Physiological and Molecular Characterization of Aristolochic Acid Transport by the Kidney

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Nonstandard abbreviations: AA, aristolochic acid; AA-I, aristolochic acid I; AA-II, aristolochic acid II; AAN, aristolochic acid nephropathy; CYP1A1/2, cytochrome P450 1A1/2; OAT, organic anion transporter; mOAT, murine organic anion transporter; CHO, Chinese hamster ovary; AA-Ia, aristolochic acid Ia; EV, empty vector; HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin; dA, deoxyadenosine; dG, deoxyguanosine

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ABSTRACT

Consumption of herbal medicines derived from *Aristolochia* plants is associated with a progressive tubulointerstitial disease known as aristolochic acid nephropathy. The nephrotoxin produced naturally by these plants is aristolochic acid I (AA-I), a nitrophenanthrene carboxylic acid that selectively targets the proximal tubule. This nephron segment is prone to toxic injury due to its role in secretory elimination of drugs and other xenobiotics. Here we characterize the handling of AA-I by membrane transporters involved in renal organic anion transport. Uptake assays in heterologous expression systems identified murine organic anion transporters (mOats) 1, 2 and 3 as capable of mediating transport of AA-I. Kinetic analyses showed that all three transporters have an affinity for AA-I in the sub-micromolar range and thus are likely to operate at toxicologically relevant concentrations *in vivo*. Structure-activity relationships revealed that the carboxy group is critical for high-affinity interaction of AA-I with mOats 1, 2 and 3, whereas the nitro group is required only by mOat1. Furthermore, the 8-methoxy group, while essential for toxicity, was not requisite for transport. Mouse renal cortical slices avidly accumulated AA-I, achieving slice-to-medium concentration ratios >10. Uptake by slices was sensitive to known mOat1 and mOat3 substrates and to the organic anion transport inhibitor probenecid, which also blocked the production of DNA adducts formed with reactive intracellular metabolites of AA-I. Taken together, these findings indicate that OAT family members mediate high-affinity transport of AA-I and are likely involved in the site-selective toxicity and renal elimination of this nephrotoxin.
INTRODUCTION

Although *Aristolochia* plants have been used as herbal remedies for centuries in both Eastern and Western cultures, their nephrotoxic properties were not recognized until the early 1990s when a group of Belgian women developed chronic renal failure following the inadvertent ingestion of the Chinese herb *Aristolochia fangchi* as part of a weight loss regimen (Vanherweghem et al., 1993). The carcinogenic properties of these plants soon became apparent when many of these same patients presented with urothelial malignancies of the renal pelvis and ureter (Nortier et al., 2000). Since then, there have been numerous independent reports of renal failure associated with the medicinal use of *Aristolochia* plants, and this nephropathy has emerged as a global health concern (Debelle et al., 2008). Recently, chronic *Aristolochia* poisoning has been linked to the etiology of another renal disease, namely Balkan endemic nephropathy (Grollman et al., 2007), where ingestion of wheat grains contaminated with *Aristolochia* seeds is the likely route of exposure.

The nephrotoxin produced by *Aristolochia* plants has been identified as aristolochic acid (AA), and the disease it causes, originally named Chinese herbs nephropathy, is known as AA nephropathy (AAN). AAN is a chronic tubulointerstitial disease characterized by cortical tubular atrophy, preserved glomeruli and a dense interstitial fibrosis that is most pronounced in the outer renal cortex (Cosyns et al., 1994a). The histologic and pathophysiologic features of AAN in humans point to the proximal tubule as the primary site of toxicity, and animal models of the disease have established that this part of the
nephron is selectively damaged by the toxin (Sato et al., 2004; Lebeau et al., 2005; Shibutani et al., 2007; Pozdzik et al., 2008).

AA is a collective term used to describe the complex mixture of structurally related nitrophenanthrene carboxylic acids produced naturally by Aristolochia plants. The principal constituents are AA-I and AA-II, two chemically identical compounds except for the presence (AA-I) or absence (AA-II) of an O-methoxy group at the 8-position (Fig.1). This functional group is a key determinant of toxicity as studies in mice have established that AA-I is nephrotoxic while AA-II is not (Sato et al., 2004; Shibutani et al., 2007). Both compounds are metabolized extensively in vivo (Fig. 1). CYP1A1/2 catalyzes demethylation of AA-I (Rosenquist et al., 2010) to yield the nontoxic metabolite AA-Ia (Shibutani et al., 2010). Enzymatic nitroreduction of AA-I and AA-II generates the corresponding aristolactams, and while these end products are nontoxic, (Sato et al., 2004), this reaction generates reactive nitrenium intermediates that form covalent adducts with purine bases in DNA (Pfau et al., 1990b). These mutagenic lesions are the basis for the carcinogenic effects of AA, but are unrelated to nephrotoxicity since both AA-I and –II cause similar patterns of DNA damage whereas only AA-I is toxic to the kidney (Sato et al., 2004; Shibutani et al., 2007).

Renal elimination of various endogenous organic metabolites and foreign compounds occurs through the processes of glomerular filtration and tubular secretion. Secretory transport, a function of the proximal tubule, is a particularly important route of excretion for chemicals whose permeation through the glomerular filtration barrier is restricted
when bound to plasma proteins. The organic anion transporter (OAT) family, a group of multi-specific membrane transport proteins with broad substrate preferences, plays a central role in the renal handling of negatively charged drugs and other organic compounds (VanWert et al., 2010). Eleven members of this family have been identified to date and nine are expressed in the kidney. OATs 1 and 3 are localized in the proximal tubule basolateral membrane where they mediate concentrative cellular uptake, the initial step in organic anion secretion, while renal localization of OAT2 remains controversial (Enomoto et al., 2002; Ljubojevic et al., 2007). The other OAT family members identified in the kidney are found in the apical membrane and their physiological roles remain to be determined.

As a low-molecular weight organic anion with a hydrophobic core, AA-I has the chemical features characteristic of known OAT substrates. We hypothesized that AA-I may be handled by members of the OAT family, and given its site of toxicity, particularly by those OATs known to be expressed in the proximal tubule. In the present study, we used radiolabeled AA-I and –II to evaluate how these two compounds are transported by the kidney, and whether membrane transport properties provide a basis for their differential toxicity. Kinetic properties were assessed to determine whether these transporters operate at toxicologically relevant concentrations of the nephrotoxin. In addition, structure-activity relationships were probed with competition studies using various AA metabolites and analogs.
METHODS

Materials. Gas mixtures composed of 95% oxygen-5% carbon dioxide were purchased from Welco-CGI (Bethlehem, PA). Invitrogen (Carlsbad, CA) supplied hygromycin. Sodium pyruvate, dimethylsulfoxide, p-aminohippuric acid, probenecid, estrone-3-sulfate, Ham’s F-12 nutrient mixture medium, Hanks’ balanced salt solution (with bicarbonate; without phenol red), Gey’s balanced salt solution, fetal calf serum, bovine serum albumin (BSA), 1,2-cyclooctadienyl iridium (I)pentane-2,4-dionate, calf thymus DNA, micrococcal nuclease and potato apyrase were obtained from Sigma-Aldrich (St. Louis, MO). Spleen phosphodiesterase was purchased from Worthington Biochemicals (Lakewood, NJ) and 3’-phosphatase-free T4 polynucleotide kinase and nuclease P1 were obtained from Roche Applied Science (Indianapolis, IN). [γ-32P]ATP (specific activity >6000 Ci/mmol) and 3H2O (specific activity 1 Ci/mmol) were purchased from GE Healthcare (Piscataway, NJ). CHO-K1 cells (parental strain) were supplied by the Cell Culture and Hybridoma Facility at Stony Brook University.

Aristolochic acids and metabolites. AA-I and –II were isolated and purified from a commercially available mixture of these two compounds (Sigma-Aldrich; St. Louis, MO) as previously described (Dong et al., 2006). Aristolochic acid Ia (AA-Ia) was isolated from rat urine as previously reported (Shibutani et al., 2010). Aristolactams-I and –II, desnitro AA-I and descarboxy AA-I were prepared using established methods (Krumbiegel et al., 1987; Priestap, 1987). Stock solutions (25 mM) for each compound were prepared in DMSO and stored at −20°C. Relative purities (AA-I, 98%; AA-II, 94%; AA-Ia, 97%; aristolactam-I, 90%; aristolactam-II, 100%; descarboxy AA-I, 83%; desnitro
AA-I, 100%) were determined by HPLC and stock solution concentrations were verified by UV spectroscopy using established extinction coefficients.

AA-I and –II were tritiated at the 2-position via a hydrogen isotope exchange reaction catalyzed by 1,2-cyclooctadienyl iridium (I)pentane-2,4-dionate (McAuley et al., 2003). The reaction components, 5 mg AA-I or –II, 5 mg iridium catalyst, and 25 mCi $^3$H$_2$O (specific activity 1 Ci/mmol), were dissolved in 0.5 ml dimethylformamide and incubated at 95°C for 4 h. Product identities were verified by HPLC with UV detection and by mass spectrometry using known reference standards. Radiochemical purity was >98% for both compounds, which had specific activities of 0.50 Ci/mmol ($[^3\text{H}]$AA-I) and 0.44 Ci/mmol ($[^3\text{H}]$AA-II). Final products were dissolved in a 1:1 mixture of aqueous 0.1% trifluoroacetic acid and acetonitrile ($[^3\text{H}]$AA-I, 233 uCi/ml; $[^3\text{H}]$AA-II, 225 uCi/ml) and stored at –20°C. Both compounds were radiolabeled at the 2-position, a site that is not modified by metabolism (Fig. 1).

**Generation of mOat-expressing transfectants.** All mOat-containing cell lines were generated using Invitrogen’s Flp-In system (Carlsbad, CA). Generation of the CHO-FRT empty vector (EV), CHO-mOat1, and CHO-mOat3 cell lines has been reported previously (Vanwert et al., 2008; VanWert and Sweet, 2008), and CHO-mOat2 cells were developed in a similar fashion. Briefly, the mOat2 cDNA clone was identified in a cDNA library of the Integrated Molecular Analysis of Genomes and their Expression Consortium (cDNA clone MGC:37316; IMAGE:4975360), purchased from the American Type Culture Collection (Manassas, VA), and amplified and purified using standard
laboratory procedures. A restriction fragment containing the mOat2 sequence was isolated from gels and ligated into vector pcDNA5/FRT, yielding the vector pcDNA5/mOat2, which was used to transfect CHO-FlpIn cells. For transfection, 2x10^5 CHO-FlpIn cells/well were plated in a 6-well culture plate, grown overnight, and then treated for 24 hr at 37°C with 1 µg pcDNA5/mOat2 DNA and 8 µg pOG44 DNA using Transfectin Lipid Reagent (2 µl Transfectin/µg DNA; Bio-Rad, Hercules, CA). The transfected cells were then given fresh medium and grown for an additional 24 h prior to trypsinization and transfer to a 25 cm² tissue culture flask containing 700 µg/ml hygromycin B (Invitrogen) in Ham’s F-12 medium with 10% serum. After approximately four weeks, selection positive clones were identified by transport assay and thereafter maintained in 125 µg/ml hygromycin B.

**AA uptake by mOat transfectants.** CHO cells stably transfected with empty vector (EV) as control, or mOats 1, 2, or 3 were grown to confluence in 24-well plates in F-12 medium supplemented with 10% fetal calf serum (FCS) and 125 µg/ml hygromycin. Hygromycin was removed from the medium 24 hours prior to transport studies. For uptake assays, cells were first rinsed with HBSS at room temperature and then 500 µl of HBSS containing varying concentrations of [³H]AA-I or [³H]AA-II were added to each well. Aliquots of the incubation solutions were reserved for liquid scintillation counting and uptake was terminated at various times by rinsing each well four times with 1 ml of ice-cold HBSS. Cells were solubilized overnight in 500 µl of 1N NaOH at room temperature. Cell extracts were neutralized with 10N HCl, and aliquots were added to Ecoscint H (National Diagnostics; Atlanta, GA) and then analyzed on a Tricarb 3100TR.
liquid scintillation counter (Perkin Elmer; Shelton, CT) with quench correction. Data were normalized to cellular protein measured with the Bradford reagent (Sigma-Aldrich; St. Louis, MO) using BSA as the standard. For dose-response studies, kinetic parameters were determined by non-linear regression fit to the Michaelis-Menten equation with GraphPad Prism version 5.02 for Windows (GraphPad Software; San Diego, CA)

**AA uptake by mouse renal cortical slices.** These experiments were conducted in accordance with institutional guidelines and with the approval of the Institutional Animal Care and Use Committee at Stony Brook University. Male C3H/HeJ mice (8-12 weeks-old; Jackson Laboratory, Bar Harbor, ME) were euthanized by CO₂ asphyxiation. The kidneys were removed and placed in ice-cold Hanks’ balanced salt solution supplemented with 10 mM Hepes (HBSS, pH 7.4). Each kidney was decapsulated, cut into cross-sectional pieces, and thin slices (~0.5 mm thick) were prepared by hand by shaving the outer cortex with a #15 scalpel blade. Single slices were placed in 2 ml of ice-cold HBSS in individual wells of a 24-well plate. Each slice was weighed in a tared weigh boat, gently blotted on the weigh boat and then returned to the 24-well plate. The weigh boat with residual fluid was reweighed, and this value was subtracted from the first weight measurement to correct for the contribution of extracellular fluid to slice weight. The average wet weight of slices used for the transport assays was 6 ± 0.3 mg, n=22. To avoid pooling slices from multiple mice, studies were designed so that one mouse provided sufficient slices for a single independent experiment with multiple variables.
Each slice was transferred to a glass vial containing 1 ml of Gey’s balanced salt solution supplemented with 1 mM pyruvate and pre-equilibrated with 95% oxygen-5% CO₂ at 37°C. Vials were placed in a 37°C orbital shaking water bath and continuously aerated with a hydrated gas mixture of 95% oxygen-5%CO₂. After preincubation for 15 min, uptake assays were initiated by the addition of [³H]AA-I or -II at a final concentration of 400 nM. When used, competitors/inhibitors were added to the incubation solution two min prior to the addition of radiochemicals. Aliquots of incubation solutions were reserved for liquid scintillation counting and uptake was terminated at various times by transferring slices to ice-cold HBSS in a 24-well plate. Slices were rinsed three times and then solubilized overnight in 500 µl of 1N NaOH. After neutralization with 10N HCl, aliquots of the slice extracts were added to Ecoscint H (National Diagnostics; Atlanta, GA) and samples were analyzed on a Tricarb 3100TR liquid scintillation counter (Perkin Elmer; Shelton, CT) with quench correction. Data are presented as slice-to-medium (S/M) ratios calculated as the quotient of slice dpm/mg wet weight divided by incubation solution dpm/µl.

**DNA adduct assay.** Mouse renal cortical slices were prepared as described above. For each sample, three slices (~20 mg wet weight) were incubated together in a glass vial containing 4 ml of Gey’s balanced salt solution supplemented with 1 mM pyruvate. Vials were placed in a 37°C orbital shaking water bath and continuously aerated with a hydrated gas mixture of 95% oxygen-5%CO₂. After preincubation for 15 min, AA-I was added to each vial to a final concentration of 2 µM. When used, probenecid (final
concentration 1 mM) was added 2 min prior to AA-I. After incubation for 1.5 h, the slices were rinsed in ice-cold HBSS and then stored at −80°C.

Genomic DNA was isolated from the renal slices with a DNeasy kit (Qiagen; Valencia, CA) according to the manufacturer’s instructions. The average yield per sample was 21 ± 1.4 µg DNA, corresponding to 1 µg genomic DNA/mg wet weight. DNA-aristolactam adduct levels in these samples were measured with a 32P-postlabeling/polyacrylamide gel electrophoresis assay previously described in detail (Dong et al., 2006). Synthetic oligodeoxynucleotide standards containing known quantities of deoxyadenosine (dA)-aristolactam or deoxyguanosine (dG)-aristolactam adducts were added to calf thymus DNA and used to identify and quantify bands corresponding to these adducts in samples.

DNA derived from samples (2.5 µg) or standards (5 µg) was digested enzymatically to mononucleotides by treatment with micrococcal nuclease and spleen phosphodiesterase at 37°C for 16 h, followed by exposure to nuclease P1 for 1 h. DNA digests were extracted with butanol, back-extracted with water and then evaporated to dryness. Digestion products were enzymatically labeled with 32P by 40 min incubation with T4 polynucleotide kinase and [γ-32P]ATP, followed by treatment with apyrase for 30 min. Labeled products in samples and standards were separated by electrophoresis on a 30% non-denaturing polyacrylamide gel. Gels were scanned on a Storm 840 phosphorimager (Amersham Biosciences; Piscataway, NJ), and sample bands corresponding to 32P-dA-aristolactam and 32P-dG-aristolactam adducts were identified.
by their position on the gel relative to the standards. Band intensities were quantified by densitometry using ImageQuant version 5.2 software (Molecular Devices; Carlsbad, CA).

**Protein binding assay.** Binding of $[^3H]$AA-I to plasma proteins was measured by ultrafiltration using Microcon centrifugal filter units (10,000 molecular weight cut-off; Millipore Corp., Bedford, MA). Preliminary studies showed that a significant fraction of $[^3H]$AA-I binds to the collection tubes in the absence of protein, resulting in overestimation of the fraction bound. Therefore, we used a method designed to reduce non-specific binding of lipophilic compounds, as reported by others (Taylor and Harker, 2006).

$[^3H]$AA-I (final concentration 2 μM) was added to 500 μl of HBSS or HBSS supplemented with either 10% FCS or 4% bovine serum albumin (BSA), and these solutions were incubated for 30 min at 37°C. Aliquots (200 μl) of each solution were transferred to the upper chambers of the filter units, which were then incubated for 30 min at 37°C. Each unit containing a radiolabeled sample was paired with a unit containing 200 μl of 4% BSA in the absence of $[^3H]$AA-I. Five-μl aliquots were taken from each upper chamber containing $[^3H]$AA-I and reserved for liquid scintillation counting, and then all units were centrifuged at 10,000 g for 7 min at room temperature. The upper chambers, with retentates, were removed and retained, and the collection tubes (pre-tared) were weighed to estimate volume. The upper chambers from each pair of units were then inverted and placed on the collection tube of their corresponding
partner. Retentates were recovered by centrifugation at 700 g at room temperature for 3 min. Collection tubes were weighed again to determine volume, and samples were taken for liquid scintillation analysis.

**Statistics.** Statistical analyses were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software; San Diego, CA). All values are presented as means ± standard error of the mean (SE); n represents the number of independent experiments. For multiple group comparisons, statistical significance (p<0.05) was assessed by analysis of variance followed by the Newman-Keuls *post hoc* test. Statistically significant (p<0.05) differences between two groups were evaluated with Student’s unpaired, 2-tailed t-test.
RESULTS

AA uptake by mOat transfectants. Initial screening of CHO cells expressing mOat1, 2, or 3 indicated that all three transfectants accumulated $[^3]$HAA-I to levels above that achieved by control EV-transfectants. For EV- and mOat1-, mOat2- and mOat-3-transfectants incubated with 1 $\mu$M $[^3]$HAA-I or –II, cellular accumulation was time-dependent and steady state was reached within 15-20 min (Fig. 2, panels A and B). Plateau values were higher for all mOat-transfectants compared to EV cells.

Uptake rates (1 min) for cells incubated with 1 $\mu$M $[^3]$HAA-I or -II are compared in Fig. 2 (panels C and D). EV-transfectants accumulated measurable quantities of both AA-I and -II, and this level of basal permeability was 1.8-fold higher (p<0.05) for AA-I compared to AA-II. Similar results were observed with parental CHO-K1 cells (data not shown), indicating that transfection and subsequent selection processes had not altered basal transport properties. AA-I uptake rates were significantly higher for all mOat-transfectants compared to EV cells (Fig. 2C). The magnitude of these increases varied among the mOat-transfectants according to the following rank order: mOat2 (six-fold) > mOat1 (three-fold) > mOat3 (two-fold). AA-II accumulation was also greater in mOat-transfectants compared to EV cells (Fig. 2D), and as was the case for AA-I, it was highest (seven-fold) in cells expressing mOat2. AA-II uptake rates were similar for mOat1- and mOat3-transfectants and on average they were three-fold higher than EV values. The uptake data presented in Figs. 2C and 2D were transformed into mOat-mediated transport rates by subtraction of the EV uptake components. As shown in Fig. 2E, comparison of AA-I and AA-II uptake rates after this correction revealed that AA-I
was more avidly accumulated in cells expressing mOats 1 or 2, whereas both compounds were handled similarly by mOat3-transfectants.

**Kinetic properties of AA transport.** Based on the time course studies (Fig. 2), we determined that 1-min uptake measurements were an accurate and practical estimate of initial uptake rates in this model, and this time-point was used for all kinetic analyses. The kinetic transport constants for AA uptake by EV- and mOat-transfected cells were evaluated at concentrations ranging from 0.125 to 2 µM. Regression analysis showed a linear relationship between the initial uptake rate and AA concentration in EV-transfectants, with average slopes of 6.4 ± 0.4 and 4.7 ± 0.9 for AA-I and AA-II, respectively (Fig. 3). These values were not significantly different from each other. In marked contrast, after subtraction of the EV background component, uptake rates in mOat-transfectants displayed saturation kinetics characteristic of carrier-mediated transport. $K_m$ values for AA-I uptake were in the sub-micromolar range (0.4-0.8 µM) with the following rank order of affinity: mOat2>mOat3>mOat1 (Table 1). High-affinity transport of AA-II was also evident, and although the $K_m$ values associated with AA-II uptake were roughly double those of AA-I for each mOat-transfectant, these differences did not reach statistical significance.

**Structure-activity relationships.** Competition studies with naturally occurring and synthetic analogs were used to identify some of the structural features of AA-I critical for its high-affinity interaction with mOats 1, 2 and 3. We tested the ability of each of these AA derivatives (5 µM) to compete with $[^3H]$AA-I (500 nM) for entry into the cell. As
expected for a carrier-mediated process, nonradiolabeled AA-I competed successfully with $[^3]$HAA-I for cellular uptake in all three of the mOat-transfectants (Fig. 4). In contrast, uptake by EV-transfectants was unaffected by nonradiolabeled AA-I, nor was it sensitive to any of the competitor compounds tested, again implicating simple diffusion as the primary route of AA-I entry in these cells.

The 8-methoxy group was not an essential structural feature for transport, as both AA-II (desmethoxy AA-I) and AA-Ia (desmethyl AA-I) reduced $[^3]$HAA-I uptake in all three mOat-transfectants (Fig. 4). In contrast, the AA-I and -II nitroreduction metabolites, aristolactams I and II, were ineffective competitors. The carboxy and nitro groups present in both AA-I and –II are absent in their corresponding aristolactams (Fig. 1). Descarboxy and desnitro AA-I analogs were used to discriminate between the relative importance of each of these side groups, and these studies showed that the carboxy group, a structure common to many OAT substrates, was essential for the interaction of AA-I with mOats 1, 2 and 3. Interestingly, the nitro group was required also by mOat 1, but not by mOats 2 or 3.

**AA-I uptake in renal cortical slices.** To relate our findings with the CHO transfectants to the native tissue, we examined transport of $[^3]$HAA-I by mouse renal cortical slices. This preparation has a long history as a model system for studies of organic anion transport by the kidney (Berndt, 1976). Proximal tubules are the most abundant nephron segment in cortical slices, and, because luminal access is restricted, cellular uptake is generally interpreted to represent secretory influx across the basolateral cell membrane.
The capacity for concentrative uptake is evaluated by measuring slice-to-medium concentration ratios of the organic anion under study.

As shown in Fig. 5, [3H]AA-I (400 nM) accumulated in slices in a time-dependent manner, such that a slice-to-medium ratio of 19 was achieved within 20 min. We used this preparation to compare the uptake of AA-I with that of AA-II. Slice-to-medium ratios were similar for both compounds (Fig. 5), validating our findings from the CHO transfectant studies and adding further evidence that OAT-mediated accumulation is not the basis for their differential toxicity.

To determine whether AA-I uptake occurs via carrier-mediated transport in this preparation, its sensitivity to known OAT inhibitors and substrates was tested. As shown in Fig. 5, probenecid (1 mM), an established OAT inhibitor, reduced AA-I influx by 71%, whereas known mOat substrates p-aminohippuric acid (1 mM) and estrone-3-sulfate (500 μM) inhibited uptake by 42% and 35%, respectively.

**Transport-dependent DNA adduct formation in renal slices.** Aristolactam-DNA adducts serve as a biomarker for intracellular accumulation of AA-I, as they are products of its intracellular metabolism (Fig. 1). To independently confirm a role for OATs in the renal handling of AA-I, we determined whether inhibition of this transport pathway affected the formation of these adducts in renal slices. Enzymatic nitroreduction of AA-I to aristolactam I generates a reactive nitrenium intermediate that forms covalent adducts with both deoxyadenosine (dA) and deoxyguanosine (dG).
residues in DNA (Pfau et al., 1990b). As shown in Fig. 6, significant quantities of both 
dG- and dA-aristolactam adducts were produced in kidney slices during 1.5 h incubation 
with 2 μm AA-I. Preliminary studies showed that the rate of adduct formation was linear 
for at least 3.5 h under these conditions (data not shown). In slices coincubated with 
AA-I and the OAT inhibitor probenecid (1 mM), adduct levels were reduced by >90% 
compared to treatment with AA-I alone. Of note, probenecid reduced AA-I transport by 
71% in this model (Fig. 5), suggesting a close association of these two processes.

**AA-I binds to plasma protein(s).** Because binding to plasma proteins is known to 
influence the distribution and elimination of many drugs (Weiner et al., 1964), we 
determined whether this might further affect the disposition of AA-I. An ultrafiltration 
assay was used to assess protein binding in two settings of physiological interest: with 
4% BSA, equivalent to 600 μM, the normal plasma concentration; and with 10% FCS, 
commonly used for tissue culture studies. As shown in Fig. 7, for solutions containing 2 
μM [3H]AA-I, the average fraction bound was 84 ± 0.7% when co-incubated with 4% 
BSA, and 78 ± 0.9% with 10% FCS. No appreciable binding was detected in protein-
free solutions. To validate the ultrafiltration assay, we calculated the percentage of 
[3H]AA-I recovered from each of these solutions, which averaged 102 ± 2% for 10% 
FCS, 99 ± 5% for 4% BSA, and 79 ± 4% in the absence of protein. Incomplete recovery 
from protein-free solutions is likely due to nonspecific binding of [3H]AA-I to the filter.
DISCUSSION

AA-I is a remarkably selective toxin. The kidney is the only known target organ, and within the kidney, the proximal tubule is the exclusive site of necrotic and apoptotic injury (Mengs, 1987; Sato et al., 2004; Lebeau et al., 2005; Shibutani et al., 2007; Pozdzik et al., 2008). In this study, we found that AA-I is a high-affinity substrate for mOats 1 and 3, transport proteins that are expressed in the proximal tubule and mediate concentrative uptake across the basolateral membrane as the initial step in organic anion secretion. While this transport pathway provides one basis for site-specific toxicity, it likely works in concert with other factors, as the relative susceptibility of proximal tubule segments S1, S2, and S3 to acute AA-I exposure varies among species. In mice, cortical proximal tubules, mainly S2, are the primary site of toxicity (Sato et al., 2004; Shibutani et al., 2007), whereas S3 is most vulnerable in rats (Lebeau et al., 2005; Pozdzik et al., 2008), and all segments may be affected with chronic AA treatment in rabbits (Cosyns et al., 2001). These species differences may be due to variations in OAT activity or expression levels along the proximal tubule, or they may reflect regional expression of specific intracellular target(s), yet to be identified, that interact with AA-I and lead to its toxicity. It is also possible that variations in intrarenal metabolism of AA-I along the nephron influences toxicity in a species-dependent manner.

In addition to mOats 1 and 3, we determined that AA-I is a substrate for mOat2, a proximal tubule transporter reported to be expressed in the basolateral membrane in humans and in the apical membrane in rodents (Enomoto et al., 2002; Ljubojevic et al.,
Because the physiological counteranion(s) and gradients driving OAT2 transport have not been identified, it is unclear whether it functions in a secretory or reabsorptive mode. This transporter is also highly expressed in liver, the primary site of AA-I detoxification (Xiao et al., 2008). Hepatic Oat2 is localized to the basolateral (sinusoidal) membrane (Simonson et al., 1994), where it could conceivably mediate influx of AA-I or efflux of its metabolites, such as AA-Ia.

The OATs are polyspecific transporters with both overlapping and distinct substrate preferences (VanWert et al., 2010). In the present study, AA-I was shown to be a high-affinity substrate for mOats 1, 2 and 3. Although AA-I transport was not directly measured, two groups (Bakhiya et al., 2009; Babu et al., 2010) recently used competition studies in heterologous expression systems to evaluate the interaction of AA-I with human (h) OATs. They found AA-I to be an effective inhibitor of substrates transported by hOATs 1 and 3, whereas it competed poorly with substrates transported by hOAT4. The reported $K_i$ values for transport mediated by hOATs 1 and 3 were in the submicromolar range and are comparable to the $K_m$ values reported in the present study, indicating that high-affinity transport of AA-I by these two transporters is conserved across species. In contrast, the $K_i$ value for hOAT4 was 20-60 $\mu$M, suggesting that high-affinity transport of AA-I is not a universal property of the OATs.

Genetic predisposition, dose and duration of exposure are important factors in the development of AAN in humans and in animal models. Reported and extrapolated values for plasma AA concentrations associated with nephropathy in humans and in
animal models (see Supplemental Table 1 for values and associated literature references) show that circulating levels of AA often fall within the affinity range of the OATs, even when protein binding is considered. During preclinical testing, 80% of human subjects developed acute renal failure (Jackson et al., 1964), often lethal, following high dose treatment with AA (1 mg/kg by intravenous bolus daily for three days), corresponding to a peak plasma level of 67 μM. In contrast, ~5% of the Belgian women who were inadvertently exposed to AA developed chronic end-stage renal disease, and on average, they ingested a much lower daily dose (25 μg/kg) for a longer period of time (13 months) (Cosyns, 2003). This dosing regimen would result in a maximal plasma concentration of 2 μM. There are several reports of Fanconi syndrome associated with the extended use of Chinese herbal remedies that contain much lower doses of AA-I (Supplemental Table 1) and in these cases we estimate plasma AA concentrations as ranging from 18-391 nM. In animal models, acute toxicity is associated with high peak plasma concentrations of 26-100 μM (Supplemental Table 1).

Various physicochemical properties are involved in substrate recognition by OATs, including size, hydrophobicity, and capacity for hydrogen bonding, along with the chemical nature, number and relative location of charged groups (Ullrich and Rumrich, 1988). Using a series of substituted compounds, our competition studies provide insight into some of the structural features of AA-I that are important for its high-affinity interaction with mOats 1, 2, and 3. For example, oxygen-containing functional groups at the 8-position have little effect on transport, whereas the anionic carboxy group at the 3-position is an absolute structural requirement for all three mOats. The carboxy group is
the main charge-bearing residue in AA-I and is one of the more common (but not required) features of known OAT substrates. Deletion of the nitro group at the 5-position unmasked an important difference in substrate preferences among the three mOats, as this group was needed for the interaction of AA-I with mOat1, but not with mOats 2 and 3. Consistent with these findings, (Ullrich and Rumrich, 1988) reported that addition of electron withdrawing side groups such as hydroxy, aldehyde or nitro groups to the monocarboxylate benzoic acid markedly increases its affinity for the \( p \)-aminohippuric acid transporter. This was assessed by stop-flow peritubular capillary perfusion in rat kidneys, a model system that predominately reflects Oat1 transport under the experimental conditions reported.

We observed time- and dose-dependent accumulation of AA-I and –II in control CHO transfectants, indicating that these compounds can enter cells through pathways other than OATs. Uptake was linear with concentration and not inhibited by non-radioactive AA, suggesting that OAT-independent transport in these cells occurs by simple diffusion. These results were not unexpected since, in addition to being a nephrotoxin, AA-I is a known carcinogen with multiple sites of action. Following \textit{in vivo} exposure, DNA adducts derived from AA-I and AA-II metabolites are found in several rodent organs including bladder, stomach, kidney, intestine, liver, lung and spleen (Schmeiser et al., 1988; Pfau et al., 1990a; Dong et al., 2006; Shibutani et al., 2007), and tumors arise at multiple sites in rodents exposed to AA (Mengs, 1982; Mengs, 1988; Schmeiser et al., 1990). In humans, AA exposure is associated with urothelial cancers uniquely localized to the renal pelvis and upper ureters (Cosyns et al., 1994b; Vanherweghem et
al., 1995). In those tissues not known to express OATs, diffusional uptake may be an important way for these carcinogens to enter cells.

In the present study we demonstrated that AA-I avidly binds to plasma proteins such as albumin, which will influence its distribution in body compartments and its elimination. The normal plasma albumin concentration is ~600 μM, which greatly exceeds plasma concentrations reported for AA-I (Supplemental Table 1), and suggests that a considerable quantity of the nephrotoxin may be sequestered by albumin in the circulation. Protein binding will also restrict AA-I excretion through glomerular filtration, which adds significance to the role that mOat-mediated secretion may play in renal clearance of AA-I. The results of our binding studies also have practical implications for cell culture studies of AA-I toxicity; we have found that 10% FCS, equivalent to 60 μM albumin, significantly attenuates cytotoxicity in response to AA-I concentrations up to 50 μM (K.G. Dickman, unpublished observations). This effect is likely due to reduced free levels of AA-I in solution because of protein binding.

No studies have addressed the renal disposition of AA-I, and while we provide evidence that AA-I is handled by OATs involved in tubular secretion, renal clearance studies will be necessary to determine whether this compound undergoes net secretion or reabsorption. In rats, 46% of orally administered AA-I is excreted in urine and 37% in feces (Krumbiegel et al., 1987). Metabolites, presumably derived from extra- and intrarenal reactions, predominate in urine, which contains little, if any, AA-I. Major urinary metabolites include AA-Ia, aristolactams I and Ia, phase II conjugates of AA-Ia
and aristolactam Ia, along with small amounts of desnitro and descarboxy AA-I (Krumbiegel et al., 1987; Chan et al., 2006).

Given the broad range of substrates accepted by the renal OATs, drug-drug interactions at the transporter level have the potential to influence the toxicity and excretion of AA-I (Sweet, 2005). For example, concomitant use of therapeutic drugs known to be substrates for OATs 1/3, such as diuretics, nonsteroidal anti-inflammatory agents, and certain antibiotics, may mitigate nephrotoxicity by competing with AA-I for cellular entry. Furthermore, in the present study we found that AA-II and some AA metabolites effectively vie with AA-I for mOat-mediated transport. This may be an important determinant of the nephrotoxic potential of various Aristolochia-based herbal medicines, as the relative proportion of AA-I (toxic) to AA-II (non-toxic) varies among Aristolochia species (Chan et al., 2003).
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Dickman
Conducted experiments: Dickman
Performed data analysis: Dickman
Wrote or contributed to the writing of the manuscript: Dickman, Sweet
Contributed new reagents or analytic tools: Sweet, Bonala, Ray, Wu
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induces proximal tubule apoptosis and epithelial to mesenchymal transformation. 


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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Primary pathways of aristolochic acid I (AA-I) and II (AA-II) metabolism. Demethylation of AA-I by cytochrome P450 (CYP) 1A1/2 produces AA-Ia, a non-toxic metabolite. Enzymatic nitroreduction (NR) of AA-I or AA-II generates a reactive nitrenium intermediate capable of forming adducts with DNA. The aristolactam end products of NR are not toxic and are subject to further metabolism through phase II reactions, as is AA-Ia (not shown). Minor pathways leading to desnitro (aristolic acid) and descarboxy metabolites of AA-I are denoted by broken arrows. *indicates sites in AA-I and AA-II that were radiolabeled with tritium for use in the present study.

Figure 2. Murine organic anion transporters (mOats) mediate transport of aristolochic acids I and II (AA-I, AA-II). Uptake of 1 μM [3H]AA-I or [3H]AA-II was measured in CHO cells transfected with mOats 1, 2, or 3, and in control empty vector (EV) transfectants. A representative study (panel A, AA-I; panel B, AA-II) shows that uptake was time-dependent, and that steady state levels of both compounds were higher in all mOat transfectants compared to EV control cells. One-minute uptake values, shown in panels C (AA-I) and D (AA-II), were significantly higher in all mOat expressing cells compared to control EV cells, and varied among the different mOats. Values in panels C and D are means ± SE, n = 12 independent experiments. Within a panel, bars with different letters are significantly different from each other (p<0.05) as determined by analysis of variance with Newman-Keuls post hoc test; bars with the same letter are not significantly different from each other. Panel E depicts data from panels C and D minus the corresponding EV values to represent mOat-mediated transport. Values in panel E
are means ± SE, n = 12 independent experiments. Statistically significant differences (p<0.05) between AA-I and AA-II transport for each mOat were determined by unpaired, 2-tailed Student's t-test. NS, not significantly different.

Figure 3. Kinetic analysis of aristolochic acid I (AA-I) and II (AA-II) transport by CHO cells expressing murine organic anion transporters (mOats). Initial uptake rates (1 min) were measured in control empty vector (EV) transfectants (panel A) and in mOat transfectants incubated with varying concentrations (0.125-2 μM) of [3H]AA-I (panel B) or [3H]AA-II (panel C). Transport was linearly related to AA concentration in EV transfectants. mOat uptake values were corrected for diffusional uptake by subtraction of EV values at each concentration. Kinetic parameters for saturable transport by mOat transfectants were determined by non-linear regression fit to the Michaelis-Menten equation and are presented in Table 1. Values are means ± SE, n = 3 independent experiments.

Figure 4. Structural determinants of aristolochic acid I (AA-I) transport by murine organic anion transporters (mOats). Structure-activity relationships were assessed by competition studies using AA metabolites as structural analogs. One-minute uptake values were measured in mOat-transfectants and in control, empty vector (EV) cells incubated with [3H]AA-I (0.5 μM) alone or in the presence of 10-fold excess (5 μM) of non-radiolabeled competitor. Competitors were: AA-I (positive control), AA-II (desmethoxy AA-I), AA-Ia (demethylated AA-I), aristolactams I and 2, descarboxy AA-I, and desnitro AA-I. Chemical structures of the competitors are shown in Fig. 1. mOat-
mediated transport values were corrected for diffusional uptake by subtraction of EV values. Data, presented as percent of control (no competitor) uptake values, are means ± SE, n = 3-5 independent experiments. *denotes values significantly different (p<0.05) from 100% as determined by single sample t-test.

Figure 5. Organic anion transporter (OAT)-dependent uptake of aristolochic acids I (AA-I) and II (AA-II) by mouse renal cortical slices. Left panel: time-dependent uptake by slices incubated with 400 nM [³H]AA-I. Corresponding slice-to-medium concentration ratios (S/M) are noted next to each time point. Panel B: slice-to-medium concentration ratios were measured following incubation for 7.5 min with 400 nM [³H]AA-I or [³H]AA-II, and were comparable for both compounds. The OAT inhibitor probenecid (1 mM) and known OAT substrates (para-aminohippuric acid (PAH, 1 mM); estrone-3-sulfate (ES, 500 µM)), inhibited AA-I uptake. Bars with different letters are significantly different from each other (p<0.05) as determined by analysis of variance with Newman-Keuls post hoc test; bars with the same letters are not significantly different from each other.

Figure 6. Inhibition of organic anion transport blocks formation of DNA adducts with aristolochic acid I (AA-I) metabolites. Mouse renal cortical slices were incubated with 2 µM AA-I for 1.5 h in the presence and absence of the OAT inhibitor probenecid (Prob, 1 mM). Enzymatic nitroreduction of AA-I generates a reactive aristolactam (AL) metabolite that forms adducts with deoxyguanosine (dG, panel B) and deoxyadenosine (dA, panel C) residues in DNA. Adduct levels in 2.5 µg DNA were measured with the ³²P-postlabeling/polyacrylamide gel assay described in Methods. Standards in panel A
represent 15 fmoles per lane, equivalent to 1 adduct/10^6 nucleotides in 5 μg DNA. Values are means ± SE, n = 3 independent experiments. *denotes statistically significant difference (p<0.05) from AA-I as determined by unpaired, 2-tailed Student’s t-test.

Figure 7. Aristolochic acid I (AA-I) binds to serum protein(s). Binding was measured by ultrafiltration (10,000 molecular weight cut-off) of Hanks balanced salt solution containing [^3]HAA-I (2 μM) and either 10% fetal calf serum (FCS), 4% bovine serum albumin (BSA), or no protein. Values are means ± SE, n = 3 independent experiments. Bars with different letters are significantly different from each other (p<0.05) as determined by analysis of variance with Newman-Keuls post hoc test; bars with the same letters are not significantly different from each other.
Table 1. Kinetic parameters for aristolochic acid transport.

<table>
<thead>
<tr>
<th></th>
<th>mOat1</th>
<th>mOat2</th>
<th>mOat3</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td><strong>K_m</strong>&lt;sub&gt;(µM)&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-I</td>
<td>0.791 ±0.007</td>
<td>0.356 ±0.050</td>
<td>0.514 ±0.057</td>
<td>mOat1 vs. mOat2: p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mOat2 vs. mOat3: p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mOat1 vs. mOat3: p&lt;0.05</td>
</tr>
<tr>
<td>AA-II</td>
<td>1.498 ±0.371</td>
<td>0.673 ±0.192</td>
<td>1.047 ±0.360</td>
<td>mOat1 vs. mOat2: NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mOat2 vs. mOat3: NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mOat1 vs. mOat3: NS</td>
</tr>
<tr>
<td><strong>p-value</strong>&lt;br&gt;AA-I vs. AA-II</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

|        |             |             |             |                                  |
| **V_max**<sub>(pmol·min<sup>-1</sup>·mg<sup>-1</sup>)</sub> |             |             |             |                                  |
| AA-I   | 28 ±2       | 43 ±9       | 12 ±0.4     | mOat1 vs. mOat2: NS              |
|        |             |             |             | mOat2 vs. mOat3: p<0.05          |
|        |             |             |             | mOat1 vs. mOat3: NS              |
| AA-II  | 29 ±3       | 34 ±8       | 25 ±2       | mOat1 vs. mOat2: NS              |
|        |             |             |             | mOat2 vs. mOat3: NS              |
|        |             |             |             | mOat1 vs. mOat3: NS              |
| **p-value**<br>AA-I vs. AA-II | NS | NS | p<0.05 |                                  |

Kinetic parameters for uptake of aristolochic acid I (AA-I) or II (AA-II) by CHO cells transfected with mouse organic anion transporters (mOats) 1, 2, or 3. Values were derived by non-linear regression fit to the Michaelis-Menten equation using data presented in Figure 3, and represent the means ± SE of 3 independent experiments. p-value refers to statistically significant (p<0.05) differences between AA-I and AA-II kinetic parameters for each mOAT as determined by unpaired, 2-tailed Student’s t-test, and to statistically significant (p<0.05) differences between kinetic parameters among mOats 1, 2, and 3 as determined by analysis of variance with Newman-Keuls post hoc test. NS, not significantly different.
Figure 2

(A) AA-I uptake (pmol/mg protein) over time (min) for mOat2, mOat3, mOat1, and EV.

(B) AA-II uptake (pmol/mg protein) over time (min) for mOat2, mOat3, mOat1, and EV.

(C) AA-I uptake (pmol/mg protein/min) for EV, mOat1, mOat2, and mOat3.

(D) AA-II uptake (pmol/mg protein/min) for EV, mOat1, mOat2, and mOat3.

(E) AA uptake (pmol/mg protein/min) for AA-I and AA-II with mOat1, mOat2, and mOat3, with statistical significance indicated by p<0.05 and NS.
Figure 7

% Bound

No protein  10% FCS  4% BSA

(a)  (b)  (b)
Supplemental Table 1. Reported and extrapolated values for plasma aristolochic acid concentrations associated with nephropathy in humans and in animal models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Daily dose</th>
<th>Route</th>
<th>Duration</th>
<th>Peak plasma concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>1 mg/kg</td>
<td>IV</td>
<td>3 d</td>
<td>67 ( \mu \text{M} )</td>
<td>S1</td>
</tr>
<tr>
<td>Human</td>
<td>25 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>13 mo</td>
<td>2 ( \mu \text{M} )</td>
<td>S2</td>
</tr>
<tr>
<td>Human</td>
<td>6.7 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>5 mo</td>
<td>391 nM</td>
<td>S3</td>
</tr>
<tr>
<td>Human</td>
<td>3.9 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>12 mo</td>
<td>226 nM</td>
<td>S4</td>
</tr>
<tr>
<td>Human</td>
<td>3.9 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>72 mo</td>
<td>226 nM</td>
<td>S4</td>
</tr>
<tr>
<td>Human</td>
<td>1.5 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>8 mo</td>
<td>88 nM</td>
<td>S3</td>
</tr>
<tr>
<td>Human</td>
<td>1.5 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>22 mo</td>
<td>88 nM</td>
<td>S5</td>
</tr>
<tr>
<td>Human</td>
<td>0.8 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>2 mo</td>
<td>49 nM</td>
<td>S6</td>
</tr>
<tr>
<td>Human</td>
<td>0.3 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>10 mo</td>
<td>18 nM</td>
<td>S5</td>
</tr>
<tr>
<td>Human</td>
<td>0.3 ( \mu \text{g/kg} )</td>
<td>PO</td>
<td>24 mo</td>
<td>18 nM</td>
<td>S3</td>
</tr>
<tr>
<td>Mouse</td>
<td>10 mg/kg</td>
<td>IP</td>
<td>Once</td>
<td>100 ( \mu \text{M} )</td>
<td>S7</td>
</tr>
<tr>
<td>Mouse</td>
<td>10 mg/kg</td>
<td>IP</td>
<td>Once</td>
<td>65 ( \mu \text{M} )</td>
<td>S8</td>
</tr>
<tr>
<td>Mouse</td>
<td>10 mg/kg</td>
<td>IP</td>
<td>Once</td>
<td>26 ( \mu \text{M} )</td>
<td>S9</td>
</tr>
<tr>
<td>Rat</td>
<td>12 mg/kg</td>
<td>PO</td>
<td>Once</td>
<td>30 ( \mu \text{M} )</td>
<td>S10</td>
</tr>
<tr>
<td>Rat</td>
<td>10 mg/kg</td>
<td>IG</td>
<td>Once</td>
<td>21 ( \mu \text{M} )</td>
<td>S11</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.4 mg/kg</td>
<td>IV</td>
<td>Once</td>
<td>12 ( \mu \text{M} )</td>
<td>S12</td>
</tr>
</tbody>
</table>

Human peak plasma concentration values are extrapolated and represent peak levels based on full distribution into a 3 L plasma volume. *based on 60 kg as an average body weight. IP, intraperitoneal; IV, intravenous; IG, intragastric; PO, oral.

Supplemental Table 1 References