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

Marie Stiborova [1], Jaroslav Mares [2], Kateřina Levova [1], Jana Pavlickova [1], Frantisek Barta [1], Petr Hodek [1], Eva Frei [3], Heinz H Schmeiser [4]

1 Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic
2 Institute of Biology and Medical Genetics, 2nd Medical School, Charles University and University Hospital Motol, Prague, Czech Republic
3 Division of Preventive Oncology, National Center for Tumour Diseases, German Cancer Research Center, Heidelberg, Germany
4 Research Group Genetic Alterations in Carcinogenesis, German Cancer Research Center, Heidelberg, Germany

Correspondence to: Prof. RNDr. Marie Stiborova, DSc.
Department of Biochemistry, Faculty of Science, Charles University
Albertov 2030, 128 40 Prague 2, Czech Republic.
TEL: +420-221 951; FAX: +420-221 951 283; E-MAIL: stiborov@natur.cuni.cz

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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

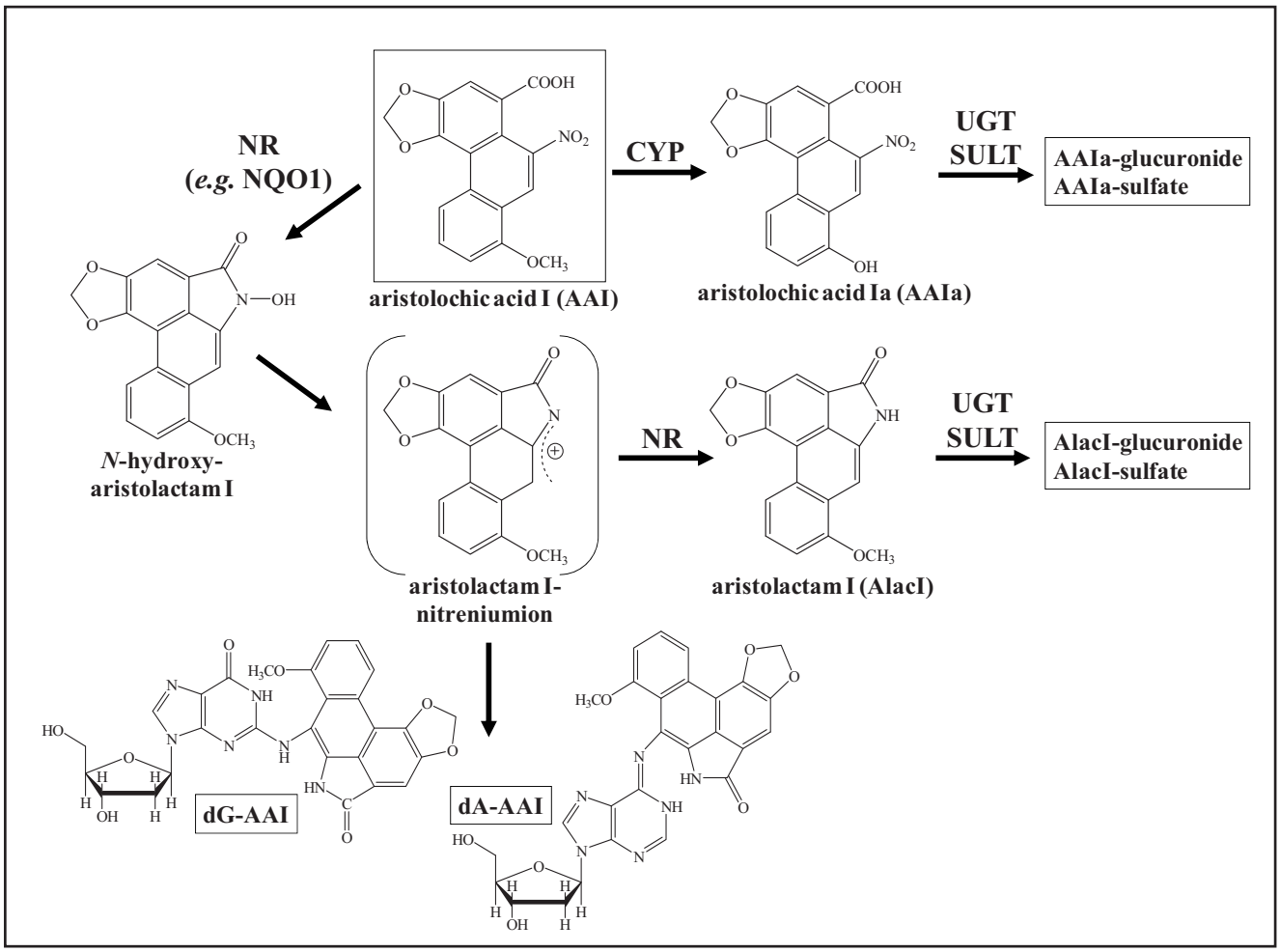

Fig. 1. Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin-*N*^{6}-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-*N*^{2}-yl)aristolactam I; NR, nitroreduction; UGT, UDP glucuronosyl transferase; SULT, sulfotransferase.

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-

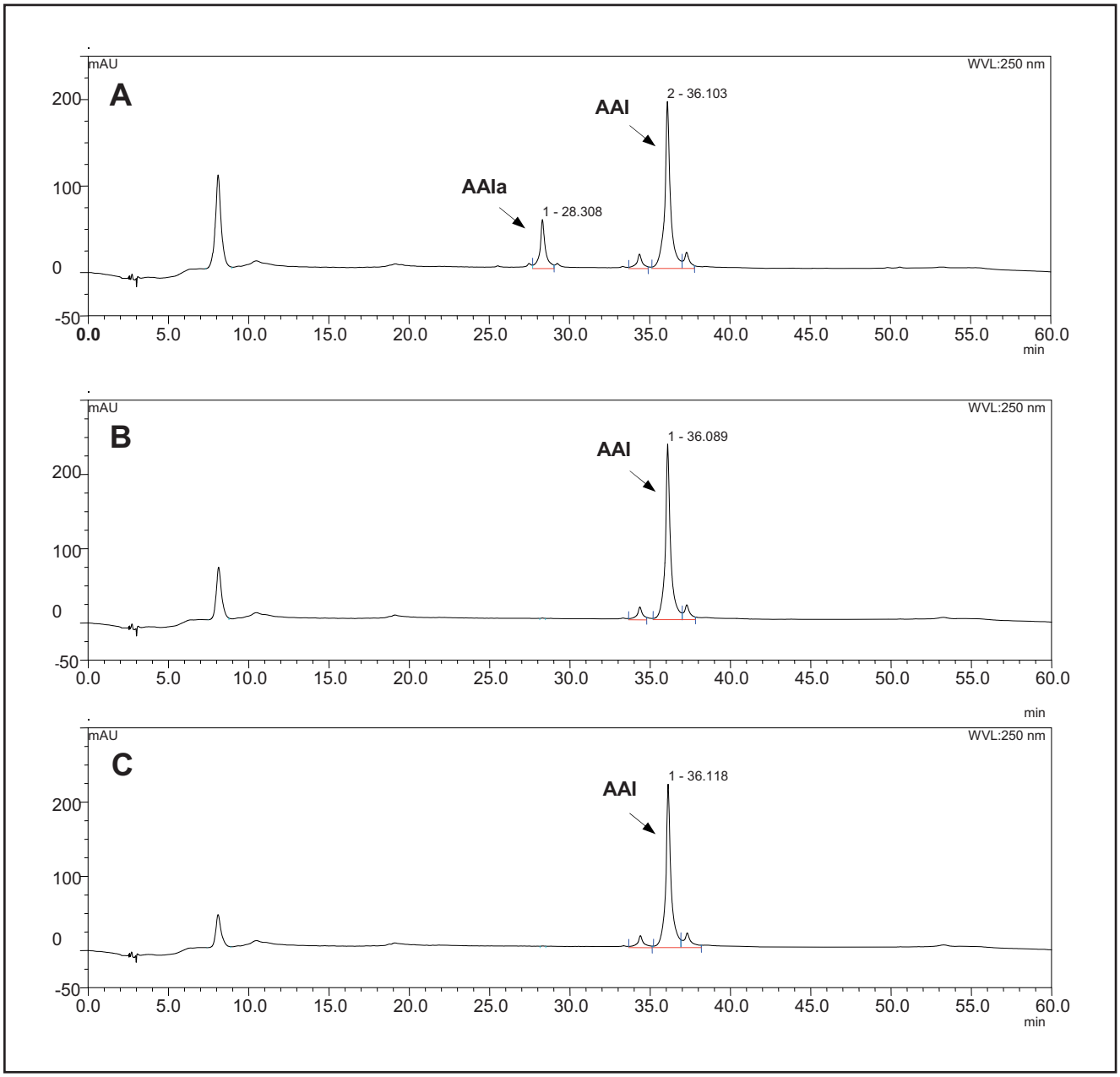

Fig. 2. HPLC chromatograpH of AAIa metabolite (peak r.t. at 28.3 min) and AAI (peak r.t. at 36 min) produced by hepatic microsomes of control (untreated) rats incubated with AAI and NADPH (A), by rat hepatic microsomes incubated with AAI without NADPH (B) and by NADPH incubated with AAI without rat hepatic 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.

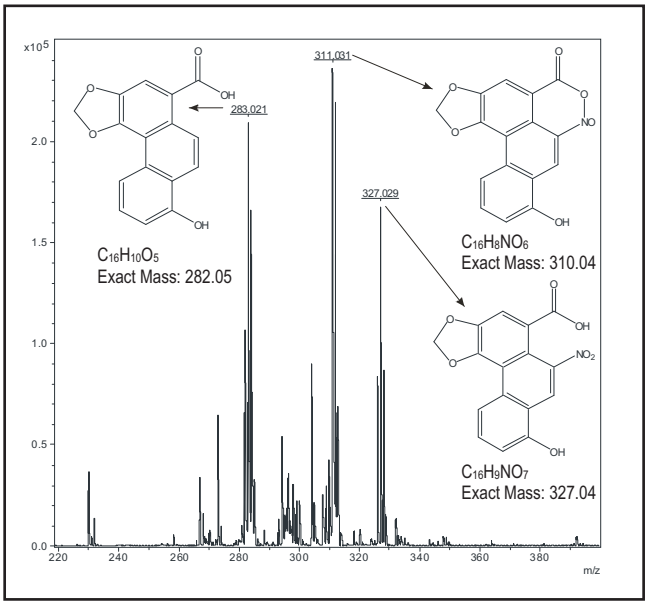

Fig. 3. Identification of AAI metabolite as AAIa by positive MALDI-TOF/TOF

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.

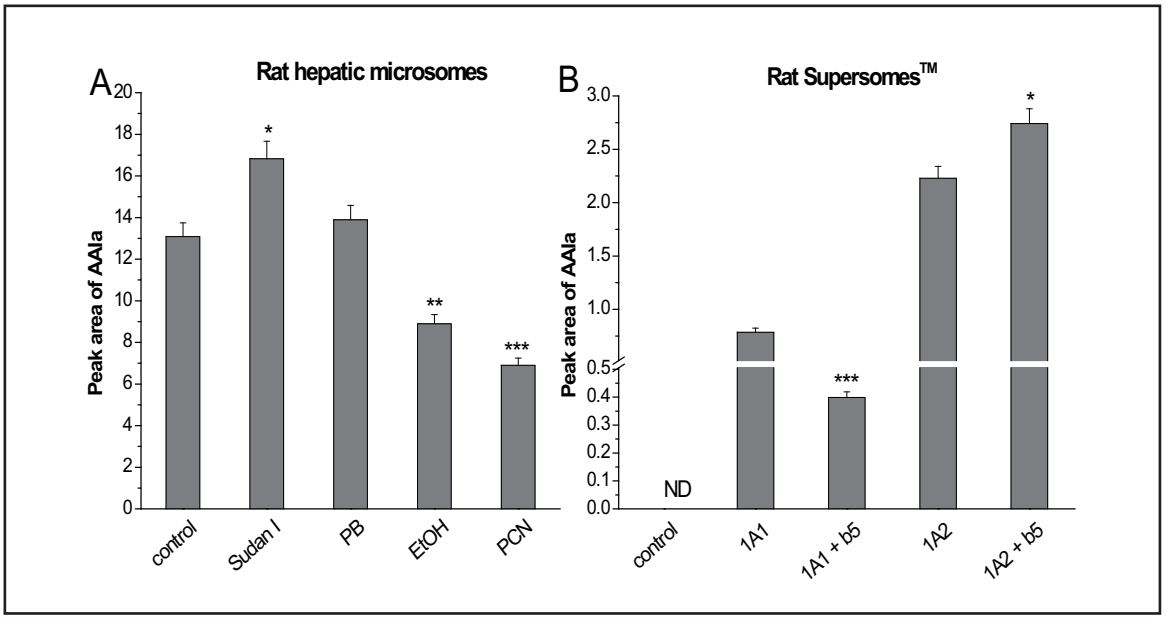

Fig. 4. Oxidation of AAI by rat hepatic microsomes (A) or rat recombinant CYP1A1 and 1A2 (B). Values are given as means ± standard deviations (n = 3). Values significantly different from hepatic microsomes of control (untreated) rats or from CYP1A1 or 1A2 without cytochrome b_5: *$p<0.05$, **$p<0.01$, ***$p<0.001$ (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.

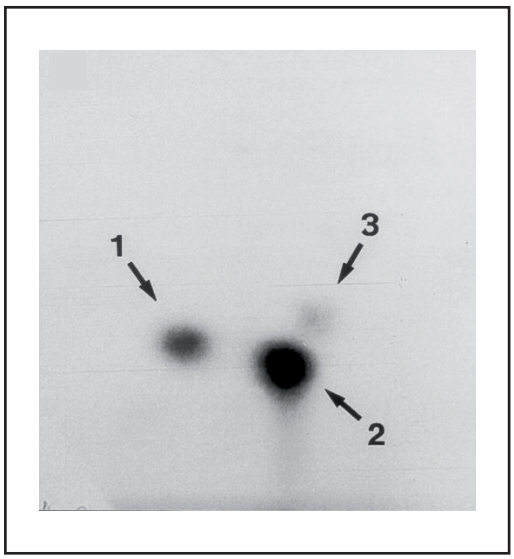

Fig. 5. Autoradiographic profile of AA-DNA adducts formed by incubation of AAI with rat recombinant CYP1A2 by using the nuclease P1 enrichment version of the ^{32}P-postlabeling assay. The origin, in the bottom left-hand corner, was cut off before exposure. Spot 1, dG-AAI; spot 2, dA-AAI; spot 3, 7-(deoxyadenosin-N^6-yl)aristolactam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

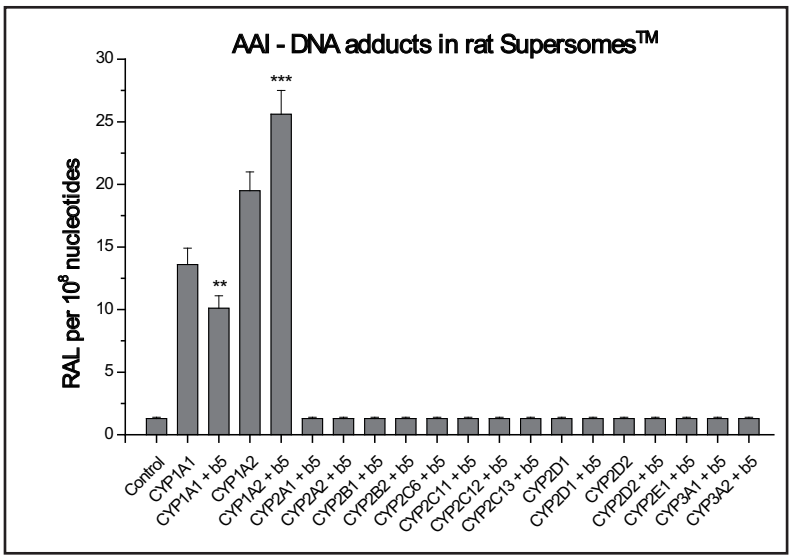

Fig. 6. DNA binding of AAI after activation with Supersomes™ containing different rat recombinant CYPs (50 pmol in a final volume of 750 μl incubations) and NADPH:CYP reductase or NADPH:CYP reductase alone (control). The nuclease P1-enrichment procedure of the ^{32}P-postlabeling assay was used for analysis. 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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