Kidney International, Vol. 67 (2005), pp. 1797–1805

Structure activity relationships of aristolochic acid analogues: Toxicity in cultured renal epithelial cells

PREMALATHA BALACHANDRAN, FENG WEI, RUI-CHAO LIN, IKHLAS A. KHAN, and DAVID S. PASCO

National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, University of Mississippi, Oxford, Mississippi; Department of Pharmacognosy, School of Pharmacy, University of Mississippi, Oxford, Mississippi; and National Institute for the Control of Pharmaceutical and Biological Products, State Food and Drug Administration, Beijing, People's Republic of China

Structure activity relationships of aristolochic acid analogues: Toxicity in cultured renal epithelial cells.

Background. Aristolochia species are nephrotoxic and carcinogenic. Recent studies showed that aristolochic acid (AA) could induce acute renal failure and tubular lesions in several species and available evidences demonstrate the unequivocal role of AA in so called Chinese herbs nephropathy.

Methods. A series of AA derivatives isolated from *Aristolochia* spp. were analyzed for their nephrotoxic potential using the neutral red dye exclusion assay in cultures of LLC-PK_1 cells. The structural relationships between AA I and its analogues were compared with their cytotoxic effects to predict structural determinants for AA toxicity. Further, caspase-3 assay was performed on toxic compounds to determine if caspases, the enzymes that play a critical role in apoptosis are involved in AA-induced cytotoxicity.

Results. AA I was found to be most toxic followed by AA II, AA VIIIa, and AA Ia in decreasing levels of toxicity. The other compounds, nitrophenanthrene carboxylic acid analogues of AA I, aristolactams, and other derivatives did not exhibit considerable toxicity. The results showed significant relationships between cytotoxicity of AA compounds and the localization of functional groups in their structure. Analogues containing hydroxyl groups diminished cytotoxicity. The demethylated analogues of AA I are markedly less active. The negative impact on cytotoxicity was found on nitroreduction of AA I. AA induced caspase activation was also observed.

Conclusion. These cytotoxic data suggest that the nitro and methoxy groups are critical determinants of nephrotoxicologic potency of AA.

Aristolochic acid (AA) and their derivatives are structurally related nitrophenanthrene carboxylic acids isolated from *Aristolochia* spp. (e.g., *Aristolochia fangchi, Aristolochia clematis, Aristolochia manshuriensis, Aristolochia contorta*). Herbal drugs derived from *Aristolochia* plants have been used as medicine in obstetrics and in the treatment of snake bites [1]. The plant extracts have also been used for the therapy of arthritis, gout, rheumatism, and festering wounds [2, 3]. The anti-inflammatory properties of AA have encouraged its use in various drug formulations in Germany [4, 5] until it was identified as a potential carcinogen in rodents by Mengs [6, 7] in late 1980s. Acute AA intoxication resulted in acute renal failure, whereas chronic administration induced multisystemic tumors in rats.

In the early 1990s, ingestion of AA was incriminated in the outbreak of the so-called "Chinese herbs nephropathy" (CHN), a severe tubulointerstitial nephritis initially described in Belgium women who had followed a particular slimming regimen that included the Chinese herbs, *Stephania tetrandra* and *Magnolia officinalis* [8, 9]. Because of name similarities, one of the herbs *S. tetrandra (*known in traditional Chinese medicine by the Pin Yin name *Han fang ji*) was inadvertently replaced in weight-reducing pills by *A. fangchi* (Pin Yin name *Guang fang ji*), which contains nephrotoxic AA [10]. In addition to a rapidly progressive interstitial renal failure due to particularly severe fibrosis, the clinical course of CHN is complicated by tumoral transformations in the urothelium [11]. The observation of typical renal interstitial fibrosis and urothelial malignancy in patients from other European [12, 13] and Asian countries [14, 15] and also in the United States [16] who were exposed to *Aristolochia* spp. containing AA conclusively demonstrated the etiologic role of AA in the genesis of the disease [17]. Therefore, it has been proposed to designate the interstitial nephropathy in which the unequivocal role of AA has been fully documented as aristolochic acid nephropathy (AAN) [18].

Several studies have established AA as a strong nephrotoxin and genotoxic mutagen [19–23]. Subsequently, all pharmaceutical preparations containing AA have been withdrawn from the market in many countries. Recently, the federal Food and Drug Administration (FDA) has issued an alert advising the consumers to

Key words: aristolochic acid, structure, toxicity.

Received for publication July 21, 2004
and in revised form October 7, 2004
Accepted for publication November 30, 2004

immediately discontinue the use of any botanical products containing AA [24].

Inasmuch as AA nephrotoxicity and carcinogenicity represents a serious health risk, there exists increasing demand to understand the structural requirements of various AA compounds derived from *Aristolochia* spp., for their nephrotoxicity. The rationale behind the present toxicological study is to compare the cytotoxic potential of AA I with a series of its analogues in order to investigate the structural determinants of AA I toxicity.

The involvement of AA in the early dysfunction of proximal tubular cells during CHN and their causal relationship between DNA adduct formation have been reported [25] and also reviewed [11]. Several studies have employed cultures of LLC-PK_1 cell lines, which are proximal tubular cells from pig kidney as in vitro system for investigating nephrotoxicity of various compounds [26–28]. We report here the results of studies designed to evaluate the cytotoxicity of various compounds isolated from *A. fangchi* and *A. contorta* with respect to their chemical structure on LLC-PK_1 cells. This in vitro model system could be somewhat reflective of the clinical situation with regard to AA-induced nephrotoxicity.

AA might have direct cytotoxic effect, inducing renal tubular lesions and subsequent acellular interstitial fibrosis. Alternatively, AA DNA adducts could induce mutations responsible not only for the development of AAN-associated malignancies [17] but also for the fibrotic process [22, 29]. Concerning the possible mechanism of AA-mediated acute tubular injury, pervious reports showed that cell apoptosis played an important role in the development of the insult [30–32]. The increase in intracellular calcium ion concentration has been suggested as one of the reasons for AA I–induced apoptosis in LLC-PK_1 cells [30]. Cells generally require specialized machinery to undergo apoptosis. The central component of this machinery is a proteolytic system involving a family of proteases called caspases. These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in the disassembly of the cell [33]. When apoptosis is induced, procaspases are proteolytically cleaved and reassemble to form active caspases. In the present study, the role of caspases in AA-induced apoptosis in proximal tubular cells has also been investigated to understand the mechanism of AA toxicity.

METHODS

Cell line maintenance

Cells used in this assay were LLC-PK_1 cells, renal epithelial cells from pig, and BT-549 cells purchased from American Type Culture Collection (Bethesda, MD, USA). Cells were maintained in RPMI 1640 medium (Gibco Invitrogen Corporation, Carlsbad, CA, USA) containing fetal bovine serum (FBS) (Atlanta Biologicals, Inc., Atlanta, GA, USA) (5% for LLC-PK_1 and 10% for BT-549) and penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Table 1. Physical chemical properties of test compounds

Name	Color	Solubility
AA-I	Yellow	Methanol, DMSO
AA-II	Yellow	Methanol, DMSO
AA VIIIa	Yellow	Methanol, DMSO
AA Ia	Yellow	Methanol, DMSO
Aristolic acid	Yellow white	$CHCl_3$, DMSO
AA-III	Yellow	Methanol, DMSO
7-OH AA-I	Yellow	Methanol, DMSO
AA VIa	Yellow	Methanol, DMSO
AA-C	Yellow red	Methanol, DMSO
AA-D	Yellow red	Methanol, DMSO
Aristofolin B	Yellow white	$CHCl_3$, DMSO
Aristolactam I	Yellow	Methanol, DMSO
Aristolactam II	Yellow	Methanol, DMSO
Aristolactam I-N-glu	Yellow	Methanol, DMSO
Aristolactam C-N-glu	Yellow	Methanol, DMSO
Aristolactam BIV	Yellow	Methanol, DMSO
Aristolactam A IIIa	Yellow	Methanol, DMSO
Ariskanin B	Yellow	Methanol, DMSO
p- OH benzoic acid	White	Methanol, DMSO
3-OH 4-methoxy benzoic acid	White	Methanol, DMSO
4-OH 2,6, dimethoxy benzoic acid	White	Methanol, DMSO
Emodin	Yellow red	Methanol, DMSO

Abbreviations are: AA, aristolochic acid; DMSO, dimethyl sulfoxide.

AA compounds investigated in this study

The AA compounds used in this nephrotoxic study were isolated in our laboratory from *Aristolochia* plants, *A. contorta* and *A. fangchi* by standard isolation protocols and their chemical structure have been identified and confirmed by spectroscopic methods (data not shown). Based on their structure, these compounds were categorized into nitrophenanthrene carboxylic acid derivatives, aristolactams, and benzoic acid derivatives. The chemical structures of these compounds are available elsewhere [34, 35]. The physical chemical properties of the test compounds are given in Table 1.

Eleven nitrophenantherene caroboxylic acid derivatives of AA, namely AA I, AA II, AA III, AA Ia, 7-OH AA I, AA IVa, AA VIIIa, AA C, AA D, aristolic acid, and aristofolin B (Table 1), and seven compounds of aristolactams derivatives, namely aristolactam I, aristolactam II, aristolactam N-beta-D-glucoside, aristolactam-C-N-glu, aristolactam B IV, aristolactam A IIIa, and ariskanin B, and benzoic acid derivatives, p-OH benzoic acid, 3-OH-4-methoxy benzoic acid, 4-OH-2,6-dimethoxy-benzoic acid, and emodin, were selected for the evaluation of cytotoxicity.

Sample preparation

The test compounds were originally suspended in dimethyl sulfoxide (DMSO) at a concentration of

20 mg/mL and they were further diluted to required concentration with cell culture media. The graded concentrations from 1 μmol/L to 300 μmol/L were used to determine the toxicity. An IC_{50} value of 300 μmol/L or above was considered as nontoxic.

Neutral red assay

An important aspect of this assay has been its utilization in establishing structure activity relationships for the series of related compounds [36, 37]. This assay procedure was adapted from Babich and Borenfreund [38]. The principle of this assay, which is based on lysosomal membrane integrity, has been previously described [39]. Briefly, the cells were seeded at the density of 2.5×10^4 cells in a volume of 0.25 mL media per well in 96-well microplate. The cell number per well was selected based on the density found to display linear growth during 24 hours of incubation in pilot studies (data not shown). Twenty-four hours after seeding, the cells were replaced with 0.25 mL of fresh media and graded concentrations of test compounds were added. DMSO was used as the control. Doxorubicin was used as the positive control and hederasaponin C was used as the negative control. After the cells are exposed to test compounds for 48 hours, the medium was replaced with 0.1 mL of fresh serum-free media containing neutral red (16 6μg/mL) (Sigma Chemical Co., St. Louis, MO, USA) and incubation with dye continued for another 90 minutes to allow for uptake of vital dye into lysosomes of viable, uninjured cells. Cells were then washed with saline and incorporated dye from viable cells was liberated by lysis with 0.33% HCl in isopropanol and the absorbance was read at 540 nm which directly correlates with cell viability. Quantitation of the extracted dye was shown to be linear with cell numbers, both by direct cell counts and by protein determination of cell populations [36, 37, 39].

Data analysis

The mean absorbance of toxicant treated wells was divided by mean absorbance of control wells and multiplied by 100 to yield the percent viability. The intra- and interassay variations for each compound were determined ($N = 3$ and $N = 2$, respectively). The dose response curve of relative cell viability was plotted to delineate the concentrations of AA compounds that inhibited cell growth to 50% (IC_{50} value).

Caspase 3/7 activity assay

Caspase 3/7 activity was estimated using Caspase Glo™ 3/7 luminescent assay kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. A total of 0.01×10^6 cells were seeded in white walled 96-well luminometer plate and incubated for 24 hours at 37°C. The cells were replaced with fresh media containing the test compounds and the incubation was continued for another 48 hours. Doxorubicin was used as the positive control and DMSO-treated cells were used as negative control for the assay. Blank contained DMSO and cell culture medium without cells. The assay provides a proluminescent caspase 3/7 substrate, which contains the tetrapeptide sequence Asp-Glu-Val-Asp (DEVD), in a reagent optimized for caspase activity, luciferase activity, and cell lysis. The addition of equal volume of this single reagent in "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal, produced by luciferase. The luminescence was measured at 30 minutes after the addition of the reagent using a Packard microplate scintillation counter in single photon mode and the luminescense is proportional to the amount of caspase activity present. DEVD-fmk was used as caspase 3/7 inhibitor. The data are expressed as mean ± SD. Comparisons were performed by unpaired Student *t* test analysis.

Control experiments from both neutral red assay and caspase 3/7 activity assay demonstrated that DMSO had no toxic effect on this cell line.

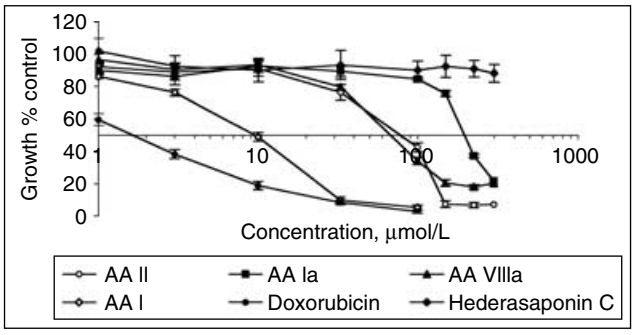


Fig. 1. Comparison of dose-response curve of toxic aristolochic acid (AA) derivatives with controls. LLC-PK_1 cells were treated with test compounds at given concentrations for 48 hours and cytotoxic potential was compared with dimethyl sulfoxide (DMSO)-treated controls using neutral red assay as described in the **Methods** section. Doxorubicin was used as positive control and hederasaponin C was used as negative control. IC_{50} values were as follows: doxorubicin 1.7 μmol/L, AA I 10 μmol/L, AA II 80 μmol/L, AA VIIIa 70 μmol/L, and AA Ia 200 μmol/L. Values are mean ± SD ($N = 6$).

RESULTS

Effect of nitrophenantherene caroboxylic acid derivatives on LLC-PK_1 cell line

Cytotoxic effects of eleven compounds of nitrophenantherene caroboxylic acid derivatives on LLC-PK_1 cell viability are shown in Figures 1 and 3. Out of these AA I, AA II, AA VIIIa, and AA Ia exhibited dose-dependent effects and the ranking by IC_{50} was AA I >AA VIIIa > AA II >AA Ia (Fig. 1). AA I was found to be an intense cytotoxic compound that elicits maximum toxicity (IC_{50} value 10 μmol/L) among all the compounds

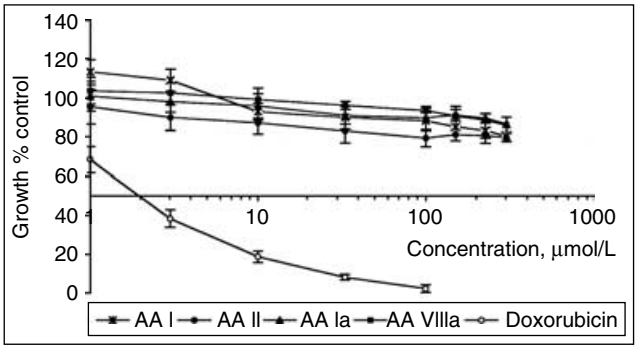


Fig. 2. Comparison of dose-response curve of aristolochic acid (AA) compounds in non-renal cell line. BT-549 cells were treated with test compounds at given concentrations for 48 hours and cytotoxic potential was compared with dimethyl sulfoxide (DMSO)-treated controls using neutral red assay as described in the **Methods** section. IC_{50} of doxorubicin 2 µmol/L. Values are mean ± SD ($N = 6$).

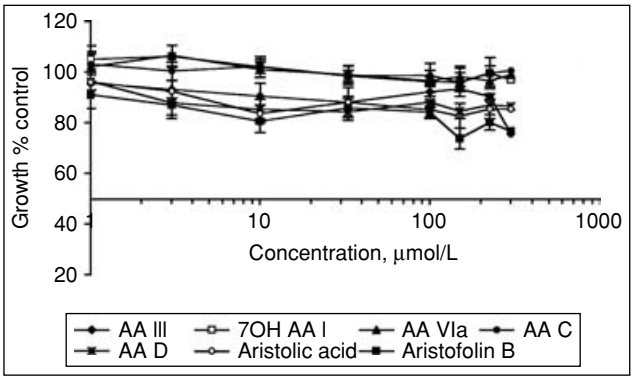


Fig. 3. Comparison of dose-response curve of non-toxic aristolochic acid (AA) derivatives. LLC-PK_1 cells were treated with AA compounds at given concentrations for 48 hours and cytotoxic potential was compared with dimethyl sulfoxide (DMSO)-treated controls using neutral red assay as described in the **Methods** section. Values are mean ± SD ($N = 6$).

examined on this cell line. AA VIIIa have an IC_{50} value of 70 µmol/L followed by AA II (IC_{50} value 80 µmol/L) and AA Ia (IC_{50} value 200 µmol/L). Doxorubicin, a known cytotoxic agent (as a positive control) showed very high toxicity with IC_{50} value of 1.7 µmol/L. Hederasaponin C isolated from *Clematis* spp. was not toxic to this cell line (negative control).

The above compounds that showed toxicity to renal cell line were tested on human epithelial breast cell line BT-549, to evaluate their kidney specific cytotoxic action. All these compounds have failed to show toxic effect on this breast cell line (Fig. 2), indicating their specificity towards nephrotoxic action. From Figure 3, it is evident that the rest of the nitrophenantherene derivatives from *Aristolochia* spp. were not toxic to LLC-PK_1 cell line.

Effect of aristolactam derivatives on LLC-PK_1 cell line

Seven aristolactam derivatives were evaluated for their toxic properties on the LLC-PK_1 cell line in graded concentrations and none of them were found to be toxic in this cell line (Fig. 4).

Effect of other minor AA derivatives on LLC-PK_1 cell line

Several other compounds present in *Aritolochia* plants in minor quantities like benzoic acid derivatives, p-OH benzoic acid, 3-OH-4-methoxy benzoic acid, 4-OH-2,6-dimethoxy-benzoic acid, and emodin were also nontoxic (Fig. 5).

Cell apoptosis and caspase activation

To investigate whether caspases play a role in AA-induced proximal tubular cell injury and apoptosis, caspase-3/7 assay was performed using AA compounds. The activity of caspase-3/7 was estimated using labeled substrate, DEVD-luciferin. The caspase activity was dramatically induced by AA I, AA II, AA Ia, and AA VIIIa and also by the mixture of AA I and II (Fig. 6) in a dose dependent manner (data not shown). The doses of these compounds that showed 100% inhibition of cell growth in neutral red assay induced maximum stimulation of caspase activity on this cell line. AA mixture induced activation of caspase was reduced in the presence of inhibitor, suggesting the specificity of caspase induction by these compounds. None of the other AA compounds (at the concentration of 300 µmol/L) were able to induce caspase activities significantly.

DISCUSSION

Renal proximal tubular cells isolated from humans and animals can serve as useful tools for assessing the biochemical and physiologic functions of the kidney [26]. These cells participate in the excretion of biogenic metabolites, xenobiotics, and drugs and represent a primary target site for several toxic compounds including AA in vivo [6], in vitro [25], and also in humans [40, 41]. In CHN, the proximal tubular cells are characterized by interstitial fibrosis [22] and the presence of extensively flattened cells has also been reported in acute toxic conditions [40, 41]. The cellular mechanisms of AA toxicity have also been delineated in the opossum kidney cell line, another model of proximal tubular cells [25].

LLC-PK_1 is an established cell line derived from normal porcine kidneys that has been widely used to study renal functions [42]. Studies indicate that LLC-PK_1 cells retain many properties of native proximal tubular epithelial cells [43]. In culture, these cells form an oriented monolayer with microvilli at their apical side and exhibit typical activities of renal proximal tubular cells such as transport of hexose, amino acids, phosphate, and organic cations [44]. In the present study, the LLC-PK_1 cell line has been used as an in vitro model system to evaluate the toxicity of AA derivatives.

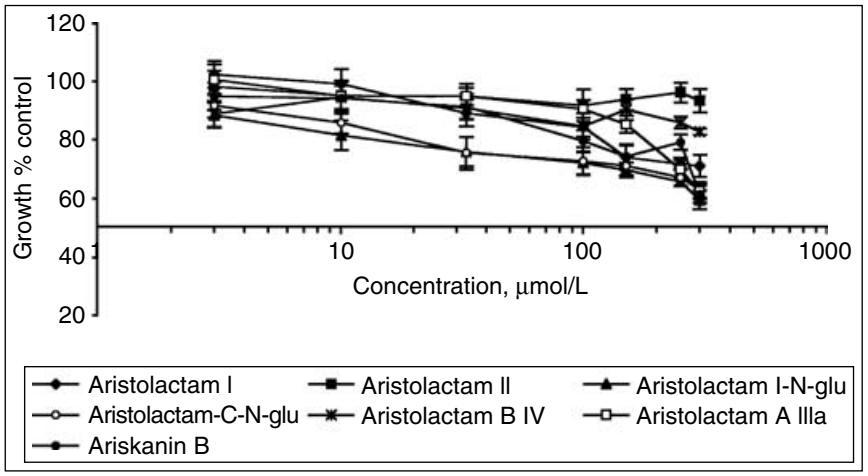


Fig. 4. Comparison of dose-response curve of aristolactam derivatives. LLC-PK_1 cells were treated with aristolochic acid (AA) compounds at given concentrations for 48 hours and cytotoxic potential was compared with dimethyl sulfoxide (DMSO)-treated controls using neutral red assay as described in the **Methods** section. Values are mean ± SD ($N = 6$).

Nitroarenes are common environmental contaminants that may represent a risk to human health. Consequently, observations regarding the toxic potential of nitrophenanthrene derivatives are of substantial interest. AA, the active principle extracted from *Aristolochia* spp., contain predominantly AA I and AA II, which are nitrophenanthrene carboxylic acid derivatives. *Aristolochic* spp. also contain several other derivatives such as 7-hydroxy AA I, AA Ia, AA-C, AA D, AA III, AA VIa, AA VIIIa, aristolic acid, aristofolin B, aristolactams, and few benzoic acid derivatives in various quantities [11, 45]. From the results of the present study, we observe some correlation between the chemical structure and the toxicity profile of AA and their derivatives. Structure-toxicity analysis shows that the ring structures, the side chains and even their localization are critical determinants for toxic properties. Table 2 depicts the structure of nitrophenanthrene carboxylic acid compounds. In this structure, the "nitro" ($-NO_2$) group in R_4 position and "methoxy" ($-OCH_3$) group in R_1 position are critical determinants for maximum toxicity as observed for AA I, which is 8-methoxy-6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid. Any modification from AA I structure, namely the addition, deletion, substitution, or replacements of the position of these side chains, drastically reduce toxicity. Since AA I is the most toxic compound identified thus far, its toxicity was compared with that of its structural analogues isolated from *Aristolochia* spp. Further discussion about toxicity is based on AA I structure.

In AA II, the removal of "methoxy" group from R_1 position reduces the toxicity. AA II, which differs from AA I by one "methoxy" group, has an IC_{50} value of 80 μmol/L and is far less toxic than AA I (IC_{50} 10 μmol/L). AA VIIIa has an IC_{50} value of 70 μmol/L. Reduced toxicity could be due to the combined effect of the rearrangement of the methoxy group position (from R_1 to R_2) and the addition of the hydroxyl group at R_1 position. In AA Ia, O-demethylation (i.e., by substitution of "hydroxyl" group in the place of "methoxy" group at R_1 position), reduces the toxicity to 200 μmol/L. In aristolic acid reductive replacement of nitro group from R_4 position renders the compound nontoxic. In aristofolin B reductive replacement of nitro group from R_4 position and addition of hydroxyl group at R_2 position results in nontoxic compound. In AA C, the deletion of a "methoxy" group from R_1 and substitution of a "hydroxyl" group at R_3 renders the compound nontoxic. In AA III, the shifting of "methoxy" group from R_1 position to R_3 also makes the compound nontoxic.

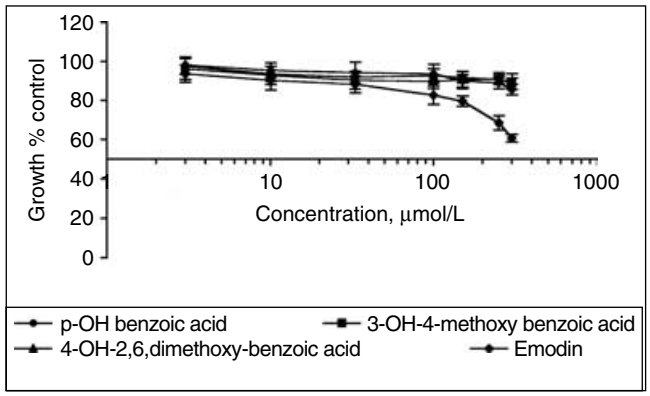


Fig. 5. Comparison of dose-response curve of benzoic acid derivatives and emodin. LLC-PK_1 cells were treated with aristolochic acid (AA) compounds at given concentrations for 48 hours and cytotoxic potential was compared with dimethyl sulfoxide (DMSO)-treated controls using neutral red assay as described in the **Methods** section. Values are mean ± SD ($N = 6$).

Addition of one hydroxyl group eliminates the toxic nature of AA I. Toxicity is absent when a "hydroxyl" group is added to either the R_2 position (e.g., 7-OH AA I), R_3 position (e.g., AA-D) or R_5 position (e.g., AA VIa).

During the process of nitroreduction, AA I loses a major portion of its toxicity as evidenced from the nontoxic nature of aristolactam compounds in LLC-PK_1 cells.

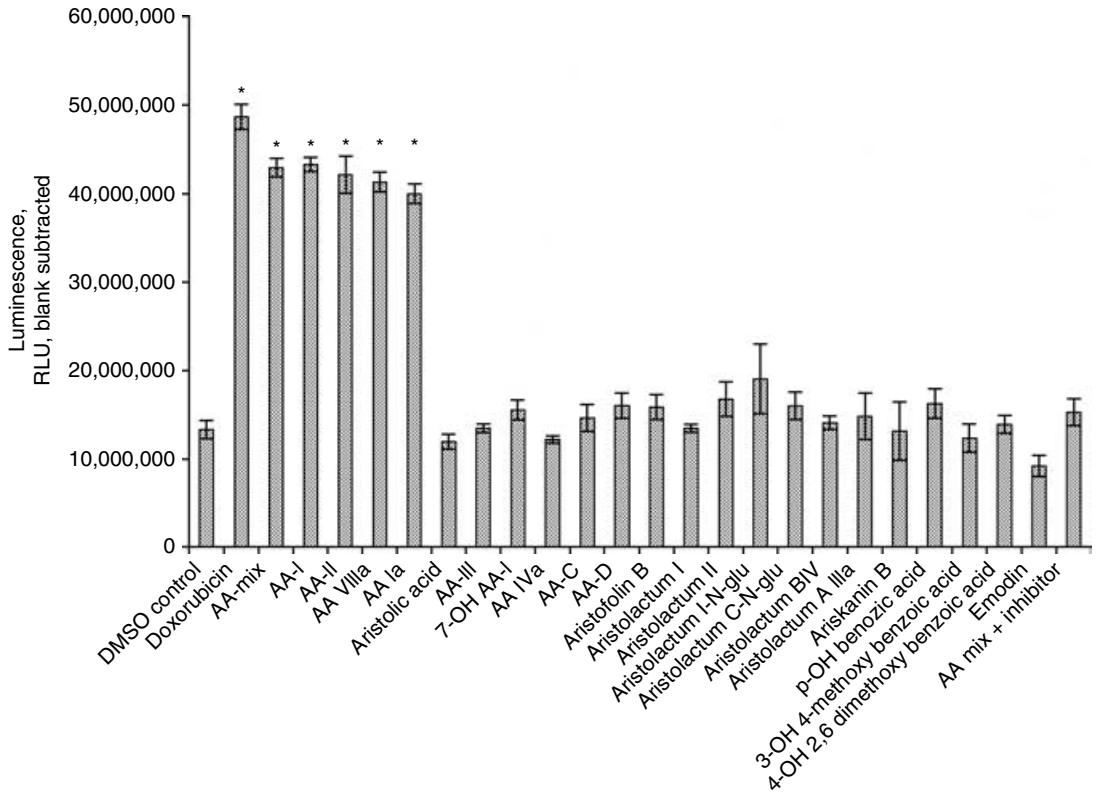


Fig. 6. Induction of caspase activity by aristolochic acid (AA) compounds. LLC-PK$_1$ cells were treated with test compounds (AA I 10 μmol/L; AA II 160 μmol/L; AA VIIIa 140 μmol/L; AA Ia 300μmol/L; AA mixture 33 μg/mL; all other compounds 300 μmol/L) for 48 hours and caspase 3/7 activity in cell lysates was compared with dimethyl sulfoxide (DMSO)-treated controls as described in the **Methods** section. Results are mean ± SD ($N = 3$). $^*P < 0.001$ compared with DMSO-treated controls.

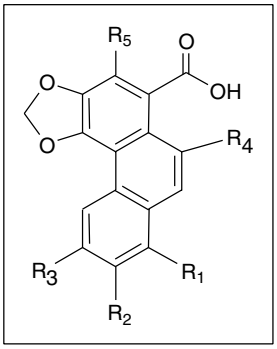
Reduction/methylation of dioxalane ring structure also showed marked loss of toxicity.

Consistent with previous studies [46] we have found that AA I is the most toxic compound in this renal cell line. Aristolic acid and AA D, reported as less toxic compound against P388 cell lines and *Salmonella* strains, are also found to be non-toxic to LLC-PK$_1$ cells. Aristolactam I and aristolactam-N-beta-D-glucoside, which have previously been shown to be toxic in cultured P388 and human epidermoid carcinoma (KB) cells [46] were not toxic in LLC-PK$_1$ cells. This could be due to the variation in cell lines and aristolactams might not be nephrotoxic. Our observation of AA I as the most toxic compound is in agreement with recent in vivo studies by Sato et al [47]. They have also observed strongest nephrotoxic effect exerted by AA I and mild nephrotoxic effects by AA II in mice. AA IVa and aristolactam I caused no renal abnormality indicated by blood chemistry or histologic change.

Mutagenic and carcinogenic effects of AA are associated with the formation of AA-DNA adducts and the extent of adduct formation largely depends on the structure of the compound. Several reports have identified AA I and II as the major components of AA and they are known to be genotoxic mutagens forming DNA adducts after metabolic transformation [48, 49]. This is in line with our observation of AA I and II as toxic compounds. Nortier and Vanherwegham [50] have analysed tissue samples from CHN patients and found the presence of AA-related DNA adducts in 38/39 cases with high prevalence of urothelial carcinoma (46%). They found these adducts to be most persistent as it was detected in kidney tissue 89 months after the discontinuation of pill presumably containing *Aristolochia* spp. The lifelong persistence of AA-DNA adducts in AA toxic conditions makes their detection in tissues a valid biologic marker ever years after the cessation of AA exposure [11].

Nitro group is one of the functional group that has received considerable attention from toxicity point of view. Several studies have confirmed that the nitro group is important for the mutagenic activity of AA in *Salmonella* and this mutagenecity was due to enzymic reduction of the NO_2 group to yield the hydroxylamino or even amino analogues [51, 52]. Nitroreduction has been reported to be the crucial step in the pathway of metabolic activation of AA to their ultimate mutagenic species and this indicates the importance of nitro group in AA I moiety for its toxic and carcinogenic properties. In the case of similar nitro compounds like nitrobenzene [53] and nitrotoluene [54] derivatives, the number and position of nitro group

Table 2. Structural details of nitrophenantherene carboxylic compounds



Name	R1	R2	R3	R4	R5	Chemical Name
AA I	OCH_3	H	H	NO_2	H	8-methoxy-6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
AA II	H	H	H	NO_2	H	6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
AA III	H	H	OCH_3	NO_2	H	10-methoxy-6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
AA Ia	OH	H	H	NO_2	H	8-hydroxy-6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
7-OH AA I	OCH_3	OH	H	NO_2	H	8-methoxy-9-hydroxy-6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
AA VIa	OCH_3	H	H	NO_2	OH	8-methoxy-4 hydroxy-6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
AA VIIIa	OH	OCH_3	H	NO_2	H	8-hydroxy-9-methoxy-6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
AA C	H	H	OH	NO_2	H	10-hydroxy-6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
AA D	OCH_3	H	OH	NO_2	H	8-methoxy-10-hydroxy-6-nitro-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
Aristolic acid	OCH_3	H	H	H	H	8-methoxy-phenanthro- (3,4-d)-1,3-dioxolo-5-carboxylic acid
Aristofolin B	OCH_3	OH	H	H	H	8-methoxy-9-hydroxy–phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid

AA is aristolochic acid.

affects the toxicity. Even in the case of benzamines the position of nitro group affected their mutagenic properties [55]. In all these compounds, the reduction of aromatic nitro group gives rise to toxic species and this shows the functional importance of "nitro group" in toxicity.

Although several reports have suggested the functional importance of the nitro group in AA mediated mutagenicity/toxicity, the results from the present study suggest that NO_2 is not the only structural requirement for AA-mediated cytotoxicity. The presence or absence of methoxy and hydroxyl groups also plays a major role in cytotoxicity. Although the results from Schmeiser et al [51] have shown that the methoxy group is not required for mutagenicity, our results suggest it is one of the essential structural requirements for nephrotoxicity. Pezzuto et al [46] have also suggested that ring substituents of this class of compounds could modulate biologic responses. Sierra-Alvarez and Lettinga [56] have noticed that increase in the number of methoxy, alkyl, or Cl groups can increase the toxicity of aromatic compounds. Although, this observation is in line with our finding of functional importance of methoxy group, the true mechanism of increased toxicity by the presence of methoxy group, and reduced toxicity by the introduction of hydroxyl group are still unclear and remains to be explored. But we suggest that, the presence of hydroxyl group can make the compound more water soluble and this can lead to increased detoxification resulting in reduced toxicity.

The metabolism of AA has been studied in different species, including humans, and has shown that the products of nitroreduction (aristolactams) are the major metabolites found in urine and feces [57]. Aristolactams are not mutagenic themselves and require metabolic activation by exogenous metabolic system whereas AA I and AA II are direct mutagens in *Salmonella* strains [17]. The cellular injury mechanism of aristolactams was also different from AA I [58].

On examination of the relevance of the present findings about AA toxicity on proximal tubular cells to the understanding of CHN, the proximal tubular cell damage observed in the present toxicity studies might result in inflammation and fibrosis in CHN conditions. This in vitro data can lead to the hypothesis that structure of AA compounds plays a primary role in AA toxicity and result in progressive kidney destruction in AA toxic/carcinogenic conditions. The early development of tubular proteinuria and glucosuria observed in CHN patients after AA intoxication, strongly suggest that proximal tubular cells play a key role in pathogenesis of CHN patients [11]. According to recent review by Cosyns [11], only a small fraction, about 3% to 5% of the patients who followed slimming regimen at Belgian clinic, developed CHN. He suggested that this low toxicity/carcinogenicity rate might be due to the variation in the content of AA in the batches of herbal pills. The present findings from our toxicity studies suggest that the difference in the composition of individual AA compounds (toxic versus nontoxic) in those herbal preparations could have potentiated toxicity/carcinogenicity in those cases. In several CHN patients reported worldwide, the names of the consumed herbs

are not reported [14, 59, 60] or available in Chinese and Japanese characters only [21, 61, 62], although AA has been identified in most of the herbal preparations used by these patients. In such cases, based on our present findings, we recommend, that both identification of herb as well as the content of all these individual AA compounds could be more helpful to understand the cause and extent of toxicity. The toxic nature of the compounds AA VIIIa and AA Ia reported from this data, in addition to known toxic compounds AA I and II, emphasize the necessity for identification of these compounds in CHN/AAN patients.

Role of caspases in AA-mediated nephrotoxicity

Caspases have emerged as powerful markers of cells undergoing apoptosis. Caspase 3/7 are active cell death proteases involved in the execution phase of apoptosis, where cells undergo morphologic changes such as DNA fragmentation, chromatin condensation, and apoptotic body formation [63, 64]. Both caspase 3/7 are functionally similar and have similar substrate specificities [65] and cleavage of PARP during apoptosis may be due to a combination of action of both these caspases [66]. To investigate whether these executioner caspases were involved in apoptotic mechanism caused by AA, caspase 3/7 activity assay was performed. Our data suggest that AA compounds activate these caspases, which could play a role in AA-induced cell injury. Recently, Li et al [58] have suggested the secretion of transforming growth factor-β (TGF-β) 1 in AA I stimulated apoptosis in human kidney (HK)-2 cells. As demonstrated in variety of cell lines (hepatoma [67], human gastric cancer cells [68], and lymphoma cells [69]), it is known that, caspase 3 activation is required for TGF-β–induced apoptosis. Thus, caspase activation observed in our present study could mediate apoptosis through TGF-β1 secretion. This finding may be the starting point to investigate the molecular basis for the requisite role of caspases in AA-induced nephrotoxicity. Caspases could also serve as attractive potential targets to modulate AA-induced injury in renal tubular cells.

CONCLUSION

This study gives insights into the nephrotoxic potential of AA I and its structural analogues. These observations clearly indicate the importance of functional groups in the order of nitro group ($-NO_2$) > methoxy ($-OCH_3$) in terms of their potency to exert toxic properties. The addition of hydroxyl groups also renders the compound less toxic/nontoxic. The AA I, the predominant constituent of *Aristolochia* spp., is the most active compound in terms of its cytotoxicity, and its structural analogues either have less effect or no effect. The alterations in renal cellular function could be greatly modified by the proportion of AA I and its structural analogues. Tubular cell apoptosis mediated by caspase 3/7 activation might be one of the mechanisms involved in this insult. Differences in uptake, distribution, and metabolism of the AA derivatives, however, may influence the in vivo toxicity of these compounds.

ACKNOWLEDGEMENT

This work was funded in part by the Food and Drug Administration grant " Botanical Dietary Supplements: Science-Base for Authentication," FD-U-002071–01.

Reprint requests to David S. Pasco, Ph.D., National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Oxford, MS 38677. E-mail: dpasco@olemiss.edu

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