Chemical Research in Toxicology

ARTICLE

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Role of P450 1A1 and P450 1A2 in Bioactivation versus Detoxication of the Renal Carcinogen Aristolochic Acid I: Studies in *Cyp1a1(−/−)*, *Cyp1a2(−/−)*, and *Cyp1a1/1a2(−/−)* Mice

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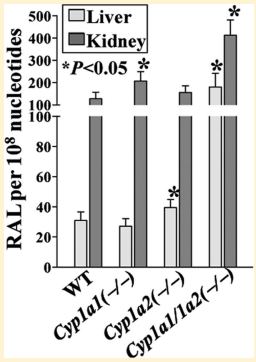
Supporting Information

ABSTRACT: Exposure to aristolochic acid I (AAI) is associated with aristolochic acid nephropathy, Balkan endemic nephropathy, and urothelial cancer. Individual differences in xenobiotic-metabolizing enzyme activities are likely to be a reason for interindividual susceptibility to AA-induced disease. We evaluated the reductive activation and oxidative detoxication of AAI by cytochrome P450 (P450) 1A1 and 1A2 using the *Cyp1a1(−/−)* and *Cyp1a2(−/−)* single-knockout and *Cyp1a1/1a2(−/−)* double-knockout mouse lines. Incubations with hepatic microsomes were also carried out *in vitro*. P450 1A1 and 1A2 were found to (i) activate AAI to form DNA adducts and (ii) detoxicate it to 8-hydroxyaristolochic acid I (AAIa). AAI-DNA adduct formation was significantly higher in all tissues of *Cyp1a1/1a2(−/−)* than *Cyp1a(+/+)* wild-type (WT) mice. AAI-DNA adduct levels were elevated only in selected tissues from *Cyp1a1(−/−)* versus *Cyp1a2(−/−)* mice, compared with those in WT mice. In hepatic microsomes, those from WT as well as *Cyp1a1(−/−)* and *Cyp1a2(−/−)* mice were able to detoxicate AAI to AAIa, whereas *Cyp1a1/1a2(−/−)* microsomes were less effective in catalyzing this reaction, confirming that both mouse P450 1A1 and 1A2 are both involved in AAI detoxication. Under hypoxic conditions, mouse P450 1A1 and 1A2 were capable of reducing AAI to form DNA adducts in hepatic microsomes; the major roles of P450 1A1 and 1A2 in AAI-DNA adduct formation were further confirmed using selective inhibitors. Our results suggest that, in addition to P450 1A1 and 1A2 expression levels in liver, *in vivo* oxygen concentration in specific tissues might affect the balance between AAI nitroreduction and demethylation, which in turn would influence tissue-specific toxicity or carcinogenicity.



INTRODUCTION

The herbal drug aristolochic acid (AA) derived from *Aristolochia* species has been proven to be the cause of so-called Chinese herbs nephropathy (CHN), now termed aristolochic acid nephropathy (AAN).[1,2] The plant extract AA is a mixture of structurally related nitrophenanthrene carboxylic acids, the major components being aristolochic acid I (AAI; Figure 1) and the demethoxy derivative aristolochic acid II. AAN is a unique type of rapidly progressive renal fibrosis that was observed initially in 1991 in a group of Belgian women after they ingested weight-loss pills containing *Aristolochia fangchi*.[3] Within a few years of taking the pills, about 50% of the AAN patients had developed upper tract urothelial carcinoma and, subsequently, bladder urothelial carcinoma.[4,5] In the meantime, similar cases of fibrosis and/or carcinoma have been reported elsewhere in Europe and Asia.[1,6,7]

Recently, exposure to AA has also been linked to Balkan endemic nephropathy (BEN) and its associated urothelial cancer.[8−10] This nephropathy is endemic in certain rural areas of Serbia, Bosnia, Croatia, Bulgaria, and Romania.

Human exposure to AA was demonstrated by the identification of specific AA-DNA adducts in urothelial tissue of AAN and BEN patients using the highly sensitive ^{32}P-postlabeling method.[4,6,8,11−13] The most abundant and persistent DNA adduct detected in patients exposed to AA is 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI); characteristic AT→TA transversions have been observed in the *TP53* tumor suppressor gene,[8−10,14] indicating a relationship between this mutation and AA-induced carcinogenesis in humans.[15−17] AA was recently classified as a Group I human carcinogen by the International Agency for Research on Cancer (IARC).[18]

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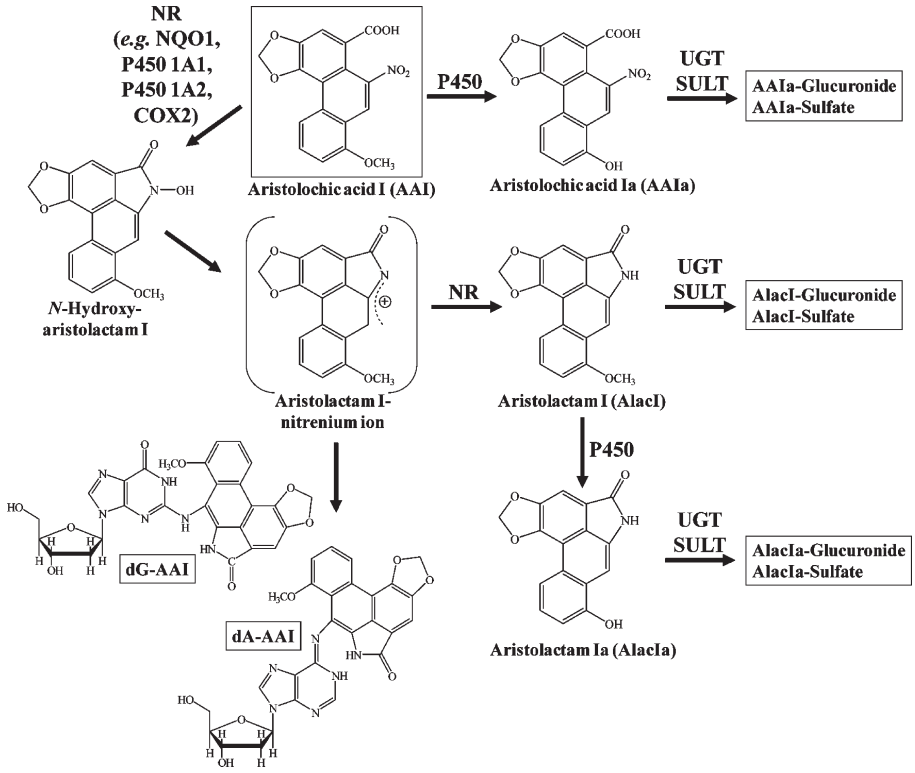

Figure 1. Pathways of AAI biotransformation and DNA adduct formation. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; NR, nitroreduction; UGT, UDP glucuronosyl transferase; SULT, sulfotransferase.

The major activation pathway for AA is nitroreduction catalyzed by both cytosolic and microsomal enzymes, NAD(P)H:quinone oxidoreductase (NQO1) being the most efficient cytosolic nitroreductase (Figure 1).[19,20] Most of the hepatic microsomal reductive activation of AA is mediated by cytochrome P450 (P450) 1A2 and, to a lesser extent, by P450 1A1; P450 oxidoreductase (POR) plays a minor role.[21,22] Prostaglandin H synthase-2 (PTGS2; cyclooxygenase-2, "COX-2") is another enzyme shown to reductively activate AA in human renal microsomes.[21,23] Whereas the enzymes catalyzing the reductive activation of AA leading to AA-DNA adducts are well characterized, those participating in AA detoxication have not been extensively examined to date. Several studies have indicated that P450 1A1 and 1A2 induction by 3-methylcholanthrene or ß-naphthoflavone protects mice from AAI-induced acute renal injury.[24,25] A major detoxication metabolite, identified as 8-hydroxy-aristolochic acid I (aristolochic acid Ia, AAIa; Figure 1), is formed following oxidative demethylation and, in turn, conjugated to the glucuronide, acetate, and sulfate.[26−28] Thus, identification of major AAI detoxication enzymes and detailed knowledge of their catalytic specificities is of major importance to understanding the etiology of AAN and BEN.

Regulation and function of P450 enzymes have been well studied *in vitro*, but to better extrapolate from *in vitro* data to the *in vivo* situation, additional factors need to be considered, such as route-of-administration, absorption, renal clearance, and tissue-specific P450 expression.[29−31]

In the present study, we evaluated P450 1A1- and 1A2-mediated oxidative detoxication of AAI using three mouse lines: *Cyp1a1(−/−)*,[32] *Cyp1a2(−/−)*,[33] and *Cyp1a1/1a2(−/−)*.[34] Urinary AAIa excretion was measured in AAI-treated mice by high-performance liquid chromatography (HPLC). AAI-DNA adduct formation *in vivo* and *in vitro* was investigated by the ^{32}P-postlabeling method.[35] Hepatic and renal P450-mediated formation of AAIa *in vitro* was determined by HPLC.

EXPERIMENTAL PROCEDURES

Chemicals. The natural mixture of AA consisting of 38% AAI and 58% AAII was purchased from Sigma Chemical Co. (St. Louis, MO, USA). AAI (as sodium salt) was isolated from the mixture by preparative HPLC; its purity was 98% as estimated by HPLC.[36]

Animal Treatment. Generation of *Cyp1a1(−/−)*,[32] *Cyp1a2(−/−)*,[33] and *Cyp1a1/1a2(−/−)*[34] knockout mouse lines (on a >99.8% C57BL/6J background) have previously been described. Age-matched C57BL/6J *Cyp1(+/+)* wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were approved by, and conducted in accordance with, the National Institute of Health standards for the care and use of experimental animals and the University of Cincinnati Medical Center Institutional Animal Care and Use Committee. Groups of female mice (3 months old; 25−30 g; n = 4/group) were treated with a single AAI dose of 50 mg/kg body weight by oral gavage. AAI was dissolved in water at a concentration of 5 mg/mL. Control mice received water only. Urine samples were collected for 24 h, and mice were killed 24 h after treatment. Liver, lung, kidney, bladder, spleen, and colon were removed, snap-frozen, and stored at −80 °C until analysis.

AAI-DNA Adduct Analysis by ^{32}P-Postlabeling. DNA from tissues was isolated by standard phenol/chloroform extraction. ^{32}P-postlabeling analysis[35] using the nuclease P1 enrichment version and thin-layer chromatography (TLC) and HPLC were performed as described.[12,37] Chromatographic conditions for TLC on polyethylenimine−cellulose plates (10 cm × 20 cm; Macherey-Nagel, Düren, Germany) were D1,

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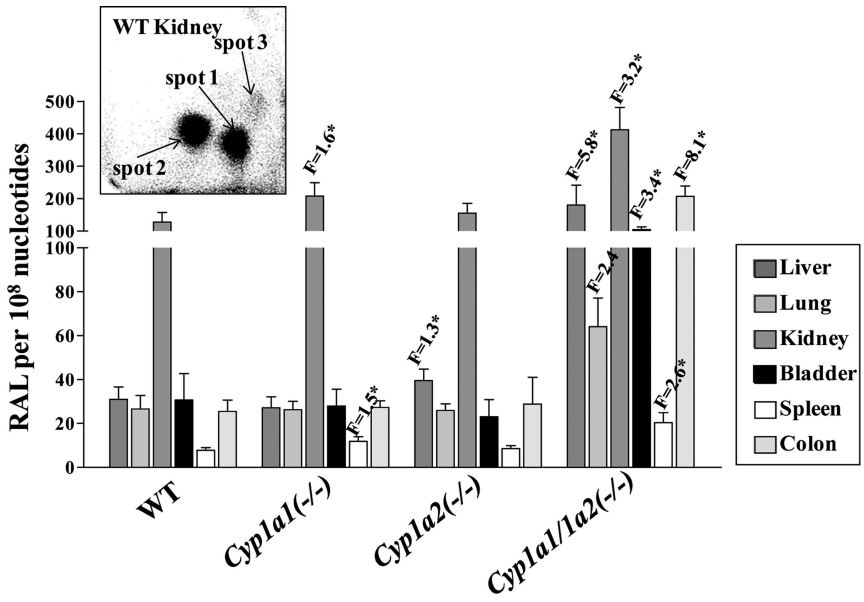

Figure 2. Quantitative TLC ^{32}P-postlabeling analysis of AAI-DNA adduct formation in organs of *Cyp1a* knockout and WT mice treated orally with 50 mg/kg body weight AAI for 24 h. "F" indicates fold-higher DNA adduct levels for *Cyp1a* knockout compared to that of WT mice. Values are given as the means ± SD ($n = 3$); each DNA sample was determined by two postlabeled analyses. RAL, relative adduct labeling. Comparison was performed by *t*-test analysis; $^*P < 0.05$, different from WT. Insert: autoradiographic profile of AA-DNA adducts in WT kidney using the nuclease P1 enrichment version of the assay. The adduct profile shown is representative of those obtained in *Cyp1a* knockout liver, lung, bladder, spleen, and colon. The origin, in the bottom left-hand corner, was cut off before exposure. Spot 1, dA-AAI; spot 2, dG-AAI; and spot 3, dA-AAII.

1.0 M sodium phosphate, pH 6.8; D3, 3.5 lithium-formate, 8.5 M urea, pH 4; D4, 0.8 M lithium chloride, 0.5 M Tris-HCl, 8.5 M urea, pH 9; and D5, 1.7 M sodium phosphate, pH 6. After chromatography, the TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA), and DNA adduct levels (RAL, relative adduct labeling) were calculated as described.[12,37] Results were expressed as DNA adducts/10^8 nucleotides. AA-DNA adducts were identified using reference compounds as described.[12] Urothelial DNA samples from AAN patients[4] were also included in the analysis for comparison.

Determination of Urinary AAIa by HPLC. Creatinine concentrations were determined spectrophotometically in urine samples collected from AAI-treated mice according to the manufacturer's Creatinine Kit protocol (Thermo Spectronics, UK) using a HELIOS Alpha spectrophotometer. For AAIa analysis, 0.5 mL of urine was mixed with 2 mL of methanol, centrifuged (1000 rpm) for 4 min, and the supernatant evaporated to dryness. The residue was dissolved in 100 μL of methanol and analyzed by HPLC. HPLC was performed with a reversed-phase column (Nucleosil 100-5 C_{18}, 25 × 4.0 mm, 5 μm; Macherey-Nagel) preceded by a C-18 guard column, using a linear gradient of 20−60% acetonitrile−100 mM triethylammonium acetate over 55 min with a flow rate of 0.6 mL/min. HPLC was carried out with a Dionex HPLC pump P580 with a UV/vis UVD 170S/340S spectrophotometer detector set at 254 nm; peaks were integrated with a CHROMELEON 6.01 integrator. The peak eluting at the retention time (r.t.) of 23.1 min corresponded to AAIa. The mass spectrum of this peak was measured on a MALDI-TOF/TOF ultraFLEX III mass spectrometer (Bruker-Daltonics, Bremen, Germany). Positive spectra were calibrated externally using the monoisotopic $[M + H]^+$ ions of the PepMixII calibrant (Bruker-Daltonics) or matrix peaks. A 10-mg/mL solution of α-cyano-4-hydroxy-cinnamic acid or a 50-mg/mL solution of 2,5-dihydrobenzoic acid in 50% MeCN/0.1% TFA was used as a MALDI matrix. A 0.5-μL sample dissolved in MeCN was directly mixed with 0.5 μL of matrix solution and dried at ambient temperature on the target. MALDI-TOF positive spectra were collected in reflector mode.

Preparation of Microsomes and Cytosols. Hepatic and renal microsomes and cytosols were isolated both from untreated mice (controls) and mice pretreated with AAI as described.[38] Pooled microsomal and cytosolic fractions ($n = 4$) were used for further analysis. Protein concentrations were assessed using the bicinchoninic acid protein assay with bovine serum albumin as standard. Each microsomal sample was analyzed for specific P450 1A1 and 1A2 activities by monitoring the following reactions: ethoxyresorufin *O*-deethylation (EROD) (P450 1A1 and 1A2) and methoxyresorufin *O*-deethylation (MROD) (P450 1A2).[39] POR activity in hepatic microsomes was measured according to Sottocasa et al.[40] using cytochrome *c* as substrate.

Microsomal Incubations Used for AAI-DNA Adduct Analysis. The deaerated and argon-purged incubation mixtures, in a final volume of 750 μL, consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg hepatic or renal microsomal protein, 0.5 mg of calf thymus DNA (2 mM dNp), and 0.5 mM AAI (dissolved in water). The reaction was initiated by adding NADPH. Incubations were carried out at 37 °C for 60 min; the microsomal-mediated AAI-DNA adduct formation was linear up to 2 h.[21] Control incubations were carried out (i) without microsomes, (ii) without NADPH, (iii) without DNA, or (iv) without AAI. After ethyl acetate extraction, DNA was isolated from the residual water phase by the phenol/chloroform extraction method, as described.[21,41]

Cytosolic Incubations for AAI-DNA Adduct Analysis. The deaerated and argon-purged incubation mixtures, in a final volume of 750 μL, consisted of 50 mM Tris-HCl buffer (pH 7.4), containing 0.2% Tween 20, 1 mM NADPH, 1 mg of hepatic or renal cytosolic protein, 0.5 mg of calf thymus DNA (2 mM dNp), and 0.5 mM AAI. The reaction was initiated by adding NADPH. Incubations were carried out at 37 °C for 60 min; cytosol-mediated AAI-DNA adduct formation was linear up to 2 h.[20] Control incubations were carried out (i) without cytosol, (ii) without NADPH, (iii) without DNA, or (iv) without AAI. After incubation and extraction, DNA was isolated as described above.

Inhibition Studies. The following chemicals were used to inhibit AAI activation in microsomes: α-naphthoflavone (α-NF), which inhibits

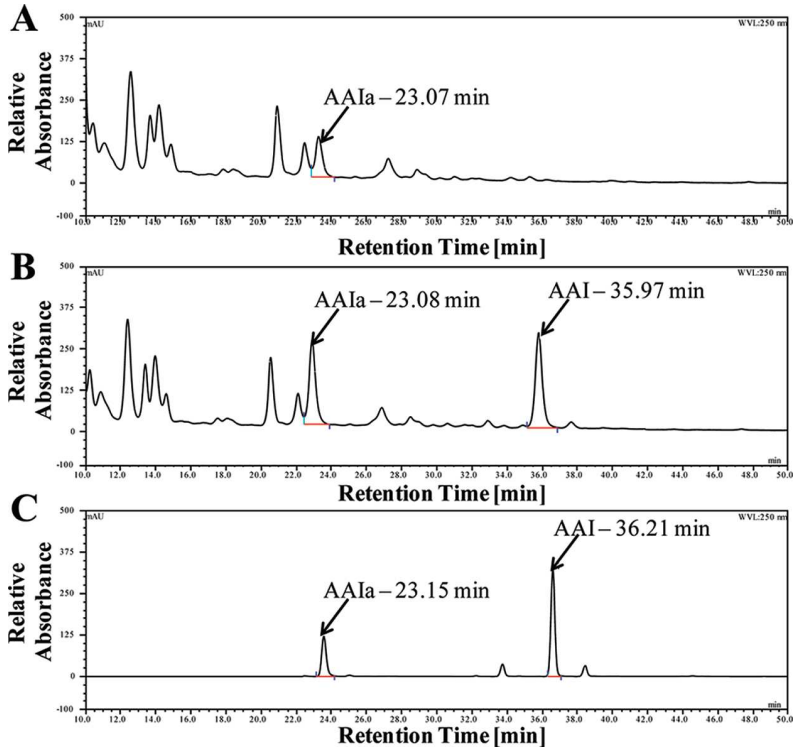

Figure 3. (A) Representative HPLC chromatograph of AAIa (peak r.t. at 23.1 min) in 24 h urine of WT mice treated orally with 50 mg/kg body weight AAI and (B) the same sample spiked with AAIa and AAI (r.t. at 36 min) standards. (C) AAI and AAIa peaks following *in vitro* incubation of hepatic microsomes from WT mice.

P450 1A1 and 1A2;[21,22] ellipticine (E), which is a competitive inhibitor of P450 1A1;[38,42] furafylline (FF), which inhibits P450 1A2;[22] and α-lipoic acid (α-LA), which inhibits POR.[21,43] Inhibitors were dissolved in 7.5 μL of methanol, to yield a final concentration of 0.1 mM in the incubation mixtures. Microsomes and inhibitors were incubated at 37 °C for 10 min with NADPH prior to adding AAI and then incubated further for 1 h at 37 °C. After incubation, DNA was isolated as above.

Determination of Microsomal AAIa Formation by HPLC. Incubation mixtures, in a final volume of 500 μL, consisted of 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg of mouse hepatic or renal microsomal protein, and 10 μM AAI. The reaction was initiated by adding NADPH. Incubations were carried out at 37 °C for 20 min. The AAI oxidative demethylation to AAIa was linear up to 25 min. Control incubations were carried out (i) without microsomes, (ii) without NADPH, or (iii) without AAI. AAI and its metabolites were extracted from incubation mixtures twice with ethyl acetate (2 × 1 mL) and dried. Residues were dissolved in 30 μL of methanol and subjected to reverse-phase HPLC as described above. Peaks for AAIa and AAI eluted with r.t.'s of 23.1 and 36 min, respectively (compare Figure 3C).

RESULTS

DNA Adduct Formation by AAI in Mice. The AAI-induced adduct patterns in various organs were qualitatively similar to those found in AAN patients, consisting of two major adduct spots (spot 1 and 2) and one minor adduct spot (spot 3) (see Figure 2, insert).[4,11] These adducts have previously been identified[11,12] as 7-deoxyadenosine-N^6-yl)aristolactam I (spot 1; dA-AAI), 7-deoxyguanosin-N^2-yl)aristolactam I (spot 2; dG-AAI), and 7-(deoxyadenosin-N^6-yl)aristolactam II (spot 3; dA-AAII). No adducts were observed in DNA of control (untreated)

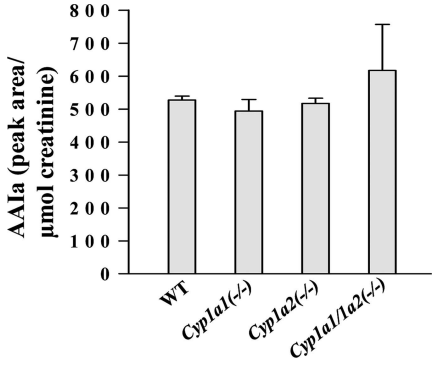

Figure 4. Excretion of AAIa in 24 h urine of *Cyp1a* knockout and WT mice treated orally with 50 mg/kg body weight AAI. All values are given as the means ± SD (n = 4).

animals (data not shown). In all mouse lines tested, the highest DNA adduct levels were observed in kidney; levels in the liver, lung, bladder, spleen, and colon were much lower (Figure 2). In *Cyp1a1/1a2*(−/−) mice, the adduct levels were 2- to 8-fold higher than those in WT mice ($P < 0.05$). Only minor changes in AAI-DNA adduct formation were found in *Cyp1a1*(−/−) and *Cyp1a2*(−/−) mice; a 1.6-fold higher adduct level in the kidney of *Cyp1a1*(−/−) mice was the largest change, followed by a 1.5-fold change in spleen, and a 1.3-fold change in kidney in *Cyp1a1*(−/−) and *Cyp1a2*(−/−) mice, respectively ($P < 0.05$).

Urinary AAIa Excretion. AAIa was detected by HPLC with a peak r.t. at 23.1 min (Figure 3A). Positive MALDI-TOF-TOF showed peaks at m/z 328.043 and 327.029, representing the

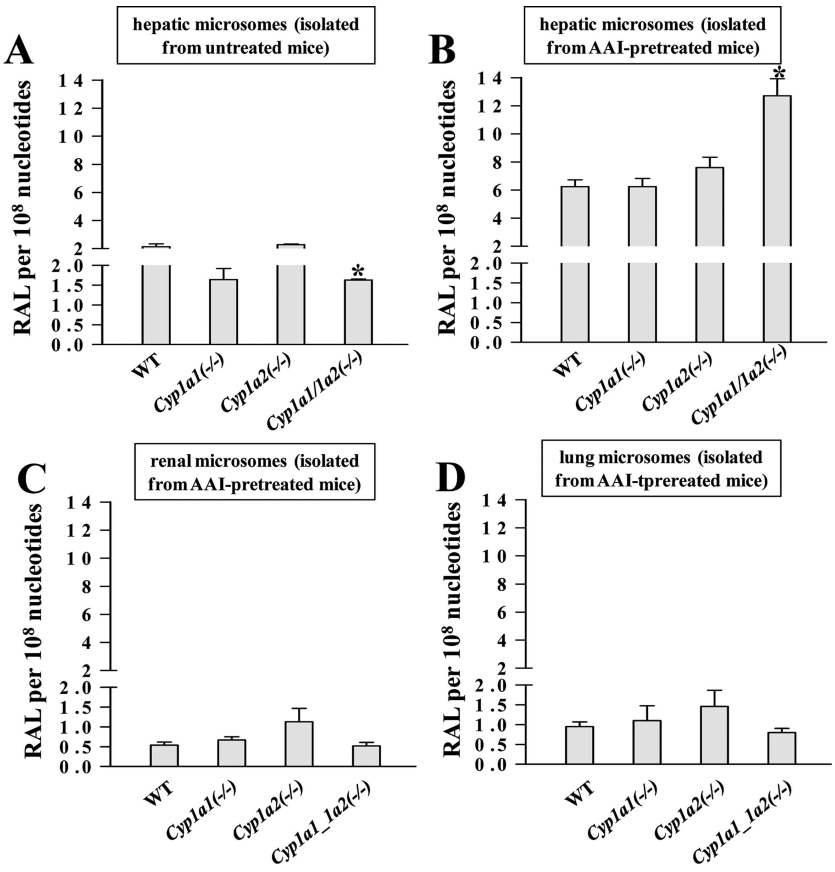

Figure 5. DNA adduct formation *in vitro* by AAI in microsomes isolated from *Cyp1a* knockout and WT mice: (A) untreated or (B−F) pretreated orally with 50 mg/kg body weight AAI for 24 h. (A,B) Hepatic, (C) renal, and (D) pulmonary microsomes were used. Values are given as the means ± SD (n = 3); each DNA sample was determined by two postlabeled analyses. RAL, relative adduct labeling. Comparison was performed by t test analysis; *P < 0.01, different from WT.

molecular ions $[M]^+$ and $[M - H]^+$ of AAIa, respectively, and the peaks at m/z 283.021 and 311.031, representing ions of AAIa fragments (Supporting Information, Figure 1), proving that the metabolite is AAIa.[44] No residual AAI was detected in the urine of any mice (Figure 3A). Unexpectedly, no significant differences in the quantity of AAIa were seen among *Cyp1a1(−/−)*, *Cyp1a2(−/−)*, or *Cyp1a1/1a2(−/−)* mice (Figure 4).

Microsomal-Mediated AAI-DNA Adduct Formation. Hepatic, renal, and pulmonary microsomes isolated from AAI-pretreated mice of all lines were incubated with AAI under hypoxic conditions (Figure 5B−D). For liver, DNA adduct formation was compared with those generated from AAI incubated with hepatic microsomes from untreated mice of all lines (Figure 5A). AAI was metabolically activated by all hepatic microsomes to generate the same pattern of AAI-DNA adducts as those obtained in the intact animal (compare Figure 2, insert). No adducts were observed in control incubations carried out in parallel without microsomes, without DNA, or without AAI. Renal and pulmonary microsomes were generally less effective in activating AAI *in vitro* to DNA adducts than hepatic microsomes (compare Figure 5B−D).

Hepatic microsomes isolated from AAI-pretreated *Cyp1a1/1a2(−/−)* mice activated AAI much more efficiently than those from AAI-pretreated WT mice, having a ∼2-fold higher DNA adduct levels (P < 0.01) (Figure 5B). In contrast, *Cyp1a1/1a2(−/−)* hepatic microsomes from mice pretreated with vehicle only formed fewer AAI-DNA adducts than microsomes from untreated WT mice (Figure 5A). More than 3-fold higher levels of AAI-DNA adducts were formed by hepatic microsomes from AAI-pretreated WT mice than from untreated WT mice (P < 0.01) (Figure 5A and B). These enhanced levels of activation by microsomes from AAI-pretreated WT mice corresponded to higher microsomal P450 1A1 and 1A2 activities (EROD and MROD) (compare Figure 7).

Inhibition of AAI-DNA Adduct Formation in Hepatic Microsomes. To characterize which enzyme is responsible for AAI-induced P450-mediated DNA adduct formation, we employed inhibitors of P450 1A1, P450 1A2, and POR (Figure 6). In all hepatic microsomes, AAI-DNA adduct formation was inhibited by α-NF (inhibits both P450 1A1 and 1A2), most effectively in WT microsomes (down to 40%), but only by 20% in *Cyp1a1/1a2(−/−)* microsomes. Ellipticine (P450 1A1 inhibitor) decreased AAI-DNA adduct formation by ∼25% in WT and *Cyp1a2(−/−)* microsomes. Furafylline (P450 1A2 inhibitor) decreased AAI-DNA adduct levels by ∼20% in WT and *Cyp1a1(−/−)* microsomes. Interestingly, even though POR activities in all liver microsomes did not differ significantly (Table 1), α-LA (POR inhibitor) showed strong inhibitory effects (down by 75%) in *Cyp1a1(−/−)* and *Cyp1a1/1a2(−/−)* microsomes, whereas no effect was observed in WT microsomes. These inhibition results with α-LA suggest that POR, in addition to P450 1A1 and 1A2, might strongly contribute to AAI-DNA adduct formation in *Cyp1a1(−/−)* and *Cyp1a1/1a2(−/−)* microsomes.

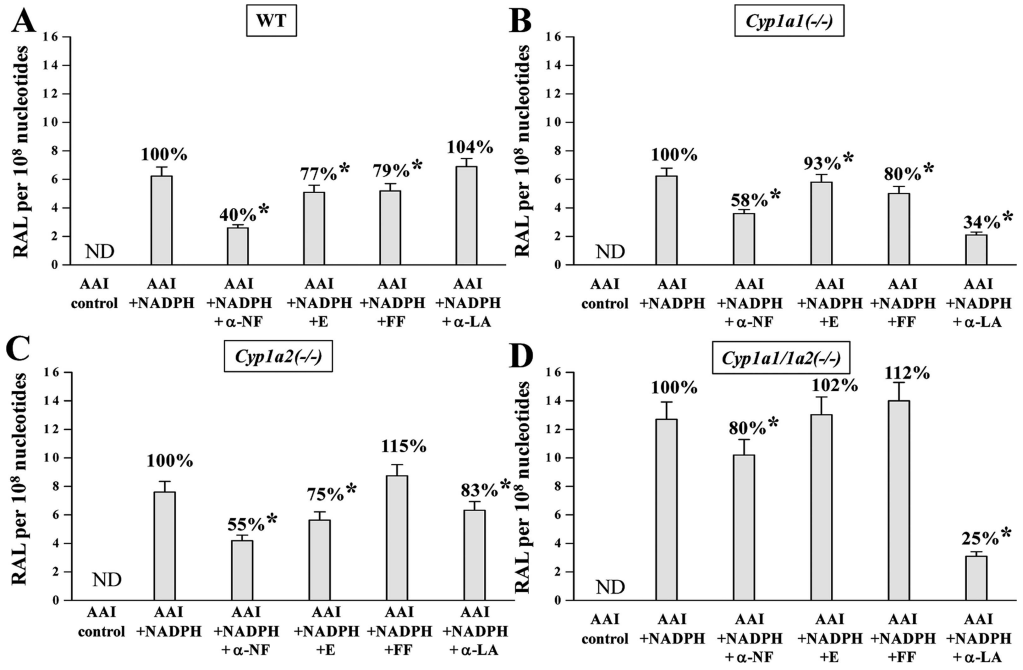

Figure 6. Effect P450 and POR inhibitors *in vitro* on AAI-DNA adduct formation in hepatic microsomes isolated from *Cyp1a* knockout and WT mice pretreated orally with 50 mg/kg body weight AAI for 24 h. Values are given as the means ± SD (n = 3); each DNA sample was determined by two postlabeled analyses. RAL, relative adduct labeling. Comparison was performed by t-test analysis; *P < 0.01, different from incubation with NADPH only. Inhibition is denoted as percentage, relative to DNA adduct levels in incubations with NADPH as cofactor only. Control, without cofactor. α-NF, α-naphthoflavone. E, ellipticine. FF, furafylline. α-LA, α-lipoic acid. ND, not detected.

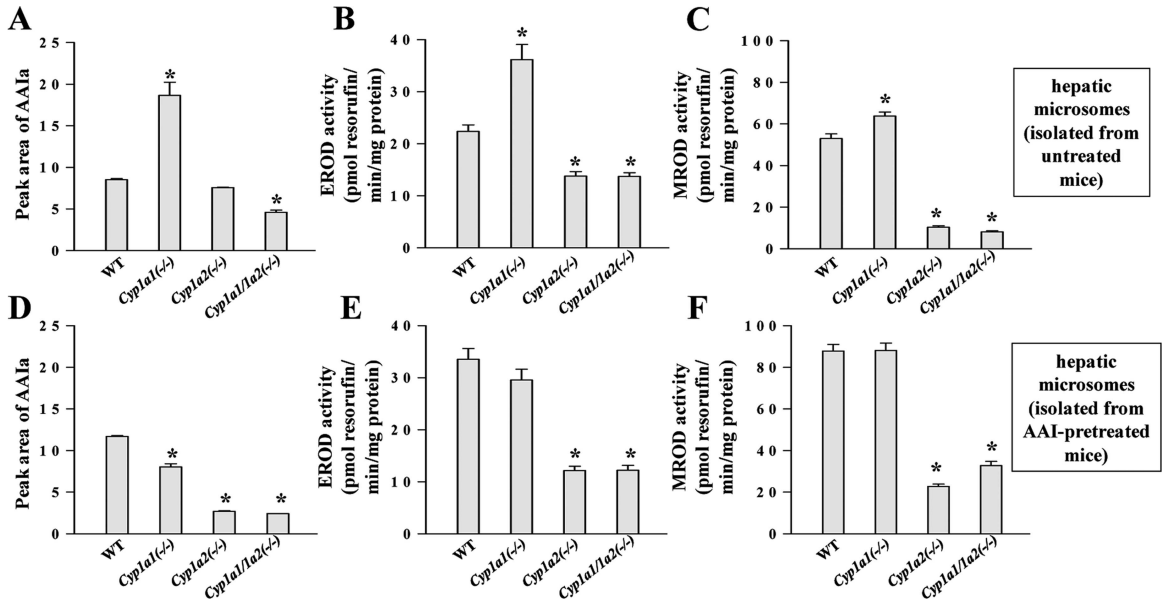

Figure 7. AAI oxidation to AAIa and P450 enzyme activity in mouse hepatic microsomes isolated from *Cyp1a* knockout and WT mice: (A−C) untreated or (D−F) pretreated orally with 50 mg/kg body weight AAI for 24 h. Formation of AAIa from AAI in hepatic microsomes (A,D). P450 1A enzyme activity in hepatic microsomes as measured by EROD (B,E) or MROD activity (C,F). All values are given as the means ± SD (n = 3). Comparison was performed by t-test analysis; *P < 0.01, different from WT.

Cytosolic-Mediated AAI-DNA Adduct Formation. Because we had previously established that NQO1 activates AAI very effectively,[20] NADPH-dependent DNA adduct formation of AAI in cytosols, a measure of AAI bioactivation by NQO1, was analyzed in the four mouse lines. Hepatic and renal cytosols from AAI-treated mice were capable of bioactivating AAI to form DNA adducts, and overall no difference in AAI-DNA adduct formation was found among the four lines; however, renal cytosols were less active than hepatic cytosols (Supporting Information, Figure 2).

Table 1. POR Activity in Hepatic Microsomes of Mouse Lines[a]

	cytochrome *c* reduction [nmol/min per mg protein] in hepatic microsomes of	
mouse line	untreated mice	mice pretreated with 50 mg/kg body weight AAI
WT	1.9 ± 0.4	1.9 ± 0.3
Cyp1a1(−/−)	1.8 ± 0.3	2.1 ± 0.2
Cyp1a2(−/−)	2.0 ± 0.3	1.8 ± 0.3
Cyp1a1/1a2(−/−)	2.1 ± 0.3	1.8 ± 0.3

[a] Values are given as the means ± SD (n = 3).

AAIa Formation in Mouse Hepatic Microsomes. To study AAI detoxication, we used the same microsomes as those in the experiments above, except that these incubations with AAI were carried out under aerobic conditions. All hepatic microsomes oxidized AAI to AAIa (Figure 7A), demonstrated by one metabolite detectable by HPLC (peak r.t. 23.1 min; see Figure 3C). The amount of AAIa formed correlated well with EROD and MROD activities (Figure 7B and C). Collectively, these findings clearly show that mouse P450 1A2 is mainly responsible for the oxidative demethylation of AAI to AAIa in mouse liver.

Microsomes from AAI-pretreated WT mice produced 1.4-fold higher amounts of AAIa than those from untreated WT mice ($P < 0.01$). In contrast, AAI pretreatment led to decreases in AAIa formation in all three knockout lines (Figure 7D). Again, the decreases in AAIa demethylation paralleled the EROD activities (compare Figure 7E). These results indicate that AAI can induce *Cyp1a1* and *Cyp1a2* gene expression in WT mice, whereas in the *Cyp1a*-knockout lines, P450 1A2 is induced in *Cyp1a1*(−/−) mice as seen by MROD activity (compare Figure 7F).

DISCUSSION

One of the common features of AAN and BEN is that not all individuals exposed to AA suffer from nephropathy and malignancy. To date, only 5% of patients treated with the weight-loss regimen in Belgium have suffered from AAN.[45] A major cause for these differences in response is highly likely to be interindividual differences in the levels of enzymes that catalyze AA biotransformation.[19] Whereas the enzymes involved in the reductive activation of AA have already been examined,[19] those participating in the oxidative detoxication of AAI to AAIa require a better understanding.

AAIa formation has been found recently to be catalyzed mainly by human and rat P450 1A1 and 1A2 *in vitro*,[44,46,47] by mouse P450 1A2,[47] and human P450 1A1 and 1A2 *in vivo* (Stiborova, M., Nebert, D. W., and Arlt, V. M., manuscript in preparation). A role of mouse P450 1A2 in AAI detoxication *in vivo* was recently reported,[47] using *Cyp1a2*(−/−) knockout mice. The authors showed that, compared with WT mice, AAI-treated *Cyp1a2*(−/−) mice had higher levels of AAI-DNA adducts in the renal cortex and an increase in microalbuminuria, which is an indicator of renal tubule dysfunction. However, they did not evaluate the role of P450 1A1 in this study; furthermore, they did not investigate the metabolic fate of AAI or the formation of AAI-DNA adducts in organs other than the kidney. Therefore, these parameters, demonstrated in the present work, represent an extension of the work in this field. Depending on the experimental conditions, P450 1A1 and 1A2 also activate AAI *in vitro*.[21,22,44,48] In the present study, we therefore have investigated in detail the balance between P450 1A1- and 1A2-mediated reductive activation and oxidative detoxication *in vitro* and *in vivo*.

We have shown that higher AAI-DNA adduct levels were found in the kidney and spleen of *Cyp1a1*(−/−) mice, in the liver of *Cyp1a2*(−/−) mice, and in all tissues examined of *Cyp1a1/1a2*(−/−), compared with those in WT mice. These data indicate that the absence of P450 1A1 and/or 1A2 leads to decreased AAI detoxication, indicating that higher amounts of AAI are available for activation to form DNA adducts. Lower AAI detoxication (demethylase activity) was also confirmed in *Cyp1a1/1a2*(−/−) liver microsomes *in vitro*. Because DNA adduct levels were higher in the kidney than the liver but activation of AAI by hepatic microsomes was much more effective than by renal microsomes, metabolites such as aristolactams or hydroxyl-lactams might be conjugated in the liver and transported to the target organ kidney to be cleaved and DNA adducts formed. However, this possibility needs to be clarified in future studies.

Ablation of either the *Cyp1a1* or *Cyp1a2* gene alone or in combination had no effect on the urinary excretion profile of AAIa. This was unexpected but at least in *Cyp1a1*(−/−) mice was reflected by higher levels of AAIa formation catalyzed by hepatic microsomes. In this case, P450 1A2 might compensate, specifically because EROD activity was higher in hepatic microsomes from *Cyp1a1*(−/−) mice than in hepatic microsomes from WT mice. In *Cyp1a1/1a2*(−/−) mice, another enzyme probably assumes responsibility for AAI detoxication. Previous studies[28,49] showed that AAIa was a major metabolite in urine, but AAIa excretion levels were not quantified; those studies also showed that AAIa can be conjugated to glucuronide, sulfate, and acetate, which again could be excreted in urine and feces, but none of these conjugates were determined in the present work. Therefore, the levels of free AAIa in urine might not reflect the total detoxication of AAI. Our *in vitro* data do indicate, however, that mouse P450 1A2 is largely responsible for AAI detoxication to AAIa, confirming the observation by Rosenquist and colleagues.[47]

AAI-DNA adduct formation found in all four mouse lines might also reflect the reductive activation of AAI by NQO1. NADPH-dependent adduct formation by AAI was found in incubations containing liver and kidney cytosols. In our previous study utilizing another mouse model, hepatic reductase null (HRN) mice in which POR is deleted specifically in the liver, expression and activity of NQO1 were found to be higher in hepatic cytosols compared to that of WT mice.[44] This higher NQO1 activity also resulted in an enhanced activation of AAI to DNA adducts in hepatic cytosols of HRN mice. However, this was not the case in the *Cyp1a* knockout mice used in the present work. It is noteworthy that a previous study showed that ablation of the *Cyp1a1* gene did not alter hepatic constitutive expression of other genes in the mouse aryl hydrocarbon receptor battery including NQO1.[32] Nevertheless, because NQO1 was found to be the most effective enzyme activating AAI in human and rodent liver and kidney,[20,50,51] its participation in this process in mouse tissues is also likely to be important. These results fit well with the proposed scheme of AAI metabolism (Figure 1). If AAI is not efficiently oxidized to AAIa, it is activated by several nitroreductases[19,52] to form a cyclic acylnitrenium ion, capable of

generating DNA adducts (Figure 1). The results found here also demonstrate that these *Cyp1a* knockout lines are excellent models to further investigate the toxic effects not only of AAI but also of other P450 1A-mediated toxic metabolites.[30,53]

However, it should be emphasized that, under hypoxic conditions, the mouse hepatic P450 1A enzymes *in vitro* were also capable of AAI-DNA adduct formation. The major role of P450 1A in AAI-DNA adduct formation by liver microsomes was further demonstrated by the use of selective enzyme inhibitors. Inhibitors of P450 1A1 and 1A2, but not of POR, decreased AAI-DNA adduct formation in WT microsomes. The finding that both P450 1A1 and 1A2 both oxidatively detoxicate and reductively activate AAI indicates that AAI must act as a ligand of P450 1A heme iron under low pO_2 concentrations. However, under aerobic conditions AAI acts as a substrate of P450 1A1 or 1A2 and takes one atom of atmospheric oxygen to O-demethylate the methoxy group of AAI to generate AAIa. Tubulointerstitial hypoxia in chronic kidney disease plays a major role in the progression to end-stage renal disease,[54] and AAN patients rapidly progress to end-stage renal disease despite the cessation of AA-containing products.[1] Hypoxia is also a key regulatory factor in tumor growth.[55] The cellular ability to survive under hypoxic conditions is one of the fundamental physiological differences between tumor cells and normal cells. Our findings suggest that, in addition to the influence of hepatic P450 1A expression, the *in vivo* pO_2 in tissues affects the balance between nitroreduction and demethylation of AAI, thereby influencing its toxicity and carcinogenicity. Thus, tubulointerstitial hypoxia could be a critical factor in the kidney.

Our *in vitro* experiments using inhibitors of hepatic microsomal P450 1A1, P450 1A2, and POR showed that, in addition to P450 1A1 and 1A2, POR also contributes to AAI-DNA adduct formation. These results are consistent with previous findings showing that purified POR alone *in vitro* can catalyze AAI-DNA adduct formation.[22,56] In hepatic microsomes from *Cyp1a1/1a2(−/−)* mice, POR or another reductase sensitive to α-LA is the major player in AAI-DNA adduct formation. The important role of POR in AAI-DNA adduct formation has also been found previously in microsomes isolated from human and HRN mouse kidney, the organ in which low levels of P450 1A are expressed.[21,44] The contributions of microsomal POR, P450 1A1, and 1A2 to reductively activate AAI therefore represent a balance between AAI concentration, spatial accessibility, and competition between the active-sites of the three enzymes and their quantitative amounts.

Taking into account previous data showing a major function of human, rat, and mouse NQO1 in AAI activation,[19,20,50,52] the current study also demonstrates roles for mouse P450 1A1 and P450 1A2 in this process. Human expression of hepatic P450 1A2 can vary 60-fold, and it is not expressed in the kidney, in contrast to P450 1A1.[57] Thus, we suggest that an individual's susceptibility to AAI is determined by AAI tissue concentrations and the oxidative versus reductive balance of P450 1A1 and P450 1A2 in the target organ kidney and in the liver where AAI is detoxicated.

■ ASSOCIATED CONTENT

Supporting Information. Positive MALDI-TOF/TOF of AAIa and DNA adduct formation *in vitro* by AAI in cytosols isolated from Cyp1a knockout and WT mice. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AA, aristolochic acid; AAI, aristolochic acid I; AAII, aristolochic acid II; AAIa, aristolochic acid Ia; AAN, aristolochic acid nephropathy; BEN, Balkan endemic nephropathy; dA-AAI, 7-deoxyadenosine-N^6-yl)aristolactam I; dA-AAII, 7-deoxyadenosine-N^6-yl)-aristolactam II; dG-AAI, 7-deoxyguanosin-N^2-yl)aristolactam I; EROD, ethoxyresorufin *O*-deethylation; FF, furafylline; MROD, methoxyresorufin *O*-deethylation; NQO1, NAD(P)H:quinone oxidoreductase; P450, cytochrome P450; POR, P450 oxidoreductase; TLC, thin-layer chromatography; WT, wild-type; α-NF, α-naphthoflavone; α-LA, α-lipoic acid

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ORIGINAL ARTICLE

Role of cytochromes P450 in metabolism of carcinogenic aristolochic acid I: evidence of their contribution to aristolochic acid I detoxication and activation in rat liver

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Abstract

OBJECTIVE: The herbal drug aristolochic acid (AA) derived from Aristolochia species has been shown to be the cause of aristolochic acid nephropathy (AAN), Balkan endemic nephropathy (BEN) and their urothelial malignancies. One of the common features of AAN and BEN is that not all individuals exposed to AA suffer from nephropathy and tumor development. One cause for these different responses may be individual differences in the activities of the enzymes catalyzing the biotransformation of AA. Thus, the identification of enzymes principally involved in the metabolism of AAI, the major toxic component of AA, and detailed knowledge of their catalytic specificities is of major importance. Therefore, the present study has been designed to evaluate the cytochrome P450 (CYP)-mediated oxidative detoxification and reductive activation of AAI in a rat model.

METHODS: DNA adduct formation was investigated by the nuclease P1 version of the ^{32}P-postlabeling method. The CYP-mediated formation of a detoxication metabolite of AAI, 8-hydroxyaristolochic acid I (AAIa), *in vitro* in rat hepatic microsomes was determined by HPLC.

RESULTS: Rat hepatic CYPs both detoxicate AAI by its oxidation to AAIa and reductively activate this carcinogen to a cyclic *N*-acylnitrenium ion forming AAI-DNA adducts *in vitro*. To define the role of hepatic CYPs in AAI demethylation and activation, the modulation of AAIa and AAI-DNA adduct formation by CYP inducers and selective CYP inhibitors was investigated. Based on these studies, we attribute the major role of CYP1A1 and 1A2 in AAI detoxication by its demethylation to AAIa, and, under hypoxic conditions also to AAI activation

to species forming DNA adducts. Using microsomes of Baculovirus transfected insect cells (Supersomes™) containing recombinantly expressed rat CYPs, NADPH:CYP reductase and/or cytochrome b_5, a major role of CYP1A1 and 1A2 in both reactions *in vitro* was confirmed.

CONCLUSION: Based on the results found in this and former studies we propose that AAI activation and detoxication in rats are dictated mainly by AAI binding affinity to CYP1A1/2 or NADPH(P)H:quinone oxidoreductase, by their turnover and by the balance between oxidation and reduction of AAI by CYP1A.

ABBREVIATIONS:

α-NF	- α-naphthoflavone
AA	- aristolochic acid
AAN	- aristolochic acid nephropathy
AAI	- 8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid
AAIa	- 8-hydroxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid
AAII	- 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid
BEN	- Balkan endemic nephropathy
CYP	- cytochrome P450
dA-AAI	- 7-(deoxyadenosin-N^6-yl)aristolactam I
dA-AAII	- 7-(deoxyadenosin-N^6-yl)aristolactam II
dG-AAI	- 7-(deoxyguanosin-N^2-yl) aristolactam I
DDTC	- diethyldithiocarbamic acid
EtOH	- ethanol
HPLC	- high performance liquid chromatography
$NADP^+$	- nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
NQO1	- NAD(P)H:quinone oxidoreductase
PB	- phenobarbital
PCN	- pregnenolone-16α-carbonitrile
PEI	- polyethylenimine
RAL	- relative adduct labeling
r.t.	- retention time
TLC	- thin layer chromatography

INTRODUCTION

The herbal drug aristolochic acid (AA) derived from Aristolochia species has been shown to be the cause of so-called Chinese herbs nephropathy, now termed aristolochic acid nephropathy (AAN) (Arlt *et al.* 2002b; Debelle *et al.* 2008; Schmeiser *et al.* 2009). The plant extract AA is a mixture of structurally related nitrophenanthrene carboxylic acids, the major components being aristolochic acid I (8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid, AAI; Figure 1) and aristolochic acid II (6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid, AAII). AAN is a rapidly progressive renal fibrosis that was observed initially in a group of Belgian women who had ingested weight loss pills containing *Aristolochia fangchi* (Vanherweghem *et al.* 1993). Within a few years of taking the pills, AAN patients also developed a high risk of upper urothelial tract carcinoma (about 50%) and, subsequently, bladder urothelial carcinoma (Nortier *et al.* 2000; Lemy *et al.* 2008). Subsequently, similar cases have been reported elsewhere in Europe and Asia (Lord *et al.* 2001; Debelle *et al.* 2008; Lai *et al.* 2010). More recently, exposure to AA has been linked to Balkan endemic nephropathy (BEN) and its associated urothelial cancer (Arlt *et al.* 2007; Grollman *et al.* 2007; Nedelko *et al.* 2009). This nephropathy is endemic in certain rural areas of Serbia, Bosnia, Croatia, Bulgaria and Romania. Exposure to AA was demonstrated by the identification of specific AA-DNA adducts in urothelial tissue of AAN and BEN patients (Schmeiser *et al.* 1996; Bieler *et al.* 1997; Nortier *et al.* 2000; Lord *et al.* 2001; Arlt *et al.* 2002a; Grollman *et al.* 2007). The most abundant DNA adduct detected in patients exposed to AA is 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI), which leads to characteristic AT→TA transversion mutations. Such AT→TA mutations have been observed in the *TP53* tumor suppressor gene in tumors from AAN and BEN patients (Lord *et al.* 2004; Arlt *et al.* 2007; Grollman *et al.* 2007; Nedelko *et al.* 2009), indicating the probable molecular mechanism of AA carcinogenesis in humans (Simoes *et al.* 2008; Arlt *et al.* 2011). As a consequence, AA was recently classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) (Grosse *et al.* 2009).

In common with other nitroaromatics the major activation pathway for AA is nitroreduction catalyzed by both cytosolic and microsomal enzymes, cytosolic NAD(P)H:quinone oxidoreductase (NQO1) being the most efficient (Stiborova *et al.* 2002b; 2003; 2008b; 2011) (Figure 1). The activation of AAI in human hepatic microsomes is mediated by CYP1A2 and, to a lesser extent by CYP1A1; NADPH:CYP reductase also plays a minor role (Stiborova *et al.* 2001b; 2001c; 2005a; 2005c). Prostaglandin H synthase (cyclooxygenase, COX) in human renal microsomes has also been shown to activate AAI (Stiborova *et al.* 2001a; 2005a). While the enzymes catalyzing the reductive activation of AAI leading to covalent DNA adducts have been widely investigated, those participating in its detoxication have not been extensively studied so far. Several studies have indicated that induction of CYP1A (e.g. by 3-methylcholanthrene and β-naphthoflavone) protect mice from AAI-induced acute renal injury (Xue *et al.* 2008; Xiao *et al.* 2008; 2009). One detoxication metabolite identified is 8-hydroxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (8-hydroxy-aristolochic acid I, aristolochic acid Ia, AAIa; Figure 1) that is formed after demethylation of AAI and is, in turn, subject to conjugation, forming glucuronide or sulfate esters (Krumbiegel *et al.* 1987; Chan *et al.* 2007; Shibutani *et al.* 2010). Human, rat and mouse CYP1A1 and 1A2 can demethylate AAI to AAIa *in vitro* (Sistkova *et al.* 2008; Rosenquist *et al.* 2010; Levova *et al.* 2011) and CYP1A2 in mice appears to mediate this reaction *in vivo* (Rosenquist *et al.* 2010). Nevertheless, CYP1A1/2 also activate AAI in human, rat and mouse livers (Stiborova *et al.*

2001b; 2005a; 2005c; 2008b; Levova *et al.* 2011). Therefore, detailed knowledge of the catalytic specificities of CYP1A and other CYP enzymes in the detoxication and activation of AAI *in vitro* and *in vivo* is essential to be elucidated.

The aim of the present study was to evaluate the CYP-mediated oxidative detoxication and reductive activation of AAI by rat CYP enzymes in detail. The formation of AAIa by rat hepatic microsomes, and by rat recombinant CYPs was determined by high performance liquid chromatography (HPLC). In addition, DNA adduct formation by AAI *in vitro* was measured by ^{32}P-postlabeling.

MATERIALS AND METHODS

Chemicals

The natural mixture of AA consisting of 38% AAI and 58% AAII was purchased from Sigma Chemical Co (St Louis, MO, USA). AAI (as sodium salt) was isolated from the mixture by preparative HPLC; its purity was 98% as estimated by HPLC (Schmeiser *et al.* 1984). Diamantane was supplied by Pliva-Lachema (Brno, Czech Republic), Other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of reagent grade purity or better.

Preparation of rat hepatic microsomes

Microsomes were prepared from livers of ten untreated Wistar rats by the procedure described previously (Stiborova *et al.* 2002b; Mizerovska *et al.* 2009; Svobodova *et al.* 2009). Microsomes were also prepared from livers of groups of ten Wistar male rats pre-treated with Sudan I, phenobarbital (PB), ethanol (EtOH) or pregnenolone-16α-carbonitrile (PCN) as described previously (Stiborova *et al.* 2002b; Mizerovska *et al.* 2009; Svobodova *et al.* 2009; Naiman *et al.* 2010).

Microsomal incubations used for AAI demethylation

Incubation mixtures, in a final volume of 250 µl, consisted of 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg rat hepatic microsomal protein and 10 µM AAI. Incubations with microsomes were carried out at 37 °C for 20 min and AAI oxidation (demethylation) to AAIa was linear up to 25 min. Control incubations were carried out (*i*) without

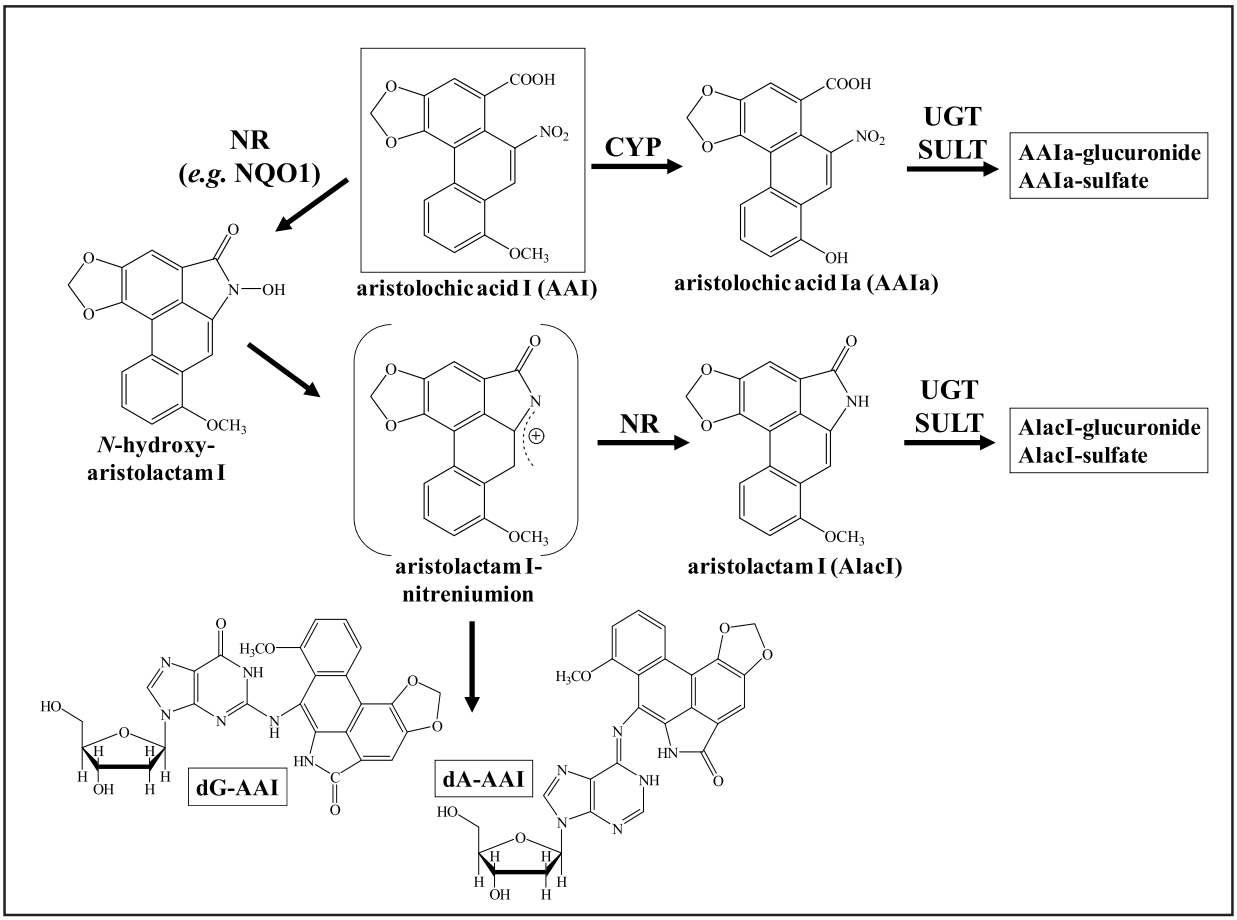

Fig. 1. Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; NR, nitroreduction; UGT, UDP glucuronosyl transferase; SULT, sulfotransferase.

microsomes, (*ii*) without NADPH or (*iii*) without AAI. Supersomes™, microsomes isolated from insect cells transfected with baculovirus constructs containing cDNA of single rat CYPs (CYP1A1 or CYP1A2), and expressing NADPH:CYP reductase were obtained from Gentest Corp. Incubation mixtures, in a final volume of 250 µl, consisted of 100 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM $MgCl_2$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase, to generate NADPH, 50 nM CYP1A1 or 1A2 in Supersomes™ and 10 µM AAI. Supersomes containing NADPH:CYP reductase alone were used for control. In experiments investigating the effect of cytochrome b_5 on AAI demethylation by recombinant CYP1A1 and 1A2, the cytochrome b_5 protein isolated from rat hepatic microsomes was added into the supersomal systems in an amount that was 3-fold higher than concentrations of CYP1A1 and 1A2. Rat liver cytochrome b_5 was isolated in our laboratory from rat hepatic microsomes by the procedure described by Roos (1996).

Determination of AAIa by HPLC

AAI and its metabolites (*i.e.* AAIa) were extracted from incubation mixtures twice into ethyl acetate (2 × 1 ml), the extracts were evaporated to dryness and the residues re-dissolved in 30 µl of methanol and subjected to reverse-phase HPLC. HPLC was performed with a reversed phase column (Nucleosil 100-5 C_{18}, 25 × 4.0 mm, 5 mm; Macherey-Nagel) preceeded by a C-18 guard column, using a linear gradient of acetonitrile (20–60% acetonitrile in 55 min) in 100 mM triethylamonium acetate with a flow rate of 0.6 ml/min. HPLC was carried out with a Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector set at 254 nm, and peaks were integrated with CHROMELEON™ 6.01 integrator. A product of AAI metabolism and AAI itself eluted with retention times (r.t.) of 28.3 and 36 min, respectively. The product eluting at 28.3 min was identified as AAIa using mass-spectroscopy analyses. Mass spectra were measured on MALDI-TOF/TOF ultraFLEX III mass spectrometers (Bruker-Daltonics, Bremen, Germany). Positive spectra were calibrated externally using the monoisotopic $[M+H]^+$ ions of PepMixII calibrant (Bruker-Daltonics, Bremen) or matrix peaks. A 10 mg/ml solution of α-cyano-4-hydroxy-cinnamic acid, or 50 mg/ml solution of 2,5-dihydrobenzoic acid in 50% MeCN/0.1% TFA were used as MALDI matrix. A 0.5 µl sample dissolved in MeCN was directly mixed with 0.5 µlL of the matrix solution and allowed to dry at ambient temperature on the target. The MALDI-TOF positive spectra were collected in reflector mode.

Inhibition studies

The following chemicals were used to inhibit AAI demethylation by rat hepatic microsomes to AAIa: α-naphthoflavone (α-NF), which inhibits CYP1A1 and CYP1A2 (Stiborova *et al.* 2001b; 2005b); furafylline, which inhibits CYP1A2 (Stiborova *et al.* 2001b); diamantane, which inhibits CYP2B (Stiborova *et al.* 2002a); sulfaphenazole, which inhibits CYP2C, quinidine, which inhibits CYP2D, diethyldithiocarbamic acid (DDTC), which inhibits CYP2A and 2E1 and ketoconazole, which inhibits CYP3A (Stiborova *et al.* 2001b). Inhibitors were dissolved in 2.5 µl methanol, except of DDTC that was dissolved in distilled water, to yield final concentrations of 1 and 10 µM in the incubation mixtures. Mixtures were incubated at 37 °C for 10 min with NADPH-generating system prior to adding AAI, and then incubated for further 20 min at 37 °C. AAI and its metabolite AAIa were extracted from incubation mixtures twice with ethyl acetate (2 × 1 ml) and analyzed using HPLC as described above.

Incubations used for analysis of AAI-DNA adduct formation by rat recombinant CYPs in Supersomes™ and DNA adduct analysis by ^{32}P-postlabeling

The deaerated and argon-purged incubation mixtures, in a final volume of 750 µl, consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 50 pmol rat recombinant CYPs in Supersomes™, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI as described previously (Stiborova *et al.* 2005a). Supersomes™, microsomes isolated from insect cells transfected with baculovirus constructs containing cDNA of single rat CYPs (CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13, CYP2D1, CYP2D2, CYP2E1, CYP3A1, and CYP3A2), and expressing NADPH:CYP reductase and/or cytochrome b_5 were obtained from Gentest Corp. Rat cytochrome b_5 was added into the mixture containing recombinant CYP1A1. 1A2, 2D1 and 2D2 in an amount that was 3-fold higher than concentrations of the CYP enzymes. Incubations with rat recombinant CYPs in Supersomes™ were carried out at 37 °C for 60 min. AAI-derived DNA adduct formation was found to be linear up to 2 hr (Stiborova *et al.* 2005a). DNA was isolated from incubation mixtures by standard phenol/chloroform extraction. ^{32}P-Postlabelling analysis (Phillips & Arlt 2007) using the nuclease P1 enrichment version, and thin layer chromatography (TLC) and HPLC were performed as described (Schmeiser *et al.* 1996; Bieler *et al.* 1997). TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Schmeiser *et al.* 1996; Bieler *et al.* 1997). Results were expressed as DNA adducts/10^8 nucleotides.

RESULTS AND DISCUSSION

Rat hepatic microsomes oxidize AAI to AAIa

Rat hepatic microsomes in the presence of NADPH were capable of metabolizing AAI to one metabolite detectable by HPLC analysis (see peak with r.t. of 28.3 min in Figure 2 for hepatic microsomes of uninduced rats).

In control incubations without microsomes or without NADPH, no AAI metabolite was found (Figure 2). Positive MALDI-TOF-TOF detected peaks at *m/z* 328.043 and 327.029, representing the molecular ions $[M-H]^+$ and $[M]^+$ of AAIa, respectively (Figure 3). The peaks at *m/z* 283.021 and 311.031, representing ions of AAIa fragments, were also found (Figure 3). These results show that the detected metabolite is the demethylation product of AAI, 8-hydroxy-aristolochic acid (AAIa) (for structure see Figure 1), which was found previously to be formed also in mouse hepatic microsomes (Levova *et al.* 2011) and by human and rat CYP enzymes (Sistkova *et al.* 2008; Levova *et al.* 2011). AAIa is supposed to be a detoxication metabolite of AAI, because it was found to be less toxic than a parental compound, AAI (Shibutani *et al.* 2010).

Involvement of rat CYP enzymes in AAI demethylation to AAIa

The capacity of different rat CYPs to demethylate AAI to AAIa was initially studied using inhibitors of individual CYP enzymes. Hepatic microsomes of control (uninduced) rats were utilized in these experiments. As shown in Table 1, a slight, but significant inhibition of AAIa formation in rat hepatic microsomes was produced by α-NF, which inhibits CYP1A1/2 and furafyl-

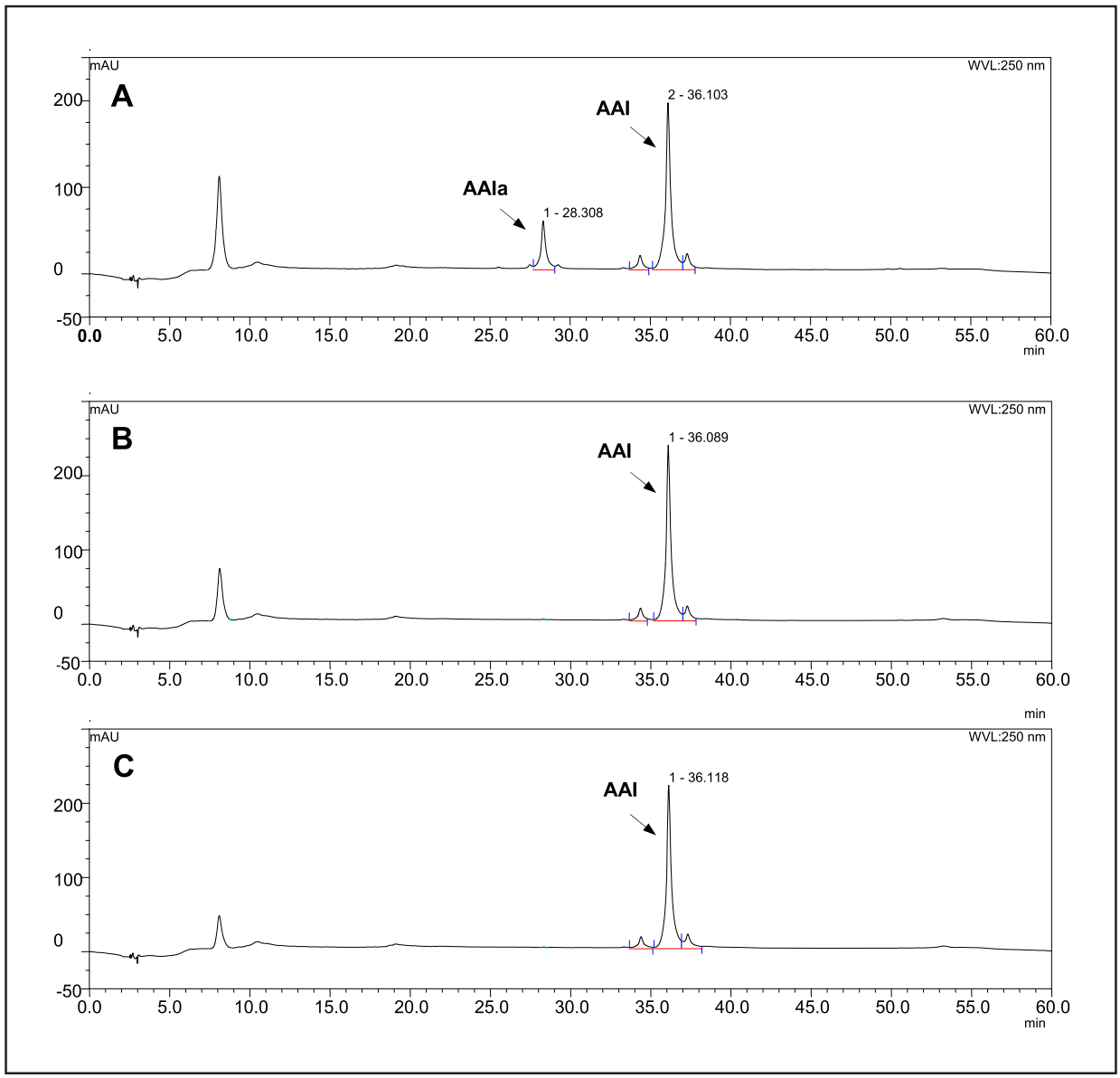

Fig. 2. HPLC chromatograpH of AAIa metabolite (peak r.t. at 28.3 min) and AAI (peak r.t. at 36 min) produced by hepatic microsomes of control (untreated) rats incubated with AAI and NADPH (A), by rat hepatic microsomes incubated with AAI without NADPH (B) and by NADPH incubated with AAI without rat hepatic microsomes (C). The peaks with the characterized AAI metabolite (AAIa) and the parent AAI are indicated in the chromatograms.

Tab. 1. The effects of CYP inhibitors on AAI demethylation to AAIa in rat hepatic microsomes.

Inhibitor	1 μM	10 μM
	AAIa formation (% of control without inhibitors)	
α-Naphthoflavone (CYP1A1/2)[a]	87.5 ± 2.5**	84.7 ± 2.2***
Furafylline (CYP1A2)	91.5 ±3.6	84.1 ±2.3***
Diamantane (CYP2B)	NI[b]	
Sulfaphenazole (CYP2C)	73.6 ± 4.8***	68.0 ±2.1***
Quinidine (CYP2D)	NI	
DDTC (CYP2A, 2E1)	68.2 ± 4.3***	52.2 ± 2.0***
Ketoconazole (CYP3A4)	96.1 ±5.2	90.2 ± 7.3

Values in the table are averages ± standard deviations (n = 3). 1 mg microsomal protein 10 mM AAI and 1 or 10 mM inhibitor were used in incubations (see Materials and Methods). Values significantly different from control incubations without inhibitors; $^{**}p<0.01$, $^{***}p<0.001$ (Student's t-test).
[a]Isoforms of CYP used in the experiment are shown in brackets.
[b]NI, no inhibition.

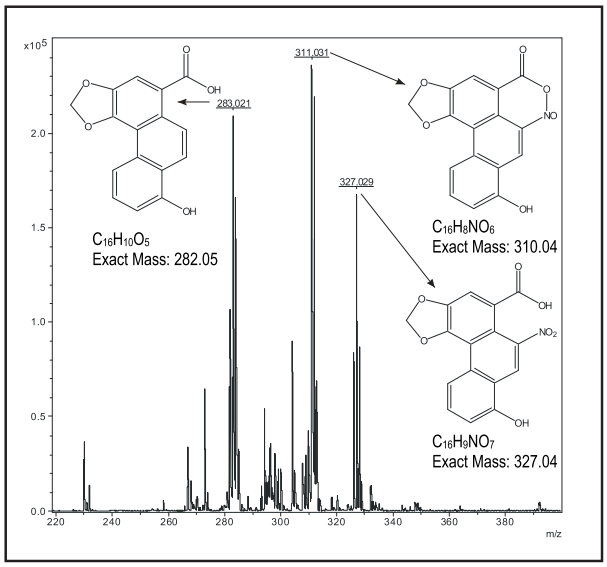

Fig. 3. Identification of AAI metabolite as AAIa by positive MALDI-TOF/TOF

line, which inhibits CYP1A2, whereas sulfaphenozole, which inhibits CYP2C and DDTC, which inhibits CYP2A and 2E1, were more effective. In contrast, the effects of inhibitors of other CYP enzymes (diamantane, an inhibitor of CYP2B, quinidine, an inhibitor of CYP2D and ketoconazole, an inhibitor CYP3A), were either negligible (ketoconazole) or these inhibitors were even without any effect (diamantane and quinidine) (Table 1). These results suggest that the rat hepatic CYP1A, 2A, 2C and 2E1 enzymes might oxidize AAI to AAIa in the microsomal system.

It should be noted, however, that the interpretation of the results from the inhibitory studies is sometimes difficult, because one inhibitor may be more effective with one substrate than another. In addition, expression levels of individual CYPs in the liver might influence the final degree of their inhibition. Indeed, the inhibition effects of CYP inhibitors on the metabolism of several xenobiotics in human livers were found to also depend, to some extent, on the levels of the CYP expression in this tissue (Lewis 2003). Therefore, further experiments were conducted using hepatic microsomes of rats treated with CYP inducers. As shown in Figure 4A, hepatic microsomes of rats treated with Sudan I (which induces CYP1A) and PB (which induces CYP2B and 2C) were 1.3 and 1.1 times more efficient to oxidize AAI to AAIa than uninduced microsomes. In contrast, other CYP inducers such as ethanol (which induces CYP2E1) and PCN (which induces CYP3A) decreased AAIa formation.

Collectively, the results found in experiments utilizing CYP inducers and inhibitors suggest that CYP1A1/2 enzymes are involved in AAI demethylation to AAIa in rat liver microsomes, and that participation of CYP2C in this reaction cannot be excluded. On the contrary, other hepatic CYPs, whose activities to demethylate AAI were inhibited in microsomes (CYP2A, 2E1) seem not to be important for AAI oxidation in rat livers. Indeed, a major role of CYP1A in AAI demethylation was confirmed in our previous work by utilizing rat recombinant CYP enzymes (Levova *et al.* 2011) Rat recombinant CYP2C enzymes that also demethylate AAI were much less effective, whereas other rat recombinant CYPs were not capable of oxidizing AAI to AAIa at all (Levova *et al.* 2011).

However, microsomes of Baculovirus transfected insect cells (Supersomes™) containing recombinantly expressed rat CYP1A1 or 1A2 and NADPH:CYP reductase used in our former study did not contain cytochrome b_5. The cytochrome b_5 is, however, one of the key proteins present in the membrane of endoplasmic reticulum that influences activities of several CYP enzymes including CYP1A1 and 1A2 (Schenkman & Jansson 2003; Duarte *et al.* 2005; Stiborova *et al.* 2006). Moreover, cytochrome b_5 is present in intact liver microsomes, and might thereby influence activities of CYP1A1 and 1A2 in this subcellular system. Therefore, to mimic the situation in microsomes, experiments in which cytochrome b_5 was added into the incubation mixtures used for demethylation of AAI to AAIa catalyzed by rat CYP1A1 and 1A2 were performed. Interestingly, cytochrome b_5 increased the efficiency of rat CYP1A2 to demethylate AAI, whereas this protein decreased formation of AAIa by rat CYP1A1 (Figure 4B). This finding indicates a major role of CYP1A2 in AAI detoxication to AAIa in rats.

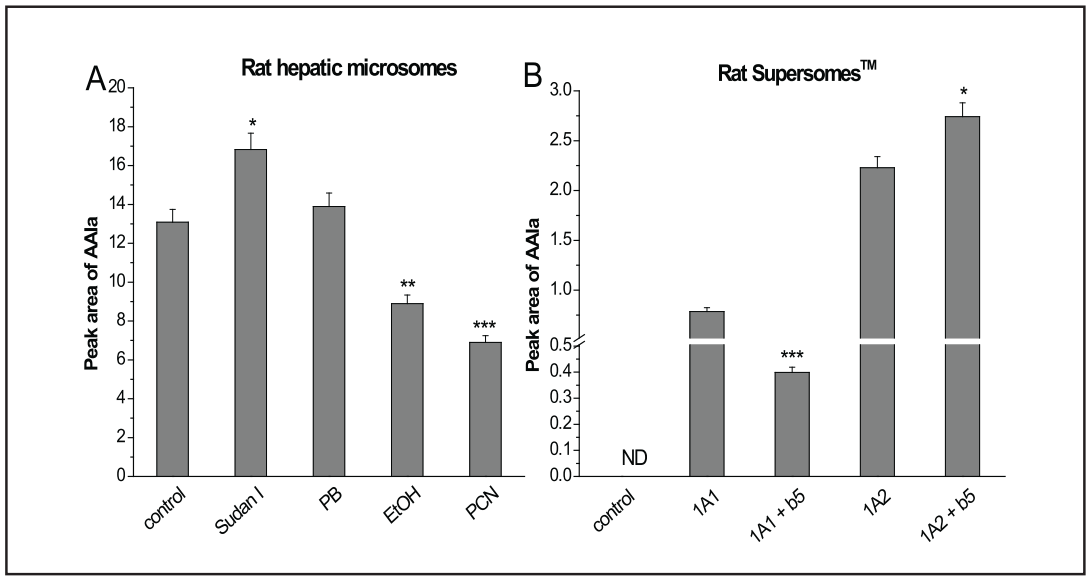

Fig. 4. Oxidation of AAI by rat hepatic microsomes (A) or rat recombinant CYP1A1 and 1A2 (B). Values are given as means ± standard deviations (n = 3). Values significantly different from hepatic microsomes of control (untreated) rats or from CYP1A1 or 1A2 without cytochrome b_5: *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test). Sudan I, phenobarbital (PB), ethanol (EtOH) and pregnenolone-16α-carbonitrile (PCN) were used as inducers of rat CYPs. 1 mg microsomal protein or 50 nM rat recombinant CYP1A1 or 1A2 and 10 μM AAI were used in incubations (see Materials and Methods). Control, control Supersomes™ containing NADPH:CYP reductase alone (50 nM). ND, not detected.

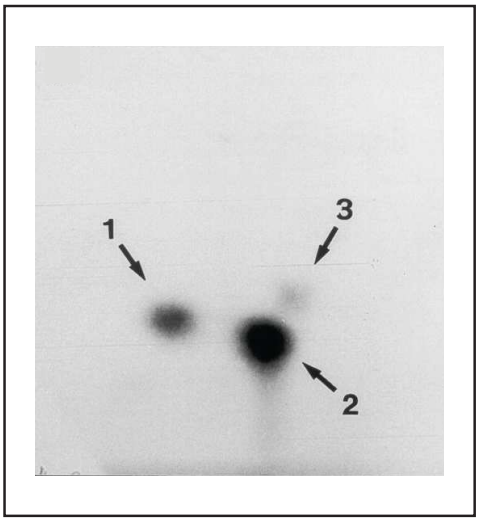

Fig. 5. Autoradiographic profile of AA-DNA adducts formed by incubation of AAI with rat recombinant CYP1A2 by using the nuclease P1 enrichment version of the ^{32}P-postlabeling assay. The origin, in the bottom left-hand corner, was cut off before exposure. Spot 1, dG-AAI; spot 2, dA-AAI; spot 3, 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII).

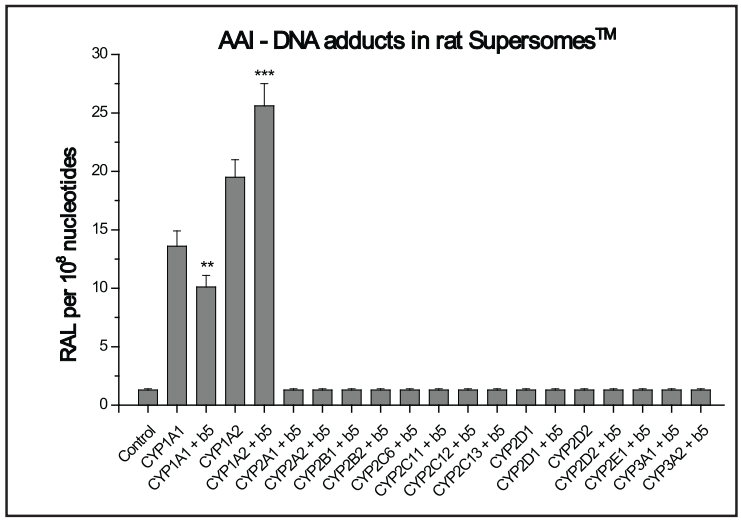

Fig. 6. DNA binding of AAI after activation with Supersomes™ containing different rat recombinant CYPs (50 pmol in a final volume of 750 μl incubations) and NADPH:CYP reductase or NADPH:CYP reductase alone (control). The nuclease P1-enrichment procedure of the ^{32}P-postlabeling assay was used for analysis. Values represent mean ± standard deviations of three separate incubations. RAL, relative adduct labeling. Values significantly different from CYP1A1 or 1A2 without cytochrome b_5: **$p<0.01$, ***$p<0.001$ (Student's t-test).

Rat cytochromes P450 1A1 and 1A2 activate AAI to species forming DNA adducts

Capability of rat hepatic microsomal CYP enzymes of activating AAI to species forming DNA adducts, suggesting a major role of CYP1A1 and 1A2 in this activation, has already been found in our earlier study (Stiborova *et al.* 2001b). Here, we identified and proved their role in AAI activation using recombinant rat CYP enzymes. The same microsomes of Baculovirus transfected insect cells (Supersomes™) containing recombinantly expressed rat CYPs, NADPH:CYP reductase and/or cytochrome b_5 as those used in the experiments

investigating efficiencies of rat CYPs in AAI demethylation (Levova *et al.* 2011 and present study) were utilized in such experiments, except that incubations were performed under hypoxic conditions. Incubation mixtures were purged with a stream of argon for 3 min before the addition of AAI. Although most of the oxygen was removed, we cannot exclude its presence in the membranes and lumen of microsomes present in the mixtures.

AAI was activated in incubations with all Supersomes™, generating the cluster of three DNA adducts as those found in AAN patients consisting of two major adduct spots (spot 1 and 2) and one minor adduct spot (spot 3) (see spots 1, 2 and 3 shown in Figure 5) (Nortier *et al.* 2000; Schmeiser *et al.* 1996). These adducts were identified previously (Bieler *et al.* 1997; Schmeiser *et al.* 1996) as 7-deoxyadenosine-N^6-yl)aristolactam I (spot 1; dA-AAI), 7-deoxyguanosin-N^2-yl)aristolactam I (spot 2; dG-AAI) and 7-(deoxyadenosin-N^6-yl) aristolactam II (spot 3; dA-AAII) (for structure see Figure 1), Low levels of AAI-DNA adducts were found in control incubations with Supersomes™ containing NADPH:CYP reductase alone (Figure 6). The same levels of AAI-DNA adducts were found also in incubations with most individual recombinant rat CYPs in Supersomes™, except of CYP1A1 and 1A2. Rat CYP1A1 and CYP1A2 were highly effective in activating AAI to species forming AAI-DNA adducts, CYP1A2 being more effective in this process than CYP1A1 (Figure 6). Cytochrome b_5 decreased levels of AAI-DNA adducts mediated by CYP1A1, whereas increased their formation in the system containing CYP1A2 (Figure 6). These results emphasize an exclusive role of rat CYP1A1 and predominantly CYP1A2 in activation of AAI to species generating AAI-DNA adducts, whereas other rat CYP enzymes are ineffective in this process.

CONCLUSIONS

In the present study we demonstrate that rat CYP1A1 and 1A2 enzymes are responsible both for AAI activation to a cyclic nitrenium ion forming DNA adducts and for demethylation of AAI to AAIa *in vitro*. The participation of rat CYP1A1/2 in oxidative detoxication of AAI (demethylation to AAIa) *in vitro* was proved using several approaches such as a use of inhibitors of CYP enzymes, specific CYP inducers and rat recombinant CYP1A1/2. The major role of rat CYP1A1 and 1A2 in reductive activation of AAI *in vitro* was identified using a panel of rat recombinant CYPs in incubations under anaerobic conditions. The results showing that CYP1A1/2 are capable of oxidizing and reducing AAI found in this work indicate that AAI acts as ligand of heme iron of the rat CYP1A1/2 enzymes under the low oxygen concentrations, being reduced instead of molecular oxygen during the CYP-mediated reaction cycle. In contrast, under the aerobic conditions it acts as a substrate of CYP1A1/2 utilizing one atom of oxygen for *O*-demethylation of a methoxy group of AAI to generate AAIa. These findings also demonstrate that besides the levels of CYP1A/12 expression in the liver and/or other tissues, the *in vivo* oxygen concentration in these tissues will affect the balance between nitroreduction and demethylation of AAI, thereby influencing its toxicity and carcinogenicity.

Taking into account the results found in this study, showing that CYP1A1/2 are capable of catalyzing both metabolic detoxication and activation of AAI, together with previous data showing a major role of human and rat cytosolic NQO1 in AAI activation (Stiborova *et al.* 2002a; 2003; 2008a; 2008b; 2011), we propose that the pathways of AAI metabolism in several organisms including rats are mainly dictated by the binding affinities of AAI to CYP1A1/2 or NQO1, and their enzymatic turnover as well as by the balance between the efficiency of CYP1A1/2 to oxidize and reduce AAI. All these enzymes exhibit polymorphisms, which are associated with different enzyme activities in human individuals. This feature may therefore be one determinant explaining an individual's susceptibility to AA. Indeed, it was reported that polymorphisms in the human NQO1 gene are important in AA-induced BEN, a disease that is associated with dietary exposure to AA (Arlt *et al.* 2007; Grollman *et al.* 2007; Toncheva *et al.* 2004; Atanasova *et al.* 2005). One of the NQO1 polymorphisms, the genotype NQO1*2/*2, was shown to predispose patients suffering from BEN to develop urothelial cancer (OR=13.75, 95%CI 1.17-166.21) (Toncheva *et al.* 2004). Therefore, the evaluation of inter-individual variations in activities of the human enzymes that play a major role in AAI activation and detoxication, including their genetic polymorphisms, remain a major challenge to explain human individual susceptibility to AA, and to predict the risk of cancer among the AAN and BEN patients.

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