Physiological and Molecular Characterization of Aristolochic Acid Transport by the Kidney

Kathleen G. Dickman, Douglas H. Sweet, Radha Bonala, Tapan Ray, and Amy Wu

ABSTRACT

Consumption of herbal medicines derived from Aristolochia plants is associated with a progressive tubulointerstitial disease known as aristolochic acid (AA) nephropathy. The nephrotoxin produced naturally by these plants is AA-I, a nitrophenanthrene carboxylic acid that selectively targets the proximal tubule. This nephron segment is prone to toxic injury because of 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 (mOat1, mOat2, and mOat3) as capable of mediating transport of AA-I. Kinetic analyses showed that all three transporters have an affinity for AA-I in the submicromolar range and thus are likely to operate at toxicologically relevant concentrations in vivo. Structure-activity relationships revealed that the carboxyl group is critical for high-affinity interaction of AA-I with mOat1, mOat2, and mOat3, whereas the nitro group is required only by mOat1. Furthermore, the 8-methoxy group, although 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 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 may be 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 after 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 uterine malignancies of the renal pelvis and ureter (Cosyns et al., 1994b; Vanherweghem et al., 1993). 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). Chronic Aristolochia poisoning has been linked to the etiology of another renal disease, 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 pro-

ABBREVIATIONS: AA, aristolochic acid; AAN, AA nephropathy; CYP 1A1/2, cytochrome P450 1A1/2; OAT, organic anion transporter; mOat, murine OAT; hOAT, human OAT; CHO, Chinese hamster ovary; EV, empty vector; HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin; FCS, fetal calf serum; dA, deoxyadenosine; dG, deoxyguanosine; NR, nitroreduction.
nounced 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 because studies in mice have established that AA-I is nephrotoxic, whereas AA-II is not (Sato et al., 2004; Shibutani et al., 2007). Both compounds are metabolized extensively in vivo (Fig. 1). CYP 1A1/2 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 although 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., 1990a). These mutagenic lesions are the basis for the carcinogenic effects of AA, but are unrelated to nephrotoxicity because both AA-I and AA-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 multispecific 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. OAT1 and OAT3 are localized in the proximal tubule basolateral membrane where they mediate concentrative cellular uptake, the initial step in organic anion secretion, whereas renal localization of OAT2 remains controversial (Enomoto et al., 2002; Ljubojević 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 AA-II to evaluate how these two compounds are transported by the kidney, and whether membrane transport properties provide a basis for their differential

Fig. 1. Primary pathways of AA-I and AA-II metabolism. Demethylation of AA-I by CYP 1A1/2 produces AA-Ia, a nontoxic 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.
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.

Materials and 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 (HBSS; with bicarbonate; without phenol red), Gey's balanced salt solution, fetal calf serum (FCS), 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 (Freehold, NJ), and 3-phosphatase-free T4 phosphorylase 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 (Chalfont St. Giles, Buckinghamshire, UK). 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 AA-II were isolated and purified from a commercially available mixture of these two compounds (Sigma-Aldrich) as described previously (Dong et al., 1995). Aristolactams I and II, desnitro AA-I, and two compounds (Sigma-Aldrich) as described previously (Dong et al., 1995) were supplied by the Cell Culture and Hybridoma Facility at Stony Brook University. Aristolochic Acids and Metabolites. AA-I and AA-II were isolated and purified from a commercially available mixture of these two compounds (Sigma-Aldrich) as described previously (Dong et al., 2006). AA-Ia was isolated from rat urine as reported previously (Shibutani et al., 2010). Aristolactam 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 dimethyl sulfoxide 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 high-performance liquid chromatography, and stock solution concentrations were verified by UV spectrophotometry using established extinction coefficients.

AA-I and AA-II were titrated at the 2-position via a hydrogen isotope exchange reaction catalyzed by 1,2-cyclooctadienyl iridium (I)pentane-2,4-dionate (McCuley et al., 2003). The reaction components, 5 mg of AA-I or AA-II, 5 mg of iridium catalyst, and 25 mCi of 3H2O (specific activity 1 Ci/mmol) were dissolved in 0.5 ml of dimethylformamide and incubated at 95°C for 4 h. Product identities were verified by high-performance liquid chromatography with UV detection and mass spectrometry using known reference standards. Radiochemical purity was >98% for both compounds, which had specific activities of 0.50 Ci/mmol ([3H]AA-I) and 0.44 Ci/mmol ([3H]AA-II). Final products were dissolved in a 1:1 mixture of aqueous 0.1% trifluoroacetic acid and acetonitrile ([3H]AA-I, 233 μCi/ml; [3H]AA-II, 225 μCi/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. Generation of the CHO-FRT empty vector (EV), CHO-mOat1, and CHO-mOat3 cell lines has been reported previously (VanWert et al., 2008; Vanwert et al., 2008), and CHO-mOat2 cells were developed in a similar fashion. In brief, 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, 2 x 10^6 CHO-FlpIn cells/well were plated in a six-well culture plate, grown overnight, and then treated for 24 h at 37°C with 1 μg of pcDNAs/mOat2 DNA and 8 μg of pOIG44 DNA using Transfectin Lipid Reagent (2 μl of Transfectin/μg DNA; Bio-Rad Laboratories, Hercules, CA). The transfected cells were then given fresh medium and grown for an additional 24 h before 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 4 weeks, selection-positve 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 EV as control or mOat1, mOat2, or mOat3 were grown to confluence in 24-well plates in F-12 medium supplemented with 10% FCS and 125 μg/ml hygromycin. Hygromycin was removed from the medium 24 h before 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 1 N NaOH at room temperature. Cell extracts were neutralized with 10 N HCl, and aliquots were added to Ecoscint H (National Diagnostics, Atlanta, GA) and then analyzed on a Tri-carb 3100TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) with quench correction. Data were normalized to cellular protein content with the Bradford reagent (Sigma-Aldrich) using BSA as the standard. For dose-response studies, kinetic parameters were determined by nonlinear regression fit to the Michaelis-Menten equation with Prism version 5.02 for Windows (GraphPad Software Inc., San Diego, CA).

AA Uptake by Mouse Renal Cortical Slices. These experiments were conducted in accordance with institutional guidelines and the approval of the Institutional Animal Care and Use Committee at Stony Brook University. Male C3H/Hej mice (8–12 weeks old; The Jackson Laboratory, Bar Harbor, ME) were euthanized with CO2 asphyxiation. The kidneys were removed and placed in ice-cold HBSS supplemented with 10 mM HEPES (pH 7.4). Each kidney was decapsulated and cut into cross-sectional pieces, and thin slices (~0.5 mm thick) were prepared by hand by shaving the outer cortex with a no. 15 scalpel blade. Slices were 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 [3H]AA-II at a final concentration of 400 nM. When used, competitors/inhibitors were added to the incubation solution 2 min before 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 1 N NaOH. After neutralization with 10 N HCl, aliquots of the slice extracts were added to Ecoscient H (National Diagnostics), and samples were analyzed on a Tri-carb 3100TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences) with quench correction. Data are presented as slice-to-medium 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 before 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 ± 4 μg DNA, corresponding to 1 μg of genomic DNA/mg wet weight. DNA-aristolactam adduct levels in these samples were measured with a 32P-postlabeling/polyacrylamide gel electrophoresis assay as described previously (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 micrococal 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% nondenaturing polyacrylamide gel. Gels were scanned on a Storm 840 phosphorimager (GE Healthcare), 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, Sunnyvale, 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 cutoff; Millipore Corporation, 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 nonspecific 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% 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-microliter aliquots were taken from each upper chamber containing [3H]AA-I and reserved for liquid scintillation counting, and then all units were centrifuged at 10,000g for 7 min at room temperature. The upper chambers, with retentates, were removed and retained, and the collection tubes (pretared) 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 700g 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. All values are presented as means ± S.E.M.; 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, two-tailed t test.

Results

AA Uptake by mOat Transfectants. Initial screening of CHO cells expressing mOat1, mOat2, or mOat3 indicated that all three transfectants accumulated [3H]AA-I to levels above that achieved by control EV transfectants. For EV, mOat1, mOat2, and mOat-3 transfectants incubated with 1 μM [3H]AA-I or AA-II, cellular accumulation was time-dependent and steady state was reached within 15 to 20 min (Fig. 2, A and B). Plateau values were higher for all mOat transfectants compared with EV cells.

Uptake rates (1 min) for cells incubated with 1 μM [3H]AA-I or AA-II are compared in Fig. 2, C and D. EV transfectants accumulated measurable quantities of both AA-I and AA-II, and this level of basal permeability was 1.8-fold higher (p < 0.05) for AA-I compared with 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 with EV cells (Fig. 2C). The magnitude of these increases varied among the mOat transfectants according to the following rank order: mOat2 (6-fold) > mOat1 (3-fold) > mOat3 (2-fold). AA-II accumulation was also greater in mOat transfectants compared with EV cells (Fig. 2D), and as was the case for AA-I, it was highest (7-fold) in cells expressing mOat2. AA-II uptake rates were similar for mOat1 and mOat3 transfectants, and on average they were 3-fold higher than EV values. The uptake data presented in Fig. 2, C and D 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 mOat1 or mOat2, 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. Km values for AA-I uptake were in the submicromolar 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 Km values associated with AA-II uptake were approximately 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 mOat1, mOat2, and mOat3. 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 [3H]AA-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, because both AA-II (desmethoxy AA-I) and AA-Ia (desmethyl AA-I) reduced [3H]AA-I uptake in all three 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.

AA-I uptake in 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 because 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 dA and dG residues in DNA (Pfau et al., 1990a). As shown in Fig. 6, significant quantities of both dG- and dA-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 with treatment with AA-I alone. It is noteworthy that 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 Proteins. 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 coincubated 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 probably caused by 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 mOat1 and mOat3, 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. Although this transport pathway provides one basis for site-specific toxicity, it probably works in concert with other factors, because 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 caused by variations in OAT activity or expression levels along the proximal tubule, or they may reflect regional expression of specific intracellular targets, 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 mOat1 and mOat3, 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; Ljubojević et al., 2007). Because the physiological counteranions 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-

Fig. 3. Kinetic analysis of AA-I and AA-II transport by CHO cells expressing mOats. Initial uptake rates (1 min) were measured in control EV transfectants (A) and mOat transfectants incubated with varying concentrations (0.125–2 μM) of [3H]AA-I (B) or [3H]AA-II (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 mOats were determined by nonlinear regression fit to the Michaelis-Menten equation and are presented in Table 1. Values are means ± S.E. (n = 3 independent experiments).
affinity substrate for mOat1, mOat2, and mOat3. Although AA-I transport was not directly measured, two groups (Bakhiya et al., 2009; Babu et al., 2010) have used competition studies in heterologous expression systems to evaluate the interaction of AA-I with human OATs (hOATs). They found AA-I to be an effective inhibitor of substrates transported by hOAT1 and hOAT3, whereas it competed poorly with substrates transported by hOAT4. The reported \( K_i \) values for transport mediated by hOAT1 and hOAT3 were in the submicromolar range and are comparable with 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 to 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 animal models. Reported and extrapolated values for plasma AA concentrations associated with nephropathy in humans and 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, after high-dose treatment with AA (1 mg/kg by intravenous bolus daily for 3 days), corresponding to a peak plasma level of 67 \( \mu \)M. 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 \( \mu \)g/kg) for

### TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mOat1</th>
<th>mOat2</th>
<th>mOat3</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_m ) (( \mu )M)</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: &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: N.S.</td>
</tr>
<tr>
<td>( V_{max} ) (pmol \cdot min^{-1} \cdot mg^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-I</td>
<td>28 ± 2</td>
<td>43 ± 9</td>
<td>12 ± 0.4</td>
<td>mOat1 vs. mOat2: N.S.</td>
</tr>
<tr>
<td>AA-II</td>
<td>29 ± 3</td>
<td>34 ± 8</td>
<td>25 ± 2</td>
<td>mOat1 vs. mOat2: N.S.</td>
</tr>
<tr>
<td>( p ) Value AA-I vs. AA-II</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>

N.S., not significantly different.
a longer period of time (13 months) (Cosyns, 2003). This dosing regimen would result in a maximal plasma concentration of 2\textsuperscript{+}/H9262M. 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 to 391 nM. In animal models, acute toxicity is associated with high peak plasma concentrations of 26 to 100 \textsuperscript{+}/H9262M (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 mOat1, mOat2, and mOat3. For example, oxygen-containing functional groups at the
8-position have little effect on transport, whereas the anionic carboxyl group at the 3-position is an absolute structural requirement for all three mOats. The carboxyl 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, because this group was needed for the interaction of AA-I with mOat1, but not with mOat2 and mOat3. Consistent with these findings, Urlich and Rumrich (1988) reported that addition of electron withdrawing side groups such as hydroxyl, 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 predominantly reflects Oat1 transport under the experimental conditions reported.

We observed time- and dose-dependent accumulation of AA-I and AA-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 nonradioactive AA, suggesting that OAT-independent transport in these cells occurs by simple diffusion. These results were not unexpected because, in addition to being a nephrotoxin, AA-I is a known carcinogen with multiple sites of action. After 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., 1990b; Dong et al., 2006; Shibutani et al., 2007), and tumors arise at multiple sites in rodents exposed to AA (Mengs et al., 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, differential 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 probably caused by reduced free levels of AA-I in solution because of protein binding.

No studies have addressed the renal disposition of AA-I, and although 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 extrarenal 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 OAT1/OAT3, 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 Oat-mediated transport. This may be an important determinant of the nephrotoxic potential of various Aristolochia-based herbal medicines, because the relative proportion of AA-I (toxic) to AA-II (nontoxic) varies among Aristolochia species (Chan et al., 2003).

Acknowledgments

We thank Penelope Stockbline and Dr. Viktoria Sidorenko for expert technical assistance with the DNA adduct assays; Dr. Horatio Priestap for valuable advice and stimulating discussions regarding aristolochic acids and their metabolites; and Dr. Ron Harvey for suggesting the synthetic method used to radiolabel AA.

Authorship Contributions

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

References


Address correspondence to: Dr. Kathleen Dickman, Department of Pharmacological Sciences, Stony Brook University, BST-8, 152, Stony Brook, NY 11794. E-mail: dickman@pharm.stonybrook.edu