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 (S.S., H.D., N.S., A.P.G.) and Department of Pathology (F.M.), State University of New York, Stony Brook, New York; and Department of Drug Information and Communication, Graduate School of Pharmaceutical Sciences, Chiba University, Yayoi-cho, Inage-ku, Chiba, Japan (S.U.)

Received January 5, 2007; accepted March 26, 2007

ABSTRACT:

Ingestion of herbal remedies containing aristolochic acids (AAs) is associated with the development of a syndrome, designated aristolochic acid nephropathy (AAN), which is characterized by chronic renal disease, designated aristolochic acid nephropathy (AAN) (Arlt et al., 2002; Cosyns et al., 2003). 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 high prevalence of urothelial cell carcinoma, which often occurred years after the onset of chronic renal disease (Cosyns et al., 1994; Nortier et al., 2000). Moreover, in contrast with 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-nitrophenanthro-(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 are available regarding the metabolic fate of AA-I and AA-II in experimental animals and humans (Krumbiegel et al., 1987). 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 Ia (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 (Schmeiser et al., 1986,1988; Krumbiegel et al., 1987; Stiborová 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 and deoxyguanosine residues in DNA (Fig. 1).

ABBREVIATIONS: AA, aristolochic acid; AAN, aristolochic acid nephropathy; 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-N2-yl) aristolactam; dG-AL, 7-(deoxyguanosin-N2-yl) aristolactam; AL, aristolactam; PAGE, polyacrylamide gel electrophoresis.
bular proteinuria, and anemia. In addition, Sato et al. (2004) observed acute nephrotoxicity and renal interstitial fibrosis in C3H/He and BALB/c mice treated i.p. or p.o. with AA. 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 AAs as a therapeutic agent was largely abandoned when Mengs (1983) demonstrated their carcinogenicity in rats. Mengs (1988) 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. 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 i.p. or p.o. 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, whereas 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 was from Worthington Biochemicals (Lakewood, NJ) and 3′-phosphatase-free T4 polynucleotide kinase and nuclease P1 was from Roche Applied Science (Indianapolis, IN). [γ-32P]ATP (specific activity, >6000 Ci/mmol) was obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK).

**Preparation of AA Materials.** A mixture of AA-I and AA-II (40:60) was purchased from Fisher Scientific Co. (Fairlawn, NJ), and the two acids were separated by preparative reverse-phase high-performance liquid chromatography on a Varian automated ProStar System (Varian, Inc., Palo Alto, CA). An X-Terra MS C18 (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 (16–25%), increased over 15 min, was used for this analysis, after a short wash of 50% acetonitrile after each run.

**Mouse Experiments.** C3H/He mice (8-week-old males), purchased from Taconic Farms (Germantown, NY), were used in compliance with guidelines established by the National Institutes of Health Office of Laboratory Animal Welfare. Animals were acclimated in temperature (22 ± 2°C)- and humidity (55 ± 5%)-controlled rooms with a 12-h light/dark cycle for at least 1 week. Regular laboratory chow and tap water were allowed ad libitum. Ten C3H/He mice were treated i.p. 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 (phosphate-buffered saline). 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 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 National Institutes of Health Office of Laboratory Animal Welfare.

**Digestion of DNA Samples.** DNA was extracted from frozen tissues using a DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. The concentration of DNA was determined by UV spectroscopy as

---

**Fig. 1.** Scheme outlining the metabolism of aristolochic acids and formation of AA-derived DNA adducts.
50 µg/ml = OD_{260 nm} 1.0. The DNA sample (5.0 µg) was enzymatically digested at 37°C for 16 h in 100 µl of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM CaCl₂, 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, and the butanol fractions were combined, back-extracted with 50 µl of distilled water, and then evaporated to dryness.

**32P-Postlabeling/PAGE Analysis.** The DNA digestion mixtures were incubated at 37°C for 40 min with 10 µCi of [γ-³²P]ATP and ³²-phosphatase-free T4 polynucleotide kinase (10 units), followed by incubation with apyrase (50 munits) for 30 min, as described previously (Terashima et al., 2002). The ³²P-labeled products were subjected to PAGE for 4 to 5 h on a nondenaturing 30% polyacrylamide gel (35 × 42 × 0.04 cm) with 1500 to 1800 V at 20 to 40 mA. The position of ³²P-labeled adducts was established by β-phosphorimager analysis (GE Healthcare). 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/10⁹ nucleotides) (Terashima et al., 2002; Dong et al., 2006). The detection limit for 5 µg of DNA was approximately 5 adducts/10⁹ nucleotides.

**Pathologic Analysis of AA-Treated Mouse Kidney.** Kidneys were fixed in 10% buffered formalin for histopathologic analysis. Sections, cut at 2 to 3 µm and stained with hematoxylin and eosin and Mallory’s trichrome were visualized at a magnification of 200×; 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, and l) tubular brush border loss (Conger et al., 1994). The scores obtained were defined as follows: 0, no histologic toxicity; 0.5 to 1.0, mild histologic toxicity; 1.0 to 2.0, moderate histologic toxicity; 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 + 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 and serum creatinine, using the Quanti Chrom Urea assay kit and Quanti Chrom Creatinine assay kit (Bio-Rad, Hercules, CA) and protein with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Supporting these results, we detected significant increases in urinary glucose and protein from mice treated with AA-I, indicating damage to the proximal tubule (Fig. 4). 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 whereas AA-II administration is not.

**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 tubule (Fig. 4). 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 whereas 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 ³²P-postlabeling/PAGE analysis. dA-AL and dG-AL adducts were detected in all of the organs collected (Table 2; Fig. 5). After i.p. treatment with AA-I, the distribution of dA- and dG-AA-I-DNA adducts ranged from 0.2 to 14.5 adducts/10⁶ 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/10⁶ nucleotides, respectively, which is 2 to 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 14.5, 13.4, and 7.2 adducts/10⁶ nucleotides, respectively, which is 2 to 70 times higher than those of 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 levels of AA-II-DNA adducts in nontarget tissues, liver, intestine, stomach, and lung, was significantly lower than those in mice receiving AA-I (Fig. 5; Table 2). In both AA-I- and AA-II-treated mice, the formation of dA-AA adducts was 3- to 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 after 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 used a mouse model developed by 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 with those from 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 pathologic changes (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 after i.p. and p.o. treatment with AA-II compared with 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 (Arlt et al., 2004; Groopman and Kensler, 2005). Using a quantitative 32P-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 32P-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 the same extent 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). The extended half-life of these AA-DNA adducts increases the probability of initiation of 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 levels 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 potentials 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, 1988; Cui et al., 2005).

In contrast with 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

**TABLE 2**

<table>
<thead>
<tr>
<th>Organs</th>
<th>dA-AA-I</th>
<th>dG-AA-I</th>
<th>dA-AA-II</th>
<th>dG-AA-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p. treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney (cortex)</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>
</tr>
<tr>
<td>Kidney (medulla)</td>
<td>12.9 ± 2.88</td>
<td>1.63 ± 0.15</td>
<td>12.5 ± 4.95</td>
<td>2.90 ± 0.64</td>
</tr>
<tr>
<td>Bladder</td>
<td>6.49 ± 1.68</td>
<td>0.71 ± 0.05</td>
<td>6.73 ± 5.1</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.
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 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 IC50 for AA-I in the LLC-PK1 cell line is 8-fold lower than that 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, although 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. We thank M. C. Torres for purifying AA-I and AA-II by high-performance liquid chromatography techniques.

References


Address correspondence to: Dr. Shinya Shibutani, 1 Nicolls Road, Stony Brook, NY 11794-8651. E-mail: shinya@pharm.stonybrook.edu