony Brook, New York 11794-8651 (S.S., H.D., N.S., A.P.G.); Department of Drug Information and Communication, Graduate School of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba 263-8522, Japan (S.U.); and Department of Pathology, School of Medicine, State University of New York, Stony Brook, New York 11794 (F.M.)
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b) *To whom correspondence and reprint requests should be addressed.
Shinya Shibutani
1 Nicolls Road, Stony Brook, New York 11794-8651
Telephone: 631-444-7849
Fax: 631-444-3218
Email: shinya@pharm.stonybrook.edu

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d) Abbreviations: AAN, aristolochic acid nephropathy; AA, aristolochic acid; AA-I, aristolochic acid I [8-methoxy-6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid]; AA-II, aristolochic acid II [6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid]; dA-AL, 7-(deoxyadenosin-\(\text{N}^6\)-yl) aristolactam; dG-AL, 7-(deoxyguanosin-\(\text{N}^2\)-yl) aristolactam; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography. PBS, phosphate buffered saline.
Abstract

Ingestion of herbal remedies containing aristolochic acids (AA) is associated with the development of a syndrome, designated aristolochic acid nephropathy (AAN), which is characterized by chronic renal failure, tubulointerstitial fibrosis and urothelial cancer. To distinguish the component(s) of AA responsible for these varied toxic effects, we administered 2.5 mg/kg/day of AA-I or AA-II for 9 days, either intraperitoneally or orally, to male C3H/He mice. Tissues were then collected and subjected to biochemical and histopathologic examination. Genotoxicity was assessed by determining quantitatively the level of aristolactam-DNA adducts in various tissues using $^{32}$P-postlabeling/polyacrylamide gel electrophoresis and an internal standard. In the primary target tissues, represented by renal cortex, medulla and bladder, we found similar levels of DNA adducts derived from AA-I and AA-II. However, in non-target tissues, the liver, stomach, intestine and lung, the levels of aristolactam-DNA adducts derived from AA-I were significantly higher than those derived from AA-II. Histopathologic analysis revealed tubular cell necrosis and interstitial fibrosis in the renal cortex of AA-I-treated mice but only minimal changes in the renal cortex of mice treated with AA-II. We conclude that AA-I and AA-II have similar genotoxic and carcinogenic potential and, while both compounds are cytotoxic, AA-I is solely responsible for the nephrotoxicity associated with AAN.
The ingestion of herbal remedies containing aristolochic acids (AAs) is associated with the development of a chronic, progressive renal disease designated aristolochic acid nephropathy (AAN) (Cosyns, 2003; Arlt et al., 2002). This clinical syndrome was reported initially in a group of women in Belgium who developed severe renal disease after ingesting slimming pills containing Aristolochia fangchi (Vanherweghem et al., 1993; Nortier and Vanherweghem, 2002). Nephrotoxicity was manifested by progressive atrophy of renal proximal tubules and development of a characteristic form of interstitial fibrosis involving the outer renal cortex and progressing toward the medulla (Cosyns et al., 1994; Depierreux et al., 1994). The process tends to spare glomeruli and is associated with less inflammation than most types of interstitial nephritis. Importantly, the syndrome was associated with a high prevalence of urothelial cell carcinoma, which often occurred years after the onset of chronic renal disease (Cosyns et al., 1999; Nortier et al., 2000). Moreover, in contrast to most other urothelial cell tumors, cancers associated with AAN tend to develop in the upper urinary tract.

AA refers to a mixture of structurally related nitrophenanthrene carboxylic acids whose major constituents include 8-methoxy-6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid (aristolochic acid I, AA-I) and its 8-demethoxylated form (aristolochic acid II, AA-II) (Kumar et al., 2003). Despite the global use of AA in herbal remedies, only limited data is available regarding the metabolic fate of AA-I and AA-II in experimental animals and humans (Krumbiegel et al., 1986). This study, and in vitro experiments (Schmeiser et al., 1986, 1988; Stiborova et al., 2001, 2002, 2005), suggest that AA-I is metabolized along two major pathways. Under aerobic conditions, it may be demethylated to form 8-OH-aristolochic acid-I (AA-Ia) which, in turn, is subject to Phase
II conjugation reactions, forming glucuronide or sulfate esters. Alternatively, the nitro group may be enzymatically reduced to generate the biologically inactive aristolactam-I (L-I), which is itself subject to Phase II conjugation. AA-II, lacking the O-methoxy group, is reduced to aristolactam-II (L-II) and then hydroxylated at C-8 to form 8-hydroxyaristolactam 1a (L-Ia) (Fig. 1) (Arlt et al., 2002).

AA-I and AA-II are mutagenic in bacteria, mammalian cells and mice (Arlt et al., 2002; Kohara et al., 2002; Mei et al., 2006). This property requires metabolic activation via partial reduction of the nitro group, an intermediate in the formation of covalent adducts with DNA (Fig. 1) (Schmeiser et al., 1988). Several mammalian enzymes are capable of activating AA-I and AA-II (Krumbiegel et al., 1987; Schmeiser et al., 1986 and 1988; Stiborova et al., 2001) by catalyzing formation of a cyclic nitrenium ion (Fig. 1). This intermediate leads to the formation of purine adducts bound to the exocyclic amino groups of deoxyadenosine (dA) and deoxyguanosine (dG) residues in DNA (Fig. 1) (Schmeiser et al., 1988; Pfau et al., 1990a and 1991). The major DNA adducts detected in rodents and humans include 7-(deoxyadenosin-N\textsuperscript{6}-yl) aristolactam I (dA-AL-I), 7-(deoxyguanosin-N\textsuperscript{2}-yl) aristolactam I (dG-AL-I), 7-(deoxyadenosin-N\textsuperscript{6}-yl) aristolactam II (dA-AL-II) and 7-(deoxyguanosin-N\textsuperscript{2}-yl) aristolactam II (dG-AL-II). These AL-DNA adducts block transcription and/or DNA replication, which, in turn, may promote cell cycle arrest or trigger apoptosis (Li et al., 2006). Such AA-DNA adducts have been detected in the kidneys of AAN patients (Schmeiser et al., 1996; Bieler et al., 1997) and experimental animals where they serve as biomarkers of exposure to AA and are directly involved in the initiation of carcinogenesis (Arlt et al., 2002). AA-initiated carcinogenicity in rodents has, in fact, been associated with activation of H-ras by
A:T→T:A transversion mutations that occur exclusively at the first adenine of codon 61 (Schmeiser et al., 1991). Similar A:T→T:A mutations also have been observed in a urothelial cancer from a patient with AAN (Lord et al., 2004).

Efforts have been made to develop a rodent model for AAN. Toxicological studies in mice, rats and rabbits reveal that treatment with AA is associated with renal tubular damage (Mengs and Stotzem, 1993; Cosyns et al., 2001), interstitial fibrosis (Cosyns et al., 2001; Debelle et al., 2002) and cancer (Li et al., 2006; Mengs, 1983 and 1988; Cui et al., 2005). Cosyns et al. used a rabbit model to study the toxicities of AAs by injecting an intraperitoneal dose of 0.1 mg/kg 5 days a week for 17 to 21 months (Cosyns et al., 2001). These investigators observed impaired growth, increased serum creatinine, glycosuria, tubular proteinuria, and anemia. In addition, Sato et al. observed acute nephrotoxicity and renal interstitial fibrosis in C3H/He and BALB/c mice treated intraperitoneally or orally with AA (Sato et al., 2004). Similar histopathologic changes were observed in experiments with rats (Debelle et al., 2002). However, as a mixture of AA-I and AA-II was used in most of these studies, it was not possible to determine whether one or both components of AA contributed to the nephrotoxic and genotoxic effects.

The proposed use of AA as a therapeutic agent was largely abandoned when Mengs demonstrated their carcinogenicity in rats (Mengs, 1983). Mengs subsequently reported the formation of tumors in multiple organs when mice were treated with a mixture of AA-I and AA-II at a dose of 5.0 mg/kg/day for 3 weeks (Mengs, 1988). Again, AA-I and AA-II were administered simultaneously in these studies, preventing any
conclusion as to whether one or both compounds are responsible for the observed carcinogenic effects.

Believing it is critical to differentiate the nephrotoxicity and genotoxicity of AA-I and AA-II, we administered each of the purified compounds intraperitoneally or orally to C3H/He mice. Tissues were then subjected to DNA adduct and histopathologic analyses. Remarkably, we found that only AA-I was capable of inducing nephrotoxicity, manifested by tubular damage and development of interstitial fibrosis, while both AA-I and AA-II were equally genotoxic, as determined by their ability to form covalent adducts in target tissues with DNA. This unexpected observation suggests that the mechanism of AA-induced nephrotoxicity proceeds via a selective reaction with a critical protein, rather than with DNA.

**Materials and Methods**

**Materials.** Micrococcal nuclease and potato apyrase were purchased from Sigma-Aldrich (St. Louis, MO), spleen phosphodiesterase from Worthington Biochemical Corp. (Lakewood, NJ) and 3'-phosphatase-free T4 polynucleotide kinase and nuclease P1 from Roche Applied Science (Indianapolis, IN). \(\gamma^{32}P\)-ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Biosciences Corp. (Piscataway, NJ).

**Preparation of AA materials.** A mixture of AA-I and AA-II (40:60) was purchased from Fisher Scientific (Fairlawn, NJ) and the two acids were separated by preparative reverse-phase HPLC on a Varian automated ProStar System (Palo Alto, CA). An X-Terra MS C-18 (5 µM with 19 × 50 mm) column (Waters, Milford, MA) was used at a flow rate of 15 ml/min and eluted with acetonitrile in 0.1 M triethylamine/acetic acid
buffer at pH 7.5. A gradient of acetonitrile (16% to 25%), increased over 15 minutes, was used for this analysis, following a short wash of 50% acetonitrile after each run.

**Mouse experiments.** C3H/He mice (8-weeks-old, male), purchased from Taconic (Germantown, NY), were used in compliance with guidelines established by the NIH Office of Laboratory Animal Welfare. Animals were acclimated in temperature (22 ± 2°C)- and humidity (55 ± 5%)-controlled rooms with a 12-hour light-dark cycle for at least 1 week. Regular laboratory chow and tap water were allowed *ad lib*. Ten C3H/He mice were treated intraperitoneally with 2.5 mg/kg/day of AA-I or AA-II for 9 days. Control mice were treated with an identical volume of vehicle (PBS). Body weight was measured daily. Urine was collected over a 24-h period beginning after the final treatment on day 10. The mice were euthanized on day 10 or 24, and tissues were collected for biochemical and histopathologic analysis. Blood was collected for determinations of plasma urea and creatinine levels. The left kidney of each mouse was removed and stored in 10% neutral buffered formalin for histopathologic analysis. All other tissues, including the contralateral kidney, were removed, frozen, and stored at -80 °C. A similar protocol was followed for studying the mice given oral administration of AA-I or AA-II (2.5 mg/kg/day for 9 days). The use of animals was in compliance with the guidelines established by the NIH Office of Laboratory Animal Welfare.

**Digestion of DNA samples.** DNA was extracted from frozen tissues using a Qiagen DNeasy Tissue kit (Valencia, CA) according to the manufacturer’s protocol. The concentration of DNA was determined by UV spectroscopy as 50 µg/ml = O.D.\textsubscript{260 nm} 1.0. The DNA sample (5.0 µg) was enzymatically digested at 37 °C for 16 hours in 100 µl of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM CaCl\textsubscript{2}, micrococcal nuclease
(30 units) and spleen phosphodiesterase (0.15 unit) (Terashima et al., 2002). The reaction mixture was then incubated for another hour with nuclease P1 (1 unit), whereupon 200 μl of water was added. The reaction samples were then extracted twice with 200 μl of butanol; the butanol fractions were combined, back-extracted with 50 μl of distilled water, and then evaporated to dryness.

**32P-postlabeling/polyacrylamide-gel electrophoresis (PAGE) analysis.** The DNA digestion mixtures were incubated at 37°C for 40 min with 10 μCi of [γ-32P]-ATP and 3'-phosphatase-free T4 polynucleotide kinase (10 units), followed by incubation with apyrase (50 milliunits) for 30 min, as described previously (Terashima et al., 2002). The 32P-labeled products were subjected to PAGE for 4-5 hours on a nondenaturing 30% polyacrylamide gel (35 x 42 x 0.04 cm) with 1500-1800 V/20-40 mA. The position of 32P-labeled adducts was established by β-phosphorimager analysis (Molecular Dynamics Inc.). To quantify the 32P-labeled products, integrated values were measured using a β-phosphorimager and compared with the standards. Known amounts (0.152–0.000152 pmol) of dA-AL-I- or dG-AL-I-modified oligodeoxynucleotide, prepared by a chemical procedure, were mixed with 5 μg of calf thymus DNA (15,200 pmol) and served as the internal standard (characterized as 0.001-1 adduct/10^5 nucleotides) (Terashima et al., 2002; Dong et al., 2006). The detection limit for 5 μg DNA was approximately 5 adducts/10^9 nucleotides.

**Pathologic analysis of AA-treated mouse kidney.** Kidneys were fixed in 10% buffered formalin for histopathologic analysis. Sections, cut at 2-3 microns, stained with H&E and Mallory’s trichrome were visualized at a magnification of x200; the cortical tissues were then quantitatively scored for injury. The scorer was blinded as to
treatment. A total of 12 pathologic changes were used to quantify renal toxicity: (a) alterations in vasa recti; (b) tubular necrosis; (c) tubular regeneration; (d) tubular mitoses; (e) increase in Bowman’s space; (f) tubularization of Bowman’s space; (g) calcification or calcium salt deposition; (h) interstitial inflammation; (i) casts; (j) apoptosis; (k) edema; (l) tubular brush border loss (Conger et al., 1994). The scores obtained were defined as follows: 0, no histologic toxicity; 0.5-1.0, mild histologic toxicity; 1.0-2.0, moderate histologic toxicity; above 2.5, severe histologic toxicity. The histologic score for each mouse was determined using a weighted formula that included all of the 12 pathologic changes, such that the total score = a + 2b + c + d + e + f + 2g + 2h + 2i + j + k + l.

**Blood and urine chemistry.** Blood collected from mice in all treatment groups was used for analysis of blood urea nitrogen (BUN) and serum creatinine, using the Quanti Chrom Urea assay kit and Quanti Chrom Creatinine assay kit (Bioassay Systems, Hayward, CA), respectively. Urinary glucose was determined using the Liquid Glucose (Oxidase) Regent Set (Canton, MI) and protein with the Bio-Rad Protein Assay (Hercules, CA).

**Results**

**Mouse model of AA nephropathy.** To determine the nephrotoxicity of AA-I and AA-II, C3H/He mice were treated intraperitoneally with AA-I or AA-II (2.5 mg/kg/day for 9 days) and were sacrificed on the 1st day (day 10) and 14th day (day 24) after ending the treatment. Control mice were treated only with vehicle. One kidney of each mouse was used for histologic examination and the contralateral kidney was used for DNA adducts
analysis. Control mice steadily gained body weight, while AA-I-treated mice lost 5-6 g of body weight in 9 days (Fig. 2). After the final treatment, the body weight of AA-I-treated mice continued to decrease and 4 of the 10 mice died before or after the end of the treatment period, indicating the potent nephrotoxicity of AA-I.

In contrast, mice treated with AA-II lost relatively little body weight during the experiment. In fact, the body weight of AA-II-treated mice began to increase after the final treatment and all mice survived until the experiment ended on day 24. The kidneys of all AA-I-treated mice appeared pale at day 10, compared to the controls. No differences in appearance were observed between the kidneys of AA-II-treated and control mice. Similar results were obtained when the same doses of AA-I and AA-II were administered orally (data not shown).

**Histopathologic analysis of kidneys of AA-treated mice.** To establish the renal toxicity of AA-I or AA-II, kidneys collected at day 10 from mice treated p.o. with AA-I or AA-II were subjected to pathologic examination. AA-I was found to cause acute tubular necrosis (Fig 3B) and extensive cortical interstitial fibrosis (Fig. 3D). Other signs of toxicity, including mild interstitial inflammation and occasional tubular apoptosis, also were observed. Kidneys from control mice showed no necrosis or fibrosis. Among AA-II-treated mice, relatively little acute necrosis (Fig 3A) and no interstitial fibrosis (Fig 3C) was observed in the renal cortex. High overall pathologic scores were obtained in kidneys collected from mice treated with AA-I given either i.p. or p.o. (Table 1); no significant histologic differences between the control and AA-II-treated kidneys were observed. Supporting these results, we detected significant increases in urinary glucose and protein from mice treated with AA-I, indicating damage to the proximal
However, these functional parameters in AA-II-treated mice were not significantly different from those of the controls. Thus, AA-I administration is associated with strong nephrotoxic effects in mice while AA-II administration is not.

**Formation of AA-derived DNA adducts in mice treated with AA-I or AA-II.** To investigate DNA damage induced by AA-I or AA-II, organs were collected at day 10 and DNA adduct formation in each tissue was determined using $^{32}$P-postlabeling/PAGE analysis. dA-AL and dG-AL adducts were detected in all of the organs collected (Table 2 and Fig. 5). Following i.p. treatment with AA-I, the distribution of dA- and dG-AA-I-DNA adducts ranged from 0.2 to 14.5 adducts/10$^6$ nucleotides. The highest level of total AA-DNA adducts was observed in the kidney, with the next highest level occurring in the bladder. The levels of AL-I-DNA adducts in the kidney cortex, medulla and bladder were 14.5, 13.4 and 7.2 adducts/10$^6$ nucleotides, respectively, which is 2-70 times higher than those of other organs except for the liver (Table 2). Among mice treated i.p. with AA-II, the highest amounts of AA-II-DNA adducts were similarly found in the kidney and bladder. The levels of total AA-II-DNA adducts in the kidney medulla, cortex and bladder were 6-90 times higher than those in other organs (Table 2). The levels of AA-DNA adduct in kidney and bladder were similar in mice treated with AA-I and AA-II. However, the level of AA-II-DNA adducts in non-target tissues, liver, intestine, stomach and lung, was significantly lower than those in mice receiving AA-I (Fig. 5 and Table 2). In both AA-I- and AA-II-treated mice, the formation of dA-AA adducts was 3-10-fold higher than that of dG-AA adducts in all organs. Animals treated p.o. with AA-I or AA-II (2.5 mg/kg/day for 9 days) had higher levels of AA-DNA adducts in the renal cortex than was observed in that tissue following i.p. treatment (Table 2). The results of these
investigations indicate that the kidney is a primary target for DNA damage induced by AA.

**Discussion**

In humans, AAN initially damages the proximal renal tubule with renal interstitial fibrosis and urothelial carcinoma developing at a later stage of this nephropathy (Cosyns, 2003; Vanherweghem et al., 1993; Nortier and Vanherweghem, 2002). AA occurs naturally as a mixture of the structural analogs AA-I and AA-II. To explore the relative nephrotoxic and genotoxic potentials of AA-I or AA-II, we employed a mouse model developed by Sato et al. (Sato et al., 2004).

The systemic toxicity of AA-I is reflected in the body weight of AA-I-treated mice which decreased by more than 30% during the experiment. Four AA-I-treated mice died, two with acute renal cortical necrosis. Two other mice died during the post-treatment period and pathologic examination revealed acute tubular necrosis and renal interstitial fibrosis. In contrast, mice treated with the same dose of AA-II lost only 12% of their body weight during the treatment and then regained their weight rapidly, becoming indistinguishable from the untreated controls (Fig. 2). In addition, the gross morphology of kidneys from AA-I-treated mice differed dramatically in appearance, compared to AA-II-treated and control mice. Treatment with AA-I, given either i.p. or p.o., induced significant acute tubular necrosis and interstitial fibrosis. The extensive gross renal damage observed was documented by scoring the pathology (Table 1). Our results are generally consistent with those of an earlier study using several strains of mice in which AA-I and AA-II were administered i.p. (Sato et al., 2004). These authors reported focal
mild interstitial changes in mice treated with AA-II (Sato et al., 2004). However, in our study, no significant necrosis or interstitial fibrosis was observed following i.p. and p.o. treatment with AA-II when compared to the controls. We conclude that AA-I is the chemical species responsible for the profound nephrotoxic effects of AA, highlighting the importance of the O-methoxy group at position C-8 of the nitrophenanthrene ring.

DNA adducts have long been used as biomarkers of genotoxic damage by chemical carcinogens (Groopman and Kensler, 2005; Arlt et al., 2004). Using a quantitative $^{32}$P-postlabeling analysis and internal standards developed in our laboratory, we found the highest AA-DNA adduct levels in the renal cortex and medulla of mice treated with AA-I and AA-II, suggesting that the kidney is a primary target for AA-induced genotoxic damage. This result is consistent with our previous studies on rats treated for 7 days with AA-I or AA-II (Dong et al., 2006), but differs somewhat from reports of rats treated orally with AA-I (10 mg/kg/day) for 5 days (Pfau et al., 1990b) or with a single dose (5 mg/kg) of AA-I (Pfau et al., 1990a; Fernando et al., 1993), in which the highest AA-adduct levels were found in the forestomach. We attribute this apparent discrepancy to the use of qualitative $^{32}$P-postlabeling methods and the lack of internal standards used by other research groups (Terashima et al., 2002). In the present study, treatment with AA-I and AA-II promoted to the same extent of the formation of DNA adducts in primary target tissues in mice, as represented by renal cortex, medulla and bladder (urothelial cells), suggesting that both compounds have similar genotoxic potentials. In rats treated orally with a single dose of AA-I (5 mg/kg), AL-DNA adducts were detectable at 36 weeks (Pfau et al., 1990a); such persistence of unrepaired AL-DNA adducts also was observed in humans (Nortier et al., 2000).
of these AA-DNA adducts increases the probability of initiating mutagenic events (Kohara et al., 2002; Mei et al., 2006).

Our results strongly suggest that at least two different biochemical mechanisms are involved in the specific renal toxicities associated with AAN. The similar level of DNA adducts achieved in renal cortex, medulla and bladder in mice treated with equivalent doses of AA-I and AA-II suggest that each toxin produces similar degrees of DNA damage in renal proximal tubules and urothelial cells. Assuming that the mutagenic potential of AA-I- and AA-II-derived DNA adducts are similar, either AA-I or AA-II might initiate the cancers associated with AAN (Arlt et al., 2002; Mengs, 1983 and 1988; Cui et al., 2005).

In contrast to their DNA damaging effects, the striking difference between AA-I and AA-II with respect to their effects on the proximal renal tubule suggests that AA-I or its metabolite is responsible for nephrotoxicity. A potential candidate for such a role is AA-Ia generated by demethylation of the o-methoxy group (Arlt et al., 2002). However, the apparent lack of cytotoxicity of AA-Ia on renal epithelial cells in culture (Balachandran et al., 2005) argues against this proposal. Alternatively, AA-I, acting through its reductively activated nitrenium intermediate, could react with a specific protein to irreversibly inhibit a pathway critical to the function of renal proximal tubule cells, resulting in to cell death and fibrogenesis.

Although AA-II lacks the specific nephrotoxic properties of AA-I, as observed in vivo, both compounds possess significant cytotoxic activity, as demonstrated in vitro using epithelial cell cultures of renal origin (Balachandran et al., 2005; Wen et al., 2006). The IC_{50} for AA-I in the LLC-PK_{1} cell line is 8-fold lower than for AA-II (Balachandran et
(al., 2005). As it is likely that AL-DNA adducts are formed in cells treated with AA-II, we postulate that the cytotoxicity observed with this compound reflects inhibition of macromolecular synthesis.

In conclusion, we have shown that AA-I and AA-II have similar genotoxic and carcinogenic potential and, while both compounds are cytotoxic to cells in culture (Balachandran et al., 2005; Wen et al., 2006), AA-I is solely responsible for the nephrotoxicity associated with AAN in mice.

Acknowledgments

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Pfau W, Schmeiser HH and Wiessler M (1990b) $^{32}$P-postlabelling analysis of the DNA adducts formed by aristolochic acid I and II. *Carcinogenesis* 11:1627-1633.


Footnotes

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b) To whom correspondence and reprint requests should be addressed. Shinya Shibutani at Department of Pharmacological Sciences, State University of New York at Stony Brook, 1 Nicolls Road, Stony Brook, New York 11794-8651. shinya@phram.stonybrook.edu.
Figure legends

Figure 1. Scheme outlining the metabolism of aristolochic acids and formation of AA-derived DNA adducts.

Figure 2. Changes of body weight of C3H/He mice treated i.p. with AA-I and AA-II. The body weight was monitored every three or four days.

Figure 3. Random histolopathologic analysis of kidneys from C3H/He mice treated p.o for 10 days with AA-I (B,D), AA-II (A,C) or control vehicle and stained with haematoxylin and eosin (upper panels) or Mallory’s trichrome (lower panels). Severe acute tubular necrosis with little inflammation is observed in panel B while the renal cortical tissue in panel A is indistinguishable from the untreated controls. The trichrome stain confirms early interstitial fibrosis (green arrows) in the AA-I-treated animal while only the usual collagen is stained in the AA-II-treated mouse. All images are at identical magnification. Image bars are 100 microns.

Figure 4. Serum and urine markers of renal injury in C3H/He mice treated with AA-I or AA-II. Blood collected from mice treated p.o. with AA-I or AA-II (2.5 mg/kg/day for 9 days) was used for analysis of BUN and serum creatinine. Urinary glucose and protein determinations were performed as described in Materials and Methods.

Figure 5. Detection of AA-DNA adducts in C3H/He mice using $^{32}$P-postlabeling/PAGE.
C3H/He mice were treated i.p. with AA-I (A) or AA-II (B) at a dose of 2.5 mg/kg/day for 9 days. Tissues were collected 24 hrs after the final treatment, and the DNA was extracted. DNA adduct levels of each tissue were determined using $^{32}$P-postlabeling/PAGE. Standards represent 5 µg of calf thymus DNA ($1.52 \times 10^7$ fmol dNs) containing a known amount ($1.52 \times 10^2$ fmol dNs) of dA-AA-I- or dG-AA-I-modified oligomer, representing 1 adduct/$10^5$ dNs.
Table 1: Pathologic score of mice treated with AA-I or AA-II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Compound</th>
<th>Total score*</th>
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<tbody>
<tr>
<td>i.p.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>1.83 ± 1.15</td>
</tr>
<tr>
<td>Day 10</td>
<td>AA-I</td>
<td>15.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>AA-II</td>
<td>2.50 ± 1.32</td>
</tr>
<tr>
<td>Day 24</td>
<td>AA-I</td>
<td>18.8 ± 6.0</td>
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<tr>
<td></td>
<td>AA-II</td>
<td>0 ± 0</td>
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<tr>
<td>p.o.</td>
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<tr>
<td>Control</td>
<td></td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Day 10</td>
<td>AA-I</td>
<td>8.83 ± 0.58</td>
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<tr>
<td></td>
<td>AA-II</td>
<td>0 ± 0</td>
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<tr>
<td>Day 24</td>
<td>AA-I</td>
<td>10.6 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>AA-II</td>
<td>0 ± 0</td>
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*Data are expressed as mean values ± S.D. based on analyses of three mice. The grading system used for classifying changes in renal pathology is described in Materials and Methods.
Table 2: Formation of AA-DNA adducts in C3H/He mice treated with AA-I or AA-II

<table>
<thead>
<tr>
<th>Organs</th>
<th>AA-I</th>
<th></th>
<th>AA-II</th>
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<tbody>
<tr>
<td></td>
<td>dA-AA-I</td>
<td>dG-AA-I</td>
<td>dA-AA-II</td>
<td>dG-AA-II</td>
</tr>
<tr>
<td>i.p. treatment</td>
<td></td>
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<tr>
<td>Kidney (pelvis)</td>
<td>12.3 ± 0.90</td>
<td>1.10 ± 0.23</td>
<td>14.1 ± 6.38</td>
<td>2.47 ± 0.91</td>
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<tr>
<td>Kidney (medulla)</td>
<td>12.9 ± 2.88</td>
<td>1.63 ± 0.15</td>
<td>12.5 ± 4.95</td>
<td>2.30 ± 0.61</td>
</tr>
<tr>
<td>Bladder</td>
<td>6.49 ± 1.68</td>
<td>0.71 ± 0.05</td>
<td>6.73 ± 5.51</td>
<td>0.88 ± 0.45</td>
</tr>
<tr>
<td>Stomach</td>
<td>2.02 ± 0.86</td>
<td>0.79 ± 0.24</td>
<td>0.87 ± 0.11</td>
<td>0.31 ± 0.13</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.73 ± 0.61</td>
<td>0.46 ± 0.16</td>
<td>0.43 ± 0.30</td>
<td>0.09 ± 0.08</td>
</tr>
<tr>
<td>Liver</td>
<td>6.52 ± 3.20</td>
<td>1.15 ± 0.38</td>
<td>0.66 ± 0.53</td>
<td>0.46 ± 0.36</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.13 ± 0.10</td>
<td>0.07 ± 0.11</td>
<td>0.13 ± 0.09</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Lung</td>
<td>3.32 ± 1.42</td>
<td>0.50 ± 0.13</td>
<td>0.60 ± 0.46</td>
<td>0.14 ± 0.09</td>
</tr>
<tr>
<td>p.o. treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney (cortex)</td>
<td>17.2 ± 6.40</td>
<td>2.58 ± 0.79</td>
<td>22.1 ± 4.10</td>
<td>5.20 ± 1.57</td>
</tr>
</tbody>
</table>

*Data are expressed as mean values ± S.D. from analyses of three mice.
Figure 1
Figure 2
Figure 4
Figure 5

A

Standard

Kidney

Medulla Cortex Bladder Stomach Intestine Liver Spleen Lung

dG-AA I-
dA-AA I-

B

dG-AA II-
dA-AA II-

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