Mutagenesis vol. 29 no. 3 pp. 189–200
Advance Access publication 5 March 2014
doi:10.1093/mutage/geu004

The influence of dicoumarol on the bioactivation of the carcinogen aristolochic acid I in rats

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Received on November 21, 2013; revised on January 13, 2014; accepted on January 17, 2014

Aristolochic acid I (AAI) is the major toxic component of the plant extract AA, which leads to the development of nephropathy and urothelial cancer in human. Individual susceptibility to AAI-induced disease might reflect variability in enzymes that metabolise AAI. *In vitro* NAD(P)H:quinone oxidoreductase (NQO1) is the most potent enzyme that activates AAI by catalyzing formation of AAI–DNA adducts, which are found in kidneys of patients exposed to AAI. Inhibition of renal NQO1 activity by dicoumarol has been shown in mice. Here, we studied the influence of dicoumarol on metabolic activation of AAI in Wistar rats *in vivo*. In contrast to previous *in vitro* findings, dicoumarol did not inhibit AAI–DNA adduct formation in rats. Compared with rats treated with AAI alone, 11- and 5.4-fold higher AAI–DNA adduct levels were detected in liver and kidney, respectively, of rats pretreated with dicoumarol prior to exposure to AAI. Cytosols and microsomes isolated from liver and kidney of these rats were analysed for activity and protein levels of enzymes known to be involved in AAI metabolism. The combination of dicoumarol with AAI induced NQO1 protein level and activity in both organs. This was paralleled by an increase in AAI–DNA adduct levels found in *ex vivo* incubations with cytosols from rats pretreated with dicoumarol compared to cytosols from untreated rats. Microsomal *ex vivo* incubations showed a lower AAI detoxication to its oxidative metabolite, 8-hydroxyaristolochic acid (AAIa), although cytochrome P450 (CYP) 1A was practically unchanged. Because of these unexpected results, we examined CYP2C activity in microsomes and found that treatment of rats with dicoumarol alone and in combination with AAI inhibited CYP2C6/11 in liver. Therefore, these results indicate that CYP2C enzymes might contribute to AAI detoxication.

Introduction

The herbal drug aristolochic acid (AA) derived from *Aristolochia* species is considered to be the cause of aristolochic acid nephropathy (AAN) (1–4), which is a rapidly progressive renal fibrosis, with a high risk for the patients of developing upper urothelial tract carcinoma and, subsequently, bladder urothelial carcinoma (4,5). Exposure to AA has also been linked to inhabitants of rural areas in the Balkans who develop nephropathy—Balkan endemic nephropathy (BEN) (4,7–9). Exposure of experimental animals to AA leads to characteristic AA–DNA adducts in renal tissue after reductive activation. The same DNA adducts, mainly 7-(deoxyadenosine-N^6-yl) aristolactam I (dA-AAI) (Figure 1), were detected in kidneys of AAN and BEN patients whereby their exposure to AA was identified (5,6,8,10–12). This deoxyadenosine adduct causes characteristic AT→TA transversions in critical genes of oncogenesis (e.g. *TP53* tumour suppressor gene) and such AT→TA mutations have indeed been found in urothelial tumours in AA-exposed humans (8,9,13–16), indicating a molecular mechanism associated with AA-induced carcinogenesis (7,17). AA has been classified as a Group I carcinogen by the International Agency for Research on Cancer.

AAI is activated by nitroreduction by both cytosolic and microsomal enzymes. Of the enzymes characterised so far, cytosolic NAD(P)H:quinone oxidoreductase (NQO1) was found to be the most active enzyme both in experimental animals and in human tissue (18–22) (Figure 1). In microsomes from human liver, cytochrome P450 (CYP) 1A2 is the most active reductase followed by CYP1A1 and NADPH:CYP reductase (POR) (23–26). However, the two isoenzymes of the CYP1A family are mainly responsible for the oxidative demethylation of AAI to 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIa; Figure 1) (24–27).

Previous studies with genetically engineered mice such as *H*epatic P450 *R*eductase *N*ull mice, which lack hepatic POR and therefore essentially all CYP activity in hepatocytes, with *Cyp1a1(−/−)*, *Cyp1a2(−/−)* and *Cyp1a1/1a2(−/−)* knockouts, as well as with *CYP1A*-humanised mice demonstrated that the balance between reductive activation and oxidative detoxication of AAI depends on the expression of both CYP1A1/1A2 and NQO1 (24–26,28). The knockout of enzymes, which mainly detoxicate AAI, was not the only reason for the observed higher DNA adduct levels, but a concomitant 3-fold increase in NQO1 protein levels in the livers of these mice was also important (26). Cytosolic fractions isolated from *H*epatic P450 *R*eductase *N*ull mice activated AAI more efficiently to DNA adducts than hepatic cytosols of wild-type mice. These findings emphasised the importance of NQO1 for the activation of AAI not only *in vitro* but also *in vivo*. AAI was also found to induce NQO1 protein levels and its enzyme activity in liver, kidney and lung of mice (26,28,29) and of Wistar rats treated with this compound (19). Again, higher NQO1 enzyme activity was associated with increased AAI–DNA adduct formation in *ex vivo* incubations of cytosols with AAI and DNA (26,28). Hence, AAI induces NQO1 and thereby its own metabolic activation leading to increased genotoxicity *in vivo*.

A role of NQO1 in renal AAI nitroreduction *in vivo* was proven in mice (male C57BL/6 mice) (22) in which the modulation of AAI metabolism by dicoumarol, an inhibitor of NQO1,

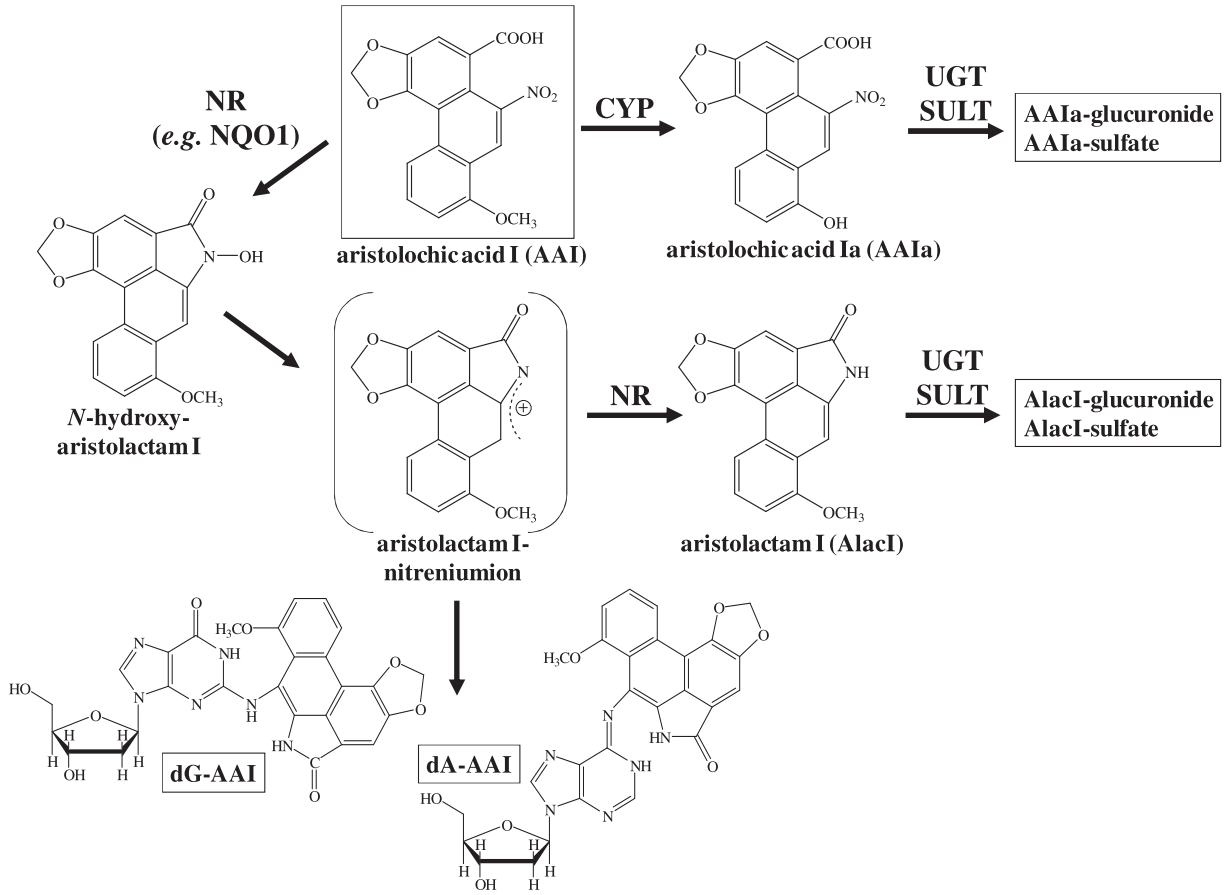

Fig. 1. Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosine-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosine-N^2-yl) aristolactam I; NR, nitroreduction; UGT, uridine diphosphate-β-D-glucuronosyltransferase; SULT, sulphotransferase.

was investigated. NQO1 activity was inhibited in dicoumarol-pretreated mice resulting in decreased levels of the reductive metabolite aristolactam I in kidney and increased amounts of AAI as well as AAIa in serum of AAI-exposed mice.

In this study, we investigated whether dicoumarol influences the genotoxicity of AAI in rats *in vivo*. In addition, the effect of this NQO1 inhibitor alone or in combination with AAI on enzymes metabolizing AAI (NQO1 and CYP1A1/2) was investigated. DNA adduct formation by AAI was evaluated by the ^{32}P-postlabelling method *in vivo* and in *ex vivo* incubations using cytosols and microsomes isolated from kidneys and livers. Further, the AAI metabolite AAIa in these *ex vivo* incubations was measured by high-pressure liquid chromatography (HPLC).

Materials and methods

Chemicals

NADPH, AAI (sodium salt), dicoumarol, Sudan I [1-(phenylazo)-2-hydroxynaphthalene], menadione (2-methyl-1,4-naphthoquinone) and calf thymus DNA were from Sigma Chemical Co (St Louis, MO, USA). 7-Ethoxyresorufin and 7-methoxyresorufin were from Fluka Chemie AG (Buchs, Switzerland). Enzymes and chemicals for the ^{32}P-postlabelling assay were from sources described (23).

Animal experiments and sample preparation

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Animals were acclimatised for 5 days and maintained at 22°C with a 12-h light/dark period. Standardised diet and water were provided *ad libitum*. Groups of 5-weeks-old-male Wistar rats (~150 g, n = 3 rats/group) were treated with dicoumarol, dissolved in sunflower oil. Dicoumarol was administered by gavage to rats twice at either 30 or 60 mg/kg body weight (bw), once at 3 p.m. and again the next day at 8 a.m. (total doses were 60 or 120 mg dicoumarol/kg bw). Another group of rats was injected intraperitoneally (i.p.) with a single dose of AAI dissolved in 1% $NaHCO_3$ (20 mg/kg bw). In the study on the effect of dicoumarol on AAI-mediated DNA adduct formation, a dose of 20 mg/kg bw of AAI was given by a single i.p. injection 2.5 h after the second dose of dicoumarol. Animals were killed 24 h after AAI treatment. Animals in the control groups received the vehicle only. Livers and kidneys were removed after killing, frozen in liquid nitrogen and stored at −80°C until analysis. DNA from livers and kidneys was isolated by extraction with phenol/chloroform (10). Microsomes and cytosols were isolated from the rat tissues by the procedure described previously (18,23). Protein concentration in the microsomal fraction was measured using bicinchoninic acid protein assay (30) with bovine serum albumin as a standard. Pooled microsomal and cytosolic samples (n = 3 rats/group) were used for the analyses.

DNA adduct analysis by ^{32}P-postlabelling

The nuclease P1 enrichment version of ^{32}P-postlabelling analysis and thin-layer chromatography on polyethyleneimine cellulose plates were carried out and DNA adduct levels (RAL, relative adduct labelling) were calculated as described previously (10,31). AA–DNA adducts were identified using reference standards as described (10).

Preparation of antibodies and estimation of CYP1A1, 1A2 and NQO1 protein content in microsomal and cytosolic fractions isolated from rat liver and kidney

The chicken anti-rat CYP1A1, anti-rabbit CYP1A2 and anti-rat NQO1 antibodies were prepared as described previously (32,33). Immunoquantification of microsomal CYP1A1 and 1A2 and cytosolic NQO1 was performed using western blotting (33). Rat CYP1A1, rabbit CYP1A2 and human NQO1 (Sigma) were used to identify the CYP1A1, 1A2 and NQO1 bands, respectively. The antigen–antibody complex was visualised with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/

nitroblue tetrazolium as dye and bands are expressed as arbitrary units (AU)/mg protein (32,33). Glyceraldehyde phosphate dehydrogenase was used as loading control and detected by its antibody (1:750; Millipore, Billerica, MA, USA).

NQO1, CYP1A1/2 and 2C6/11 enzyme activity assays

In hepatic and renal cytosols, NQO1 activity was measured using menadione (2-methyl-1,4-naphthoquinone) as a substrate; the assay was improved by the addition of cytochrome *c* and NQO1 activity expressed as nanomolar cytochrome *c* reduced (26,28). Microsomal samples were characterised for specific CYP1A1 and 1A2 activities: ethoxyresorufin *O*-deethylation (EROD; CYP1A1/2) and methoxyresorufin *O*-demethylation (MROD; CYP1A2) (34). CYP1A1 enzyme activity was also monitored by Sudan I hydroxylation to 4′-hydroxy-, 6-hydroxy- and 4′,6-dihydroxy-Sudan I (22). Hepatic microsomal samples were also characterised for specific CYP2C6 and 2C11 activities with their marker substrates determining diclofenac 4′-hydroxylation and testosterone 16α-hydroxylation, respectively (35,36). In hepatic microsomes, POR activity was analysed using cytochrome *c* as a substrate (24).

Cytosolic and microsomal formation of AAI–DNA adducts

The de-aerated and nitrogen-purged incubation mixtures, in which cytosols were used to activate AAI, contained 50 mM Tris–HCl buffer (pH 7.4), 0.2% Tween 20, 1 mM NADPH, 1 mg rat hepatic or renal cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI in a final volume of 750 µl. Incubations with cytosols were performed at 37°C for 60 min; AAI-derived DNA adduct formation was found to be linear up to 2 h (18). Control incubations were performed either (i) without cytosol, (ii) without NADPH, (iii) without DNA or (iv) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (20,23,25).

The de-aerated and nitrogen-purged incubation mixtures, in which microsomes were used to activate AAI, contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg of hepatic or renal microsomal protein, 0.5 mg of calf thymus DNA (2 mM dNp) and 0.5 mM AAI in a final volume of 750 µl. Microsomal incubations were carried out at 37°C for 60 min; AAI–DNA adduct formation was found to be linear up to 2 h in microsomes (23). Control incubations were carried out either (i) without microsomes, (ii) without NADPH, (iii) without DNA or (iv) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase as described previously.

Microsomal incubations to study AAI demethylation

Incubation mixtures contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg rat hepatic or renal microsomal protein and 10 µM AAI in a final volume of 250 µl and were incubated at 37°C for 20 min; AAI *O*-demethylation to AAIa was determined to be linear up to 25 min. Control incubations were carried out either (i) without microsomes, (ii) without NADPH or (iii) without AAI. AAI and its metabolite AAIa were separated by reverse-phase HPLC, identified by mass spectrometry and quantified as described previously (26). Briefly, HPLC was carried out with an Nucleosil 100–5 C_{18}, 25 × 4.0 mm, 5 mm (Macherey-Nagel) column, using a linear gradient of acetonitrile (20–60% acetonitrile in 55 min) in 100 mM triethylammonium acetate with a flow rate of 0.6 ml/min. A Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector set at 254 nm was used. Peaks were integrated with CHROMELEON™ 6.01 integrator. A peak eluting at retention time 22.7 min was identified as AAIa using mass spectroscopy analysis (26). A typical HPLC chromatogram is shown as a supplementary Figure 1, available at *Mutagenesis* Online.

Statistical analyses

For statistical data analysis, we used Student's *t*-test. All *P*-values are two-tailed and considered significant at the 0.05 level.

Results

DNA adduct formation in rats treated with dicoumarol and AAI compared to adduct formation in rats treated with AAI alone

AAI–DNA adduct formation was determined by ^{32}P-postlabelling in liver and kidney of rats treated with a single i.p. dose of 20 mg/kg bw of AAI. Similarly, rats pretreated orally with total doses of 60 and 120 mg/kg bw of the NQO1 inhibitor dicoumarol prior to AAI injection were analysed. AAI–DNA adduct patterns in both organs were similar to those found *in vivo* in humans and consisted of two major adducts (spots 1 and 2) and one minor adduct (spot 3; see Figure 2, insert) (1,5,10,13). These adducts have been identified to be dA-AAI (spot 1), 7-(deoxyguanosine-N^2-yl)aristolactam I (dG-AAI; spot 2) and dA-AAII (spot 3). No adducts were found in DNA of control rats treated with vehicle only or in those treated with dicoumarol alone (data not shown).

In all rats, the levels of AAI–DNA adducts were higher in liver than in kidney, the target organ of AAI genotoxicity (Figure 2). Interestingly, in contrast to the strong inhibition by dicoumarol observed *in vitro* (18,26), the opposite effect upon AAI–DNA adduct formation was found *in vivo*. In both organs analysed, AAI–DNA adduct levels increased with the dicoumarol dose used in the pretreatment. Compared to adduct levels found in rats treated with AAI alone, DNA binding was 11- and 5.4-fold higher in liver and kidney, respectively, of rats pretreated with a total dose of 120 mg/kg bw of dicoumarol prior to exposure to AAI ($P < 0.001$). Therefore, dicoumarol, when administered to rats prior to AAI, seems to induce pathways activating AAI in both organs. As NQO1 and CYP1A1/2 might determine the AAI–DNA adduct levels, their protein levels and enzyme activities were investigated in the two rat organs.

The effect of dicoumarol treatment with or without AAI upon NQO1 and CYP1A1/2 protein levels and their enzymatic activities in rat liver and kidney

Treatment of rats with AAI led to a significant NQO1 protein induction in kidney cytosol (1.4-fold, $P < 0.05$), but not in liver.

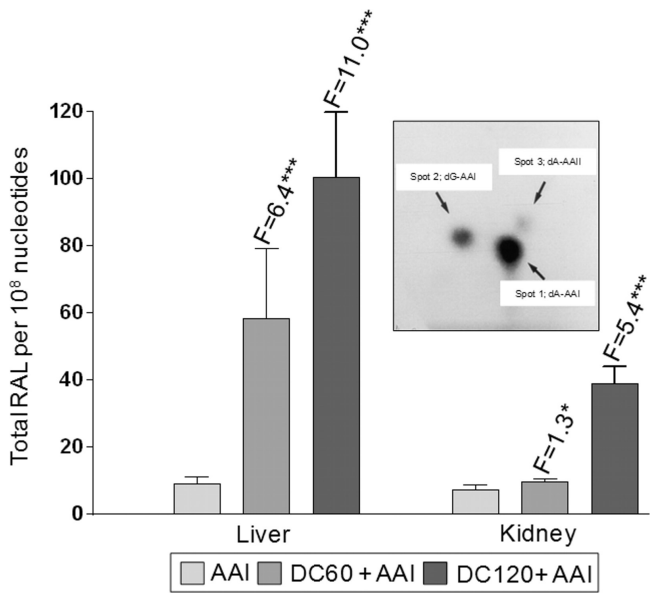

Fig. 2. Quantitative thin-layer chromatography ^{32}P-postlabelling analysis of AAI–DNA adduct levels in organs of rats treated i.p. with a single dose of 20 mg/kg bw AAI with or without the pretreatment with dicoumarol [total doses of 60 (DC60) or 120 mg (DC120) dicoumarol/kg bw; see Materials and methods for details]. Numbers above columns ('F') indicate fold changes in DNA adduct levels in AAI-treated animals pretreated with dicoumarol compared to animals treated with AAI alone. Values are given as the means ± SD ($n = 3$); each DNA sample was determined by two postlabelled analyses. RAL, relative adduct labelling. Comparison was performed by *t*-test analysis; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, different from animals treated with AAI alone. Insert: autoradiographic profile of AA–DNA adducts in rat liver using the nuclease P1 enrichment version of the assay. The adduct profile shown is representative of those obtained in other organs investigated. 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.

Likewise, a significantly higher NQO1 enzyme activity was found only in kidney of AAI-treated rats (1.5-fold, $P < 0.001$). Whereas administration of dicoumarol to rats decreased or did not change NQO1 protein levels in liver, levels increased in a dose-dependent manner in kidney. However, treatment of rats with AAI after pretreatment with dicoumarol resulted in increased NQO1 protein levels in both organs. NQO1 activities in liver cytosols were only significantly higher at the high dicoumarol dose in combination with AAI, whereas they were significantly higher in kidney cytosols from rats treated with AAI, dicoumarol alone or dicoumarol with AAI (Figure 3).

The effect of exposure to AAI and dicoumarol on the protein levels of microsomal CYP1A1/2 in rats was also examined as both CYP enzymes play a dual role in AAI metabolism. In control rats, higher protein levels of CYP1A1 were found in hepatic than in renal microsomes by western blotting (Figure 4A and B). These levels corresponded with the CYP1A enzyme marker activities; EROD for CYP1A1/2 (Figure 5C and D) and Sudan I oxidation for CYP1A1 (Figure 5G and H). As shown in Figures 4 and 5, dicoumarol treatment alone, and particularly in combination with AAI, resulted in lower hepatic CYP1A1 protein levels, but this was not reflected in decreased enzyme activity (i.e. EROD and Sudan I oxidation). In contrast, statistically significant ~3-fold higher CYP1A1 protein levels were found in kidneys of rats treated with dicoumarol relative to control rats. This was paralleled by Sudan I oxidation and this difference was even more pronounced when measuring EROD activities (Figures 4 and 5). AAI also induced renal CYP1A1 protein levels and activities, which was further enhanced in combination with the lower dicoumarol dose, but did not reach the levels induced by dicoumarol alone (Figures 4 and 5).

CYP1A2 was found to be expressed only in rat liver, and not in kidney (Figure 4D), confirming earlier studies showing that CYP1A2 is an almost exclusively hepatic enzyme (37). In concordance, MROD activity, a marker reaction of CYP1A2, was found in liver, but was negligible in kidney (Figure 5E and F). AAI and dicoumarol treatment alone as well as dicoumarol pretreatment prior to AAI administration resulted in elevated CYP1A2 protein levels and MROD activities in the liver (Figures 4C and 5E), except in the rats treated with 120 mg/kg bw of dicoumarol prior to AAI administration; CYP1A2 protein level remained, while MROD activity increased 2-fold. This suggests that the weak CYP1A2 signals detected by western blotting probably do not truly reflect protein levels and

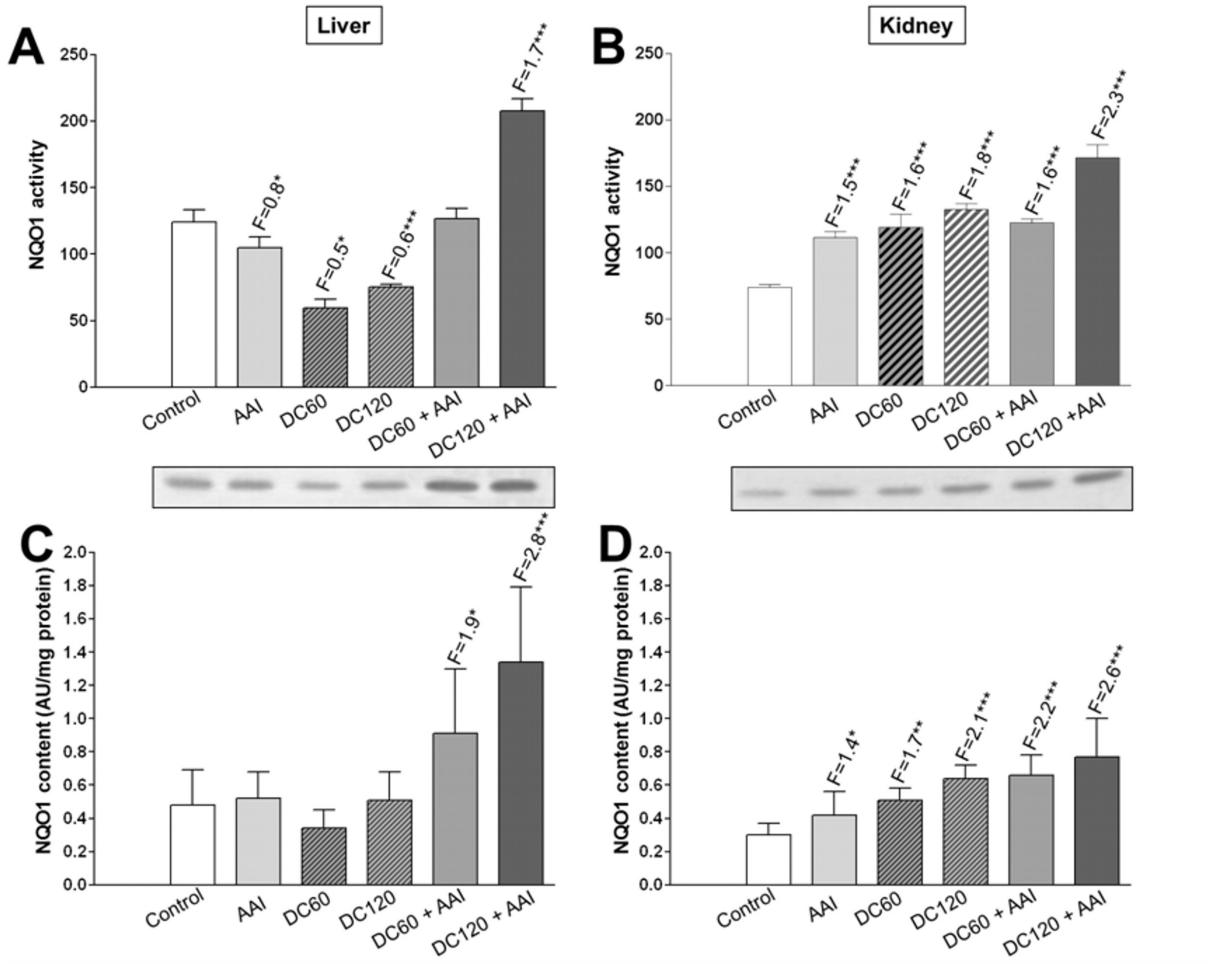

Fig. 3. NQO1 enzyme activity (**A** and **B**) and NQO1 protein levels (**C** and **D**) in rat cytosols isolated from untreated (control) animals and animals pretreated orally with dicoumarol [total doses of 60 (DC60) or 120 mg (DC120) dicoumarol/kg bw] alone or in combination with AAI (single i.p. dose of 20 mg/kg bw). NQO1 activity in hepatic (A) and renal cytosols (B) was determined using menadione and cytochrome *c* as substrate (expressed as nanomoles cytochrome *c* per minute per milligram protein). Cytosol isolated from liver (C) or kidney (D) was analysed by western blotting in the same blot (insert) and, therefore, can be compared directly. Human recombinant NQO1 (Sigma) was used to identify the rat NQO1 band in rat cytosol (data not shown). Numbers above columns ('F') indicate fold changes in protein level or enzyme activity compared to control. Values are given as the means ± SD ($n = 3$). Comparison was performed by *t*-test analysis; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from control.

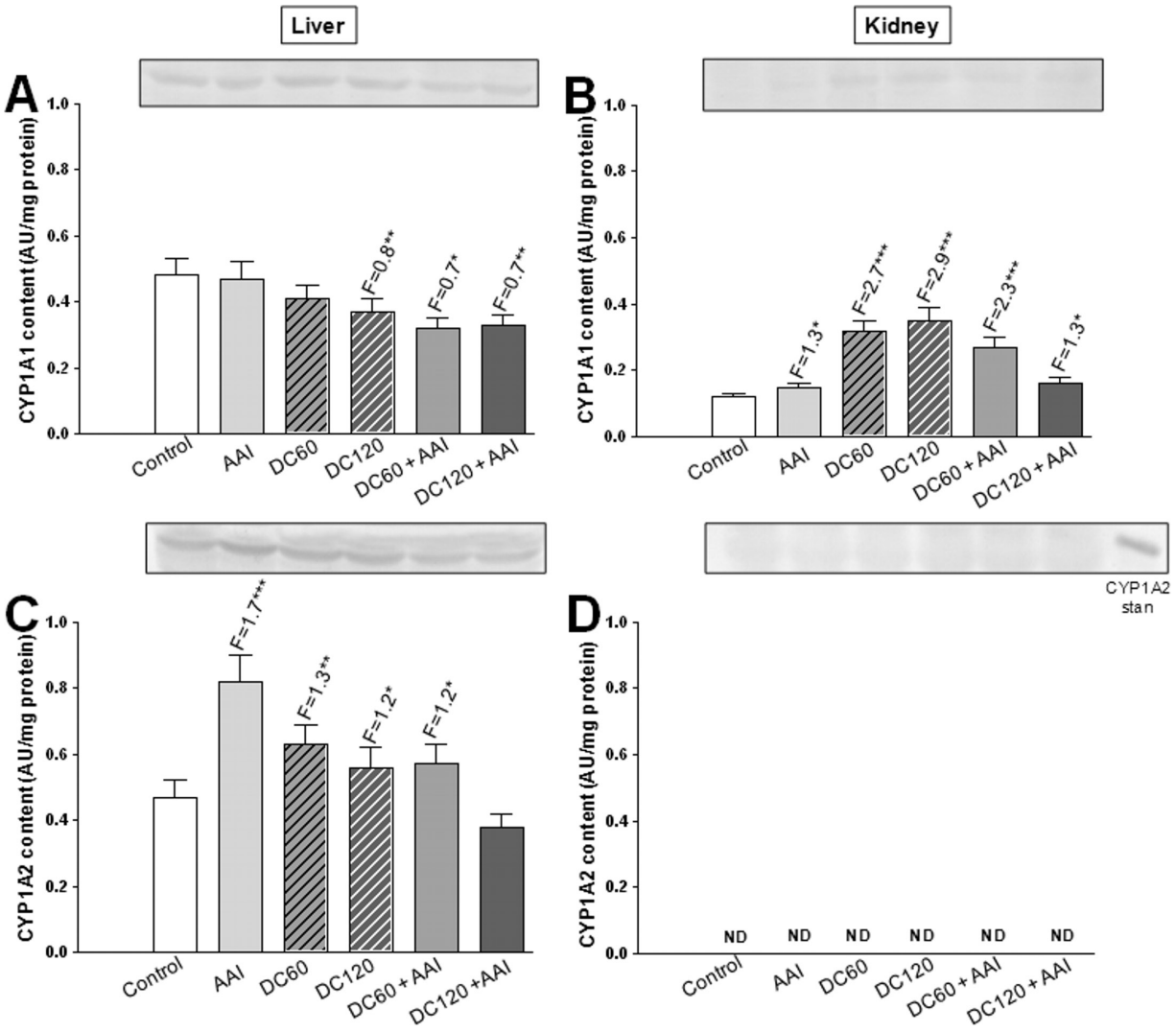

Fig. 4. CYP1A1 (**A** and **B**) and CYP1A2 (**C** and **D**) protein levels in rat microsomes isolated from untreated (control) animals and animals pretreated orally with dicoumarol [total doses of 60 (DC60) or 120 mg (DC120) dicoumarol/kg bw] alone or in combination with AAI (single i.p. dose of 20 mg/kg bw). Microsomes isolated from liver (A and C) and kidney (B and D) were analysed by western blotting in the same blot (insert) and therefore can be directly compared. Rat recombinant CYP1A2 was used to identify the rat CYP1A2 band (see panel D). Values are given as the means of arbitrary units (AU) ± SD ($n = 3$). Numbers above columns ('F') indicate fold changes in protein level or enzyme activity compared to control. ND, not detected. Comparison was performed by *t*-test analysis; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from control.

determination of enzyme activity provides a more accurate assessment of enzyme induction.

The activity of POR was increased in rat kidney by AAI or dicoumarol treatment alone as well as in combined administration, but remained essentially unchanged in liver (Figure 6). POR not only acts as an electron donor in catalytic functions of CYPs (38,39), but is also able to activate AAI to some extent (23).

Cytosolic versus microsomal activation of AAI

In further experiments, AAI–DNA adduct formation catalysed by cytosols isolated from liver and kidney of rats from all treatment groups was investigated *ex vivo*. Cytosols were incubated with AAI, DNA and the cofactor of NQO1, NADPH, and analysed for DNA adduct formation by ^{32}P-postlabelling. AAI was activated by the cytosols from both organs as evidenced by AAI–DNA adduct formation (Figure 7A and B) and the DNA adduct pattern was the same as those found *in vivo* (see Figure 2, insert). No adducts were observed in control incubations carried out in parallel (data not shown). In kidney, cytosols from all modes of treatment AAI–DNA adduct levels increased up to 3.3-fold relative to cytosols isolated from untreated animals (controls). Renal cytosols isolated from AAI-treated rats led to 2.5-fold higher adduct levels than cytosol from control animals ($P < 0.001$; Figure 7B) and corresponded to higher NQO1 protein levels in these cytosolic samples (compare Figures 3 and 7). Despite a lack of significant NQO1 induction at either protein or activity levels by AAI, DNA adduct levels catalysed by rat hepatic cytosols were 1.7-fold higher than in controls (Figure 3). These results indicate that other cytosolic nitroreductases may contribute to the increased AAI–DNA adduct formation observed in rat liver cytosol. In this context, it is noteworthy that previous studies have shown that xanthine oxidase is capable of activating AAI (18,19).

Consistent with the lower NQO1 protein and activity levels elicited by dicoumarol in liver (see Figure 3A and C), AAI–DNA adduct formation was lower in hepatic cytosol isolated from rats treated with 60 mg/kg bw of dicoumarol. However, when rats

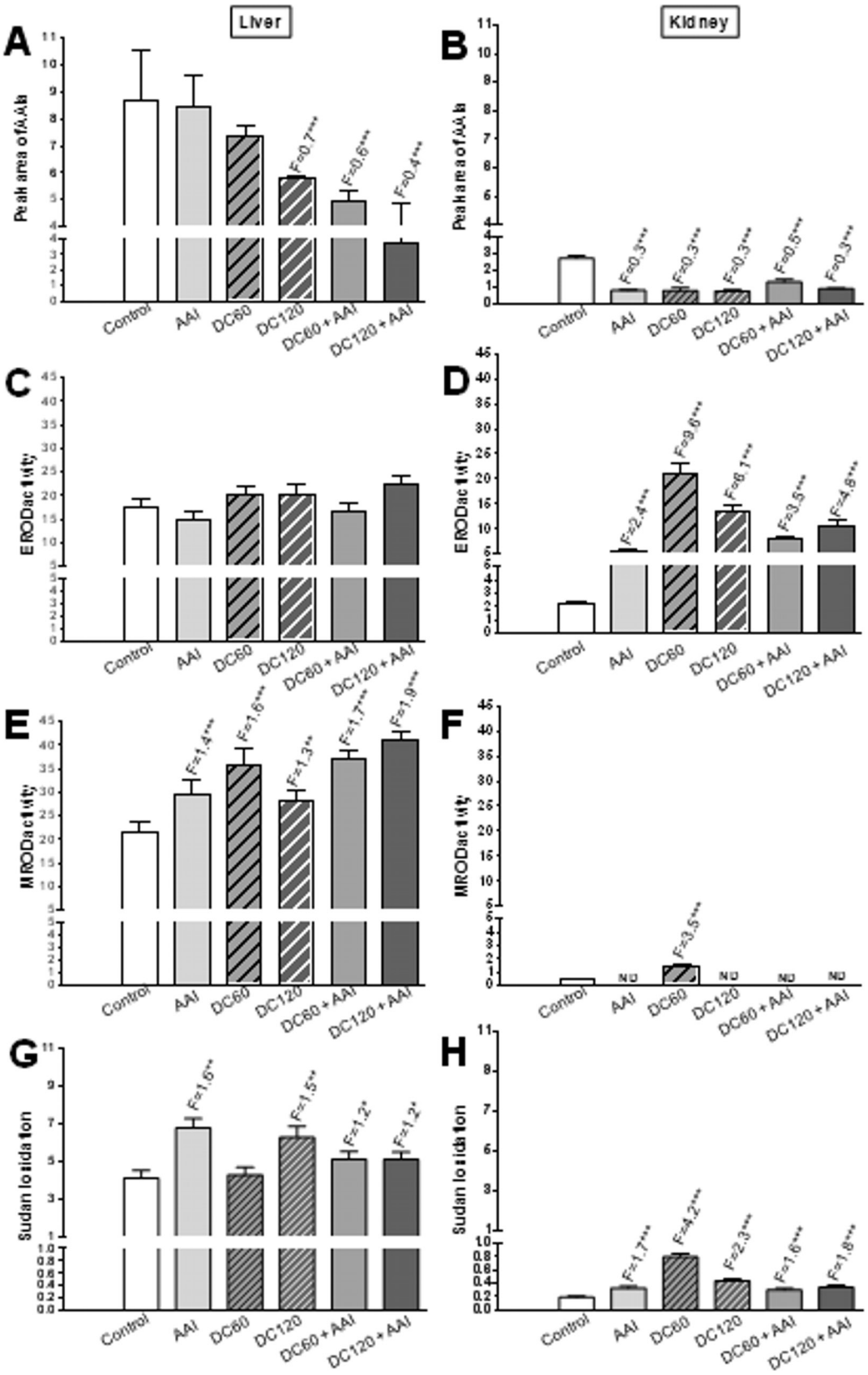

Fig. 5. AAI oxidation to AAIa and CYP1A enzyme activities in rat microsomes isolated from untreated (control) animals and animals pretreated orally with dicoumarol [total doses of 60 (DC60) or 120 mg (DC120) dicoumarol/kg bw] alone or in combination with AAI (single i.p. dose of 20 mg/kg bw). Formation of AAIa (peak area per minute per milligram protein) from AAI in hepatic (**A**) and renal (**B**) microsomes. CYP1A enzyme activity as measured by EROD activity (picomoles resorufin per minute per milligram protein; **C** and **D**), MROD activity (picomoles resorufin per minute per milligram protein; **E** and **F**) or Sudan I oxidation (nanomoles total C-hydroxylated metabolites per minute per milligram protein; **G** and **H**). All values are given as the means ± SD ($n = 3$). Numbers above columns ('F') indicate fold changes in AAIa levels or enzyme activities compared to control. ND, not detected. Comparison was performed by *t*-test analysis; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from control.

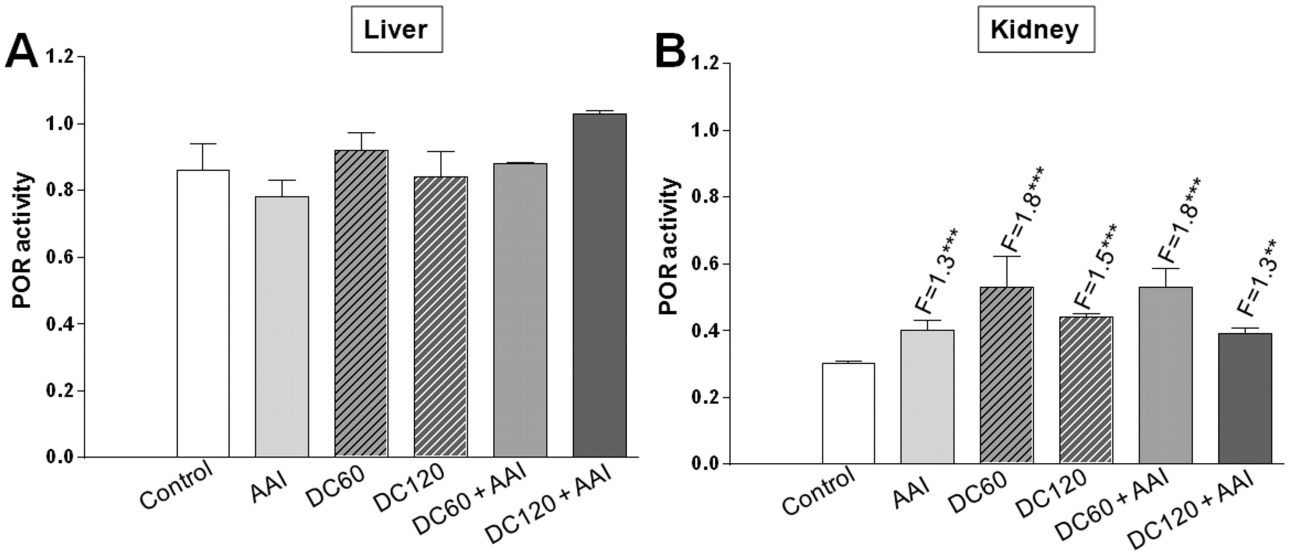

Fig. 6. POR enzymatic activity in rat microsomes isolated from liver (**A**) and kidney (**B**) of untreated (control) animals and animals pretreated orally with dicoumarol [total doses of 60 (DC60) or 120 mg (DC120) dicoumarol/kg bw] alone or in combination with AAI (single i.p. dose of 20 mg/kg bw). POR was measured using cytochrome *c* as substrate (nanomoles cytochrome *c* per minute per milligram protein). All values are given as the means ± SD ($n = 3$). Numbers above columns ('F') indicate changes in enzyme activity compared to control. Comparison was performed by *t*-test analysis; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from control.

were treated with dicoumarol prior to AAI administration, AAI–DNA adduct levels increased up to 2.6-fold relative to hepatic cytosolic incubations from control rats (Figure 7A). In kidney, cytosols from all modes of treatment AAI–DNA adduct formation was up to 3.3-fold higher relative to controls.

Because microsomal CYP1A1/2 are also able to activate AAI by nitroreduction to species forming DNA adducts, hepatic and renal microsomes of control and treated rats were analysed for their capacity to form AAI–DNA adducts in *ex vivo* incubations. AAI was reductively activated by hepatic and renal microsomes from all rats (Figure 7C and D). The DNA adduct pattern generated was the same as that found *in vivo* (see Figure 2, insert). No adducts were observed in control incubations carried out in parallel (data not shown). The only significant increase in AAI–DNA adduct levels catalysed by hepatic microsomes was seen in the two groups exposed to 60 mg/kg bw dicoumarol (Figure 7C). AAI–DNA adduct formation was up to 2.7-fold higher in kidney microsomes from all treatment groups relative to controls; except by those isolated from rats pretreated with 120 mg/kg bw dicoumarol prior to AAI administration (Figure 7D). The reason for such a finding remains to be explained.

The effect of treatment of rats with AAI and dicoumarol as well as pretreatment with dicoumarol prior to AAI administration on oxidation of AAI to AAIa by rat hepatic and renal microsomes

As microsomal CYP1A1/2 also detoxify AAI to its *O*-demethylated metabolite AAIa (23–25,40), AAIa formation by hepatic and renal microsomes was investigated *ex vivo*. Under the conditions used, liver and kidney microsomes oxidised AAI to AAIa (Figure 5A and B). A dose-dependent decrease in AAI oxidation to AAIa in hepatic and renal microsomes (up to ~40% relative to controls) was caused by treatment of rats with dicoumarol or pretreatment of rats with dicoumarol prior to AAI administration. However, this decrease did not correspond to CYP1A enzyme activities shown in Figure 5C and D. In order to explain this discrepancy, we further investigated the effect of dicoumarol on the oxidation of AAI to AAIa catalysed by rat hepatic microsomes. We found that AAIa formation was strongly inhibited by dicoumarol (with an IC_{50} value of 2 μM), but CYP1A enzyme activity as measured by EROD, MROD and Sudan I oxidation did not change (data not shown). Collectively, these results suggest that not only CYP1A1/2 but also other CYPs might contribute to the oxidation of AAI to AAIa in rat hepatic microsomes and that these enzymes are modified by dicoumarol. We hypothesised that CYPs of the 2C subfamily might be candidates as they can also oxidise AAI to AAIa to some extent (26) and because CYP2C enzymes are highly expressed in the livers of male rats accounting for ~55% of the rat liver CYP complement (41). Among them, CYP2C11 and 2C6 contribute ~50% and 20% to the total hepatic CYP2C content in rats, respectively (42,43), and both CYP2C isoenzymes have previously been shown to be able to *O*-demethylate AAI to AAIa (26). To test this hypothesis, the effect of dicoumarol treatment *in vivo* upon CYP2C6 and 2C11 activities in hepatic microsomes was analysed (Figure 8); diclofenac 4′-hydroxylation and testosterone 16α-hydroxylation were used as marker activities for CYP2C6 and 2C11, respectively (35,36). As shown in Figure 8, exposure of rats to dicoumarol, either with or without AAI, decreased testosterone 16α-hydroxylation activities up to 60% relative to control; diclofenac 4′-hydroxylation was reduced up to 20%. Therefore, the lower CYP2C enzyme activities could explain the lower oxidation rates of AAI to AAIa in these microsomes and indicate that CYP2C11 and 2C6 can contribute to AAIa formation in rat liver.

Discussion

Previously, we have demonstrated that NQO1 expressed in human, rat or mouse liver and kidney as well as purified human and rat NQO1 is the predominant enzyme responsible for the genotoxicity of AAI *in vitro* (18–21). In addition, other studies suggested that NQO1 might also contribute to AAI–DNA

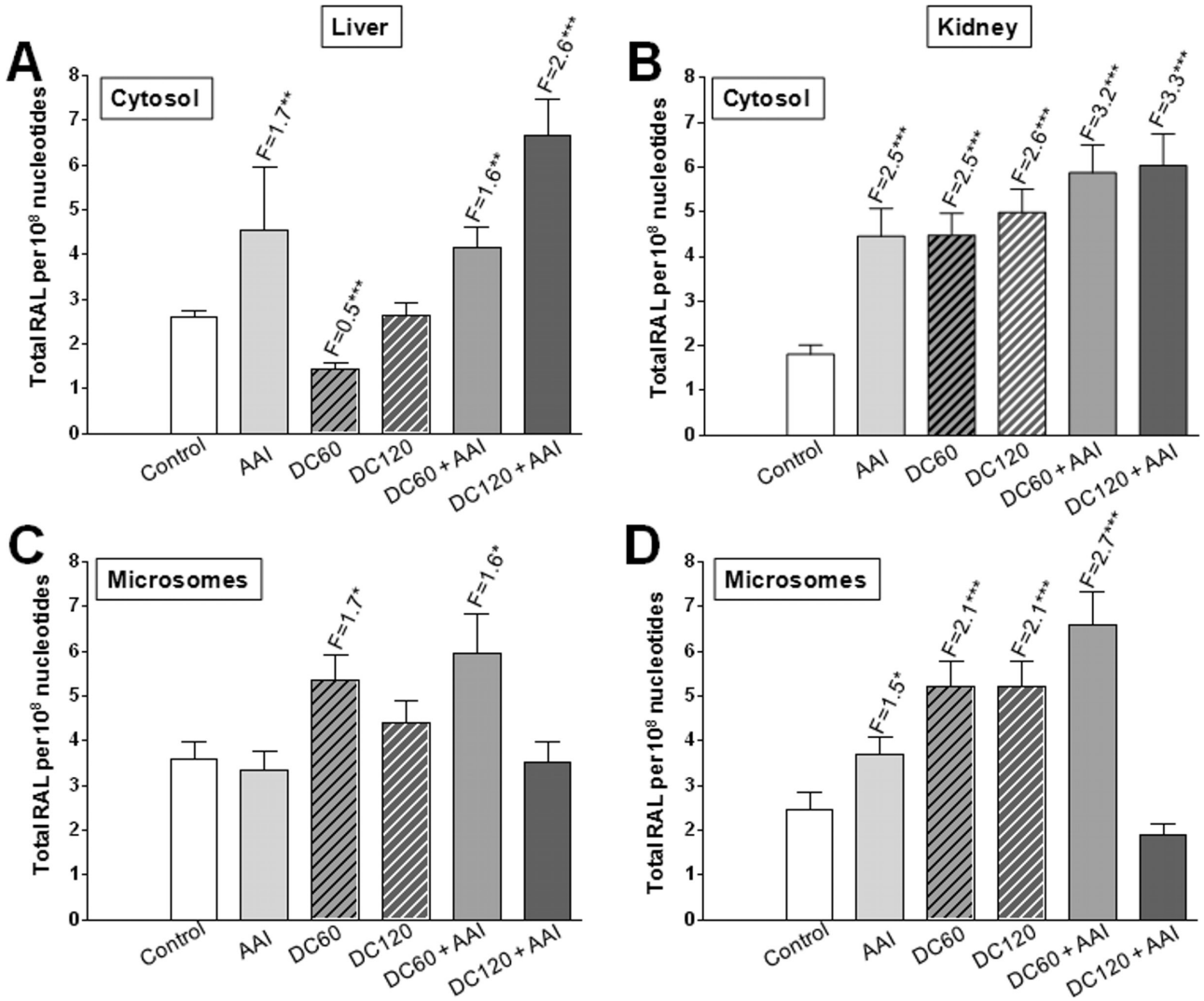

Fig. 7. DNA adduct formation *ex vivo* by AAI in rat cytosols (**A** and **B**) and microsomes (**C** and **D**) isolated from untreated (control) animals and animals pretreated orally with dicoumarol [total doses of 60 (DC60) or 120 mg (DC120) dicoumarol/kg bw] alone or in combination with AAI (single i.p. dose of 20 mg/kg bw). AAI–DNA adduct formation was determined by ^{32}P-postlabelling in hepatic (A and C) and renal (B and D) fractions. Values are given as the means ± SD ($n = 3$); each DNA sample was determined by two postlabelling analyses. RAL, relative adduct labelling. Numbers above columns ('F') indicate fold changes in DNA adduct levels compared to control. Comparison was performed by *t*-test analysis; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from control.

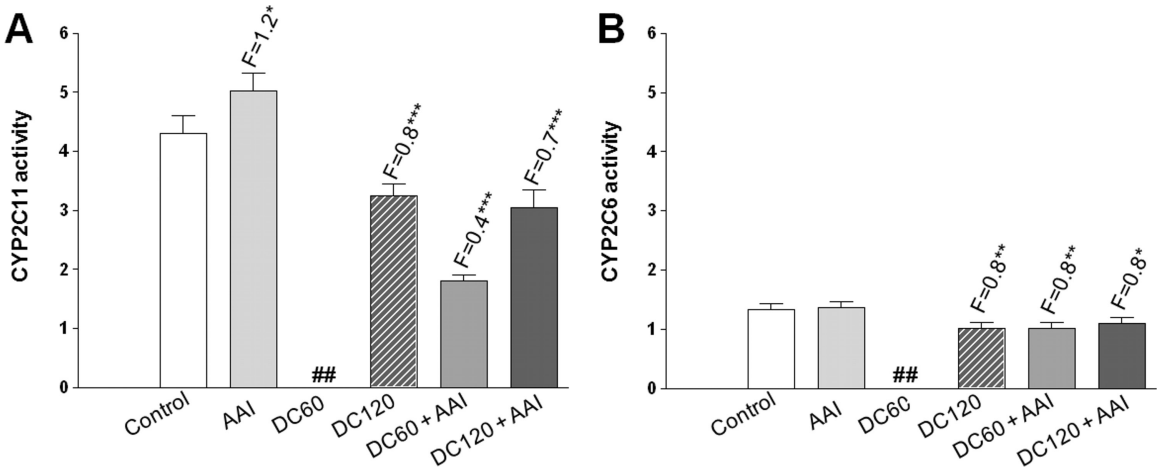

Fig. 8. CYP2C11 (**A**) and 2C6 enzyme activities (**B**) in rat hepatic microsomes isolated from untreated (control) animals and animals pretreated orally with dicoumarol (total doses of 60 [DC60] or 120 mg [DC120] dicoumarol/kg bw) alone or in combination with AAI (single i.p. dose of 20 mg/kg bw). CYP2C11 was measured as testosterone 16α-hydroxylation (nanomoles 16α-hydroxytestosterone per minute per milligram protein) and CYP2C6 as diclofenac 4′-hydroxylation (nanomoles 4′-hydroxydiclofenac per minute per milligram protein). All values are given as the means ± SD ($n = 3$). ##, sample lost. Numbers above columns ('F') indicate fold changes in enzyme activities compared to control. Comparison was performed by *t*-test analysis; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from control.

adduct formation *in vivo* (19–21,24,26,28). In this study, we utilised dicoumarol, an inhibitor of NQO1, in a Wistar rat animal model and showed that the contribution of NQO1 to AAI activation is complex when comparing *in vitro* and *in vivo* findings. Bioavailability of AAI to different organs, species differences, length of exposure to dicoumarol and AAI, and the applied doses seem to be crucial parameters in the effect of this NQO1 inhibitor upon AAI genotoxicity.

Dicoumarol had previously been administered to male C57BL/6 mice to investigate the participation of NQO1 in AAI metabolism and AAI-induced nephrotoxicity (22). The authors found that NQO1 indeed plays a crucial role in (renal) AAI nitroreduction *in vivo*. They showed that NQO1 activity was inhibited in a concentration-dependent manner in mice treated with increasing doses of dicoumarol. A single i.p. injection of AAI 2.5 h after dicoumarol pretreatment resulted in decreased levels of its reductive metabolite aristolactam I in kidney. As a consequence, levels of AAI, and particularly AAIa, in serum and kidney were higher in dicoumarol-pretreated mice. Furthermore, pretreatment with this NQO1 inhibitor decreased AAI-induced nephrotoxicity and increased the survival rate of these mice (22). Collectively, these results suggested that dicoumarol acts as an inhibitor of AAI reduction in this mouse model.

The aim of this study was to evaluate the formation of AAI–DNA adducts in rats exposed to AAI in the presence of two concentrations of dicoumarol. We administered the higher doses of dicoumarol and AAI and the same dosing schedule as Chen *et al.* (22) that had been used for mice, and AAI–DNA adduct formation was monitored as the biological end-point of AAI genotoxicity. The resultant adduct levels obtained *in vivo* and in *ex vivo* incubations were used as a surrogate measure of the activity of enzymes involved in AAI bioactivation. Previous studies in rats have shown that dicoumarol is still present up to 10 h after oral administration and concentrations probably increasing in blood due to the slow uptake from the intestine (44). In addition, dicoumarol has a long elimination half-live ranging from 5 to 25 h in rats (45). Here, we found that AAI–DNA adduct levels in kidney, and particularly in liver, were higher in rats treated with dicoumarol prior to AAI administration (Figures 2 and 9). This finding is unexpected because NQO1, the target of dicoumarol inhibition, is the primary AAI activating enzyme in rats, mice and humans.

Hepatic cytosols from rats treated with dicoumarol showed the same NQO1 protein levels as those from untreated rats, whereas the enzyme activities were decreased ~2-fold. This may be due to dicoumarol still binding to NQO1 as dicoumarol has strong protein binding properties. It is expected that due to its long elimination half-life from blood, residual dicoumarol may remain in tissues, particularly in liver as it is the main site of metabolism (45). The decreased NQO1 enzyme activity resulted in lower DNA adduct formation in *ex vivo* incubation with AAI using hepatic cytosols. The situation in the kidney was more complex. Because the effect of residual dicoumarol upon enzyme activity *ex vivo* was not observed, overall dicoumarol concentrations might be lower in the kidney compared to liver (45). In kidney, dicoumarol induced NQO1 protein levels and also activity. Furthermore, in liver and kidney, NQO1 protein levels and activity were induced by treating rats with dicoumarol prior to AAI administration and this corresponded to higher levels of AAI–DNA adducts formed *in vivo* and in *ex vivo* incubations of AAI with cytosols of these organs.

Higher adduct levels found in liver and kidney of rats pretreated with dicoumarol prior to AAI administration might be, beside increased levels and activities of NQO1, also the result of higher protein levels and activities of CYP1A1 in kidney and CYP1A2 in liver. Namely, the two CYP1A enzymes can reductively activate AAI to DNA binding (24–26). However, an increase in the levels of AAI–DNA adducts formed in *ex vivo* incubations of AAI with hepatic and renal microsomes from rats treated with dicoumarol prior to AAI was lower than that generated in *ex vivo* incubations of AAI with cytosols from these rats (see Figure 7). The reductive activation of AAI by these two CYP1A enzymes might, therefore, be less important for an increase in AAI–DNA adduct levels in rat liver and kidney *in vivo* than induction of NQO1. Furthermore, oxidation of AAI by both CYPs was found to lead to AAIa formation (Figure 1) (24–26). In this study, the detoxication metabolite AAIa generated in *ex vivo* incubations was lower in microsomes isolated from either kidney or liver from rats treated with dicoumarol relative to controls. Here again as in the case of NQO1, we expect dicoumarol to still be present in hepatic microsomes (45). The low IC_{50} value of 2 μM dicoumarol determined for the inhibition of AAIa formation in hepatic microsomes shows that only small amounts of dicoumarol need to be bound to affect AAI demethylation. This is in strong contrast to the observation of increased AAIa serum levels in dicoumarol-pretreated mice administered AAI (22). This would point to another enzyme system or another organ demethylating AAI *in vivo*, at least in mice. As dicoumarol inhibition of AAIa formation in hepatic microsomes did not correlate with CYP1A1/2 activity, other CYPs were considered. We examined the specific activities of two members of the rat CYP2C subfamily also known to *O*-demethylate AAI to AAIa (26). Indeed both CYP2C11 and 2C6 enzyme activities were inhibited by dicoumarol; the same effect was also seen in combination with AAI treatment. Dicoumarol itself is mainly metabolised in the liver and earlier work showed that human CYP2C is responsible (46), although it is noteworthy that this finding has not been further investigated. The authors used antibodies generated against rat CYP2C7 and 2C11 and found that formation of 7-hydroxy-dicoumarol in human hepatic microsomes was inhibited by 50–60% (46). Collectively, these data suggest a competitive inhibition of CYP2C activity by residual dicoumarol in our *ex vivo* system. In addition, an inhibition of *O*-demethylation of AAI to AAIa was even higher when rats were treated with dicoumarol and AAI (see Figure 5A).

All these findings indicate that higher adduct levels found in the liver of rats pretreated with dicoumarol prior to AAI administration might be, beside induction of NQO1, also caused by decreased AAI detoxication to AAIa due to CYP2C inhibition by dicoumarol and AAI. Higher levels of AAI are available for its reductive activation to form AAI–DNA adducts (Figure 9). In kidney, induction of NQO1 with dicoumarol and AAI seems to be the predominant process responsible for higher levels of AAI–DNA adducts in this organ (Figure 9).

The inductive effect of AAI on NQO1 found in this work confirmed previous studies, where NQO1 protein levels and its enzyme activity were induced by AAI in kidney of mice (26,28,29). Thus, NQO1 might also be induced in the kidneys of patients suffering from AAN and BEN, and this feature can contribute to an elevated risk for cancer. However, in this study, we found for the first time that dicoumarol also induces the NQO1 protein and its enzyme activity in the AAI target organ kidney. Because dicoumarol is used in medicine as an

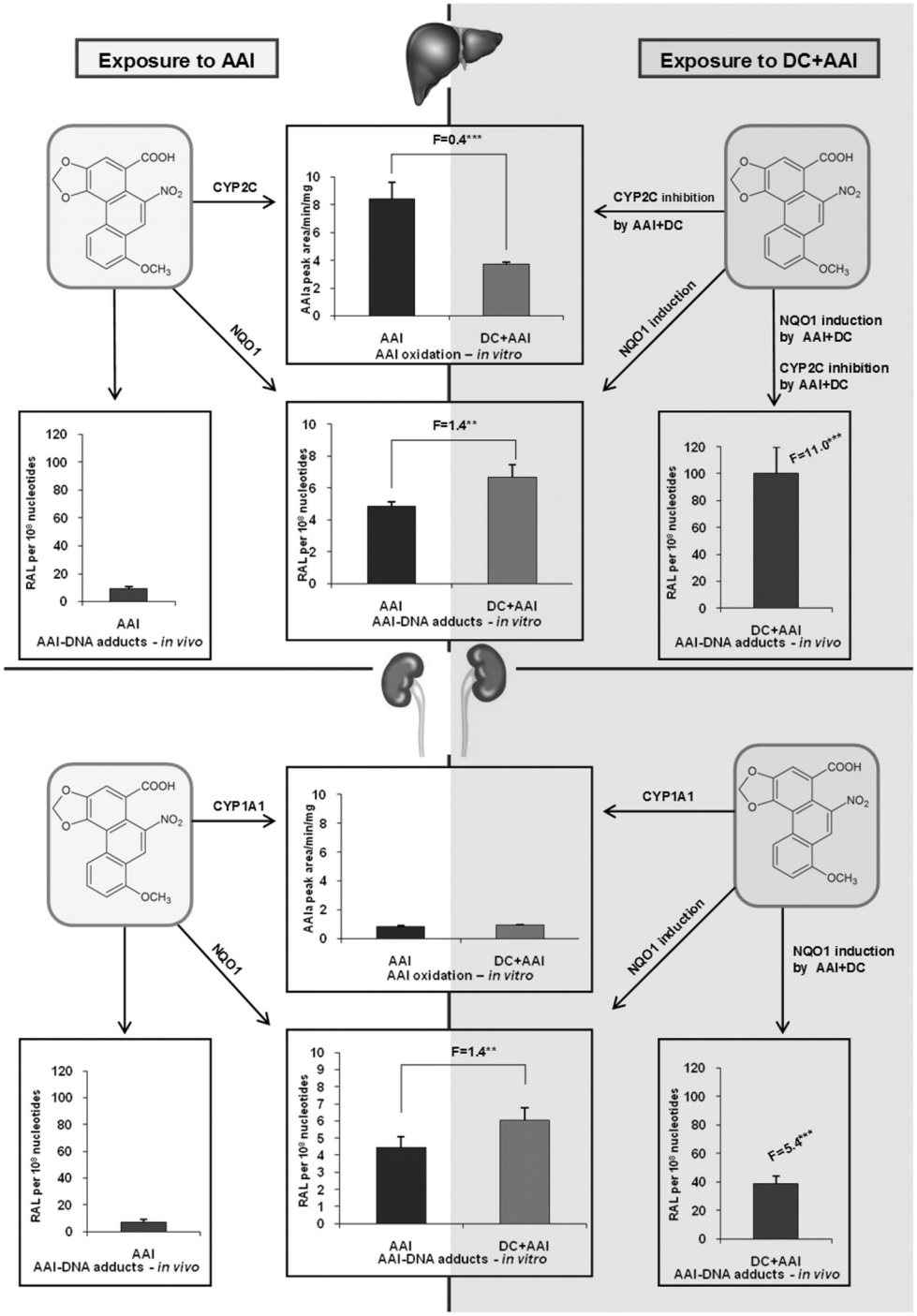

Fig. 9. Schematic summary showing the effects of dicoumarol treatment in rats on AAI metabolism. NQO1 induction with dicoumarol prior to AAI administration led to increased AAI–DNA adduct formation catalysed by rat hepatic and renal cytosols (see panel showing AAI–DNA adduct levels *in vitro*). Inhibition of CYP2C by dicoumarol treatment led to a decrease of AAIa formation in hepatic microsomes (see panel showing AAI oxidation *in vitro*). Both NQO1 induction and CYP2C inhibition increase AAI–DNA adduct formation in liver, whereas only NQO1 induction impacts on AAI–DNA binding in kidney (see panels showing AAI–DNA adduct levels *in vivo*). 'F' indicates fold increase in rats pretreated with dicoumarol compared to animals treated with AAI alone. Comparison was performed by *t*-test analysis; $^{**}P < 0.01$, $^{***}P < 0.001$. AAI, aristolochic acid I; DC, dicoumarol; CYP, cytochrome P450; NQO1, NAD(P)H:quinone oxidoreductase 1.

anticoagulant drug that functions as a vitamin K antagonist (similar to warfarin for which it was the inspiration; reviewed in refs 47 and 48), such an induction might increase AAI–DNA adduct formation leading to a higher risk of AAI-mediated development of urothelial cancer in humans exposed to AA.

Even though we demonstrate in this study that both AAI and dicoumarol increased NQO1 protein levels and activity, the mechanism(s) are not yet clear. NQO1 induction has been widely investigated in a variety of studies (reviewed in refs 49–53). Protein levels of NQO1 are induced by several chemicals, often by pathways generating reactive oxygen species (ROS). *NQO1* gene expression is primarily regulated by the KEAP1/NRF (NF-E2-related factor 2) pathway, which controls redox homeostasis and facilitates the adaptation of most cells

to oxidative stress (50–53). Because ROS have been found in some human cells treated with AAI (54,55), ROS formation caused by AAI might contribute to NQO1 induction.

NQO1 is known to be implicated in vitamin K metabolism; it is identical to the so-called dicoumarol-inhibited vitamin K reductase (53,56). Because vitamin K is redox-cycled during its metabolism by other enzymes, ROS might be generated in the one-electron redox reactions in this metabolism (53,57). As NQO1 competes with enzymes that redox cycle vitamin K and catalyses two-electron reduction of vitamin K, less semiquinone and ROS are formed. If dicoumarol inhibits NQO1 activity, a significant increase in ROS might occur (53,56) also resulting in enhanced *NQO1* expression. Because our data in rats and the results of the mouse study (22) do not unequivocally answer the question how important NQO1 is for AAI activation *in vivo*, utilization of *NQO1*-knockout animal models (58) may bring a definite proof. The elucidation of the contribution of NQO1 to AAI bioactivation resulting in its genotoxicity and nephrotoxicity is of great importance. The activity of NQO1 in humans may differ significantly among individuals, because, beside its induciblity, the gene is polymorphic. Therefore, the impact of *NQO1* genotype on AAI-induced nephropathy and urothelial cancer in humans remains still to be clarified. We propose that analyses of the expression levels and activities of enzymes metabolizing AAI in AAN and BEN patients may clarify to which extent they contribute to the development of AA-induced nephropathies and cancer.

Supplementary data

Supplementary Figure 1 is available at *Mutagenesis* Online.

Funding

Grant Agency of the Czech Republic (303/09/0472); Charles University in Prague (UNCE 204025/2012 and 570513).

Acknowledgements

Work at King's College London is supported by Cancer Research UK. V.M.A. is member of European Union Network of Excellence ECNIS (Environmental Cancer Risk, Nutrition and Individual Susceptibility).

Conflict of interest statement. None declared.

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Dicoumarol inhibits rat NAD(P)H:quinone oxidoreductase *in vitro* and induces its expression *in vivo*

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Submitted: 2014-09-23 Accepted: 2014-11-08 Published online: 2014-11-30

Key words: **aristolochic acid; aristolochic acid nephropathy; Balkan endemic nephropathy, NAD(P)H:quinone oxidoreductase; dicoumarol; protein expression; Western blotting; metabolic activation; DNA adducts; rat model**

Neuroendocrinol Lett 2014; **35**(Suppl. 2):123–132 **PMID:** 25638376 NEL351014A14

Abstract

OBJECTIVES: Dicoumarol is known to act as an inhibitor of NAD(P)H:quinone oxidoreductase (NQO1). This cytosolic reductase significantly contributes to the genotoxicity of the nephrotoxic and carcinogenic alkaloid aristolochic acid I (AAI). Aristolochic acid causes aristolochic acid nephropathy (AAN), and Balkan endemic nephropathy (BEN), as well as associated urothelial malignancies. NQO1 is the most efficient enzyme responsible for the reductive bioactivation of AAI to species forming covalent AAI-DNA adducts. However, it is still not known how dicoumarol influences the NQO1-mediated reductive bioactivation of AAI.
METHODS: AAI-DNA adduct formation was determined by ^{32}P-postlabeling. Expression of *NQO1* mRNA and NQO1 protein was determined by real-time polymerase chain reaction and Western blotting, respectively.
RESULTS: In this study, dicoumarol inhibited AAI bioactivation to form AAI-DNA adducts mediated by rat and human NQO1 *in vitro* as expected. We however, demonstrated that dicoumarol acts as an inducer of NQO1 in kidney and lung of rats treated with this NQO1 inhibitor *in vivo*, both at protein and activity levels. This NQO1 induction increased the potency of kidney cytosol to bioactivate AAI and elevated AAI-DNA adduct levels were found in *ex-vivo* incubations of AAI with renal cytosols and DNA. *NQO1* mRNA levels were induced in liver only by dicoumarol.
CONCLUSION: Our results indicate a dual role of dicoumarol in NQO1-mediated genotoxicty of AAI. It acts both as an NQO1 inhibitor mainly *in vitro* and as an NQO1 inducer if administered to rats.

ORIGINAL ARTICLE

Abbreviations:

AA	- aristolochic acid
AAI	- 8-methoxy-6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid
AAN	- aristolochic acid nephropathy
ARE	- antioxidant response element
BEN	- Balkan endemic nephropathy
bw	- body weight
CHN	- Chinese herbs 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
KEAP1	- Kelch-like ECH-associating protein 1
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
NQO1	- NAD(P)H:quinone oxidoreductase
NRF2	- nuclear factor-erythroid 2-related factor 2
PEI	- polyethylenimine
RAL	- relative adduct labeling
ROS	- reactive oxygen species
qRT-PCR	- quantitative real-time polymerase chain reaction
TLC	- thin layer chromatography
UUC	- upper urinary tract urothelial carcinoma

INTRODUCTION

Dicoumarol was found to function as a strong inhibitor of NAD(P)H:quinone oxidoreductase (NQO1) (Hosoda *et al.* 1974; Asher *et al.* 2006). Besides other functions, NQO1 plays an important role in the genotoxicity of the plant component aristolochic acid I (AAI) found in medicinal herbal remedies (Stiborova *et al.* 2003; 2008b; 2008c; 2011a; 2011b; 2012; 2013b; 2014a; 2014b; Schmeiser *et al.* 2009; Arlt *et al.* 2011; Martinek *et al.* 2011; Levova *et al.* 2011). The herbal drug aristolochic acid (AA) derived from *Aristolochia* species has been shown to be the cause of so-called Chinese herbs nephropathy (CHN), now termed aristolochic acid nephropathy (AAN) and its associated urothelial malignancies (Debele, *et al.* 2008; Schmeiser *et al.* 2009; 2014). The major component of AA, AAI, is the predominant compound responsible for this disease.

AAN is a rapidly progressive renal fibrosis that was initially observed in a group of Belgian women who had ingested weight loss pills containing *Aristolochia fangchi* (Vanherweghem *et al.* 1993; Nortier *et al.* 2000; Gökmen *et al.* 2013). Within a few years of taking the pills, AAN patients also showed a high risk (~50%) of upper urothelial tract carcinoma (UUC) and, subsequently, bladder urothelial carcinoma. Similar cases have been reported elsewhere in Europe and Asia (Schmeiser *et al.* 2009). Dietary exposure to AA has also been linked to Balkan endemic nephropathy (BEN) and its associated urothelial cancer (Arlt *et al.* 2007; Grollman *et al.* 2007; Moriya *et al.* 2011; Schmeiser *et al.* 2012); 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; 2009; Arlt *et al.* 2002a; 2002b; 2007; Grollman *et al.* 2007; Jelakovic *et al.* 2012; Yun *et al.* 2012). The most abundant DNA adduct detected in AAN patients is 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI) (Fig. 1) (Schmeiser *et al.* 2014), which causes characteristic AT→TA transversions. Such AT→TA mutations have been observed in the *TP53* tumor suppressor gene and other genes in tumours from AAN and BEN patients (Lord *et al.* 2004; Grollman *et al.* 2007; Olivier *et al.* 2012; Hoang *et al.* 2013; Poon *et al.* 2013), indicating a probable molecular mechanism associated with AA-induced carcinogenesis (Arlt *et al.* 2007; Gökmen *et al.* 2013). More recently, AA exposure was discovered to contribute to the high incidence of UUC in Taiwan, where medicinal use of *Aristolochia* plants is widespread (Chen *et al.* 2012); again, the *TP53* AT→TA transversion mutational signature in patients with UUC was predominant which are otherwise rare. AA has been classified as a Group I carcinogen in humans by the International Agency for Research on Cancer (Grosse *et al.* 2009).

The metabolic activation of AAI by nitroreduction is catalyzed by both cytosolic and microsomal enzymes and leads to formation of AAI-DNA adducts and in this process NQO1 is the most efficient cytosolic nitroreductase (Stiborova *et al.* 2003; 2005; 2008b; 2008c; 2011a; 2011b; 2012; 2013b; 2014a; 2014b; 2014c) (Fig. 1). Indeed, microsomal cytochrome P450 (CYP) 1A1/2 and NADPH:CYP oxidoreductase that also activate AAI are less efficient in AAI bioactivation than cytosolic NQO1 (Arlt *et al.* 2011; Stiborova *et al.* 2001a; 2011b; 2012; Jerabek *et al.* 2012). Results of our former studies also demonstrate that NQO1 plays an important role not only in AAI activation *in vitro* (Stiborova *et al.* 2003; 2008b; 2008c; 2011a; 2011b; 2012), but also *in vivo* (Stiborova *et al.* 2013b; 2014a; 2014b; 2014c). A role of NQO1 in renal AAI nitroreduction *in vivo* was proven by Chen and collaborators (Chen *et al.* 2011), in one mouse model (male C57BL/6 mice). In their study AAI metabolism *in vivo* was decreased by inhibitors of NQO1 such as dicoumarol (Hosoda *et al.* 1974; Asher *et al.* 2006). In contrast, we have recently found in Wistar rats, that dicoumarol treatment prior to AAI administration increased the reductive activation of AAI leading to enhanced genotoxicity (i.e. AAI-DNA adduct formation) in liver and kidney (Stiborova *et al.* 2014c). Under the experimental conditions used, higher NQO1 expression levels caused elevated levels of AAI-DNA adducts (Stiborova *et al.* 2014c).

Therefore, in the present study, the effect of dicoumarol upon NQO1-mediated reductive activation of AAI *ex vivo*, and its effect upon *NQO1* gene expression, NQO1 protein levels and NQO1 enzyme activity *in vivo*, was investigated in several organs (liver, kidney and lung) of rats treated with this compound. Expression of *NQO1* mRNA and NQO1 protein in rats was determined by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting, respectively, while the activity of NQO1 was measured with menadione as a marker substrate and by AAI-DNA adduct formation in *ex vivo* incubations with AAI and DNA.

MATERIALS AND METHODS

Animal experiments

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Groups of male Wistar rats (150 g, n=3/group) were treated with dicoumarol in a suspension in corn oil. Dicoumarol was administered by gavage to rats twice at either doses of 30 or 60 mg/kg body weight (bw), once in the afternoon (3 p.m.) and then again the next day (8 a.m.) (total doses of 60 and 120 mg/kg bw, respectively). Animals in the control groups received vehicle, corn oil, only. Animals were sacrified 24 h after the last treatment. Livers, kidneys and lungs were removed immediately after sacrifice, frozen in liquid nitrogen and stored at –80 °C until isolation of cytosolic fractions.

Preparation of cytosolic samples

Hepatic, renal and pulmonary cytosolic fractions from untreated and dicoumarol-pretreated rats were isolated by differential centrifugation as previously described (Stiborova *et al.* 2003; 2011a; 2012). Pooled cytosolic fractions (n=3 rat/group) were used for further analyses.

Determination of NQO1 protein levels by Western blotting

The chicken anti-rat NQO1 antibodies were prepared as described previously (Stiborova *et al.* 2006). Immunoquantification of cytosolic NQO1 was carried out on proteins transferred to a polyvinylidene fluoride membrane after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Stiborova *et al.* 2006). Human recombinant NQO1 (Sigma) was used to identify the NQO1 band from rat cytosols. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium as dye and bands are expressed as arbitrary units (AU)/mg protein (Stiborova *et al.* 2006). Glyceraldehyde phosphate dehydrogenase was used as loading control and detected by commercial antibody (1:750, Millipore; MA, USA).

Measurement of NQO1 enzyme activity

NQO1 activity was determined using menadione as a substrate as described (Stiborova *et al.* 2003); improved measurement was achieved by addition of cytochrome *c* (Mizerovska *et al.* 2011).

Cytosolic incubations of AA with DNA to analyze AAI-DNA adduct formation

The de-aerated and nitrogen-purged incubation mixtures, in a final volume of 750 µl, included 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Tween 20, 1 mM NADPH, 1 mg rat cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI. Incubations were carried out at 37 °C for 60 min; AAI-derived DNA adduct formation in cytosols is known to be linear up to 2 h (Stiborova *et al.* 2003). Control incubations were carried out (*i*) without cytosol, (*ii*) without NADPH, (*iii*) without DNA, or (*iv*) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the standard phenol/chloroform extraction method (Stiborova *et al.* 2007b; 2013a).

Incubations with rat and human NQO1

The incubations used to evaluate DNA adduct formation by AAI with rat NQO1 isolated from rat liver (Stiborova *et al.* 2001b) and human recombinant NQO1 (Sigma) were done as described (Stiborova *et al.* 2002; 2003). To study the effect of dicoumarol on AAI-DNA adduct formation, incubations 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, 0.1 mM AAI (dissolved in water), 0.5 mg of calf thymus DNA (2 mM dNp) and 20 µg (0.06 units) of rat or human NQO1 in the absence or presence of 10 µM dicoumarol (dicoumarol dissolved in 7.5 µl of ethanol). One unit of NQO1 reduces 1 µmol of cytochrome *c* per min in the presence of menadione as substrate at 37°C. The reaction was initiated by adding NADPH. All reaction mixtures were incubated at 37°C for 60 min; NQO1-mediated AAI-derived DNA adduct formation was found to be linear up to 90 min (Stiborova *et al.* 2011a). In control incubations NQO1 or its cofactor (NADPH) was omitted from the mixtures. After extraction with ethyl acetate, DNA was isolated from the residual water phase as described above.

DNA adduct analysis by ^{32}P-postlabelling

DNA adduct formation was analysed using the nuclease P1 enrichment version of the thin-layer chromatography (TLC) ^{32}P-postlabelling method (Schmeiser *et al.* 2013). DNA digestion, adduct enrichment and labelling were performed as described (Stiborova *et al.* 2003; 2011a). Chromatographic conditions for TLC on polyethylenimine-cellulose plates (10×20 cm; Macherey-Nagel, Düren, Germany) were: D1, 1.0 M sodium phosphate, pH 6.8; D3: 3.5 M 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; D5, 1.7 M sodium phosphate, pH 6. After chromatography TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labelling) were calculated as described (Stiborova *et al.* 2003; 2011a). Results were expressed as DNA adducts/10^8 normal nucleotides.

NQO1 mRNA contents in rat liver, kidney and lung

NQO1 mRNA expression in rat liver, kidney and lung was determined as described previously (Stiborova *et al.* 2006; 2008a). Total RNA was isolated from frozen

livers, kidneys and lungs of untreated and dicoumarol-treated rats (n=3; each group) and mRNA was quantified by qRT-PCR exactly as described (Stiborova *et al.* 2008a). Briefly, the qPCR data were analyzed by the program RotorGene v6 (Corbett Research, Sydney, Australia) and evaluated by comparative cycle threshold (c_T) method for relative quantification of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then $\Delta\Delta c_T$ was evaluated according to following equations: $\Delta c_T = c_T$ (target) $- c_T$ (internal standard), $\Delta\Delta c_T = \Delta c_{T\ treated} - \Delta c_{T\ control}$, where $\Delta c_{T\ treated}$ is Δc_T for treated rats and $\Delta c_{T\ control}$ is Δc_T for untreated rats. The induction of mRNA expression of studied target gene in pretreated animals was evaluated as $2^{-\Delta\Delta c_T}$.

RESULTS

Dicoumarol inhibits bioactivation of AAI by rat and human NQO1 to AAI-DNA adducts in vitro

As described in our former studies, rat and human NQO1 are capable of activating AAI to form AA-DNA adducts *in vitro* (Stiborova *et al.* 2002; 2003; 2008b; 2008c; 2011a; 2011b; 2014b; Martinek *et al.* 2011). In the presence of NADPH, the cofactor of NQO1, AAI was reductively activated by NQO1 to species generating the same pattern of DNA adducts as that found in patients suffering from AAN and BEN (Schmeiser *et al.* 1996; 2009; 2014; Nortier *et al.* 2000; Arlt *et al.* 2002a; 2002b). The adduct pattern formed consisted of two major adducts, 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) and 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI), and one minor adduct, 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII) (see insert Figure 1). If dicoumarol was added to the incubation mixture AAI-DNA adduct formation catalyzed by rat and human NQO1 *in vitro*, was inhibited by 96 and 98%, respectively (Table 1). These results confirmed not only the efficiency of NQO1 to activate AAI to AAI-DNA adducts, but also showed the potency of dicoumarol to inhibit the activity of this reductase *in vitro*.

Tab. 1. The effect of dicoumarol on the AAI-DNA adduct formation from AAI (0.1 mM) by NQO1 *in vitro*.

	RAL[a] (mean ± SEM/10^8 nucleotides)			
Enzymatic system	**dG-AAI**	**dA-AAI**	**dA-AAII**	**Total**
Human NQO1 without cofactor	n.d.			
+ NADPH	32.6±3.7	50.2±5.6	8.2±0.9	91.0±9.5
+ NADPH + dicoumarol[b]	0.5±0.05***	1.1±0.1***	0.2±0.02***	1.8±0.2***
				(**2%**)[c]
Rat NQO1 without cofactor	n.d.			
+ NADPH	4.2±0.4	15.4±1.6	1.0±0.1	20.6±2.1
+ NADPH + dicoumarol	0.2±0.02***	0.6±0.07***	n.d.	0.80±0.1***
				(**3.9%**)

Numbers are averages ± SEM (n=4) of duplicate *in vitro* incubations, each DNA sample was determined by two postlabeled analyzes.
[a] Relative adduct labeling; [b] 10 μM dicoumarol; [c] % of control
Significantly different from levels of AAI-DNA adducts without dicoumarol: ***p<0.001 (Student's t-test).

The effect of dicoumarol on NQO1 enzyme activity and AAI-DNA adduct formation in liver, kidney and lung cytosols of rats treated with this compound

In further experiments, we investigated the effects of dicoumarol on NQO1 activity *in vivo*, namely, the influence of treatment of rats with dicoumarol on NQO1 activity in liver, kidney and lung of these rats. Because NQO1 is a cytosolic enzyme, NQO1 activity (measured with its two substrates, menadione and AAI) was analyzed in cytosols isolated from these rat organs. Hepatic, renal and pulmonary cytosols isolated from control and dicoumarol-treated rats were analyzed for their efficiencies to reduce these substrates.

The activity of NQO1 with menadione as substrate decreased down to 50 % in liver cytosol when rats were treated with dicoumarol (Figure 2A). These results correspond to the strong inhibitory effect of dicoumarol on NQO1 enzyme activity *in vitro* (Hosoda *et al.* 1974; Asher *et al.* 2006). However, in contrast to these results, unexpectedly a 1.8- and 1.7-fold increase in the activity of this enzyme was found in kidney and lung cytosols isolated from rats treated with the higher dicoumarol dose (Figure 2A).

Since AAI is also a substrate of NQO1, its reductive bio-activation to species forming AAI-DNA adducts was analyzed. Cytosols were incubated with AAI, DNA and NADPH, and analyzed for formation of AAI-DNA adducts by ^{32}P-postlabelling. AAI was reductively activated by all rat cytosols isolated from untreated (control) animals, as evidenced by AAI-DNA adduct formation (Figure 3). The DNA adduct patterns generated were the same as those analyzed in patients suffering from AAN and BEN (Schmeiser *et al.* 1996; 2012; 2014; Nortier *et al.* 2000; Arlt *et al.* 2002a; 2002b) or *in vitro* in incubations with rat and human NQO1 (Stiborova *et al.* 2002; 2003; 2011a; Martinek 2011) (see insert Figure 1). No adducts were observed in control incubations carried out in parallel (data not shown). Analogously to NQO1 activity with menadione as substrate, a 2-fold decrease in AAI-DNA adduct levels was found in incubations of liver cytosolic fractions isolated from dicoumarol-treated rats. However, a 2.6- and 1.9-fold increase in levels of AAI-DNA adducts was detected in DNA incubations with AAI and kidney or lung cytosols, respectively, from rats exposed to 120 mg dicoumarol compared to controls (Figure 3). Changes in AAI-DNA adduct formation in cytosols from each of these organs correlated with the NQO1 activities determined with menadione in these cytosols. The absolute

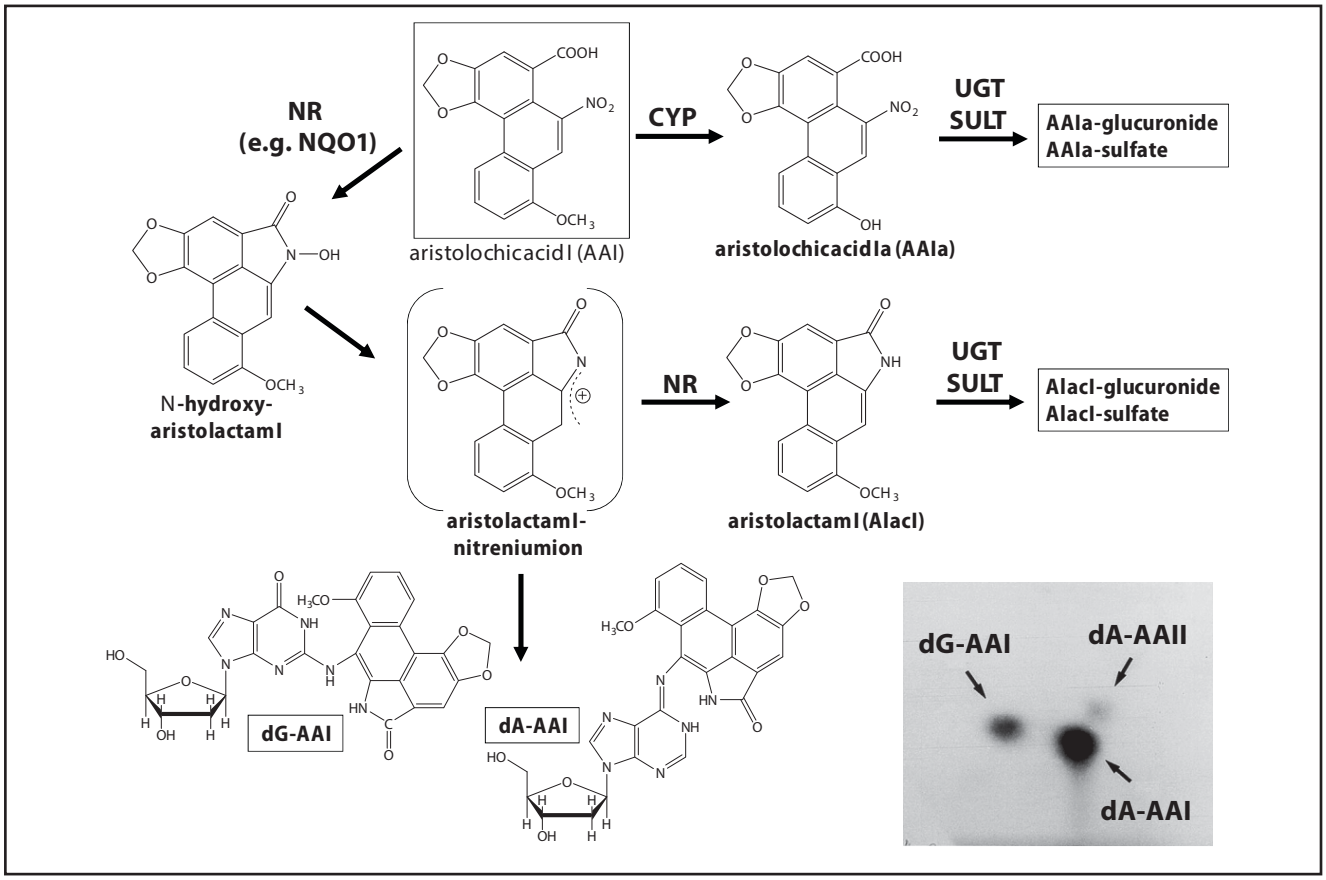

Fig. 1. Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; NR, nitro-reduction; UGT, UDP glucuronosyltransferase; SULT, sulfotransferase. Insert: Autoradiographic profile of AAI-DNA adducts formed by incubation of AAI with hepatic cytosol from control rats and DNA using the nuclease P1 enrichment version of the ^{32}P-postlabeling assay.

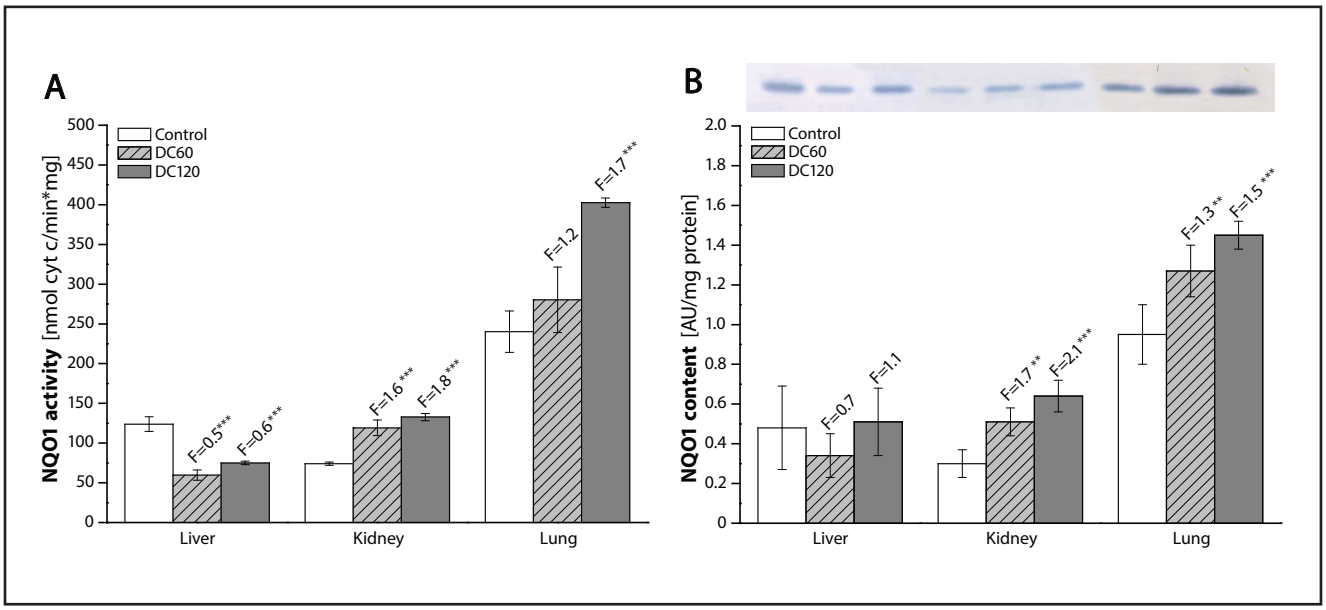

Fig. 2. NQO1 enzyme activity (A) and NQO1 protein expression (B) in cytosols isolated from liver, kidney and lung of untreated (control) rats or rats treated with total doses of 60 or 120 mg/kg bw dicoumarol (see Materials and Methods for details). NQO1 enzymatic activity in cytosols (A) was determined as described in chapter 2.4. NQO1 protein expression in cytosols (B) was determined by Western blotting (see insert). Human recombinant NQO1 (Sigma) was used to identify the rat NQO1 band in rat cytosols (data not shown). Glyceraldehyde phosphate dehydrogenase was used as loading control. All values are given as means ± SD (n = 3). Values significantly different from control rats: $^{**}p<0.01$, $^{***}p<0.001$ (Student's t-test). Numbers above columns ("F") indicate fold changes in protein level or enzyme activity compared to control. DC – dicoumarol.

NQO1 activities in the two extrahepatic organs were, however, not consistent with AAI-DNA adduct formation. Although NQO1 activity was higher in lung than kidney cytosols, the levels of AAI-DNA adducts formed by lung cytosols were almost one order of magnitude lower than by cytosols of liver or kidney (compare Figures 2 and 3).

The effect of dicoumarol on expression of NQO1 protein in liver, kidney and lung of rats treated with this compound

The activities of a variety of enzymes including NQO1 are usually determined by their expression levels, but other factors influencing their activities cannot be excluded. We, therefore, evaluated whether expression of NQO1 proteins is the cause of changes in NQO1 activities found in tested organs of rats treated with dicoumarol. A method of Western blotting, suitable to evaluate expression of proteins, was used for such investigations. Using this technique, we explained the unexpected increase in NQO1 activity in kidney and lung of rats treated with dicoumarol relative to controls.

In contrast to the rat liver, where non-significant changes in the levels of NQO1 protein were detected, this NQO1 inhibitor acted as an inducer of NQO1 protein expression in rat kidney and lung; up to 2.1- and 1.5-fold higher levels of NQO1 protein were found in kidney and lung cytosols of dicoumarol-treated rats relative to those of control animals, respectively (Fig. 2B). The levels of NQO1 protein in kidney and lung correlated with the enzyme activity in cytosols of these organs. Expression levels of NQO1 and its enzyme activities measured with menadione as a substrate in hepatic and renal cytosols essentially corresponded also to the bioactivation of AAI to species forming DNA adducts. However, such a correlation was not found in lung cytosols; low levels of AAI-DNA adducts did not correspond to the high expression of NQO1 and enzyme activity in cytosols of this organ (compare Figures 2 and 3). The reason responsible for this phenomenon is not known and remains to be explored.

NQO1 mRNA levels in rat liver, kidney and lung

Changes in *NQO1* mRNA levels were determined by qRT-PCR analysis. As shown in Table 2, treatment of rats with both doses of dicoumarol induced significant increases in *NQO1* mRNA levels in the liver; a 1.7- and 2.5-fold increase in levels of *NQO1* mRNA was found after exposure of rats to 60 and 120 mg/kg bw of dicoumarol, respectively (Table 3). The dicoumarol effect on *NQO1* mRNA in the other two organs was either none (lung) or not significant (kidney) (Table 2).

DISCUSSION

Our results show that dicoumarol, a strong inhibitor of NQO1 (Hosoda *et al.* 1974; Asher *et al.* 2006), plays a dual role on this enzyme in Wistar rats *in vivo*. We show that *in vitro* dicoumarol inhibits the reductive bioactivation of AAI catalyzed by rat and human NQO1; this reaction was inhibited up to 98% by dicoumarol. The effects of dicoumarol *in vivo* were, however, not so clear

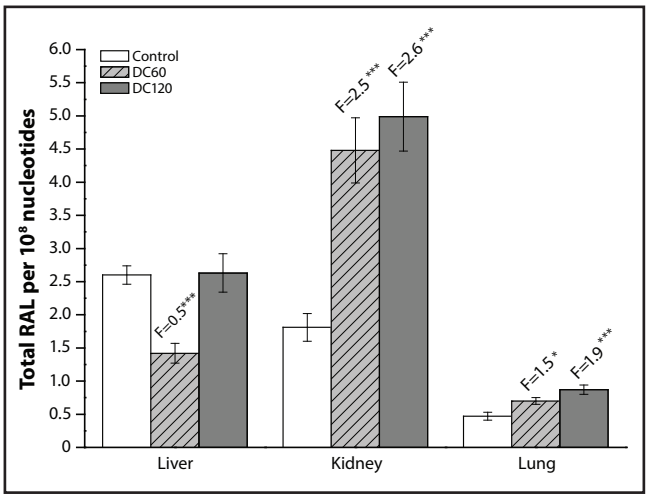

Fig. 3. DNA adduct formation *ex vivo* by AAI in cytosols isolated from untreated (control) rats or rats treated with total doses of 60 and 120 mg/kg bw dicoumarol (see Materials and Methods for details). AAI-DNA adduct formation was determined by ^{32}P-postlabelling in hepatic, renal and pulmonary cytosolic fractions. Values are given as the means SD (n = 3). RAL, relative adduct labeling. Comparison was performed by t-test analysis; $^{*}p < 0.05$, $^{***}p < 0.001$, different from control. Numbers above columns ("F") indicate fold changes in DNA adduct levels compared to control. DC – dicoumarol.

Tab. 2. Expression of *NQO1* mRNA in rats treated with dicoumarol NQO1.

Rats	Δc_T[a]	Fold Change[b]
Control rats		
Liver	6.40±0.21	
Kidney	7.82±0.47	
Lung	7.35±0.11	
Rats treated with 60 mg/kg dicoumarol		
Liver	5.61±0.22	1.7*
Kidney	7.29±0.75	1.4
Lung	7.54±0.30	0.9
Rats treated with 120 mg/kg dicoumarol		
Liver	5.09±0.15	2.5***
Kidney	7.83±0.73	1.0
Lung	7.37±0.19	1.0

[a] Results shown are mean ± SD (n = 3) of untreated (control) rats or rats treated with 60 or 120 mg/kg bw dicoumarol.
[b] The Fold Change refers to fold increase in *NQO1* mRNA expression in treated animals over control group evaluated by $2^{-(\Delta\Delta c_T)}$ method (see Material and Methods for detail).
Significantly different from controls: $^{*}p<0.05$; $^{***}p<0.001$ (Student's t-test).

cut. In liver cytosols from dicoumarol-treated rats isolated 24 h after the last administration NQO1 activity is either inhibited or unchanged (see Figure 2). It is possible that dicoumarol is still binding to liver NQO1 because it has strong protein binding properties (Asher *et al.* 2006). It is expected that due to its long elimination half-life of 5–25 h in rats (Lai *et al.* 1976) residual dicoumarol may remain in tissues, particularly in liver as its main site of metabolism. The decreased NQO1 enzyme activity resulted in lower DNA adduct formation in *ex-vivo* incubations with AAI using hepatic cytosols (see Figure 3). The patterns of protein levels, enzyme activities and AAI-DNA adduct formation also show inhibition of NQO1 at 60 mg, but a recovery at 120 mg for reasons unknown. However, the situation in the kidney and lung was more complex. In kidney and lung, dicoumarol induced NQO1 protein levels and also enzyme activity in a dose dependent manner (see Figures 2 and 3). An up to a 2-fold induction in NQO1 protein expression was observed in kidney after exposure of rats to dicoumarol. The NQO1 protein levels correlated with enzyme activity. However, reduction of AAI by rat cytosols to a cyclic acylnitrenium ions forming DNA adducts (Schmeiser *et al.* 2009; Stiborova *et al.* 2014a; 2014b; 2014c) correlated well only in liver and kidney with NQO1 expression and enzyme activity. The situation in rat lung was completely different from that in the other two organs investigated. Basal and dicoumarol-induced NQO1 protein levels in rat lung were up to 3-times higher than in liver and kidney. Likewise, the efficiency of rat lung cytosols to reduce menadione was up to 5-fold higher than those of liver and kidney cytosols. However, the activity of lung cytosols to reduce AAI to species forming DNA adducts was low. Several reasons for this observation are possible: some inhibitors that compete with AAI to binding to the NQO1 active centre might be present in rat lung cytosol or the lung NQO1 protein has undergone allosteric effects in AAI reduction. Which of these reasons might be most important in our experiments is hard to say and remains to be explained.

Dicoumarol as an NQO1 inhibitor has been employed in a mouse model (male C57BL/6 mice) by Chen and collaborators (Chen *et al.* 2011) to evaluate the role of NQO1 in AAI nitroreduction in kidney *in vivo*. In their study, modulation of the AAI metabolism by dicoumarol and phenindione, another NQO1 inhibitor (Chen *et al.* 1999), was investigated (Chen *et al.* 2011). Whereas inhibition of NQO1 activity decreased the amount of the reductive metabolite aristolactam I in kidney in dicoumarol-pretreated mice 30 min after a single *i.p.* injection of AAI, the amounts of AAI and its detoxication metabolite AAIa increased in mouse serum. Furthermore, pretreatment of mice with dicoumarol decreased AAI-induced nephrotoxicity as well as the survival rate of these mice (Chen *et al.* 2011). These results suggested that dicoumarol acts as an inhibitor of AAI nitroreduction in this mouse model. However, the effect of dicoumarol was only investigated a short period (30 min) after pretreatment of mice with it.

In our previous study using Wistar rats as the experimental model, these were treated with dicoumarol 22 h prior to AAI administration (Stiborova *et al.* 2014c). As a measure of genotoxicity we found higher levels of AAI-DNA adducts in liver and kidney. The induction of NQO1 protein and enzyme activity by dicoumarol found in the present study provides an explanation for our previous results. We demonstrated that a long exposure time to dicoumarol in Wistar rats of 24 h resulted in an increase in NQO1 protein expression and its activity in rat kidney and lung. Such an induction increased the potency of kidney cytosols to reductively activate AAI and led to elevated AAI-DNA adduct levels in *ex-vivo* incubations of AAI with renal cytosolic fractions and DNA. However, because AAI is also reductively activated to form AAI-DNA adducts or oxidatively detoxified to AAIa by CYP enzymes such as CYP1A and/or 2C (Stiborova *et al.* 2001a; 2012; 2014c; Arlt *et al.* 2011; Levova *et al.* 2011), their influencing by dicoumarol might also be important to modulate levels of AAI-DNA adducts formed *in vivo*. We have previously found that higher protein levels and activities of CYP1A1 in kidney and CYP1A2 in liver were found in rats treated with dicoumarol. But, this increase was less important for an increase in AAI-DNA adduct levels in rat liver and kidney treated with dicoumarol prior to administration to AAI *in vivo* than induction of NQO1 (Stiborova *et al.* 2014c). On the contrary, inhibition of CYP2C6 and 2C11 enzymatic activities in rats treated with dicoumarol was found and led to a decrease in AAI oxidative detoxification to AAIa. This finding indicates that higher adduct levels found in the liver of rats pretreated with dicoumarol prior to AAI administration might be, beside induction of NQO1, also caused by decreased AAI detoxification to AAIa due to the dicoumarol-mediated CYP2C inhibition (Stiborova *et al.* 2014c). Because dicoumarol is used as an anticoagulant drug that functions as a vitamin K antagonist also in human medicine (for a review, see Wallin *et al.* 2008; Rajan & Moliterno 2012), an induction of NQO1 and inhibition of CYP activities might increase AAI-DNA adduct formation leading to a higher risk of AAI-mediated development of urothelial cancer in humans.

Besides analyses of the effect of dicoumarol on NQO1 enzyme activity and NQO1 protein levels, we also investigated its influence on *NQO1* mRNA expression. Although NQO1 protein levels essentially correlated with enzyme activity, no correlation between these parameters was found with regards to *NQO1* mRNA expression. A significant increase in *NQO1* mRNA expression was found only in the livers of dicoumarol-treated rats, in contrast to protein and activity, but no effect of dicoumarol on mRNA expression of this enzyme was observed in kidney and lung at both dicoumarol doses, despite increases in NQO1 protein levels in these organs (see Table 2 and Figure 2).

Similar discrepancies between induction of mRNAs of NQO1 or several other enzymes and their protein levels were observed previously (Dickins, 2004; Stiborova et al, 2006; 2007a; Aimova *et al.* 2007). It is known that some inducers might prolong half-lives of mRNAs, while others increase transcription. Therefore, in our experiments, where the rats were sacrified 24 h after the last dicoumarol administration, mRNA levels may have returned to normal, since half-lives of mRNAs are usually much shorter than those of proteins (for a review, see Dickins, 2004). Furthermore, the NQO1 protein stabilization in kidney and lung might be another explanation for the discrepancy observed. However, the studies investigating the stability of NQO1 protein and the half-life of its mRNA in individual rat organs were not carried out in our laboratory and are also not described in the literature. In addition, detailed analyses of the time dependence of the mRNA expression levels and NQO1 protein levels were not performed in the present study. Therefore, future investigations should address the questions whether the transient induction of *NQO1* mRNAs or the different half-lives and/or stabilities for its mRNA and protein are the rational for our observation, and/or how dicoumarol impacts on the stability of mRNA and protein of this enzyme.

Even though we here demonstrated that dicoumarol increased NQO1 protein expression and enzyme activity, the mechanisms of this induction effect are still a matter of debate. NQO1 induction has been widely investigated in a variety of studies (for a review, see Jaiswal 2000; Dinkova-Kostova *et al.* 2004; Ross 2004; Dinkova-Kostova & Talalay 2010). Protein levels of NQO1 enzyme are influenced by several chemicals. NQO1 expression is regulated by two distinct regulatory elements in the 5' flanking region of the *NQO1* gene, the antioxidant response element (ARE) and the xenobiotic response element involving ligand-activated aryl hydrocarbon receptor (Jaiswal 2000; Dinkova-Kostova *et al.* 2004; Ross, 2004; Dinkova-Kostova and Talalay, 2010). In ARE-mediated *NQO1* induction, nuclear factor-erythroid 2-related factor 2 (NRF2) and the cytoskeletal-binding protein KEAP1 (Kelch-like ECH-associating protein 1) play an important role (*i.e.* the NRF2-KEAP1 mechanism of NQO1 induction). ARE-mediated *NQO1* gene expression is increased by a variety of antioxidants, tumour promoters and reactive oxygen species (ROS) (Jaiswal, 2000; Dinkova-Kostova *et al.* 2004; Ross, 2004; Dinkova-Kostova & Talalay, 2010). NQO1 is known to be implicated into the vitamin K metabolism, and was demonstrated to be identical to the so called dicoumarol-inhibited vitamin K reductase (Dinkova-Kostova & Talalay, 2010). Because vitamin K is redox cycled during its metabolism, ROS might be generated in some one-electron redox reactions involved in this metabolism (Gong *et al.* 2008; Tie *et al.* 2011). NQO1 competes with enzymes that redox cycle vitamin K and catalyzes two-electron reduction of vitamin K to hydroquinone. This prevents the formation of the semiquinone and ROS. If dicoumarol inhibits NQO1 activity, ROS formation could increase significantly (Gong *et al.* 2008; Dinkova-Kostova & Talalay, 2010) and might be the cause of ARE activation leading to NQO1 induction. Hence, an increase in dicoumarol-mediated ROS formation caused by NQO1 inhibition of two-electron redox reactions of vitamin K metabolism might be one mechanism by which dicoumarol induces NQO1. However, the question whether ROS formation during these redox cycling reactions is the means by which NQO1 is induced, or by other mechanisms, remains to be explored in future studies.

In conclusion, in this study we demonstrated that dicoumarol, a strong inhibitor of NQO1, increases expression of NQO1 protein and enzyme activity in kidney and lung of rats exposed to this compound and that these effects lead to increased bioactivation of AAI to species binding to DNA mainly in the kidney, the target organ of AAI. Because dicoumarol is a drug used in human medicine, such an NQO1 induction might increase AAI-DNA adduct formation leading to a higher risk of AAI-mediated development of urothelial cancer in humans treated with this drug.

ACKNOWLEDGEMENTS

Financial support from GACR (grant 14-18344S in panel P301) and Charles University in Prague (UNCE 204025/2012) is highly acknowledged. Work at King's College London is supported by Cancer Research UK.

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Mutation Research 768 (2014) 1–7

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

The effect of aristolochic acid I on expression of NAD(P)H:quinone oxidoreductase in mice and rats—A comparative study☆

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ARTICLE INFO

Article history:
Received 9 August 2013
Received in revised form
22 December 2013
Accepted 15 January 2014
Available online 24 April 2014

Keywords:
Aristolochic acid nephropathy
NAD(P):quinone oxidoreductase
Protein expression
Metabolic activation
DNA adducts

ABSTRACT

Aristolochic acid is the cause of aristolochic acid nephropathy (AAN) and Balkan endemic nephropathy (BEN) and their associated urothelial malignancies. Using Western blotting, we investigated the expression of NAD(P)H:quinone oxidoreductase (NQO1), the most efficient cytosolic enzyme that reductively activates aristolochic acid I (AAI) in mice and rats. In addition, the effect of AAI on the expression of the NQO1 protein and its enzymatic activity in these experimental animal models was examined.

We found that NQO1 protein levels in cytosolic fractions isolated from liver, kidney and lung of mice differed from those expressed in these organs of rats. In mice, the highest levels of NQO1 protein and NQO1 activity were found in the kidney, followed by lung and liver. In contrast, the NQO1 protein levels and enzyme activity were lowest in rat-kidney cytosol, whereas the highest amounts of NQO1 protein and activity were found in lung cytosols, followed by those of liver.

NQO1 protein and enzyme activity were induced in liver and kidney of AAI-pretreated mice compared with those of untreated mice. NQO1 protein and enzyme activity were also induced in rat kidney by AAI. Furthermore, the increase in hepatic and renal NQO1 enzyme activity was associated with AAI bioactivation and elevated AAI-DNA adduct levels were found in *ex vivo* incubations of cytosolic fractions with DNA and AAI. In conclusion, our results indicate that AAI can increase its own metabolic activation by inducing NQO1, thereby enhancing its own genotoxic potential.

1. Introduction

The herbal drug aristolochic acid (AA) derived from *Aristolochia* species has been shown to be the cause of so-called Chinese herbs nephropathy (CHN), now termed aristolochic acid nephropathy (AAN) [1,2]. The plant extract containing AA is a mixture of structurally related nitrophenanthrene carboxylic acids, the major components being aristolochic acid I (AAI) (Fig. 1) and aristolochic acid II (AAII). AAN is a rapidly progressive renal fibrosis that was initially observed 20 years ago in a group of Belgian women who had ingested slimming pills containing *Aristolochia fangchi* [3,4]. Within a few years of taking the pills, AAN patients also showed a high risk (~50%) of upper urothelial tract carcinoma and, subsequently, bladder urothelial carcinoma. In the meantime, it has become clear that AAN is a world-wide environmental and iatrogenic disease associated with urothelial cancer in humans [2,5,6]. Dietary

Abbreviations: AA, aristolochic acid; AAI, aristolochic acid I; AAIa, aristolochic acid Ia; AANAAII, aristolochic acid II; AAN, aristolochic acid nephropathy; BEN, Balkan endemic nephropathy; CYP, cytochrome P450; dA-AAI, 7-(deoxyadenosin-N^6-yl)-aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; dA-AAII, 7-(deoxyadenosin-N^6-yl)aristolactam II; HRN, Hepatic P450 Reductase Null; IARC, International Agency for Research on Cancer; NQO1, NAD(P)H:quinone oxidoreductase; POR, NADPH:P450 oxidoreductase; RAL, relative adduct labeling; SDS, sodium dodecyl sulphate; S.E.M., standard error of the mean; TLC, thin-layer chromatography; WT, wild-type.

☆ This work was supported in part by the Grant Agency of the Czech Republic, Grant 301/09/0472 and Charles University in Prague (UNCE 204025/2012). Work at King's College London is supported by Cancer Research UK.

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http://dx.doi.org/10.1016/j.mrgentox.2014.01.012
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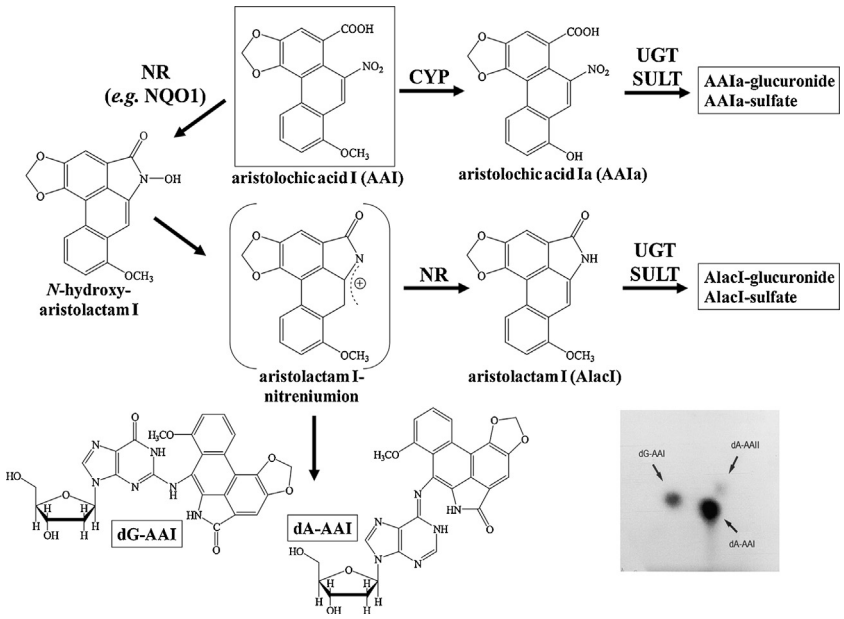

Fig. 1. Pathways of biotransformation and DNA-adduct formation by AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; NR, nitro-reduction; UGT, UDP glucuronosyltransferase; SULT, sulfotransferase. Insert: Autoradiographic profile of AAI-DNA adducts formed by incubation of AAI with hepatic cytosol from mice, determined with the nuclease-P1 enrichment version of the ^{32}P-postlabelling assay. The adduct profile shown here is representative of those obtained in cytosolic fractions of other organs of mice and rats analyzed.

exposure to AA has also been linked to Balkan endemic nephropathy (BEN) and its associated urothelial cancer [6–8]; this nephropathy is endemic in certain rural areas of Serbia, Bosnia, Croatia, Bulgaria and Romania.

Exposure to AA was demonstrated by identification of specific AA-DNA adducts in renal tissue of AAN and BEN patients [2,5–13]. The most abundant DNA adduct detected in patients is 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI) (Fig. 1), which causes characteristic AT→TA transversions. Such an AT→TA mutational signature was predominant in the *TP53* tumor-suppressor gene of urothelial tumors from AAN and BEN patients [4,8,14,15], a class of mutations accounting otherwise for only approximately 5% of all the *TP53* mutations in non-AA-associated human urothelial tumors, according to the International Agency for Research on Cancer (IARC) TP53 database [16]. The same AT→TA transversions have been induced experimentally in human *TP53* in mouse embryonic fibroblasts from Hupki (Human *TP53* knock-in) mice treated *in vitro* with AAI [17,18] thus indicating a probable molecular mechanism associated with AA-induced carcinogenesis [6,19]. AA has been classified as a Group-1 carcinogen in humans by the IARC [20]. The National Toxicology Program has listed AA as *known to be a human carcinogen* for the first time in its 12th Report on Carcinogens.

The activation pathway for AAI is *via* nitro-reduction, catalyzed by both cytosolic and microsomal enzymes; in this process NAD(P)H:quinone oxidoreductase (NQO1) is the most efficient cytosolic nitroreductase [21–26] (Fig. 1). In contrast to NQO1, we found that conjugation enzymes such as human sulfotransferases or *N*,*O*-acetyltransferases did not significantly activate AAI [21,25,26]. AAI is also activated by microsomal enzymes: human, rat and mouse cytochrome P450 (CYP) 1A1 and 1A2 reductively activate AAI to form DNA adducts both *in vitro* and *in vivo* [27–33]. Besides NQO1 and CYP1A1/2, microsomal NADPH:P450 oxidoreductase (POR) also activates AAI, but this enzyme plays a minor role [32,33]. Human and rodent CYP1A1 and 1A2 are also the principal enzymes involved in oxidative detoxification of AAI to the *O*-demethylated metabolite 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIa, Fig. 1) *in vitro* and *in vivo* [34–37]. Previous studies also suggested that, in addition to CYP1A1 and 1A2 expression levels, oxygen concentrations in specific organs or cells may affect the balance between AAI nitro-reduction (activation) and demethylation (detoxification), which in turn would influence tissue-specific toxicity or carcinogenicity of AA [22,23,27–30]. However, reductive activation of AAI in organisms may not only be dictated by CYP1A1/2, but also by the expression of the major AAI activating enzyme, NQO1. Indeed, we observed that higher AAI-DNA adduct levels in *Cyp1a1(−/−)* and *Cyp1a2(−/−)* single-knockout and *Cyp1a1/1a2(−/−)* double-knockout mice [29] relative to wild-type (WT) mice were not only the result of the lack of AAI demethylation by the CYP-dependent system, but also by higher NQO1 activity, which activates AAI [27,29,30,38]. Similar results were obtained in Hepatic P450 Reductase Null (HRN) mice, in which the *Por* gene is deleted specifically in hepatocytes, resulting essentially in the absence of CYP activity in the liver [27]. Expression of the NQO1 protein in hepatic and/or renal cytosolic samples was higher in several of these mouse lines compared with the expression in WT mice [27,38]; this was paralleled by increased NQO1 activity and AAI-DNA-adduct formation in *ex vivo* cytosolic incubations with DNA and AAI. Collectively, these results suggested that deletion of *Cyp1a1/2* or *Por*, and therefore the absence of the corresponding enzymes, is partially compensated by increased expression of the cytosolic nitroreductase NQO1 [27,29,30].

Because of the importance of NQO1 in reductive activation of AAI, the aim of this study was to assess NQO1 protein expression and the effect of AAI on this expression in a mouse and a rat model of AAN, which are currently used in standardized experimental protocols. By use of Western blotting we measured the expression levels of this enzyme in hepatic, renal and pulmonary cytosols isolated from rats and mice that were either untreated (control) or treated with AAI. In addition, NQO1 enzyme activity and DNA-adduct formation in *ex vivo* cytosolic incubations with DNA and AAI were measured in these sub-cellular fractions.

2. Materials and methods

2.1. Animal experiments

Age-matched C57BL/6J mice, purchased from The Jackson Laboratory (Bar Harbor, ME, USA), were of the same strain as those used in our previous studies [29,30]. Groups of female mice (3 months old; weight, 25–30 g; $n = 4$/group) were treated with a single dose of AAI as sodium salt in water (50 mg/kg body weight) by oral gavage as described previously [29,30]. Control mice received water only. Animals were killed 24 h after the treatment.

Groups of male Wistar rats (weight, ~150 g; $n = 4$/group) were treated with a single *i.p.* injection of AAI dissolved in 1% $NaHCO_3$, at a dose of 20 mg/kg body weight. Control rats receive the solvent only. Animals were killed 24 h after the treatment. The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki.

2.2. Preparation of cytosolic samples

Hepatic, renal and pulmonary cytosolic fractions from untreated and AAI-pretreated mice and rats were isolated as previously described [27–30]. Pooled cytosolic fractions ($n = 4$ mice or rats/group] were used for subsequent analyses.

2.3. Determination of NQO1 protein levels by Western blotting

NQO1 antibodies were prepared as described previously [39]. Immunoquantification of cytosolic NQO1 was carried out on proteins transferred to a polyvinylidene-fluoride membrane after separation by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis [39–41]. Human recombinant NQO1 (Sigma) was used to identify the NQO1 band in gels of mouse and rat cytosols. Glyceraldehyde-phosphate dehydrogenase was used as loading control and detected by a specific antibody (1:750, Millipore; MA, USA).

2.4. Measurement of NQO1 enzyme activity

NQO1 activity was determined with menadione as a substrate, as described by Ernster [42], but improved by addition of cytochrome *c* [43].

2.5. Measurement of cytosolic AAI-DNA-adduct formation by ^{32}P-postlabelling

Incubation mixtures, in a final volume of 750 μl, included 50 mM Tris–HCl buffer (pH 7.4) containing 0.2% Tween20, 1 mM NADPH, 1 mg mouse or rat cytosolic protein, 0.5 mg calf-thymus DNA (2 mM dNp) and 0.5 mM AAI [21]. Incubations were carried out at 37 °C for 60 min; AAI-derived DNA adduct formation is known to be linear up to 2 h [25]. Control incubations were carried out (i) without cytosol, (ii) without NADPH, (iii) without DNA, or (iv) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the standard phenol/chloroform extraction method. The ^{32}P-postlabelling assay with the nuclease-P1 enrichment version, and thin-layer chromatography (TLC) for analysis of AAI-DNA-adduct formation, were performed as described [10,22]. TLC sheets were scanned with a Packard Instant Imager (Dowers Grove; USA). DNA adduct levels (RAL, relative adduct labeling) were calculated as described [11,21,25]. Results were expressed as DNA adducts/10^8 nucleotides.

3. Results

By use of Western blotting, we investigated the organ specificity of NQO1 protein expression and the effects of AAI treatment on its expression in two experimental animal models, mice and rats. Liver, kidney and lung cytosolic samples isolated from control (untreated) animals and AAI-treated animals were used for analyses.

3.1. Determination of NQO1 protein expression and evaluation of NQO1 enzyme activity in the liver, kidney and lung of mice and rats

Expression of NQO1 protein was detected in all cytosolic samples in both animal models. Expression levels of this enzyme were different in the tissues (organs) investigated, both in mice and rats. In mice, the highest expression of NQO1 protein was found in the kidney; levels were 2.5- ($p < 0.001$) and 1.3-fold ($p < 0.05$) higher in kidney and lung relative to liver, respectively (Fig. 2A, upper panel). Likewise, the highest NQO1 activity, measured with menadione as a substrate, was found in mouse kidney, followed by lung and liver (Fig. 2A, lower panel).

Interestingly, in rats the highest NQO1 protein levels were observed in lung, followed by liver and kidney; levels were 2.0- ($p < 0.001$) and 1.3-fold ($p < 0.05$) higher in pulmonary and hepatic cytosols relative to renal cytosols, respectively (Fig. 2B, upper panel). NQO1 protein levels in individual rat organs were paralleled by the specific activity of this enzyme: NQO1 activity was 3.2- and 1.6-fold (both $p < 0.001$) higher in lung and liver cytosols relative to cytosols of kidney, respectively (Fig. 2B, lower panel).

3.2. The effect of AAI treatment on NQO1 protein expression and NQO1 enzyme activity in liver, kidney and lung of mice and rats

Treatment of mice with AAI increased NQO1 protein expression and enzyme activity in liver and kidney; protein levels were 2.1- and 1.8-fold higher in hepatic and renal cytosols of AAI-treated mice than in control (untreated) mice, respectively. In contrast, no effect of AAI treatment was observed in mouse lung (Fig. 2A, upper panel). The increase in NQO1 protein expression in liver and kidney was paralleled by an increase in NQO1 activity, however, no enzyme activity was detectable in pulmonary cytosol isolated from AAI-treated mice (Fig. 2A, lower panel).

In contrast to these results, treatment of rats with AAI led to a significant increase in NQO1 protein expression only in kidney cytosol (1.4-fold, $p < 0.05$); no increased expression was observed in cytosols isolated from liver and lung (Fig. 2B, upper panel). Likewise, a significant increase in NQO1 enzyme activity caused by AAI-treatment was found only in rat kidney (1.5-fold, $p < 0.001$) (Fig. 2B, lower panel).

3.3. AAI-DNA-adduct formation mediated by mouse and rat cytosols

The efficiencies of hepatic, renal and pulmonary cytosols from AAI-treated mice and rats to activate AAI generating DNA adducts were compared with those from untreated animals (Fig. 3). AAI was metabolically activated in hepatic cytosolic samples of mice and rats as well as renal and pulmonary cytosols of rats only. Always the same pattern of AAI-DNA adducts was generated, consisting of three DNA adduct spots (see Fig. 1, insert). The same DNA adduct pattern by ^{32}P-postlabelling has been observed in renal tissue from AAN and BEN patients [4,10,11,14,15] and adducts were previously identified [2,11] as 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI) and 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII) (Fig. 1). No DNA adducts were observed in control incubations carried out in parallel without cytosols, without DNA, or without AAI (data not shown).

Similar levels of AAI-DNA adducts were formed during *ex vivo* incubations with hepatic cytosols of mice and rats with AAI and DNA, and also with rat renal cytosols. Adduct levels practically corresponded with the NQO1 activity in these cytosolic samples (compare Figs. 2 and 3). However, even though the highest NQO1 activity in rats was found in lung cytosols (see Fig. 2B, lower panel), the lowest levels of AAI-DNA adducts were formed in these lung samples (Fig. 3B). Likewise, only background levels of AAI-DNA adducts were found in incubations containing mouse pulmonary and renal cytosols (not quantified).

Higher AAI-DNA adduct levels (>1.7-fold, $p < 0.01$) were formed in hepatic cytosols from AAI-treated mice and rats compared with those isolated from untreated animals (Fig. 3). A 2.5-fold ($p < 0.001$) increase in AAI-DNA adduct formation was also found in renal cytosol isolated from AAI-treated rats compared with control (untreated) animals (Fig. 3B), while no effect of AAI treatment on DNA-adduct formation was found in lung cytosol. The higher AAI-DNA adduct levels in mouse-liver and rat-kidney cytosols

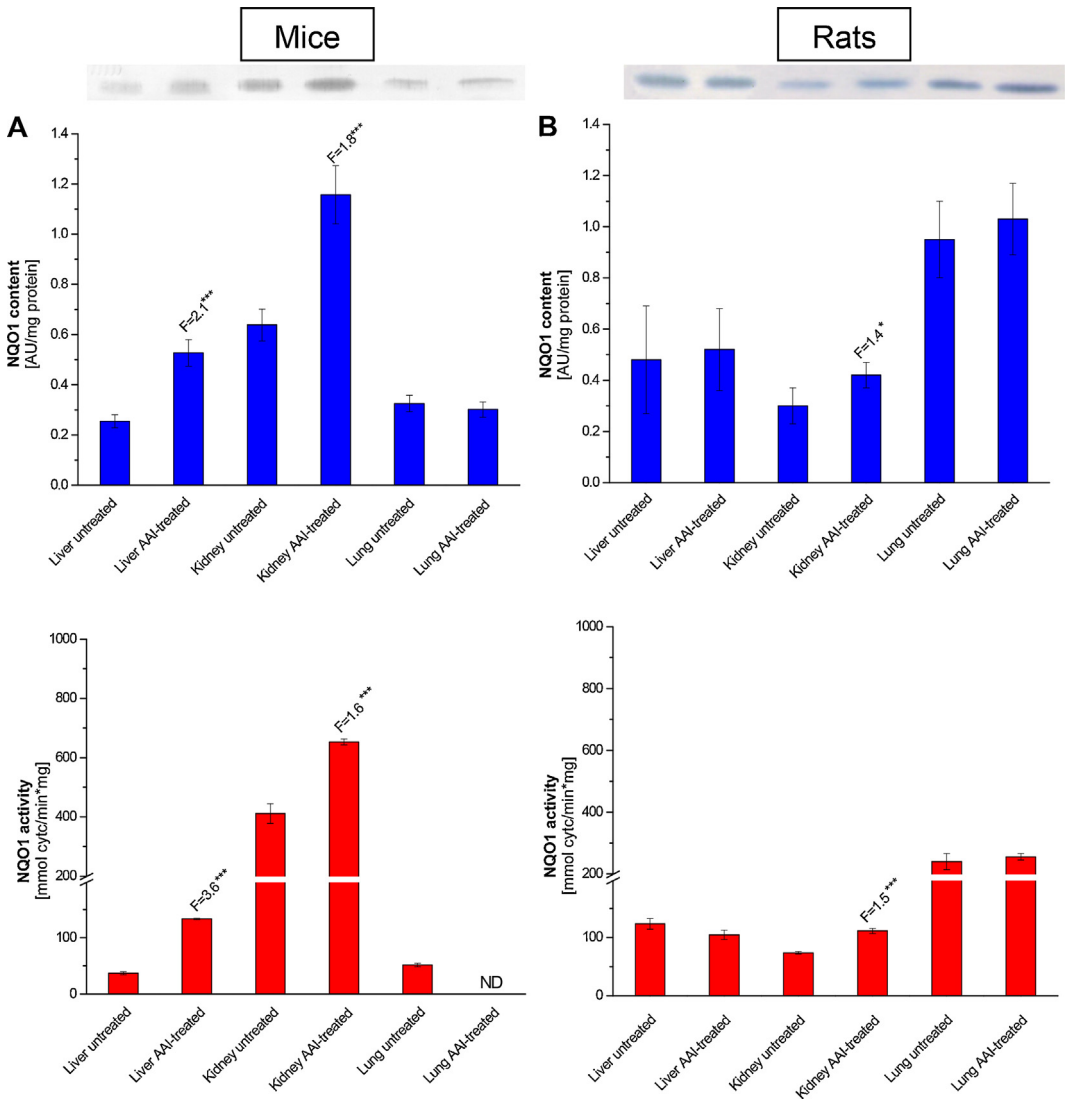

Fig. 2. NQO1-protein expression (blue columns) and NQO1-enzyme activity (red columns) in cytosols isolated from liver, kidney and lung of mice (A) and rats (B). Mice were treated with a single oral dose of 50 mg/kg body weight AAI or left untreated (control). Rats were treated with a single *i.p.* dose of 20 mg/kg body weight AAI or left untreated (control). NQO1-protein expression in cytosols (upper panel) was determined by Western blotting (see insert). Human recombinant NQO1 was used to identify the mouse and rat NQO1 band in murine and rat cytosols, respectively (data not shown). NQO1-enzyme activity in cytosols (lower panel) was determined as described in chapter 2.4. All values are given as means ± S.E.D. ($n = 4$). ND, not detected. Comparison was performed by *t*-test analysis; $^{*}p < 0.05$, $^{***}p < 0.001$, different from control.

corresponded with higher cytosolic NQO1-protein levels in these cytosolic samples (compare Figs. 2 and 3). However, the increase in adduct levels in rat hepatic cytosol (>1.7-fold) was higher than the observed change in NQO1-protein expression (1.1-fold) and NQO1-enzyme activity in hepatic cytosol was even not increased by AAI-treatment of rats (Figs. 2 and 3). We can only speculate to explain this finding, but it is possible that another rat hepatic nitroreductase such as xanthine oxidase, which is also able to activate AAI [21–23], may be responsible for this phenomenon.

4. Discussion

Previously we found that AAI, in the presence of NADPH (a cofactor of NQO1), is activated by cytosolic fractions of human liver and kidney, as well as by purified human NQO1, to DNA adducts identical to those found in humans diagnosed with AAN and BEN [21–23,25,26]. These results suggested that NQO1 may be the principal enzyme responsible for AAI activation. In the present study we used Western-blot analysis to determine NQO1-protein levels in cytosols from liver, kidney and lung of mice and rats, two animal species sensitive to the toxic and carcinogenic effects of AAI, and utilized in the AAN experimental protocols [2,21,22,35–38], NQO1-enzyme activity and AAI-DNA adduct levels were also determined. Furthermore, we evaluated not only basal NQO1 expression, but also NQO1 expression following AAI treatment. This study is a continuation of our previous work, which investigated the expression of NQO1 in several genetically modified mouse lines [38] and aims to enhance our understanding on the role of NQO1 in AAI bio-activation.

Our results demonstrate that expression of NQO1 protein and enzyme activity was species- and organ-specific. In mice, cytosolic NQO1-protein levels and enzyme activity were highest in kidney, which is in line with the nephrotoxic properties of AA. In rats, however, the highest levels of NQO1 protein and NQO1 activity were found in lung, whereas they were the lowest in kidney.

Our study also indicates that treatment with AAI enhanced NQO1-protein levels and enzyme activity in mouse-liver and mouse-kidney cytosol and in rat-kidney cytosol. We found that increased NQO1-protein levels in kidney and liver correlated with

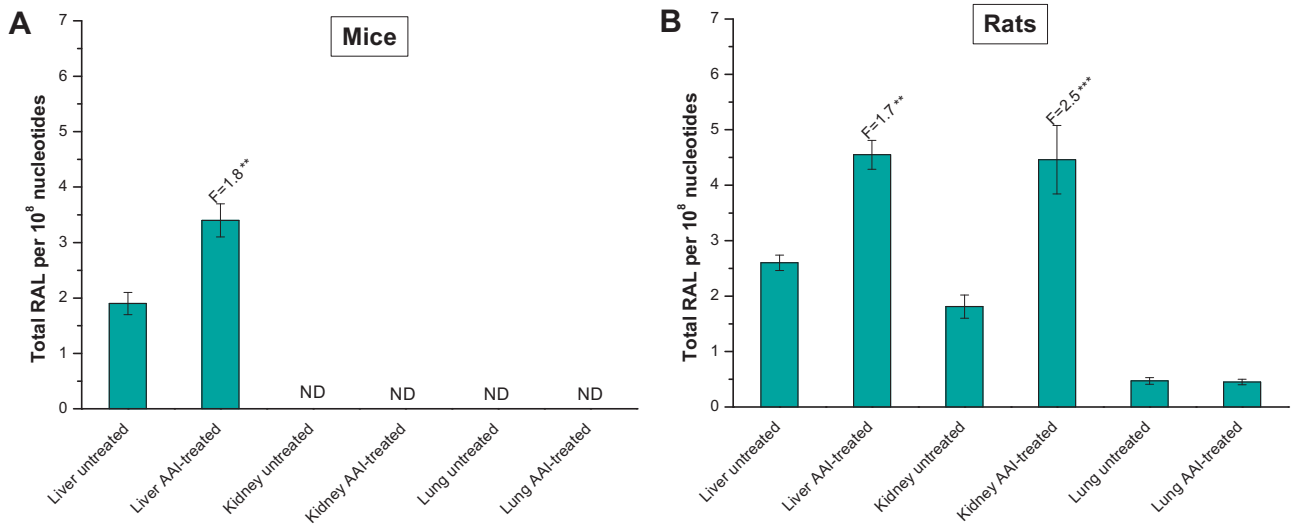

Fig. 3. DNA-adduct formation *ex vivo* by AAI in mouse (A) and rat cytosols (B) determined by ^{32}P-postlabelling. Mice were treated with a single oral dose of 50 mg/kg body weight AAI or left untreated (control). Rats were treated with a single *i.p.* dose of 20 mg/kg body weight AAI or left untreated (control). Values are given as the means ± S.E.D. ($n = 4$). RAL, relative adduct labeling. ND, not determined (*i.e.*, levels of AAI-DNA adducts were at the detection limit). Comparison was performed by *t*-test analysis; $^{**}p < 0.01$, $^{***}p < 0.001$, different from control.

NQO1-enzyme activity in these two organs. However, only in mouse-liver cytosol, and in cytosol of rat kidney, the increase in NQO1-protein levels also paralleled higher levels of AAI-DNA adducts formed in *ex vivo* incubations of AAI and DNA with these cytosols. In contrast, the increase in AAI-DNA adduct levels formed in *ex vivo* incubations of rat hepatic cytosol with AAI was higher than the elevated NQO1 protein expression, and the NQO1 enzyme activity in this cytosol was not even increased by treatment of rats with AAI. In this context, it is noteworthy that previous studies have shown that xanthine oxidase is capable of activating AAI [21,22]. Thus, only the AAI-mediated induction of NQO1 in mouse liver and rat kidney enhances the reductive bio-activation of AAI to form DNA adducts, thereby enhancing its own genotoxic potential in these organs.

The near absence of significant formation of AAI-DNA adducts detected after *ex vivo* incubation of AAI with mouse kidney and lung cytosolic fractions contrasted with results of corresponding experiments with rat systems. Moreover, the situation in mouse lung is completely different from that in the other two mouse organs investigated and from that in rat lung. Basal and induced NQO1-protein levels in mouse lung, as measured by Western blotting, were similar to that in liver, but NQO1-enzyme activity was not detectable in lung after AAI treatment. Several reasons for this observation and for the low AAI-DNA adduct formation in *ex vivo* incubation of AAI with mouse lung and kidney cytosols are possible: (i) the NQO1 protein expressed in lung is inactive; (ii) menadione and AAI are not good substrates for lung NQO1; (iii) an as-yet unknown inhibitor is present in mouse lung and kidney cytosol, thereby decreasing reduction of menadione and/or AAI; or (iv) pulmonary and renal NQO1 proteins have undergone allosteric effects due to interactions with different substrates including menadione or AAI. Which of these reasons is most important in our experiments is difficult to estimate, but it is noticeable that no NQO1-enzyme activity was seen in the mouse lung after AAI treatment [38]. Further, also in rat-lung cytosols some discrepancies in NQO1 expression and its activity remain to be explained. Even though NQO1-protein expression in lung cytosols both from untreated as well as AAI-treated rats correlated well with NQO1-enzyme activity, low efficiencies of these lung cytosols to catalyze AAI-induced DNA-adduct formation were found.

The differences in NQO1-protein expression, its enzyme activity and reductive activation of AAI in individual test organs of mice and rats suggest potentially different metabolism, nephrotoxicity and carcinogenicity patterns of AAI in these animal models. This suggestion is supported by several former studies: different responses of mice and rats to AAI to mediate development of interstitial nephropathy and associated cancer of the upper urinary tract, as well as biotransformation of AAI were found previously [22,24,36,44–50].

It is noteworthy that the increase in NQO1-protein expression and enzyme activity due to AAI treatment was lower in rats than in mice. This is likely caused by the different dosing protocols used for AAI treatment in rats and mice; not only the administered dose of AAI was different (20 *vs* 50 mg/kg bw in rat and mice) but also the route of AAI administration (*i.p.* in rats *vs* gavage in mice). As the exposure route was different for the two animal models, the toxicokinetics are most probably different between the models and a direct comparison is thus not possible.

The highest levels of protein and enzyme activity of NQO1 in mouse kidney, and the efficient induction of this enzyme by AAI in this organ are consistent with the finding that AAI-DNA adduct formation was the highest in mouse kidney *in vivo* [27,29,30]. In other words, AAI can induce NQO1 in kidney, which is the target organ of AAI-induced toxicity, and increased DNA-adduct formation contributes to the observed toxicity in this organ, which is less pronounced in other organs of mice exposed to AAI [27,29,30]. The stimulating effects of AAI treatment on NQO1 induction found here in mice and rats also confirmed results of previous studies [38,51,52]. Therefore, this enzyme is likely to be induced in the kidneys of AAN and BEN patients, which may contribute to their increased risk for urothelial cancer.

Besides NQO1, the CYP1A1/2 enzymes were also found to be involved in AAI metabolism [27–33]. By use of *Cyp1a*-knock-out (single and double knock-outs) and *CYP1A*-humanized mouse lines, the crucial role of CYP1A1 and 1A2 enzymes in AAI metabolism *in vivo* was unambiguously proven [29,30,36]. Human and rodent CYP1A1 and 1A2 play a dual role in the metabolism of AAI. Under anaerobic conditions they reductively activate AAI, while under oxidative conditions they are the predominant enzymes catalyzing *O*-demethylation of AAI to AAIa (*i.e.* detoxification). This AAI oxidation finally leads to a decrease in AAI-induced renal injury. Based on

current knowledge we propose that AAI metabolism *in vivo* is determined by the binding affinity of AAI to CYP1A1/2 or NQO1, by their enzymatic turnover, as well as by the oxygen levels in the organs [53]. However, the extent to which these enzymes contribute to AAI-mediated nephropathy and upper urothelial tract carcinoma in humans is still a matter of debate, and remains to be investigated. Unfortunately, studies investigating a possible association between genetic polymorphisms of enzymes metabolizing AAI with the risk of developing AAN/BEN and/or upper urothelial tract carcinoma have led to controversial results. It was reported that polymorphisms in the human *NQO1* gene are important in AA-induced nephropathy [54,55]. The *NQO1*2/*2* genotype (*NQO1 C609T* polymorphism), resulting in very low NQO1-expression levels, has been shown to predispose BEN patients to a much higher incidence of urothelial cancer (OR = 13.75; 95% CI, 1.17–166.21) [54,55]. This finding appears to be opposite to what one might expect, given our demonstration herein of the importance of NQO1 in AAI activation; however, diminished NQO1 metabolism of AAI could lead to an enhanced body burden which might lead to increased risk of tumorigenesis over time in BEN patients [38]. However, no significant association was found between this *NQO1 C609T* polymorphism and the risk of developing AAN [56]. This discrepancy shows that the developments of the nephropathies (AAN and BEN) and upper urothelial tract carcinoma by AAI seem to follow different paths. Maybe AAI as such is nephrotoxic, but reductive activation, *e.g.* catalyzed by NQO1 is needed for genotoxicity leading to cancer.

In contrast to NQO1, even though the *CYP1A1* and *CYP1A2* genes are also polymorphic [57–60], no relationships between the polymorphisms of CYP1A1 and AA-induced nephropathy were found in AAN/BEN patients [55,56] and the changes in the *CYP1A2* gene have not been investigated yet.

5. Conclusions

By use of Western-blot analysis, NQO1-protein levels were analyzed in liver, kidney and lung of untreated and AAI-treated mice and rats. Our study demonstrated that AAI has the potential to induce the activity of the cytosolic nitroreductase NQO1 in liver and kidney in both animal models. Our studies and the findings of others [54,55] indicate that certain *NQO1* genotypes appear to be linked to an increased risk of urothelial cancer in BEN patients, underscoring the potential clinical importance of NQO1 activity in AAI-exposed humans. However, because studies evaluating an association of genetic polymorphisms of the enzymes metabolizing AAI with a risk of developing AAN, BEN and upper urinary tract urothelial carcinoma have provided contradictory results, another approach should be utilized to evaluate the contribution of the enzymes metabolizing AAI in these processes. We propose that analyses of the expression levels of these enzymes (NQO1, CYP1A1 and 1A2) and their phenotyping in AAN and BEN patients should bring more valuable data on their real contribution to the development of AA-induced nephropathies and cancer risk among these patients.

Conflict of interests statement

The authors declare that there are no conflicts of interests in relation to this work.

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International Journal of
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A Mechanism of *O*-Demethylation of Aristolochic Acid I by Cytochromes P450 and Their Contributions to This Reaction in Human and Rat Livers: Experimental and Theoretical Approaches

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Received: 14 October 2015 ; Accepted: 5 November 2015 ; Published: 18 November 2015
Academic Editor: Ute Roessner

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Abstract: Aristolochic acid I (AAI) is a plant alkaloid causing aristolochic acid nephropathy, Balkan endemic nephropathy and their associated urothelial malignancies. AAI is detoxified by cytochrome P450 (CYP)-mediated *O*-demethylation to 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIa). We previously investigated the efficiencies of human and rat CYPs in the presence of two other components of the mixed-functions-oxidase system, NADPH:CYP oxidoreductase and cytochrome b_5, to oxidize AAI. Human and rat CYP1A are the major enzymes oxidizing AAI. Other CYPs such as CYP2C, 3A4, 2D6, 2E1, and 1B1, also form AAIa, but with much lower efficiency than CYP1A. Based on velocities of AAIa formation by examined CYPs and their expression levels in human and rat livers, here we determined the contributions of individual CYPs to AAI oxidation in these organs. Human CYP1A2 followed by CYP2C9, 3A4 and 1A1 were the major enzymes contributing to AAI oxidation in human liver, while CYP2C and 1A were most important in rat liver. We employed flexible *in silico* docking methods to explain the differences in AAI oxidation in the liver by human CYP1A1, 1A2, 2C9, and 3A4, the enzymes that all *O*-demethylate AAI, but with different effectiveness. We found that the binding orientations of the methoxy group of AAI in binding centers of the CYP enzymes and the energies of AAI binding to the CYP active sites dictate the efficiency of AAI oxidation. Our results indicate that utilization of experimental and theoretical methods is an appropriate study design to examine the CYP-catalyzed reaction mechanisms of AAI oxidation and contributions of human hepatic CYPs to this metabolism.

Keywords: plant nephrotoxin and carcinogen aristolochic acid I; cytochrome P450-mediated detoxification of aristolochic acid I; contribution of cytochromes P450 in detoxification of aristolochic acid I in human and rat livers; molecular modeling

1. Introduction

Aristolochic acid (AA) is an herbal drug prepared from plants of the *Aristolochia* genus, where two alkaloids aristolochic acid I (AAI) (Figure 1) and AAII are the predominant chemical components [1]. Over twenty years ago, AA was shown to be the cause of a unique kidney disease Chinese herbs nephropathy (CHN), which is now assigned as aristolochic acid nephropathy (AAN) (reviewed in [1–3]). This specific renal fibrosis is associated with development of upper urothelial tract carcinoma (UUC) and, finally, bladder urothelial carcinoma [3–6]. AA is a Group I carcinogen as declared by the International Agency for Research on Cancer [7]. This plant alkaloid is also considered to participate in development of another kidney disease, Balkan endemic nephropathy (BEN), and its associated urothelial malignancy [8,9]. This disease is endemic in certain rural areas of Balkan countries which are localized closed to the tributaries of the Danube river basin [10].

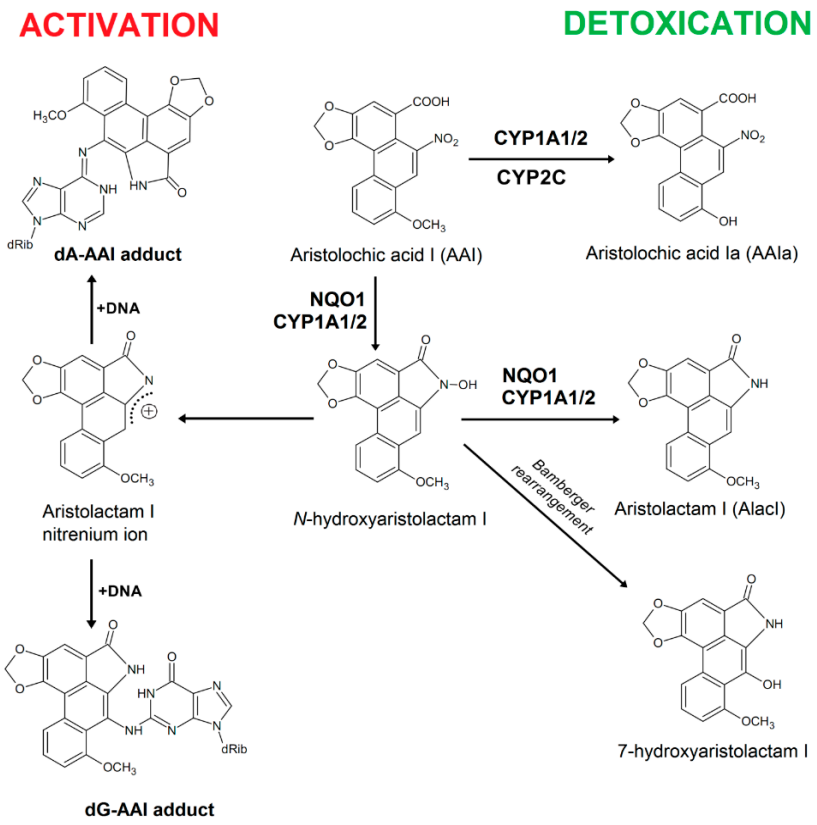

Figure 1. Activation and detoxification pathways of AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; CYP1A1/2, cytochrome P450 1A1 and 1A2; CYP2C, cytochromes P450 of the 2C subfamily; and NQO1, NAD(P)H:quinone oxidoreductase.

In contrast to the findings that AAI might directly cause interstitial nephropathy, enzymatic activation of AAI to intermediates that bind to DNA is a necessary reaction leading to AA-mediated malignant transformation [8,9,11–15]. Indeed, exposure to AA has been proven by the detection of unique DNA adducts formed by AA in many tissues of patients suffering from AAN and BEN [9,10,16–18]. Specific AA-DNA adducts found in kidneys of patients are considered as biomarkers of exposure to AA; among them the 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) adduct is formed most frequently and is the long persistent adduct [1,5,10,18,19]. This DNA lesion produces specific A to T transversion mutations that were detected in the *TP53* tumor suppressor gene in tumors of patients suffering from AAN and BEN [8,9,20] and in immortalized Hupki (human *TP53* knock-in) mouse fibroblast cells (HUFs) treated with AAI [21], suggesting a molecular mechanism of

AA-induced carcinogenic processes [8,22]. Interestingly, these A to T transversions have also been detected in other loci by whole-genome and exome sequencing analyzing AA-mediated UUC and AAI-exposed HUFs [23–26].

Nitroreduction of AAI, the compound that is considered as the major factor causing the AAN and BEN development, is required to exert its carcinogenic properties (*i.e.*, UUC development). Such nitroreduction results to the generation of *N*-hydroxyaristolactam I that leads to the formation of a cyclic acylnitrenium ion, the intermediate that either form DNA adducts or rearranges to 7-OH-aristolactam I (Figure 1) [2]. The product of AAI oxidation, 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIa), is a detoxification metabolite of this carcinogen. It is generated by *O*-demethylation of the methoxy group of AAI, and is excreted from organisms either in its free or conjugated forms [27–30] (Figure 1).

The concentration of AAI in organisms is crucial for both renal injury and induction of malignant transformations initiated by activated AAI. In addition to the quantities of AAI ingested by organisms, conversion of this chemical determines its actual concentration, thereby modulating also the clinical consequences of exposure. Hence, the characterization of enzymes that are mainly responsible for both detoxification and activation of AAI in humans as well as characterization of efficiencies of these enzymes in these reactions is of major importance.

Various enzymes metabolize AAI. Many studies demonstrated that NAD(P)H:quinone oxidoreductase (NQO1) acts as one of the most effective cytoplasmic nitroreductases reducing AAI to a cyclic acylnitrenium intermediate forming adducts in DNA [12–15,31–37]. In human and rodent liver microsomes AAI is reductively activated by cytochrome P450 (CYP) 1A2 and, to a lesser extent, by CYP1A1. Microsomal NADPH:CYP oxidoreductase (POR) also activates AAI in these organs [12,15,29,30,34,35,38–45]. However, CYP1A1 and 1A2 play a dual role in the metabolism of AAI. Whereas under anaerobic conditions they reductively activate AAI, under aerobic conditions these enzymes catalyze *O*-demethylation of the methoxy group of AAI forming AAIa (*i.e.*, detoxification) [12–15,29,30,34,38–43,46,47]. Recent studies have indicated that human and rodent CYPs of the 1A subfamily are the major enzymes oxidizing AAI under aerobic (*i.e.*, oxidative) conditions *in vitro* and *in vivo* (reviewed in [14,15]). Besides CYP1A/2, human and rat CYPs of the 2C subfamily also oxidize AAI [30,43,47,48] (Figure 1). The CYP-mediated AAI oxidation results to a decrease in AAI-mediated kidney injury [49,50].

However, there is still little information available about the impact of individual CYP enzymes on the oxidative AAI detoxification to AAIa in liver, the major organ for xenobiotic metabolism including AAI [30] in humans or animal models. Therefore, we evaluated contribution of individual CYP enzymes expressed in human liver to AAIa formation and compared it with that of CYPs expressed in livers of several animal models including rats that might, to some extent, mimic the fate of AAI in humans [35,48,51–56]. In order to compare efficiencies of hepatic microsomes of several species to oxidize AAI to AAIa, we previously analyzed generation of AAIa by human, rat, mouse, and rabbit liver microsomes [30,43]. The subcellular fractions from livers of all tested species were capable of catalyzing the oxidation of AAI to AAIa. The reaction was dependent on NADPH, a cofactor of POR-mediated CYP catalysis; without NADPH no oxidation of AAI was found. These results indicated that AAI oxidation by hepatic microsomes is mediated by CYP enzymes. Among the used microsomes, human and rat hepatic microsomes produced the most similar amounts of AAIa [30,43], indicating that rat hepatic microsomes seem to be an appropriate model to mimic oxidation of AAI in human hepatic microsomes. Therefore, human and rat enzymatic systems were utilized in this study.

2. Results and Discussion

To identify contributions of individual hepatic CYPs to AAI oxidation, three approaches were employed: (i) use of selective CYP enzyme inhibition in human and rat microsomes; (ii) use of human and rat recombinant CYPs; and (iii) analysis of the data on formation of AAIa by individual human

and rat CYPs in these systems as well as those on the CYP enzyme expression levels in human and rat livers. Molecular modeling capable of evaluating interactions of AAI with the binding center of human CYPs was utilized to identify the molecular mechanisms of AAI *O*-demethylation catalyzed by human CYP enzymes that *O*-demethylate AAI in human liver.

2.1. Effect of CYP Enzyme Inhibitors on AAI O-Demethylation Catalyzed by Human and Rat Hepatic Microsomes

Under the experimental conditions used the individual CYP inhibitors were used in equimolar concentrations to that of AAI (10 μM). As shown in Table 1 and in our previous study [30], we found that AAIa formation in human microsomes was inhibited by α-napththoflavone, an inhibitor of CYP1A1/2, furafylline, an inhibitor of CYP1A2, and ketoconazole, an inhibitor of CYP3A, while inhibitors of other CYPs such as diamantane, an inhibitor of CYP2B, sulfaphenazole, an inhibitor of CYP2C, quinidine, an inhibitor of CYP2D, and diethyldithicarbamate (DDTC), an inhibitor of CYP2A and 2E1, were ineffective. In the present study we show that the same compounds inhibit AAIa formation also in rat hepatic microsomes, but in rat microsomes sulfaphenazole and DDTC also significantly decreased AAI oxidation (Table 1). The latter findings confirm that AAIa formation is catalyzed by hepatic CYP enzymes of both species and that human and rat CYP1A and 3A enzymes besides rat CYP2C, 2A, and 2E1 might be effective in the AAI *O*-demethylation reaction. These results also demonstrate a relatively weak potency of the CYP inhibitors under equimolar concentrations to that of AAI suggesting a high binding affinity of AAI to these CYP enzymes. However, although human and rat livers contained various CYPs, some of them are present in this human organ at limited amounts (*i.e.*, CYP1A1, 1B1, and 2B) [57,58]. Hence, this phenomenon may affect the degree of enzyme inhibition. Furthermore, it is important to mention that data found with inhibitors are sometimes difficult to be interpreted. Namely, the inhibitor can act more efficiently with one enzyme substrate than another.

Table 1. Effects of cytochrome P450 (CYP) inhibitors on AAI oxidation to aristolochic acid Ia (AAIa) by human and rat liver microsomes.

Inhibitor [a]	AAIa Formation (% of Control without Inhibitor)	
	Human Microsomes	Rat Microsomes
α-Napththoflavone (CYP1A1/2)	89 ± 5 *	85 ± 5 **
Furafylline (CYP1A2)	75 ± 4 **	84 ± 5 **
Diamantane (CYP2B)	NI [b]	NI
Sulfaphenazole (CYP2C)	NI	68 ± 3 ***
Quinidine (CYP2D)	NI	NI
DDTC (CYP2A, CYP2E1)	96 ± 5	52 ± 4 ***
Ketoconazole (CYP3A)	74 ± 4 **	90 ± 4 *

[a] CYPs for compounds that act as their specific inhibitors are in brackets. Equimolar concentrations of individual inhibitors and AAI (10 μM) and 0.1 nmol of CYP were in incubation mixtures. The data are the mean ± SD of three parallel measurements (n = 3); [b] NI, no inhibition; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, statistically different from data of controls, without inhibitors (Student t-test).

2.2. O-Demethylation of AAI to AAIa by Human and Rat Recombinant CYPs in Supersomes™

In former studies of our laboratory, we examined the activity of individual human and rat CYPs to oxidize AAI to its *O*-demethylation metabolite utilizing recombinant enzymes heterologously expressed in microsomal fractions of baculovirus-infected insect cells (Supersomes™) (Gentest Corp., Woburn, MI, USA) in combination with their reductase, POR [30,43]. However, this CYP system is not optimally corresponding to the natural microsomal system. In order to better mimic the situation in hepatic microsomes, individual CYPs were not only expressed with POR, but also with cytochrome b_5, a known modulator of enzymatic activity of several CYPs [59–72]. We previously analyzed the efficiencies of human and rat CYP enzymes [30,41] to oxidize AAI to AAIa in the presence of

this microsomal protein (Figure 2). In the experimental systems used, cytochrome b_5 was either expressed in Supersomes™ together with CYPs and POR or Supersomes™ were reconstituted with purified cytochrome b_5. Comparison of activities of individual CYPs to oxidize AAI to AAIa in the presence of cytochrome b_5 with those determined previously without this microsomal protein [30,43] indicated that cytochrome b_5 influences AAIa formation catalyzed by several CYPs. The strongest effect of cytochrome b_5 was found on this reaction catalyzed by rat CYP1A1 and 1A2. The addition of cytochrome b_5 to the incubation mixtures decreased AAIa formation catalyzed by rat CYP1A1, up to 50% relative to control ($p < 0.001$), and increased this reaction catalyzed by CYP1A2, by 1.2-fold ($p < 0.01$) (compare Figure 6 in [43] and data shown in Figure 2B). The potency of human CYP3A4 to oxidize AAI was also increased, by 1.4-times ($p < 0.01$) by cytochrome b_5. In addition, an increase in AAIa formation catalyzed by CYP1A2 and 2C9 by cytochrome b_5 was also found, but this increase was not significant [30].

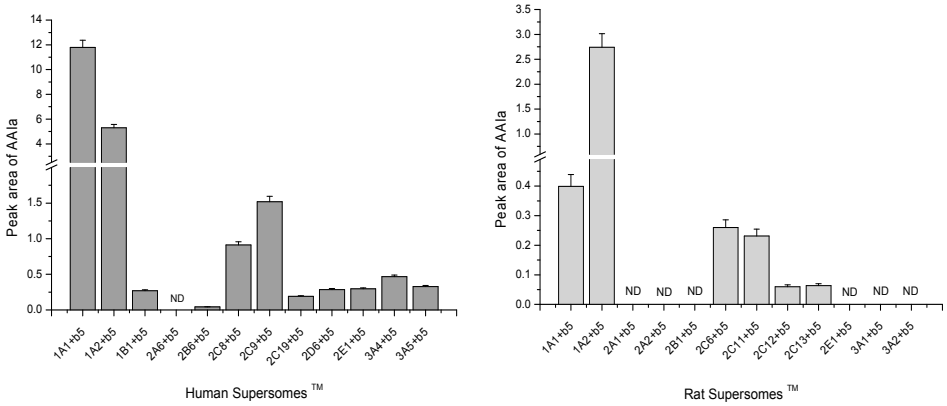

Figure 2. AAI *O*-demethylation to AAIa catalyzed with Supersomes™, each with a different human recombinant CYP (**A**) and rat recombinant CYP; (**B**) having these CYPs in combination with cytochrome b_5 (b_5). Data are averages ± SD of three parallel measurements ($n = 3$). ND, not detected. Data previously published in [30,41,43].

Our results demonstrate that under the presence of the microsomal cytochrome b_5 human CYPs are more effective in AAI oxidation than their rat orthologs. Human and rat CYP1A enzymes are the major enzymes oxidizing AAI. Other CYPs such as human and rat CYPs of the 2C subfamily and human CYP3A (CYP3A4/5), 2D6, 2E1, and 1B1, also form AAIa, but with much lower efficiency than CYP1A (Figure 2). For example, 7.9- and 3.5-times higher levels of AAIa were formed by human CYP1A1 and 1A2 than by the most efficient CYP enzymes of the 2C subfamily (*i.e.*, human CYP2C9), respectively. Likewise, human CYP1A1 and 1A2 were more than 13- and 5.8-fold more effective to oxidize AAI than another member of the CYP2C subfamily, human CYP2C8, respectively (Figure 2). Only rat CYP1A and 2C enzymes oxidize AAI of which CYP1A enzymes are more active than CYP2C enzymes (Figure 2B).

It should be emphasized that human/rat CYP1A1 and 1A2 orthologs show species-species differences in AAI preference, reaction velocities of its oxidation and the effects of cytochrome b_5. Human CYP1A1 was found to be more effective to *O*-demethylate AAI than human CYP1A2, whereas rat CYP1A2 oxidizes this compound more efficiently than rat CYP1A1 (Figure 2).

2.3. Contributions of Individual CYPs to AAIa Formation in Human and Rat Livers

Employing the results showing the velocities of AAI oxidation to AAIa by the Supersomal CYP enzyme systems containing cytochrome b_5 (Figure 2) and the amounts of CYP enzymes expressed in human and rat livers [57,58,73–83], the contributions of individual CYPs to AAI oxidation in

human and rat liver microsomes were evaluated. The highest contribution to AAI oxidation in human liver is attributed to CYP1A2 (~47.5%), followed by CYP2C9 (~15.8%), CYP3A4 (~10.5%), and CY1A1 (~8.3%). Even though the activity of human recombinant CYP1A1 to oxidize AAI is highest among all tested human CYPs (see Figure 2A), the low amounts of CYP1A1 in human livers (<0.7%) [75,83–85] caused that its contribution to the reaction in this human organ is lower than contributions of CYP1A2, 2C9 and 3A4 (Figure 3A). Of the other CYPs, CYP2E1, 2C8, and 2C19 also partially contributed to AAI oxidation, but only by ~1.1%, ~1.0% and ~0.6%, respectively. Contributions of other human CYPs (CYP1B1, 2B6, 2D6, and 3A5) in AAI oxidation in human livers are negligible.

In rat liver the highest contribution to AAI oxidation to AAIa is attributed to the CYP2C subfamily (~83%), mainly to CYP2C11 (~42%) and CYP2C6 (~17%), followed by CYP1A subfamily (~17%) (Figure 3B). Since the level of CYP1A1 expression in rat liver is around 10-fold lower than that of CYP1A2, the contribution of this CYP to AAI oxidation in rat liver is negligible (~1.7%).

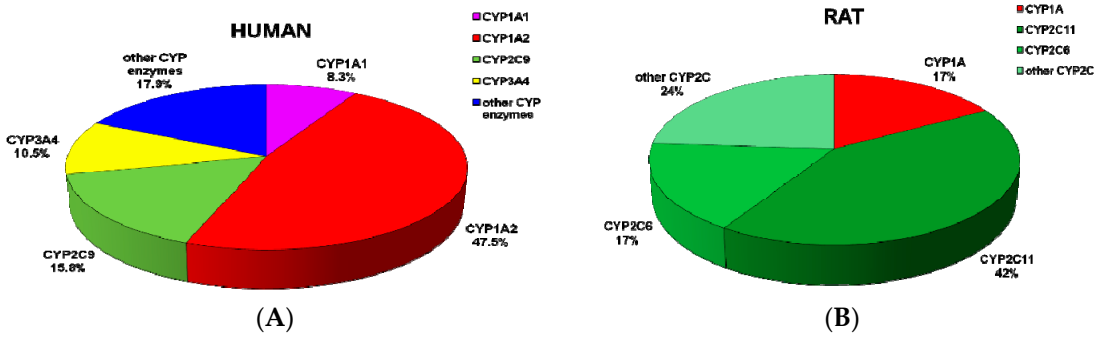

Figure 3. Contributions of CYP enzymes to AAIa formation in human (**A**); and rat livers (**B**).

2.4. Binding of AAI to the Active Sites of Compounds I of Human CYP1A1, 1A2, 2C9, and 3A4

O-Demethylation of AAI proceeds via the CYP-mediated attack of the carbon atom of the methoxy group by oxygen, which leads to the formation of the α-C-hydroxylation product that additionally decomposes to formaldehyde and AAIa (Figure 4). Therefore, the binding orientation of the methoxy group of AAI in the binary complex of AAI with the CYP active sites, which is a prerequisite process for *O*-demethylation of AAI, should dictate the efficiency of individual CYPs to catalyze this reaction. Thus, differences among abilities of the CYP enzymes to *O*-demethylate AAI (Figure 2) might be caused by the affinities of AAI to these enzymes and the binding orientation of the methoxy group of this compound in their active sites.

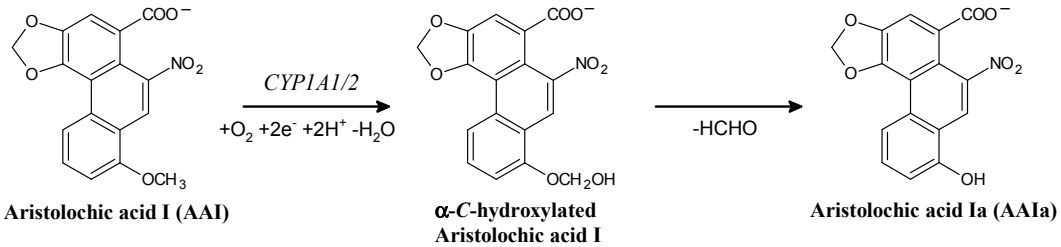

Figure 4. The CYP-mediated *O*-demethylation of AAI to AAIa.

In order to investigate this suggestion, molecular modeling (*in silico* docking, employing soft-soft, flexible, docking procedure [84]) was used in this study. Using this method, we investigated binding of AAI to the active site of the Compounds I of human CYP1A1, 1A2, 2C9, and 3A4, the CYPs that all *O*-demethylate AAI but with different effectiveness and contribute efficiently to this reaction

in human liver (Figure 5). The AAI molecule was successfully docked into the active sites of these CYPs. Every docking predicted several binding orientations of the AAI molecule. Positions showing short distances (3.7–4.9 Å) between the methoxy group of AAI and the activated oxygen atom in Compounds I of CYPs was found for all the CYP enzymes examined (Figure 5, Table 2). However, CYP2C9 and 3A4 enzymes are predicted to bind the AAI molecule with a significantly lower affinity than CYPs of the 1A subfamily (see values of free energies of binding shown in Table 2). The predicted binding free energy of AAI to human CYP1A1 is slightly lower than that to CYP1A2, but the distance between the carbon in the methoxy group of AAI and the oxygen atom on heme iron in the binary complex of CYP1A1 with AAI is shorter by 0.5 Å (Table 2). The larger distance of the reacting groups would result in a decreased reaction rate during CYP1A2-catalyzed AAI demethylation. Collectively, these results provide a suggestion why CYP1A1 and 1A2 are most efficient in AAI oxidation, while other CYPs (CYP2C9 and 3A4) are less active to catalyze this reaction (Figure 2A).

Table 2. The predicted binding free energies and distances facilitating *O*-demethylation of AAI bound in selected CYPs complexes.

Simulated System	The Most Stable Productive Orientations of AAI in the Complex with CYP	
	Estimated Free Energy of Binding (kcal/mol)	O(Comp I)-OCH_3 (AAI) Distance [Å] [a]
CYP1A1	−7.0	4.4
CYP1A2	−7.7	4.9
CYP2C9	−5.3	4.3
CYP3A4	−6.0	3.7

[a] Distance between the carbon in the methoxy group of AAI and oxygen atom on heme iron in the complex of an activated CYP enzyme (Compound I) with AAI, see Figure 5.

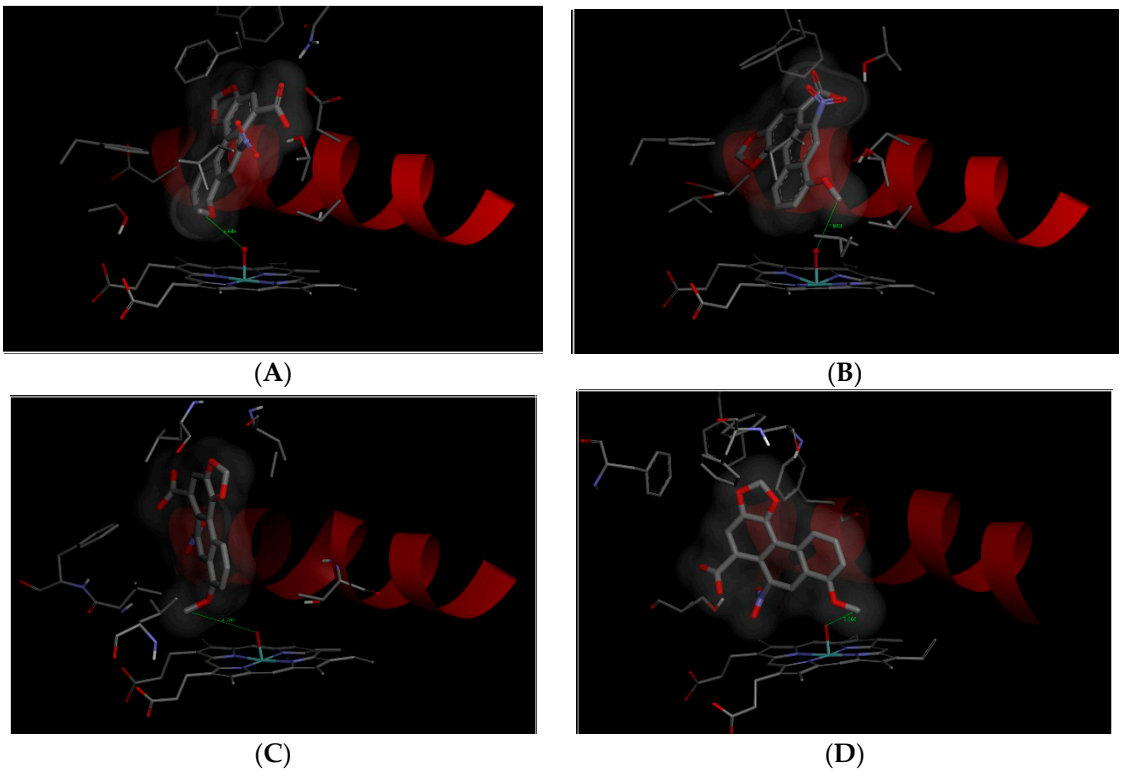

Figure 5. The binding orientations found in molecular docking calculations facilitating *O*-demethylation of AAI bound in human CYP1A1 (**A**); CYP1A2 (**B**); CYP2C9 (**C**); and CYP3A4 (**D**). AAI, heme and amino acids residues interacting ligand are rendered as bold sticks and sticks, respectively. Red ribbon represents a part of the I helix.

3. Experimental Section

3.1. Supersomes™

Microsomes (Supersomes™) prepared from insect cells transfected with baculovirus constructs containing cDNA of single human CYP (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) or their rat orthologs (CYP1A1, 1A2, 2A1, 2A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1, and 3A2), and expressing POR and cytochrome b_5 were purchased from Gentest Corp. (Woburn, MI, USA). Supersomes™ containing rat CYP1A1/2 were reconstituted with cytochrome b_5 (CYP: cytochrome b_5, 1:5) isolated from rat liver microsomes by the procedures as described [80,85,86].

3.2. Preparation of Rat Hepatic Microsomes

Microsomal fractions were prepared from livers of ten male Wistar rats (AnLab, Prague, Czech Republic) by differential centrifugation as described previously [87].

3.3. Microsomal Incubations to Study AAI O-Demethylation

Incubation mixtures (250 μL) contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg human or rat liver microsomal protein and 10 μM AAI [30,43]. Incubations were performed at 37 °C for 20 min; AAI oxidation (demethylation) to AAIa was determined to be linear up to 25 min. Control incubations were performed (i) without microsomal proteins; (ii) without NADPH; or (iii) without AAI. The optimum pH of the reaction mediated by human and rat liver microsomes was found to be pH 7.4; a decrease or an increase in pH to 6.4 and 8.4 lead to up to a 1.7- or 1.9-fold decrease in AAIa oxidation, respectively. This pH was therefore used in additional experiments. For Supersomes™, incubation mixtures (final volume 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 (NADPH-generating system), 50 nM CYPs in Supersomes™, and 10 μM AAI. Supersomes™ with POR alone were utilized as controls. AAI and its metabolites (*i.e.*, AAIa) were extracted from incubation mixtures with 2 × 1 mL of ethyl acetate and evaporated to dryness; residues were dissolved in 30 μL of methanol and analyzed with reverse-phase HPLC as described [43,46].

3.4. Inhibition Studies

Inhibition studies in human and rat liver microsomes were conducted similarly as shown previously [30]. α-Naphthoflavone (α-NF), which inhibits CYP1A1 and 1A2; furafylline, which inhibits CYP1A2; diamantane, which inhibits CYP2B; sulfaphenazole, which inhibits CYP2C; quinidine, which inhibits CYP2D; diethyldithiocarbamate (DDTC), which inhibits CYP2E1 and CYP2A; and ketoconazole (KC), which inhibits CYP3A, were employed to analyze inhibition of AAI oxidation by human and rat liver microsomes. Compounds were dissolved in 2.5 μL methanol (except for DDTC that was dissolved in distilled water) to yield final concentrations of 10 μM in the incubation mixtures. Inhibitors (10 μM) were incubated at 37 °C for 10 min with the NADPH-generating system prior to addition of 10 μM AAI, and then incubated for an additional 20 min at 37 °C. AAIa formation was analyzed by HPLC as described above.

3.5. Contributions of CYP Enzymes to O-Demethylation of AAI in Human and Rat Livers

In order to calculate the contributions of individual CYPs to AAI oxidation in human and rat livers, we utilized the velocities of AAI oxidation to AAIa by the Supersomal CYP enzyme systems containing cytochrome b_5 (Figure 2) in the combination with the data on the expression levels of CYPs in human and rat livers [57,58,73,74]. The contributions of these enzymes were calculated by relative activity factor because the activities of CYP in Supersomes™ should be considered in addition to the relative contents of CYPs in the livers. Therefore, the contributions of each CYP that oxidizes AAI in

livers were calculated by dividing of the relative activity of each of such CYPs oxidizing AAI [r.a.$_{cypi}$] (rate of AAI oxidation multiplied by amounts of this CYP in tissues examined), by the total relative activities ($\sum$[r.a.$_{cypi}$]) of all CYPs oxidizing this substrate. Of human liver CYPs, CYP3A4 is the major enzyme present in this human organ (~30% of the CYP hepatic complement), followed by CYP2C9 and 1A2 (~15% and ~13%, respectively), while CYP2C19, 2E1 2A6, 2D6, 2C8, and 3A5 are present in human liver in levels of the range of ~8.5%–~2.5% of the liver CYPs (see [58] for an overview). CYPs of the 2C subfamily (CYP2C6, 2C11, 2C12, and 2C13) are the major enzymes expressed in rat livers accounting of ~55% of the CYP complement [57]. Of the CYP2C complement, ~50% and ~20% correspond to CYP2C11 and 2C6, respectively [73,74,83]. Of the other CYPs, CYP2E1, 3A, 2D, 2A, 2B, and 1A enzymes are also present in rat livers, expressed in levels of ~15%, ~10%, ~7%, ~6%, ~5%, and ~2%, respectively [57].

3.6. Molecular Docking of AAI into Compounds I of Human CYP1A1, 1A2, 2C9, and 3A4

The X-ray based coordinates of human CYP1A1 (2.6 Å resolution, PDB ID 4I8V) [88], human CYP1A2 (1.95 Å resolution, PDB ID 2HI4) [89], CYP2C9 (2.45 Å resolution, PDB ID 4NZ2), and CYP3A4 (2.74 Å resolution, PDB ID 1W0G) were used as starting structures for modeling of AAI interactions with the ground state of CYP enzymes. During structure preparation, hydrogen atoms were added and crystallographic water and ligand molecules were removed, usual protonation states and Gasteiger partial charges were assigned to all residues, except for the atomic charge of the ferric ion of the heme cofactor, for which a value more consistent with a metal in octahedral coordination was used [90]. The geometries and charges of a ligand (AAI) were predicted using *ab initio* calculations on the Hartree-Fock level of theory in conjunction with the basis set 6-31+G(d). All *ab initio* calculations were carried out with program Gaussian 03 [91].

We utilized a hybrid global-local Lamarckian genetic algorithm implemented in Autodock v4.2.6 program [84] suite to evaluate binding free energies and preferred binding modes for studied compounds. The Autodock v4 combines two procedures to find the most preferable binding modes, rapid grid-based energy evaluation and efficient search of torsional freedom, together with optional soft-soft docking. During the flexible docking procedure, both the position of the ligand and the orientations of the selected flexible side-chains are optimized simultaneously. In order to allow the enzyme to adapt to a new ligand, we ran soft-soft docking calculations. All rotatable bonds of the ligands and 10-11 selected amino acid side chains, CYP1A1 (S122, F123, N221, F224, F258, D313, D320, T321, V382, L496, T497), CYP1A2 (T124, F125, T223, F226, F260, D313, D320, T321, L382, L497, T498), CYP2C9 (V113, F114, I205, L208 T301, L366), and CYP3A4 (F108, S119, F213, F215, F241, F304) were allowed to rotate freely. We carried out an extensive search (2000 docking runs per system) of the most preferred binding modes of an AAI molecule within a 57 × 47 × 47 grid-box centered on the substrate binding cavity. Similar resulting structures (RMSD lower than 1.0 Å) were grouped and finally sorted by binding free energy of the best binding structure within each cluster. A set of binding modes with similar binding energies was found for every system as a result. We assume that only the orientations with a sufficiently short distance between carbon of the methoxy group of AAI and the activated oxygen atom in the CYP Compound I would facilitate the AAI oxidation.

3.7. Statistical Analyses

Statistical analyses were performed with Student's *t*-test and $p < 0.05$ was considered significant.

4. Conclusions

The data presented in this study advance our knowledge on the oxidative detoxification of the human carcinogen AAI by human and rat CYPs and explain the differences in efficiency of human CYP1A1, 1A2, 2C9, and 3A4 enzymes to oxidize AAI. Human and rat CYP1A1 and 1A2 are the major enzymes oxidizing AAI. Other CYPs, such as human and rat CYPs of the 2C subfamily and human CYP3A4/5, 2D6, 2E1, and 1B1 also form AAIa but with much lower efficiency than CYP1A

enzymes. Based on the amounts of AAIa formed by the tested human and rat CYP enzymes and the levels of CYP expression in human and rat livers, their contributions to AAIa formation in these organs were determined. The highest contribution to AAI oxidation in human liver is attributed to CYP1A2 (almost 50%), followed by CYP2C9, CYP3A4, and CYP1A1 (approximately 10%–15% each). In rat liver, the CYP2C subfamily contributes more than 80% to AAI oxidation, mainly CYP2C11 (roughly 40%) and CYP2C6, followed by CYP1A (nearly 20% each). The importance of these CYP enzymes to oxidize AAI in human and rat liver were confirmed by inhibition studies utilizing selective inhibitors of individual CYPs in hepatic microsomes of both species. These results are also in concordance with data found in *in vivo* studies utilizing *Cyp1a/Por*-knockout- or *CYP1A*-humanized mouse lines [15,29,30,42,43,47], as well as rat models [35,48] indicating the importance of human ad rodent CYP1A and 2C in AAI oxidation *in vivo*.

Flexible *in silico* docking modeling studies helped to understand the enzymatic differences in AAI oxidation by human CYP1A1, 1A2, 2C9, and 3A4 indicating that the binding orientations of the methoxy group of AAI in the CYP active centers and the energies of AAI binding dictate the efficiencies of these CYP enzymes in AAI oxidation. These results demonstrate that both the activities of individual human and rat CYPs and their expression levels in the liver dictate the degree of AAI detoxification in this organ. Therefore modulation of levels and activities of hepatic CYPs mediated by their polymorphisms or internal regulation, including their induction or inhibition by endogenous and exogenous compounds, determines AAI (geno)toxicity. The utilization of experimental and theoretical approaches is a useful tool to investigate the CYP-catalyzed reaction mechanisms, as demonstrated here for AAI.

Acknowledgments: This work was supported by the Grant Agency of the Czech Republic (grant 14-18344S in panel P301) and Charles University (Prague, Czech Republic) (UNCE204025/2012). Work at King's College London (London, UK) is also supported by Cancer Research UK (London, UK) (grant number C313/A14329).

Author Contributions: Conception and design: Marie Stiborová, Václav Martínek, Petr Hodek, Volker M. Arlt, Heinz H. Schmeiser; Analysis and interpretation of the data: Marie Stiborová, František Bárta, Kateřina Levová, Václav Martínek; Drafting of the article: Marie Stiborová, Volker M. Arlt, Václav Martínek; Critical revision of the article for important intellectual content: Marie Stiborová, Volker M. Arlt.

Conflicts of Interest: The authors declare no conflict of interest.

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Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Induction of cytochromes P450 1A1 and 1A2 suppresses formation of DNA adducts by carcinogenic aristolochic acid I in rats *in vivo*

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ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form 21 January 2016
Accepted 29 January 2016
Available online 1 February 2016

Keywords:
Aristolochic acid I
Cytochromes P450 1A1 and 1A2
Oxidative detoxification
Reductive activation
DNA adducts

ABSTRACT

Aristolochic acid I (AAI) is a natural plant alkaloid causing aristolochic acid nephropathy, Balkan endemic nephropathy and their associated urothelial malignancies. One of the most efficient enzymes reductively activating AAI to species forming AAI-DNA adducts is cytosolic NAD(P)H:quinone oxidoreductase 1. AAI is also either reductively activated or oxidatively detoxified to 8-hydroxyaristolochic acid (AAIa) by microsomal cytochrome P450 (CYP) 1A1 and 1A2. Here, we investigated which of these two opposing CYP1A1/2-catalyzed reactions prevails in AAI metabolism *in vivo*. The formation of AAI-DNA adducts was analyzed in liver, kidney and lung of rats treated with AAI, Sudan I, a potent inducer of CYP1A1/2, or AAI after pretreatment with Sudan I. Compared to rats treated with AAI alone, levels of AAI-DNA adducts determined by the ^{32}P-postlabeling method were lower in liver, kidney and lung of rats treated with AAI after Sudan I. The induction of CYP1A1/2 by Sudan I increased AAI detoxification to its *O*-demethylated metabolite AAIa, thereby reducing the actual amount of AAI available for reductive activation. This subsequently resulted in lower AAI-DNA adduct levels in the rat *in vivo*. Our results demonstrate that CYP1A1/2-mediated oxidative detoxification of AAI is the predominant role of these enzymes in rats *in vivo*, thereby suppressing levels of AAI-DNA adducts.

1. Introduction

Aristolochic acid (AA) is a herbal drug prepared from plants of the *Aristolochia* genus containing nitrophenanthrene carboxylic acids, of which 8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (aristolochic acid I, AAI) (Fig. 1) and 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAII) are the predominant components (Arlt et al., 2002b). Over twenty years ago, AA was shown to be the cause of a unique renal disease formerly called Chinese herbs nephropathy, now referred to as aristolochic acid nephropathy (AAN) (for a review, see Arlt et al., 2002b; Schmeiser et al., 2009; Gökmen et al., 2013). AAN is a rapidly progressive renal fibrosis with a high risk for upper urothelial tract carcinoma (UUC) and, subsequently, bladder urothelial carcinoma (Vanherweghem et al., 1993; Nortier et al., 2000; Arlt et al., 2002b; Yun et al., 2012; Gökmen et al., 2013). AA has been classified as a Group I carcinogen by IARC (IARC, 2012). Exposure to AA has also been found to be the cause of a similar type of renal disease, Balkan endemic nephropathy (BEN) and its associated occurrence of urothelial malignancy (Arlt et al., 2007; Grollman et al., 2007). This disease is endemic in certain rural areas of Balkan countries near the tributaries of the Danube river (Schmeiser et al., 2012).

Abbreviations: AA, aristolochic acid; AAI, aristolochic acid I; AAII, aristolochic acid II; AAIa, aristolochic acid Ia; AAN, aristolochic acid nephropathy; BEN, Balkan endemic nephropathy; bw, body weight; c_T, cycle threshold; CYP, cytochrome P450; 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; HPLC, high performance liquid chromatography; HUFs, Hupki (human *TP53* knock-in) mouse fibroblasts; MROD, methoxyresorufin *O*-demethylation; NQO1, NAD(P)H: quinone oxidoreductase; POR, P450 oxidoreductase; PEI-cellulose, polyethylenimine-cellulose; RAL, relative adduct labeling; RT-PCR, real-time polymerase chain reaction; r.t., retention time; SD, standard deviation; TLC, thin-layer chromatography; UUC, upper urothelial tract carcinoma; UV–vis, ultraviolet–visible.

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http://dx.doi.org/10.1016/j.tox.2016.01.011
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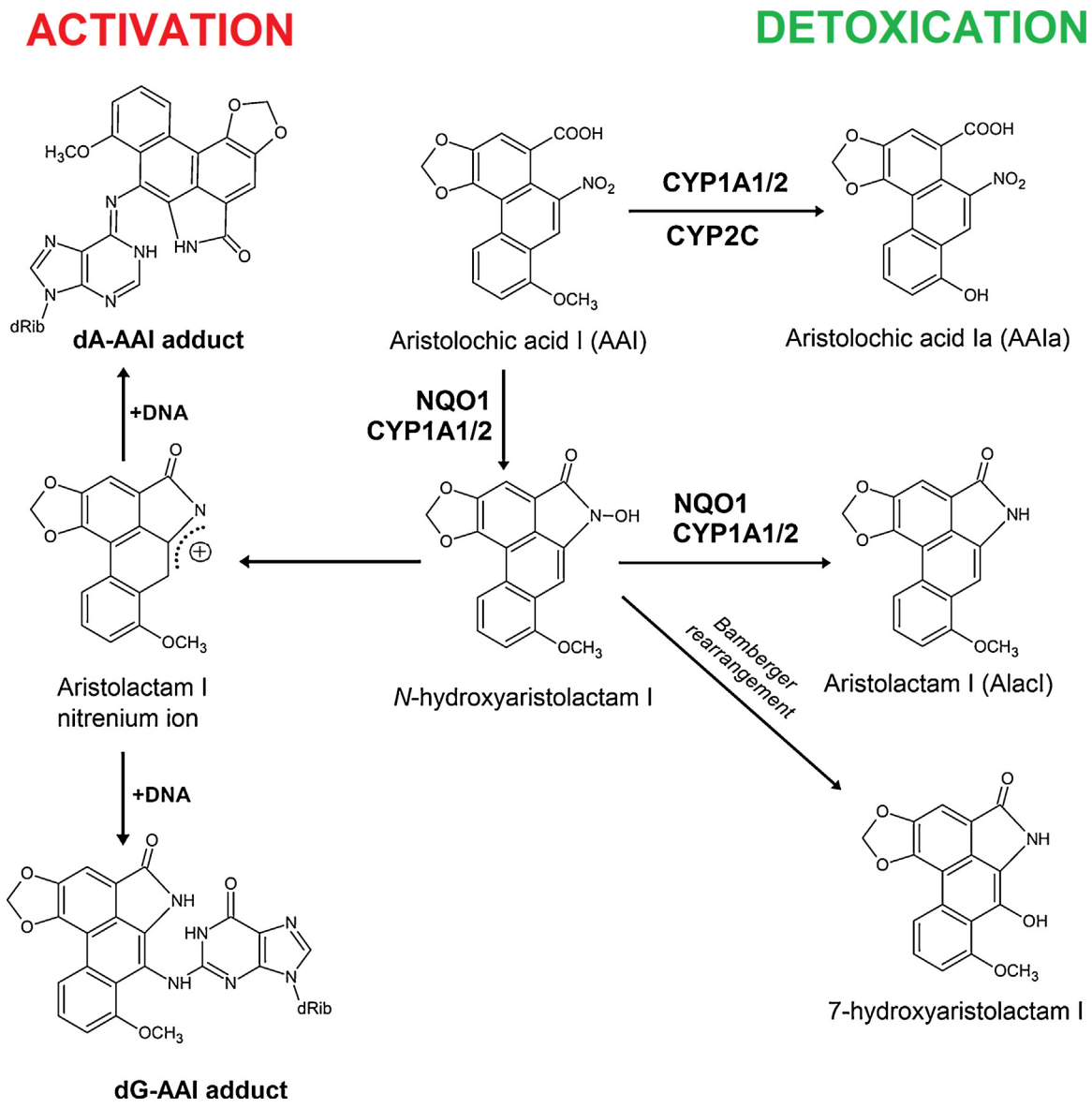

Fig. 1. Activation and detoxification pathways of AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl) aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl) aristolactam I; CYP1A1/2, cytochrome P450 1A1 and 1A2; CYP2C9, cytochrome P450 2C9; CYP3A4, cytochrome P450 3A4; NQO1, NAD(P)H:quinone oxidoreductase.

Characteristic AA-DNA adducts in renal tissue of AAN and BEN patients are biomarkers of exposure to AA even long after AA exposure, the 7-(deoxyadenosin-N^6-yl) aristolactam I (dA-AAI) adduct being the most abundant adduct formed and the most persistent (Nortier et al., 2000; Arlt et al., 2002a,b; Schmeiser et al., 2012, 2014). This deoxyadenosine adduct causes characteristic A–T transversion mutations and such mutations were found in the *TP53* tumour suppressor gene in tumors from AAN and BEN patients (Lord et al., 2004; Grollman et al., 2007) and in immortalized Hupki (human *TP53* knock-in) mouse fibroblasts (HUFs) exposed to AAI (Nedelko et al., 2009). This feature indicates a molecular mechanism of AA-mediated carcinogenesis (Arlt et al., 2007; Kucab et al., 2010). More recently, these A–T transversion mutations were also observed in loci of other genes by whole-genome and exome sequencing analyzing AA-associated UUC and AAI-treated HUFs (Poon et al., 2013; Hoang et al., 2013; Nik-Zainal et al., 2015).

Nitro-reduction of AAI, the compound that is considered as the major cause for AA-mediated development of AAN and BEN, is required to exert its carcinogenic properties (*i.e.* UUC development) (Schmeiser et al., 1996, 2009; Arlt et al., 2002b; Stiborová et al., 2008a; Gökmen et al., 2013). Such nitro-reduction leads to the formation of *N*-hydroxylated aristolactam I which either converts to a reactive cyclic acylnitrenium ion generating DNA adducts or rearranges to 7-hydroxyaristolactam I (Schmeiser et al., 2009). The product of AAI oxidation, 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIa), is formed by *O*-demethylation of the methoxy group (Fig. 1), and is a detoxification product of this carcinogen. AAIa is excreted either in its free form or conjugated (Chan et al., 2006; Shibutani et al., 2010; Arlt et al., 2011; Stiborová et al., 2012).

Various enzymes are involved in the metabolism of AAI. A variety of studies by us and others have shown that the cytosolic nitroreductase, NAD(P)H:quinone oxidoreductase (NQO1), is the

most efficient enzyme activating AAI to DNA adducts (Stiborová et al., 2002a, 2003, 2008a,b, 2011a, 2013b, 2014a,b,c; Martinek et al., 2011; Chen et al., 2011). In human and rodent hepatic microsomes AAI is also activated by cytochrome P450 (CYP) 1A2 and, to a lesser extent, by CYP1A1 and NADPH:cytochrome P450 oxidoreductase (POR) (Stiborová et al., 2001, 2005a,b, 2008a, b, 2011b, 2012, 2013b, 2014a,b,c; Arlt et al., 2011, 2015; Levová et al., 2011, 2012; Jerabek et al., 2012) (Fig. 1). However, human and rodent CYP1A1 and 1A2 play a dual role in the metabolism of AAI. Under anaerobic conditions they reductively activate AAI, while under oxidative conditions they are the predominant enzymes catalyzing *O*-demethylation of AAI to AAIa (*i.e.* detoxication) (Stiborová et al., 2001, 2005a,b, 2008a,b, 2011b, 2012, 2013b, 2014a,b,c; Sistkova et al., 2008; Rosenquist et al., 2010; Arlt et al., 2011; Levová et al., 2011). Beside CYP1A/2, rat and human CYPs of the 2C and 3A subfamilies also oxidize AAI (Sistkova et al., 2008; Rosenquist et al., 2010; Levová et al., 2011; Stiborová et al., 2012, 2015a,b) (Fig. 1). The CYP-mediated AAI oxidation leads to a decrease in AAI-induced renal injury (Xiao et al., 2008; Xue et al., 2008).

The crucial role of CYP1A1 and 1A2 enzymes in AAI metabolism *in vitro* was unambiguously proven using several systems containing these enzymes [*i.e.* microsomal systems, inhibitors of these enzymes and correlation analyses, recombinant human and rat CYP1A1/2 heterologously expressed in microsomes of insect cells (Supersomes^{TM}), purified enzymes reconstituted with POR and other components of the monooxygenase system] (Stiborová et al., 2001, 2005a,b, 2011b, 2012, 2013b, 2014a,b,c; Sistkova et al., 2008; Arlt et al., 2011; Levová et al., 2011). In addition, the importance of CYP1A1 and 1A2 in AAI metabolism has been demonstrated *in vivo* using *Cyp1a1/2*-knock-out (single and double knock-outs) and *CYP1A*-humanized mouse lines (Rosenquist et al., 2010; Arlt et al., 2011; Stiborová et al., 2012, 2014a,b,c). Based on current knowledge we proposed that AAI metabolism by CYP1A1/2 *in vivo* is determined by the binding affinity of AAI to these CYPs, and their enzymatic turnover as well as by the oxygen levels in the organs (Stiborová et al., 2012, 2013b, 2014a,b). Even though several studies considered CYP1A1/2 to be enzymes that detoxify AAI *in vivo* (Xiao et al., 2008; Rosenquist et al., 2010; Arlt et al., 2011; Stiborová et al., 2012, 2014a,b,c), the question which of their two opposing roles in AAI metabolism (AAI detoxification to AAIa *versus* activation of AAI to form AAI-DNA adducts) prevails *in vivo* remains to be answered.

To elucidate the roles of CYP1A this study was performed. AAI was administered to Wistar rats pretreated with Sudan I (1-phenylazo-2-naphthol), a strong inducer of CYP1A1 and CYP1A2 (Refat et al., 2008; Stiborová et al., 2013a), and AAI-DNA adduct levels in target and non-target organs were determined by ^{32}P-postlabeling and compared to those in organs of rats treated with AAI only. The amounts of CYP1A1/2 enzymes expressed in rats at transcriptional and translational levels were analyzed by real-time polymerase chain reaction (RT-PCR) and Western blotting, and their activities determined with their marker substrates. The formation of AAIa, the detoxification metabolite of AAI, was analyzed using high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Chemicals

NADPH, AAI (sodium salt), Sudan I [1-(phenylazo)-2-hydroxynaphthalene], menadione (2-methyl-1,4-naphthoquinone), cytochrome *c* and calf thymus DNA were from Sigma Chemical Co. (St. Louis, MO, USA). 7-Methoxyresorufin was purchased from Fluka Chemie AG (Buchs, Switzerland). All these and other chemicals were reagent grade or better. Enzymes and chemicals for the ^{32}P-postlabeling assay were from sources already described (Stiborová et al., 2005a).

2.2. Animal experiments and sample preparation

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Animals were purchased from AnLab (Prague, Czech Republic), acclimatized for 5 days and maintained at 22 °C with a 12 h light/dark period. Standardized diet and water were provided *ad libitum*. One group of five weeks old male Wistar rats (~125–150 g, $n = 3$/group) was treated *i.p.* with a single dose of AAI dissolved in 1% $NaHCO_3$ (20 mg/kg body weight, bw), the second group with two doses of Sudan I dissolved in maize oil (*i.p.*, always with 30 mg/kg bw) in two consecutive days, and the third group, where rats were treated *i.p.* with two doses of Sudan I (always with 30 mg/kg bw in two consecutive days) and with AAI (20 mg/kg bw) 24 h after the second dose of Sudan I-treatment. Three control rats received the same volume of both vehicles only. Animals were killed 1 day after the treatment by cervical dislocation. Livers, kidneys and lungs were removed, immediately after sacrifice, frozen in liquid nitrogen and stored at −80 °C. DNA from livers, kidneys and lungs was isolated by extraction with phenol/chloroform (Schmeiser et al., 1996). Total RNA was isolated from another aliquot of frozen organs using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the procedure supplied by the manufacturer. The quality of isolated RNA was verified by horizontal agarose gel electrophoresis, RNA quantity was assessed by UV–vis spectrophotometry on a Carry 300 spectrophotometer (Varian, Palo Alto, CA, USA). Microsomes and cytosols were isolated from the rat tissues by a procedure described previously (Stiborová et al., 2003, 2005a). Protein concentration in the microsomal and cytosolic fractions was measured using bicinchoninic acid protein assay (Wiechelman et al., 1988) with bovine serum albumin as a standard. Pooled microsomal and cytosolic samples ($n = 3$ rats/group) were used for analyses. All microsomal and cytosolic samples were free of residual Sudan I, AAI or their metabolites as determined by HPLC (Stiborová et al., 1988, 2002b, 2005c; Levová et al., 2011).

2.3. DNA adduct analysis by ^{32}P-postlabeling

The nuclease P1 enrichment version of ^{32}P-postlabeling analysis, and thin-layer chromatography (TLC) on polyethylenimine-cellulose (PEI) plates were carried out and DNA adduct levels (RAL, relative adduct labeling) were calculated as described previously (Schmeiser et al., 1996, 2013). AAI-DNA adducts were identified using reference standards as described (Schmeiser et al., 1996).

2.4. CYP1A and NQO1 mRNA content in rat livers, kidneys and lungs

RNA samples (1 μg) were reverse transcribed using 200 U of reverse transcriptase per sample with random hexamer primers utilizing RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The prepared cDNA was used for real-time (RT) polymerase chain reaction (PCR) performed in RotorGene 2000 (Corbett Research, Sydney, Australia) under the following cycling conditions: incubation at 50 °C for 2 min and initial denaturation at 95 °C for 10 min, then 50 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min, and elongation for 30 s at 72 °C. Gain was set to 7 and fluorescence was acquired after elongation step. The PCR reaction mixtures (20 μl) contained 9 μl cDNA diluted 10-times in Milli-Q ultrapure water (Biocel A10, Millipore, Billerica, MA, USA),

10 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 1 μl TaqMan Gene Expression Assay Mix (commercially available unlabeled PCR primers and FAM™ dye-labelled probe for rat *CYP1A1/2* or *NQO1* as target genes and β-*actin* as reference internal standard gene). Each sample was analysed in two parallel aliquots. Negative controls had the same compositions as samples but cDNA was omitted from the mixture. Data were analyzed by the program RotorGene v6 (Corbett Research, Sydney, Australia) and evaluated by comparative cycle threshold (c_T) method for relative quantitation of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then $\Delta\Delta c_T$ was evaluated according to following equations: $\Delta c_T = c_T$ (target) $- c_T$ (internal standard), $\Delta\Delta c_T = \Delta c_{T\text{treated}} - \Delta c_{T\text{control}}$, where $\Delta c_{T\text{treated}}$ is Δc_T for treated rats and $\Delta c_{T\text{control}}$ is Δc_T for untreated rats. Δc_T is positive if the target is expressed at a lower level than the internal standard (β-*actin*), and negative if expressed at a higher level. The induction of mRNA expression of studied target genes in treated animals was evaluated as $2^{-(\Delta\Delta cT)}$.

2.5. Preparation of antibodies and estimation of CYP1A1, 1A2, and NQO1 protein content in microsomal and cytosolic fractions isolated from rat liver and kidney

The chicken anti-rat CYP1A1, anti-rabbit CYP1A2 and anti-rat NQO1 antibodies were prepared as described previously (Stiborová et al., 2002b, 2006). Immunoquantification of microsomal CYP1A1 and 1A2 and cytosolic NQO1 was performed using Western blotting (Stiborová et al., 2006). Rat CYP1A1, rat CYP1A2 and human NQO1 (Sigma) were used to identify the CYP1A1, 1A2 and NQO1 bands, respectively. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium as dye and bands are expressed as arbitrary units (AU)/mg protein (Stiborová et al., 2002b, 2006). Glyceraldehyde phosphate dehydrogenase was used as loading control and detected by its antibody (1:750, Millipore; MA, USA).

2.6. NQO1, CYP1A1/2 and 2C6/11 enzyme activity assays

In hepatic, renal and pulmonary cytosols NQO1 activity was measured using menadione (2-methyl-1,4-naphthoquinone) as a substrate; the assay was improved by the addition of cytochrome *c* and NQO1 activity expressed as nmol cytochrome *c* reduced (Levová et al., 2011, 2012). Microsomal samples were characterized for specific CYP1A1 and 1A2 activities: Sudan I hydroxylation to 4′-hydroxy-, 6-hydroxy-, and 4′,6-dihydroxy-Sudan I (CYP1A1) (Stiborová et al., 1988, 2002b, 2005c) and methoxyresorufin *O*-demethylation (MROD) (CYP1A2) (Burke et al., 1994). Hepatic microsomal samples were also characterized for specific CYP2C6 and 2C11 activities with their marker substrates determining diclofenac 4′-hydroxylation and testosterone 16α-hydroxylation, respectively (Kobayashi et al., 2002; Yamazaki et al., 2006).

2.7. Microsomal incubations to study AAI demethylation

Incubation mixtures contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg rat hepatic, renal or pulmonary microsomal protein and 10 μM AAI in a final volume of 250 μl and were incubated at 37 °C for 20 min; AAI *O*-demethylation to AAIa was determined to be linear up to 25 min. Control incubations were carried out either (i) without microsomes, (ii) without NADPH or (iii) without AAI. AAI and its metabolite AAIa were separated by reverse-phase HPLC, identified by mass spectrometry and quantified as described previously (Levová et al., 2011). Briefly, HPLC was carried out with an Nucleosil 100-5C_{18}, 250 × 4.0 mm, 5 mm (Macherey-Nagel) column, using a linear gradient of acetonitrile (20–60% acetonitrile in 55 min) in 100 mM triethylammonium acetate with a flow rate of 0.6 ml/min. A Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector set at 254 nm was used. Peaks were integrated with CHROMELEON™ 6.01 integrator. A peak eluting at retention time (r.t.) 22.7 min was identified as AAIa using mass-spectroscopy analysis (Levová et al., 2011). A typical HPLC chromatogram is shown in Supplementary Fig. 1.

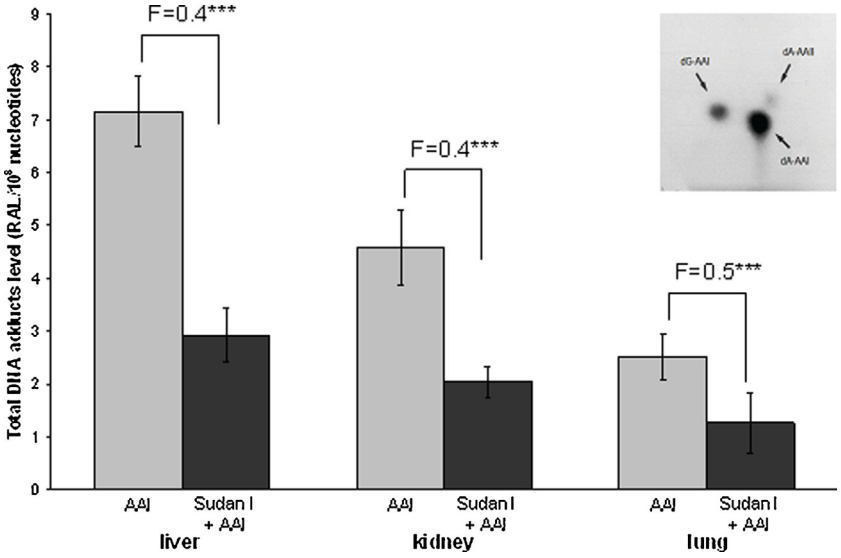

Fig. 2. Quantitative TLC ^{32}P-postlabeling analysis of AAI-DNA adduct levels in organs of rats treated with AAI, Sudan I or AAI after exposure to Sudan I. Numbers above columns ("F") indicate fold changes in DNA adduct levels in animals treated with AAI combined with Sudan I compared to animals treated with AAI alone. 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; $^{***}P < 0.001$, different from animals treated with AAI alone. Insert: Autoradiographic profile of AAI-DNA adducts formed in liver of rats treated with AAI, determined by the nuclease P1 enrichment version of the ^{32}P-postlabeling assay.

2.8. Microsomal and cytosolic formation of AAI-DNA adducts

The de-aerated and nitrogen-purged incubation mixtures, in which microsomes were used to activate AAI contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg of hepatic or renal microsomal protein, 0.5 mg of calf thymus DNA (2 mM dNp) and 0.5 mM AAI in a final volume of 750 μl. Microsomal incubations were carried out at 37 °C for 60 min; AAI-DNA adduct formation was found to be linear up to 2 h in microsomes (Stiborová et al., 2005a). Control incubations were carried out either (i) without microsomes, (ii) without NADPH, (iii) without DNA or (iv) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase as described above (Stiborová et al., 2005a, 2011a, 2012; Arlt et al., 2011).

The de-aerated and nitrogen-purged incubation mixtures, in which cytosols were used to activate AAI contained 50 mM Tris–HCl buffer (pH 7.4), 0.2% Tween 20, 1 mM NADPH, 1 mg rat hepatic or renal cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI in a final volume of 750 μl. Incubations with cytosols were performed at 37 °C for 60 min; AAI-derived DNA adduct formation was found to be linear up to 2 h (Stiborová et al., 2003). Control incubations were performed either (i) without cytosol, (ii) without NADPH, (iii) without DNA or (iv) without AAI. After extraction with ethyl acetate DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described above.

2.9. Statistical analyses

For statistical data analysis we used Student's *t*-test. All *P*-values are two-tailed and considered significant at the 0.05 level.

3. Results

3.1. DNA adduct formation in rats treated with AAI and Sudan I compared to adduct formation in rats treated with AAI alone

AAI-DNA adduct formation was determined by ^{32}P-postlabeling in liver, kidney and lung of male Wistar rats treated *i.p.* with AAI, Sudan I, or AAI after pretreatment with Sudan I. Using the nuclease P1 version of ^{32}P-postlabeling assay, all liver, kidney and lung samples from rats treated with AAI showed an adduct pattern similar to that found in kidney tissue from AAN and BEN patients (Arlt et al., 2002b; Nortier et al., 2000; Schmeiser et al., 1996, 1997, 2012). As shown in Fig. 2, the adduct pattern consisted of three adduct spots. These spots have been identified as 7-(deoxyguanosin-N^2-yl) aristolactam I (dG-AAI), 7-(deoxyadenosin-N^6-yl) aristolactam I (dA-AAI) and 7-(deoxyadenosin-N^6-yl) aristolactam II (dA-AAII). We have shown previously that the dA-AAII adduct can also be generated from AAI, probably *via* a demethoxylation reaction of AAI or dA-AAI (Stiborová et al., 1994; Schmeiser et al., 1997). No AAI-derived DNA adducts were found in DNA of control rats treated either with vehicle or Sudan I only (data not shown).

Generally, AAI-DNA adduct levels were higher in liver, the organ predominantly responsible for biotransformation of xenobiotics including AAI, as well as kidney, the target organ of AAI genotoxicity (Stiborová et al., 2008a,b, 2014a,b), than in lung (Fig. 2). In all organs of rats treated with AAI after pretreatment with Sudan I, the levels of AAI-DNA adducts were only half of those in rats exposed to AAI alone (Fig. 2 and Supplementary Table 1). Therefore, Sudan I, when administered to rats before their exposure to AAI, shifts the metabolic pathway of AAI that finally leads to a decrease in AAI-DNA adduct levels in all three organs.

Because CYP1A1/2 enzymes both oxidize (*i.e.* detoxify AAI) and reduce (*i.e.* activate AAI to form to AAI-DNA adducts) AAI, their expression might determine the balance between activation and detoxification pathways of AAI (Stiborová et al., 2008a,b, 2013b, 2014a,b). Therefore, we investigated whether expression levels of these enzymes influence AAI-DNA adduct formation found *in vivo* (Fig. 2 and Supplementary Table 1).

3.2. The effect of AAI treatment with or without Sudan I upon CYP1A1/2 and NQO1 mRNA and protein levels and their enzymatic activities in rat liver, kidney and lung

The effect of exposure to AAI, Sudan I and both compounds on expression of CYP1A1 and 1A2 at the mRNA and protein levels, was examined in liver, kidney and lung.

The mRNA and protein of CYP1A1 (Table 1 and Fig. 3) were expressed in all organs of control rats. Sudan I oxidation, a marker for CYP1A1 enzyme activity (Stiborová et al., 2002b, 2005c), was also detectable in all organs studied, but only very low Sudan I oxidation was measurable in kidney and lung, the organs expressing the lower protein levels of CYP1A1 than liver (Fig. 3).

The *CYP1A2* mRNA was expressed mainly in liver (Table 1), whereas the CYP1A2 protein expression levels were higher in liver

Table 1
Relative expression of mRNA of hepatic, renal and pulmonary CYP1A1, CYP1A2 and NQO1 in liver, kidney and lung from untreated (control) animals and animals treated with AAI, Sudan I or AAI combined with Sudan I.

		Liver		Kidney		Lung	
		Δc_T[a]	Fold change over control	Δc_T[a]	Fold change over control	Δc_T[a]	Fold change over control
CYP1A1	Control	12.84 ± 0.44	*1.00*	7.22 ± 0.22	*1.00*	15.20 ± 0.15	*1.00*
	AAI	9.93 ± 0.44	7.54***	7.56 ± 0.27	0.791	13.53 ± 0.29	3.19**
	Sudan I	0.36 ± 0.06	5710***	4.53 ± 0.35	6.45***	2.08 ± 0.04	8930***
	Sudan I + AAI	1.56 ± 0.31	2490***	4.53 ± 0.17	6.63***	2.05 ± 0.22	9090***
CYP1A2	Control	0.75 ± 0.34	*1.00*	16.38 ± 0.42	*1.00*	19.89 ± 0.18	*1.00*
	AAI	−2.22 ± 0.08	7.86***	14.60 ± 0.32	3.43**	12.26 ± 0.26	198***
	Sudan I	−5.23 ± 0.44	63.2***	7.85 ± 0.25	370***	7.30 ± 0.26	6170***
	Sudan I + AAI	−5.76 ± 0.16	91.5***	8.72 ± 0.82	202***	10.67 ± 0.43	595***
NQO1	Control	6.03 ± 0.24	*1.00*	7.51 ± 0.16	*1.00*	5.98 ± 0.46	*1.00*
	AAI	2.10 ± 0.29	15.2***	7.27 ± 0.18	1.18	5.66 ± 0.27	1.25
	Sudan I	1.06 ± 0.22	31.2***	5.88 ± 0.28	3.10**	2.97 ± 0.08	8.06***
	Sudan I + AAI	1.47 ± 0.28	23.5***	6.05 ± 0.31	2.76**	3.42 ± 0.44	5.92***

[a] Values relative to β-actin are means ± S.D. from data found for three male rats ($n = 3$) (control and treated with AAI, Sudan I and AAI with Sudan I). The induction of mRNA expression of studied target genes in treated animals was evaluated as $2^{-(\Delta\Delta cT)}$ (see Section 2). Comparison was performed by Student's *t*-test analysis.

** $P > 0.01$.

*** $P > 0.001$ significantly different from controls.

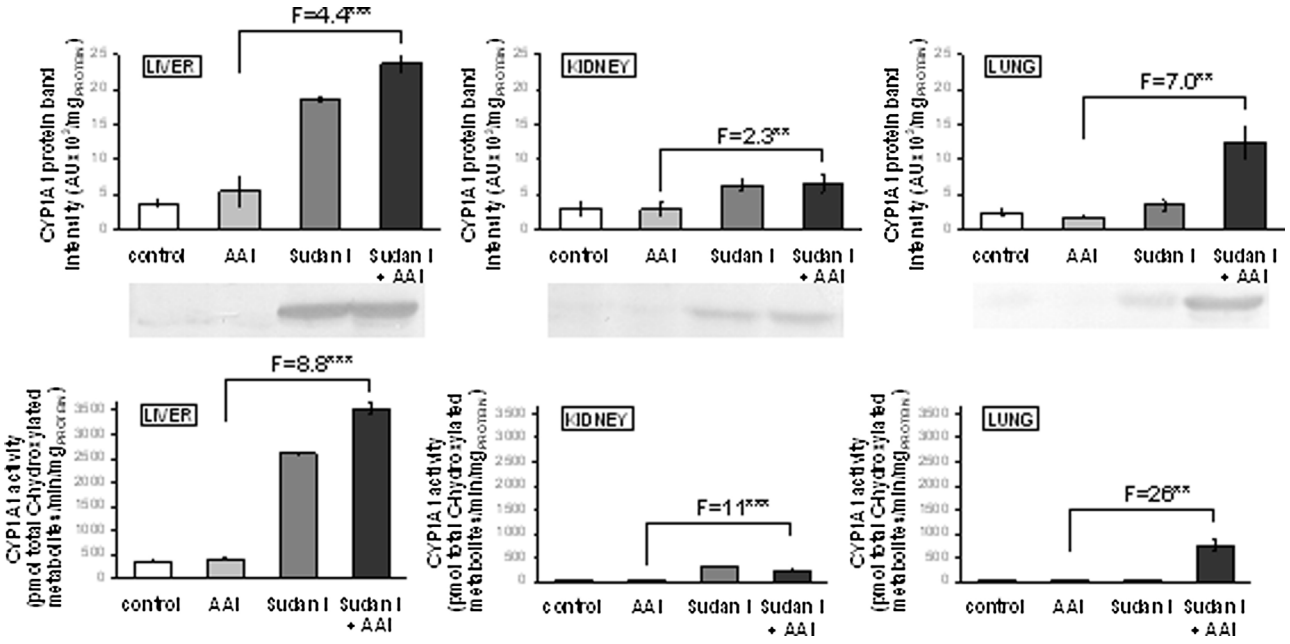

Fig. 3. CYP1A1 protein levels (upper panels) in rat microsomes isolated from untreated (control) animals and animals treated with AAI, Sudan I or AAI after exposure to Sudan I. Microsomes isolated from liver, kidney and lung were analyzed by Western blotting in the same blot (insert) and, therefore, can be compared directly. Values are given as the means of arbitrary units (AU per mg protein) ± SD ($n = 3$). CYP1A1 enzyme activity as measured by Sudan I oxidation (nmol total C-hydroxylated Sudan I metabolites/min × mg protein) (lower panels). All values are given as the means ± SD ($n = 3$). Numbers above columns ("F") indicate fold changes in protein level or enzyme activity in microsomes of rats treated with AAI with Sudan I compared to those with AAI alone. Comparison was performed by *t*-test analysis; $^{**}P < 0.01$, $^{***}P < 0.001$, different from data found in microsomes form rats treated with AAI alone.

and lung than in kidney (Fig. 4). In concordance, MROD activity, a marker reaction of CYP1A2, was found in liver and lung, with no activity in kidney (see Fig. 4).

As shown in Table 1, treatment of rats with Sudan I alone or with this compound before exposure to AAI induced expression of *CYP1A1* mRNA in all tested organs. Treatment of rats with AAI alone induced mRNA levels of this CYP only in the liver and lung. The effect of both compounds combined was either the same as of Sudan I alone (lung and kidney) or led to lower mRNA levels in the liver. The most drastic effect was seen in the lung where Sudan I

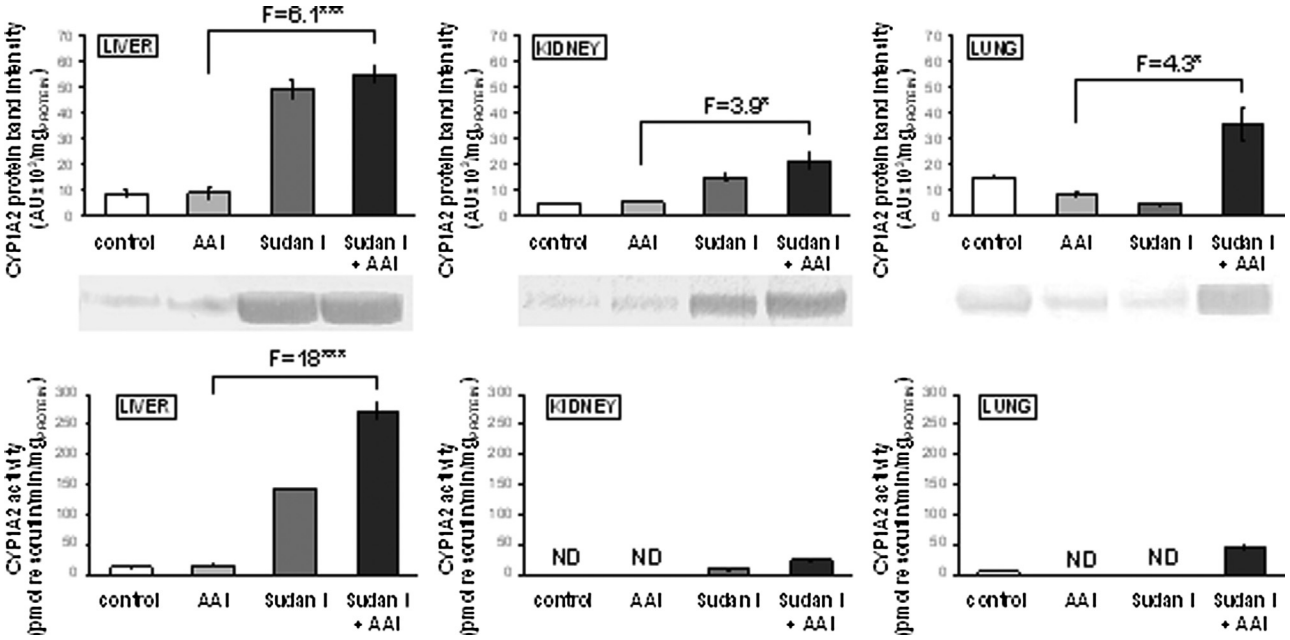

Fig. 4. CYP1A2 protein levels (upper panels) in rat microsomes isolated from untreated (control) animals and animals treated with AAI, Sudan I or AAI after exposure to Sudan I. Microsomes isolated from liver, kidney and lung were analyzed by Western blotting in the same blot (insert) and, therefore, can be compared directly. Values are given as the means of arbitrary units (AU per mg protein) ± SD ($n = 3$). CYP1A2 enzyme activity as measured by MROD (pmol resorufin/min × mg protein) (lower panels). All values are given as the means ± SD ($n = 3$). Numbers above columns ("F") indicate fold changes in protein level or enzyme activity in microsomes of rats treated with AAI with Sudan I compared to those with AAI alone. Comparison was performed by *t*-test analysis; $^{*}P < 0.05$, $^{***}P < 0.001$, different from data found in microsomes form rats treated with AAI alone.

alone or in combination with AAI increased levels of *CYP1A1* mRNA 2900-times as compared to AAI alone (Table 1). Expression of CYP1A1 protein and oxidation of Sudan I, a marker for CYP1A1, were always higher in organs of rats treated with AAI after pretreatment with Sudan I than with AAI alone (Fig. 3).

Expression of mRNA and protein of CYP1A2 was also induced by treatment of rats with AAI, Sudan I or their combined administration (Table 1 and Fig. 4). In liver the mRNA, protein and CYP1A2 enzyme activities ran parallel, in kidney activities were detectable only in microsomes of rats treated with Sudan I or Sudan I combined with AAI. In lung the very high mRNA induction was not reflected in the phenotype; a decrease in amounts of CYP1A2 protein found in lung of rats treated with AAI or Sudan I did not correspond to a 198- or 6170-fold increase in the *CYP1A2* mRNA expression levels (Fig. 4).

The results found confirmed that Sudan I is a strong inducer of CYP1A1/2 in rats and indicate that a combined treatment of rats with Sudan I and AAI leads to even higher enzyme levels than with Sudan I alone.

Treatment of rats with Sudan I and Sudan I combined with AAI also led to an increased expression of cytosolic NQO1, again at the mRNA, protein and enzyme activity levels in liver, kidney and lung (Table 1 and Fig. 5). Similarly to CYP1A, at the doses used, Sudan I resulted in greater increases at the protein level. Expression of mRNA, protein and enzyme activity of NQO1 measured with menadione as a substrate ran parallel in all three organs and were always higher in organs of rats treated with AAI and Sudan I than in those treated with AAI alone (Fig. 5). However, the efficacy of NQO1 induction by AAI with Sudan I compared to AAI alone was lower than that for CYP1A expression (compare Figs. 3–5). These findings indicate that both compounds administered to rats act as moderate inducers of NQO1.

3.3. The effect of treatment of rats with AAI, Sudan I and both agents in combination on oxidation of AAI to AAIa by rat hepatic, renal and pulmonary microsomes

Since microsomal CYP1A1 and 1A2 detoxify AAI to its oxidative *O*-demethylated metabolite AAIa (Sistkova et al., 2008; Rosenquist et al., 2010; Arlt et al., 2011; Levová et al., 2011; Stiborová et al., 2012, 2013b, 2014a,b, 2015b), AAIa formation from AAI was investigated *ex vivo* in hepatic, renal and pulmonary microsomes of all treatment groups. AAIa was formed by liver microsomes from the AAI plus Sudan I group at moderately higher levels as compared to microsomes of rats treated with AAI alone. But in kidney only Sudan I treatment alone increased AAIa formation 1.6-fold ($P < 0.01$), AAI had no effect or even inhibited oxidation of AAI (Fig. 6). In lung the low activity of CYP1A enzymes detectable essentially only in microsomes of rats exposed to both Sudan I and AAI (see the CYP1A1/2 activities determined with their marker substrates shown in Figs. 3 and 4) was confirmed also by formation of AAIa, as AAIa was only detectable at low levels in pulmonary microsomes of this group (Fig. 6). These results indicate that CYP1A1/2 enzymes catalyze AAI demethylation to AAIa in test rat organs, but this activity does not seem to be very effectively induced by Sudan I either alone or in combination with AAI.

A probable reason for this observation is that not only CYP1A1/2, but also enzymes of the 2C subfamily, which are highly expressed in the livers of male rats, accounting for approximately 55% of the rat liver CYP complement (Nedelcheva and Gut, 1994), can oxidize AAI. CYP2C11 with ~50% and CYP2C6 at ~20% are the main members of the hepatic CYP2C family in rats (Večeřa et al., 2011; Zachařová et al., 2012). Both have been shown to be capable of efficiently oxidizing AAI to AAIa (Levová et al., 2011; Stiborová et al., 2014c, 2015a,b), and the contribution of the CYP2C enzymes to AAIa formation in rat liver

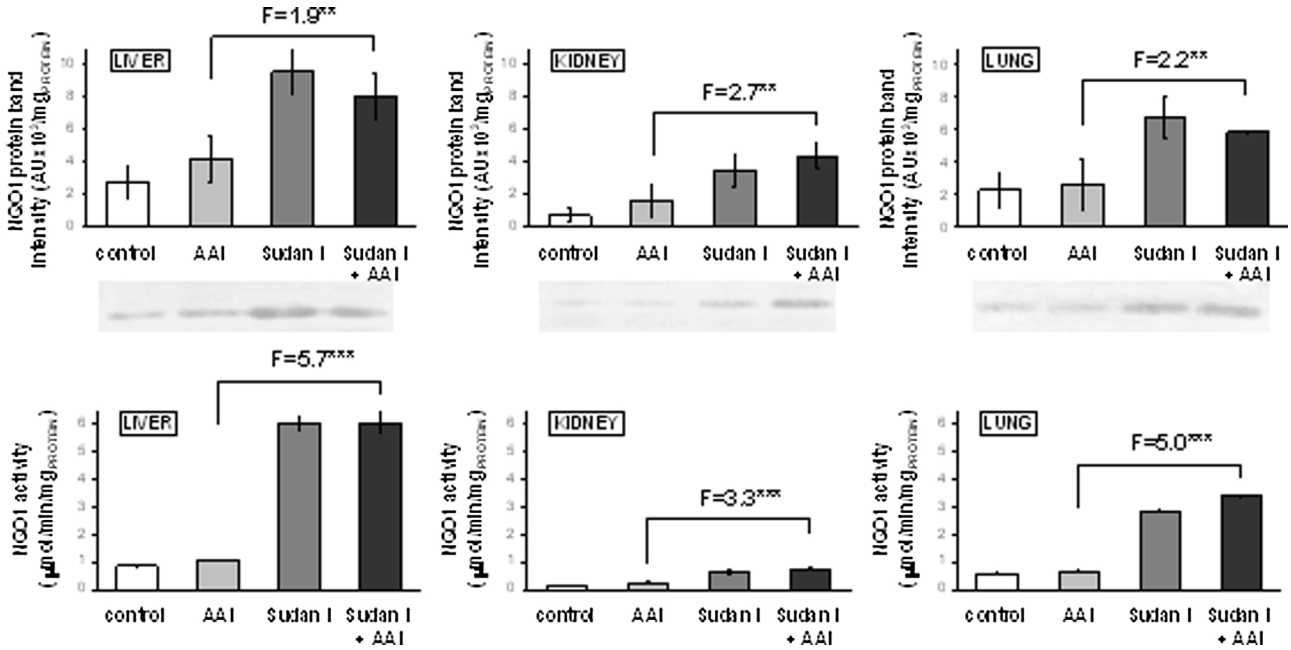

Fig. 5. NQO1 protein levels (upper panels) and NQO1 enzyme activity (lower panels) in rat cytosols isolated from untreated (control) animals and animals treated with AAI, Sudan I or AAI after pretreatment with Sudan I. Cytosol isolated from liver, kidney or lung was analyzed by Western blotting in the same blot (insert) and, therefore, can be compared directly. Human recombinant NQO1 was used to identify the rat NQO1 band in rat cytosol (data not shown). Values are given as the means of arbitrary units (AU per mg protein) ± SD ($n = 3$). NQO1 activity in hepatic, renal and pulmonary cytosols was determined using menadione and cytochrome *c* as substrate (expressed as nmol cytochrome *c* reduced/min × mg protein). Numbers above columns ("F") indicate fold changes in protein level or enzyme activity in cytosols of rats treated with AAI with Sudan I compared to those with AAI alone. Values are given as the means ± SD ($n = 3$). Comparison was performed by *t*-test analysis; $^{**}P < 0.01$, $^{***}P < 0.001$, different from data found in cytosols of rats treated with AAI alone.

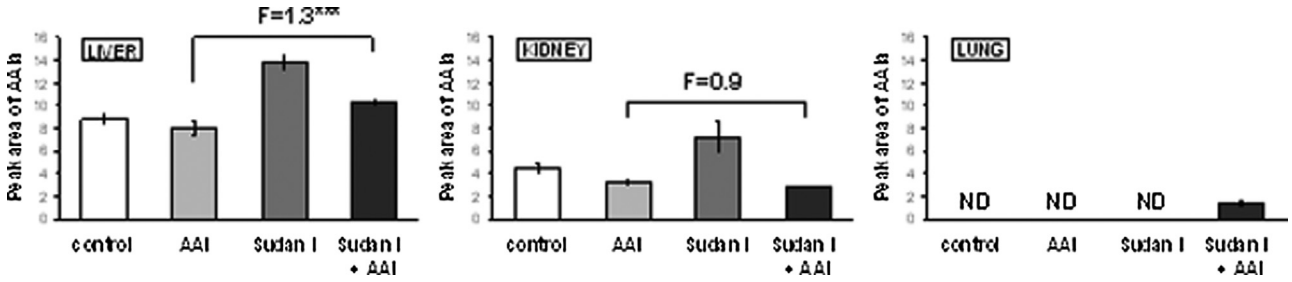

Fig. 6. Formation of AAIa (peak area per minute per miligram protein) in rat microsomes isolated from untreated (control) animals and animals treated with AAI, Sudan I or AAI after exposure to Sudan I with AAI as a substrate. All values are given as the means ± SD ($n = 3$). Numbers above columns ("F") indicate fold changes in AAIa levels in microsomes of rats treated with AAI with Sudan I compared to those with AAI alone. ND, not detected. Comparison was performed by *t*-test analysis; $^{***}P < 0.001$, different from data found in microsomes of rats treated with AAI alone.

microsomes is more than 4-times higher than that of CYP1A (Stiborová et al., 2015b). Upon induction of CYP1A with Sudan I the relative amount of the CYP2C enzymes in the microsomes will decrease leading to lower CYP2C activity if analyzed based on mg protein, as was the case in our study. To test this, CYP2C activity was also analyzed in hepatic microsomes using diclofenac 4′-hydroxylation for CYP2C6 and testosterone 16α-hydroxylation as a marker for CYP2C11 (Kobayashi et al., 2002; Yamazaki et al., 2006). As shown in Fig. 7 exposure of rats to Sudan I, either with or without AAI, decreased testosterone 16α-hydroxylation activities based on mg protein up to 33% relative to control while diclofenac 4′-hydroxylation was marginally lower. Therefore, decreased relative CYP2C activity could explain why AAIa formation in liver microsomes of rats treated with AAI, Sudan I or with a combination of both compounds did not run parallel to CYP1A induction tested with their marker activities, namely, Sudan I oxidation and MROD.

3.4. Microsomal versus cytosolic activation of AAI

In further experiments we investigated whether induction of microsomal CYP1A1/2 and cytosolic NQO1 also influences the reductive activation of AAI to AAI-DNA adducts catalyzed by rat microsomal and cytosolic fractions *ex vivo*. For the investigations we focused on the liver and kidney (target organ for AAI genotoxicity).

AAI-DNA adduct formation was analyzed in *ex-vivo* incubations under hypoxic conditions. Incubation mixtures were purged with a stream of nitrogen for 2 minutes before the addition of AAI. AAI was reductively activated by both hepatic and renal microsomes from all treatment groups (Fig. 8). The adduct pattern generated was the same as that found *in vivo* (see Fig. 2). No adducts were observed in control incubations carried out in parallel (data not shown). A significant two to three-fold increase in AAI-DNA adduct formation was seen in incubations of DNA with AAI and hepatic or renal microsomes of rats exposed to Sudan I alone or in combination with AAI (Fig. 8). Overall, the increases in AAI-DNA adduct formation *ex vivo* corresponded to the induction of CYP1A1/2 at protein levels in rats and confirmed the participation of these CYPs in the reductive activation of AAI found previously (Stiborová et al., 2001, 2005a,b, 2012, 2014b). The AAI-DNA adduct formation by microsomes under the oxidative (*i.e.* aerobic) conditions was not analyzed in this study. Namely, under these conditions the oxidation of AAI in microsomes (see Fig. 6) should compete with its reduction, which finally result in decreased levels of AAI-DNA adducts. Indeed, as shown in our previous study, an inhibition of AAI-DNA adduct formation occurred in the microsomal system under the aerobic conditions (Schmeiser et al., 1997).

Cytosols, where NQO1 is expressed, were also incubated with AAI, calf thymus DNA and NADPH, the cofactor of NQO1, and analyzed for DNA adduct formation by ^{32}P-postlabeling. AAI was activated by hepatic cytosols as evidenced by specific AAI-DNA adduct formation (Fig. 8). No DNA adducts were observed in control incubations carried out in parallel (data not shown). Liver cytosols from rats treated with AAI, Sudan I and AAI after pretreatment with Sudan I produced AAI-DNA adduct levels which were 1.2-, 4.3- and 4.5-fold higher, respectively, relative to cytosols isolated from untreated animals (Fig. 8). The increase in AAI-DNA adduct formation ran parallel to higher NQO1 activity in these cytosols (compare Fig. 5). Renal cytosols isolated from AAI-treated rats, rats treated with Sudan I and rats treated with Sudan I plus AAI led to 1.1-, 3.9- and 4.2-fold higher AAI-DNA adduct levels relative to cytosols from control animals, respectively. Again, the

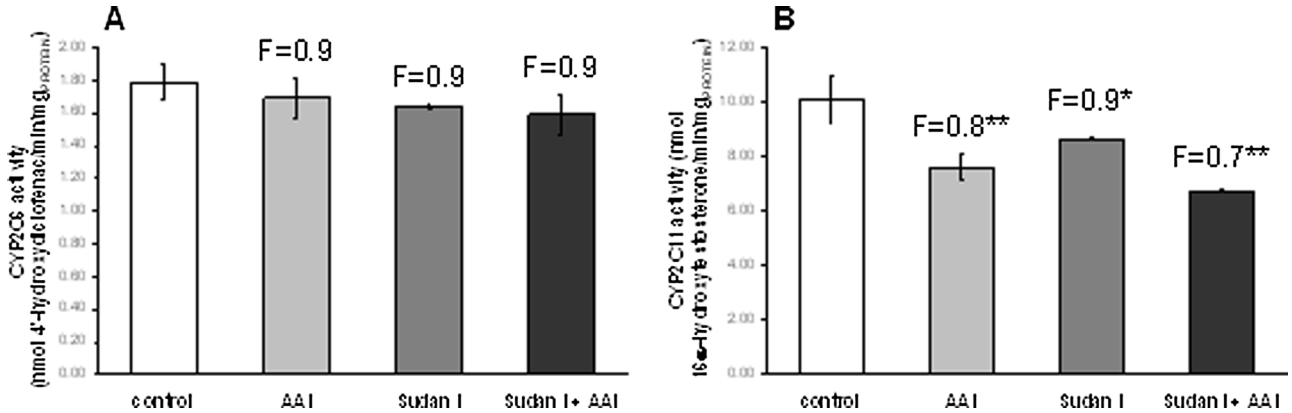

Fig. 7. CYP2C6 (A) and CYP2C11 enzyme activities (B) in rat hepatic microsomes. CYP2C6 was measured as diclofenac 4′-hydroxylation (nmol 4′-hydroxydiclofenac/min × mg protein) and CYP2C11 as testosterone 16α-hydroxylation (nmol 16α-hydroxytestosterone/min × mg protein). All values are given as the means ± SD ($n = 3$). Numbers above columns ("F") indicate fold changes in enzyme activities compared to control. Comparison was performed by *t*-test analysis; $^{***}P < 0.001$, different from control.

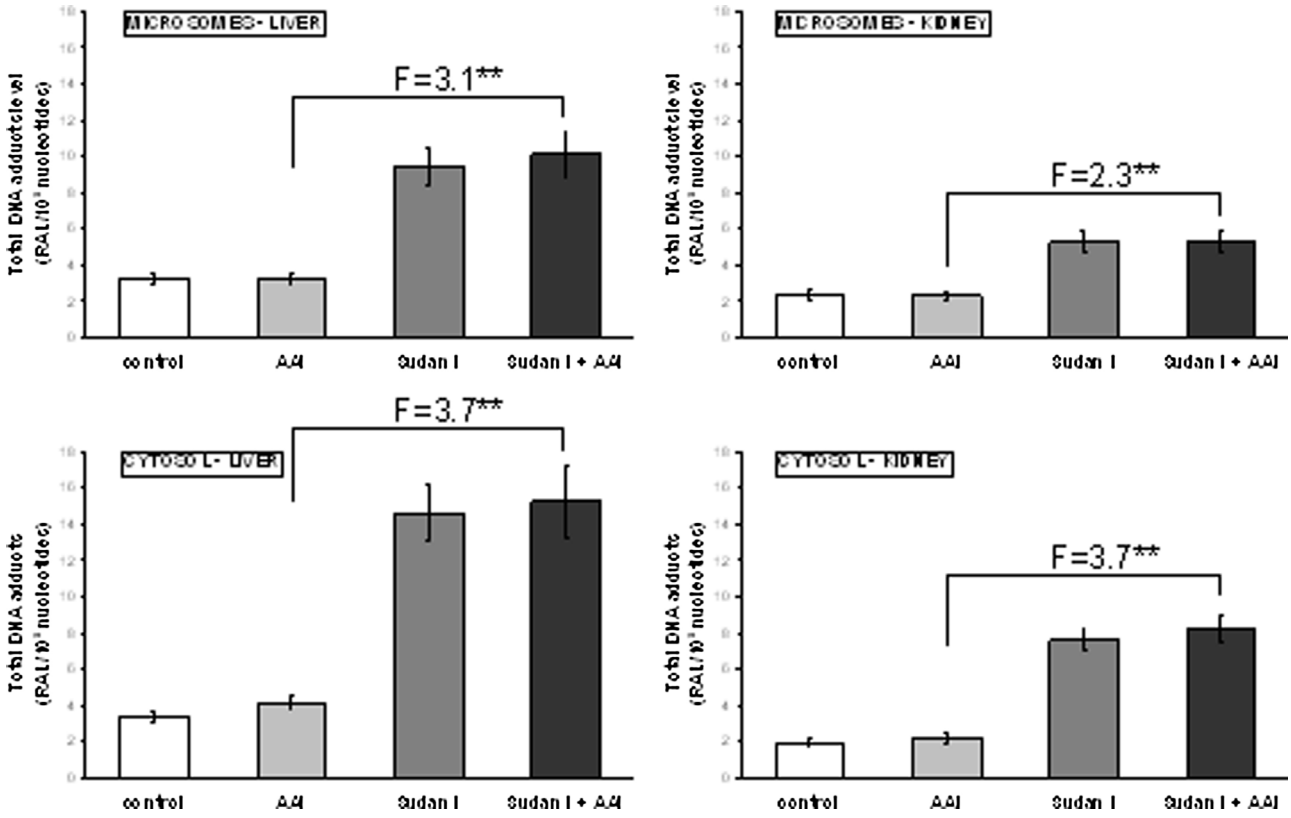

Fig. 8. DNA adduct formation *ex vivo* by AAI in rat microsomes (upper panels) and cytosols (lower panels) isolated from liver and kidney of untreated (control) animals and animals treated with AAI, Sudan I or AAI after exposure to Sudan I and incubated with DNA, AAI and NADPH. AAI-DNA adduct formation was determined by ^{32}P-postlabeling. Values are given as the means ± SD ($n = 3$); each DNA sample was determined by two postlabeling analyses. RAL, relative adduct labeling. Numbers above columns ("F") indicate fold changes in AAI-DNA adduct levels in microsomes and cytosols of rats treated with AAI with Sudan I compared to those with AAI alone. Comparison was performed by *t*-test analysis; $^{***}P < 0.001$, different from data found with microsomes or cytosols of rats treated with AAI alone.

observed adduct levels was consistent with the observed NQO1 enzyme activity (compare Figs. 5 and 8).

4. Discussion

CYP1A1 and 1A2 have the dual function to catalyze AAI detoxification to AAIa and the activation of AAI to form AAI-DNA adducts. The aim of this study was to evaluate which of the two opposing functions prevails in an experimental rat model *in vivo*. Here we modulated the expression of CYP1A1/2 by Sudan I treatment which is a strong inducer of these enzymes (Stiborová et al., 2013a; Refat et al., 2008). As a measure of genotoxicity the formation of AAI-DNA adducts was determined. The formation of AAIa was used as a measure for AAI detoxification.

The results of this study demonstrate that AAI-DNA adducts are formed *in vivo* in all organs tested (liver, kidney and lung), both in rats treated with AAI alone or in combination with the inducer Sudan I. These findings suggest that AAI is distributed *via* the blood stream and that these tissues have the metabolic capacity to reductively activate this carcinogen. The levels of AAI-DNA adducts in individual organs therefore depend both on a distribution of AAI to individual organs and on the activities of enzymes catalyzing either its oxidative detoxification or its reductive activation to species forming AAI-DNA adducts. Indeed, our results demonstrate that expression levels of CYP1A enzymes modulate the metabolism of AAI in the rat organs, thereby dictating AAI-DNA adduct formation *in vivo*. Furthermore, it is probable that enhanced clearance of AAI in the liver of induced animals is also altering the levels of AAI-DNA adducts in the kidney.

In our study rats were exposed to AAI for 24 h only to resolve the role of CYP1A1/2 in AAI oxidative or reductive metabolism *in vivo*. We had previously shown the formation of AAI-DNA adducts in liver and kidney 24 h after administration (Pfau et al., 1990; Stiborová et al., 1994, 2014c; Arlt et al., 2002b). Therefore, for these experimental purposes and to study the acute effects we used this short exposure, in order to resolve the role of CYP1A1/2 in AAI oxidative or reductive metabolism *in vivo*. Our results indicate that under these conditions AAI genotoxicity (*i.e.* AAI-DNA adduct formation) is reduced after administration of the CYP1A1/2 inducer Sudan I. However, it is important to note that the doses to which humans are exposed to are orders of magnitude lower than the AAI dose administered to rats in this study and its effect at lower but chronic and life-long doses may be different. We found that only half of the AAI-DNA adduct levels were formed in liver, kidney and lung of rats treated with AAI after exposure to Sudan I, than in rats treated with AAI alone (see Fig. 2). These findings demonstrate that induction of CYP1A1 and 1A2 by Sudan I might increase AAI detoxification, leading to lower amounts of AAI available for activation. However, only 1.3-fold higher AAI detoxification (*O*-demethylation activity) was found *ex vivo* in microsomes of treated rats. Previous studies have shown that CYP2C enzymes are also capable in *O*-demethylating AAI (*i.e.* AAI detoxification), and are even more efficient than the CYP1A enzymes to catalyze this reaction in rat liver microsomes (Stiborová et al., 2014c, 2015b). CYP2C enzymes constitute about 55% of hepatic CYPs in male rats, Sudan I alone or in combination with AAI induces CYP1A about 4-fold, thereby reducing the relative amount of the other CYP enzymes. In microsomes from CYP1A induced rats, the

contribution of CYP2C is therefore lower by a factor of approximately 4 explaining the relatively weak induction of AAIa formation we observed in such microsomes.

The results of the present study fit with the proposed scheme of AAI metabolism (see Fig. 1). If AAI is oxidized to AAIa, lower amounts of AAI are available to be activated by enzymes with nitroreductase activity like NQO1 (for a review, see Stiborová et al., 2008b, 2014a,b,c) which generate cyclic acylnitrenium ions that bind to DNA (*i.e.* DNA adduct formation) (Fig. 1). Our results are in accordance with two previous studies showing that AAI detoxification is lower in *Cyp1a* knockout mice (*i.e. Cyp1a1(-/-)*, *Cyp1a2(-/-)* and *Cyp1a1/2(-/-)* mouse lines) leading to an increase in AAI (geno) toxicity (Rosenquist et al., 2010; Arlt et al., 2011).

Our results of the *ex-vivo* experiments also confirm previous findings (Stiborová et al., 2001, 2012; Arlt et al., 2011; Levová et al., 2011) that under hypoxic (anaerobic) conditions, rat hepatic and renal CYP1A enzymes are capable of reducing AAI to species forming DNA adducts. Induction of CYP1A proteins and their enzyme activities correlated with increased AAI-DNA adduct formation *ex vivo* (Fig. 8). Therefore, induction of CYP1A1 and 1A2 leads to both oxidation and reduction of AAI which indicates that in case of hypoxia AAI must act as a ligand of CYP1A heme iron under low pO_2. Indeed, reduction of AAI as a ligand of heme iron of CYP1A1 and 1A2 could be confirmed by molecular modeling (Jerabek et al., 2012; Stiborová et al., 2014b). On the other hand, under aerobic conditions AAI acts as a classical substrate of CYP1A1 or 1A2, and takes one atom of atmospheric oxygen to *O*-demethylate the methoxy group of AAI to generate AAIa. In line with this suggestion is the finding that binding of AAI to the active site of the Compounds I of CYP1A1 and 1A2 indeed favors *O*-demethylation of AAI to AAIa (see Fig. 5 in Stiborová et al., 2015b). However, as shown in Fig. 2, the increased reductive activation of AAI *ex vivo* had no apparent impact on the reductive metabolism of AAI *in vivo*; AAI-DNA adduct formation was attenuated by induction of CYP1A enzymes. Likewise, induction of cytosolic NQO1, which led to an increase in AAI-DNA adduct formation *ex vivo*, had no significant effect *in vivo*, as a decrease in AAI-DNA adduct levels was observed. These findings demonstrate that *in vivo* the oxygen concentrations in rat tissues are sufficient to facilitate the process of the oxidative *O*-demethylation of AAI, which is thereafter the predominant reaction of CYP1A1/2 in AAI metabolism *in vivo*. Therefore, in addition to the influence of CYP1A expression, the *in vivo* pO_2 in tissues is an important factor that affects the balance between nitroreduction and *O*-demethylation of AAI, thereby influencing its (geno) toxicity and carcinogenicity. Indeed, the presence of oxygen in the *in-vitro* incubations of AAI with DNA and microsomal or cytosolic enzymes strongly inhibits the levels of AAI-DNA adducts formed in these systems (Schmeiser et al., 1997).

Based on the present study and taking into account previous results obtained in *Cyp1a*-knock-out and *CYP1A*-humanized mouse lines (Rosenquist et al., 2010; Arlt et al., 2011; Stiborová et al., 2012, 2014a,b,c), we conclude that the efficiency of the CYP1A family to protectively oxidize AAI to AAIa prevails over its reducing activation *in vivo*. The evaluation of inter-individual variations in the human CYP1A enzymes, including their genetic polymorphisms, remains a major challenge to explain human individual susceptibility to AAI, and to predict the risk of cancer among patients suffering from AAN and BEN.

Conflict of interest

The authors declare that there are no conflicts of interest.

Funding

Financial support from Grant Agency of the Czech Republic (grant 14-18344S) and Charles University in Prague (grants UNCE 204025/2012 and 570513) is highly acknowledged. Work at King's College London is supported by Cancer Research UK (grant C313/A14329).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.01.011.

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Arch Toxicol (2015) 89:2141–2158
DOI 10.1007/s00204-014-1360-1

GENTOXICITY AND CARCINOGENICITY

The influence of ochratoxin A on DNA adduct formation by the carcinogen aristolochic acid in rats

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Received: 3 June 2014 / Accepted: 28 August 2014 / Published online: 11 September 2014

Abstract Exposure to the plant nephrotoxin and carcinogen aristolochic acid (AA) leads to the development of AA nephropathy, Balkan endemic nephropathy (BEN) and upper urothelial carcinoma (UUC) in humans. Beside AA, exposure to ochratoxin A (OTA) was linked to BEN. Although OTA was rejected as a factor for BEN/UUC, there is still no information whether the development of AA-induced BEN/UUC is influenced by OTA exposure. Therefore, we studied the influence of OTA on the genotoxicity of AA (AA–DNA adduct formation) in vivo. AA–DNA adducts were formed in liver and kidney of rats treated with AA or AA combined with OTA, but no OTA-related DNA adducts were detectable in rats treated with OTA alone or OTA combined with AA. Compared to rats treated with AA alone, AA–DNA adduct levels were 5.4- and 1.6-fold higher in liver and kidney, respectively, of rats treated with AA combined with OTA. Although AA and OTA induced NAD(P)H:quinone oxidoreductase (NQO1) activating AA to DNA adducts, their combined treatment did not lead to either higher NQO1 enzyme activity or higher AA–DNA adduct levels in ex vivo incubations. Oxidation of AA I (8-methoxy-6-nitrophenanthro[3,4-*d*]-1,3-dioxole-5-carboxylic acid) to its detoxification metabolite, 8-hydroxyaristolochic acid, was lower in microsomes from rats treated with AA and OTA, and this was paralleled by lower activities of cytochromes P450 1A1/2 and/or 2C11 in these microsomes. Our results indicate that a decrease in AA detoxification after combined exposure to AA and OTA leads to an increase in AA–DNA adduct formation in liver and kidney of rats.

Keywords Balkan endemic nephropathy · Aristolochic acid nephropathy · Aristolochic acid · Ochratoxin A · DNA adducts

Electronic supplementary material The online version of this article (doi:10.1007/s00204-014-1360-1) contains supplementary material, which is available to authorized users.

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Introduction

Balkan endemic nephropathy (BEN) is a chronic tubulointerstitial nephropathy characterized by an insidious onset and gradual progression to end-stage renal disease (ESRD) which was first described more than 60 years ago (Danilovic et al. 1957; Tanchev et al. 1956). BEN remains to be an important medical, social and economic burden for all countries harboring this devastating disease. It affects residents of rural farming villages located along tributaries of the Danube river in Bosnia and Herzegovina, Bulgaria, Croatia, Romania and Serbia (Pfohl-Leszkowicz 2009; Radovanovic 2002; Stefanovic 1983). A characteristic feature of BEN is its close association with

upper urothelial carcinomas (UUC) of the renal pelvis and ureter (Miletić-Medved et al. 2005; Radovanovic 2002; Stefanovic 1983; Stefanović and Radovanović 2008). Both BEN and UUC exhibit a familial but not inherited association, suggesting the importance of environmental factors as well as genetic determinants in this disease (Ceović et al. 1985; Radovanovic 2002; Toncheva et al. 1998). For the past decades, a variety of environmental agents have been investigated (Arlt et al. 2002a, b, 2007; Grollman et al. 2007; Ivic 1969; Radovanovic 2002; Voice et al. 2006); among them are various heavy metals, mycotoxins such as ochratoxin A (OTA) and organic chemicals, and recently the carcinogenic and nephrotoxic plant product aristolochic acid (AA) was identified as the main cause for the development of BEN-associated UUCs (Arlt et al. 2002b, 2007; Chen et al. 2012; Gökmen et al. 2013; Grollman et al. 2007; Hoang et al. 2013; Hranjec et al. 2005; Jelakovic et al. 2012, 2013; Long and Voice 2007; Olivier et al. 2012; Pfohl-Leszkowicz 2009; Pfohl-Leszkowicz et al. 2002, 2007; Poon et al. 2013; Schmeiser et al. 2009, 2012; Tatu et al. 1998; Voice et al. 2006). Even though exposure to OTA, a common food contaminant and nephrotoxin, was rejected as an important factor for BEN/UUC by the EU Committee on Food Safety (EFSA 2006), its role in the development of BEN cannot be ruled out. Nevertheless, dietary exposure to AA is considered the major risk factor for BEN/UUC where AA is likely ingested via home-baked bread prepared from flour contaminated by seeds of *Aristolochia clematitis* (Arlt et al. 2002b, 2007; Chen et al. 2012; Gökmen et al. 2013; Grollman and Jelakovic 2007; Grollman et al. 2007; Hranjec et al. 2005; Jelakovic et al. 2012, 2013; Schmeiser et al. 2009, 2012). The detection of AA–DNA adducts in renal tissue of BEN patients with and without UUC and the identification of AT → TA transversion mutations, the mutational signature of AA, in the *TP53* gene in BEN/UUC describe the molecular mechanism of AA carcinogenesis in BEN (Arlt et al. 2002a, 2007; Chen et al. 2012; Gökmen et al. 2013; Grollman et al. 2007; Jelakovic et al. 2012, 2013; Schmeiser et al. 2009, 2012, 2014).

Clinical manifestations and pathophysiology of BEN are very similar to another nephropathy, aristolochic acid nephropathy (AAN), which has been unambiguously proven to be caused by AA exposure (Arlt et al. 2002a; Bieler et al. 1997; Gökmen et al. 2013; Lord et al. 2004; Schmeiser et al. 1996, 2009). Further, the formation of AA–DNA adducts, mainly 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) (Fig. 1), which lead to the induction of characteristic AT → TA transversion mutations in critical genes of oncogenesis in AAN-associated UUC, indicates the molecular mechanism of AA-induced carcinogenesis (Arlt et al. 2002a, b, 2007; Chen et al. 2012; Cosyns et al. 1994; Debelle et al. 2008; De Broe 2012; Gökmen et al. 2013; Grollman et al. 2007; Hoang et al. 2013; Jelakovic et al. 2012, 2013; Lord et al. 2004; Moriya et al. 2011; Nortier et al. 2000; Olivier et al. 2012; Poon et al. 2013; Schmeiser et al. 2009, 2012; Vanherweghem et al. 1993; Yun et al. 2012). Thus, AA has been classified as a Group I human carcinogen by the International Agency for Research on Cancer. BEN and AAN have the same etiology, and depending on AA dose and duration of AA exposure, one can develop rapidly progressing renal disease as seen in many AAN patients or a more slowly progressing phenotype as was found in BEN (Arlt et al. 2002a, b, 2007; Cosyns et al. 1994; Debelle et al. 2008; De Broe 2012; Grollman and Jelakovic 2007; Grollman et al. 2007; Vanherweghem et al. 1993).

Even though AA has been proven to be the main cause of BEN, it is still possible that OTA is involved in the development of BEN/UUC. This may be an important factor for human risk assessment as exposure to the mycotoxin OTA via contaminated food has been demonstrated by high blood concentrations of OTA in residents of endemic villages for BEN (Long and Voice 2007; Peraica et al. 2008a, b; Pfohl-Leszkowicz 2009; Pfohl-Leszkowicz et al. 2002). Drug–drug interactions, in which a drug affects the pharmacological activity of another drug when humans are exposed to both drugs, are common (Long and Voice 2007; Thomas-Schoemann et al. 2014; Viau 2002). Thus, OTA may influence the biological effects of AA including its (geno) toxicity.

In the present study, we investigated the influence of OTA on the genotoxicity of the plant extract AA, a natural mixture of 8-methoxy-6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid (AAI, Fig. 1) and 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAII, Fig. 1). For this purpose, a rat model, which is currently used in standardized experimental protocols of AAN (Debelle et al. 2002; Mengs and Stotzem 1993; Priestap et al. 2012; Stiborová et al. 2014b), was used and the formation of AA–DNA adducts was investigated in vivo. Rats were either treated separately with AA and OTA or with AA in combination with OTA. The dose of OTA and AA used for the studies was higher than to be expected for human exposure, but is justified for this subacute animal study, since the aim was to determine AA–DNA adduct levels high enough to see effects upon these levels exerted by OTA. Since metabolic activation and detoxification pathways of AA might both influence AA-induced kidney damage, the expression and activities of enzymes involved in AA metabolism and crucial for the development of BEN/UUC were investigated. Thus, the influence of such treatment on cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1), which is the most efficient nitroreductase activating AA (Bárta et al. 2014; Chen et al. 2011; Levová et al. 2011; Martinek et al.

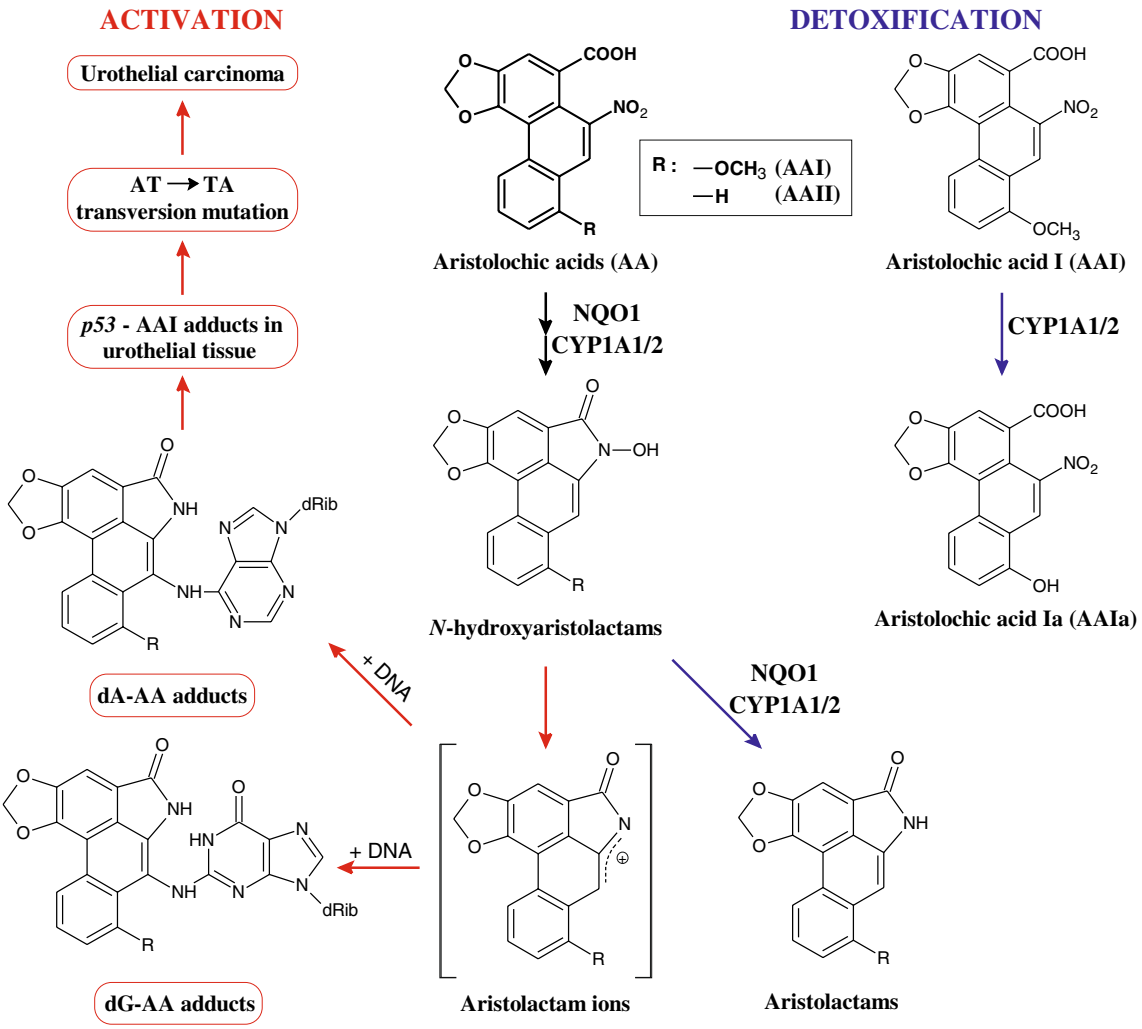


Fig. 1 Metabolic activation and detoxification pathways of aristolochic acid I and II (AAI and AAII). AAI: R = OCH_3; AAII: R = H. dA-AA; 7-(deoxyadenosin-N^6-yl)aristolactam I or 7-(deoxyadenosin-N^6-yl)aristolactam II; dG-AA, 7-(deoxyguanosin-N^2-yl)aristolactam I or 7-(deoxyguanosin-N^2-yl)aristolactam II; CYP1A1/2, cytochrome P450 1A1 and 1A2; NQO1, NAD(P)H:quinone oxidoreductase

2011; Stiborová et al. 2002a, 2003, 2008a, 2011, 2013b, 2014b), and microsomal cytochrome P450 (CYP) 1A1/2, which can both reductively activate AA and oxidatively detoxify AAI to 8-hydroxyaristolochic acid (AAIa; Fig. 1; Arlt et al. 2011a; Chen et al. 2011; Levová et al. 2011; Rosenquist et al. 2010; Sistkova et al. 2008; Stiborová et al. 2012, 2013b, 2014a, b), was examined. DNA adduct formation by AA was evaluated by the ^{32}P-postlabeling method in vivo in rat liver and kidney and in ex vivo incubations using hepatic and renal cytosols and microsomes. Further, the detoxication metabolite AAIa was measured in microsomal ex vivo incubations using high-performance liquid chromatography (HPLC).

Materials and methods

Chemicals

NADPH, the plant extract AA (~33 and ~64 % of AAI and AAII, respectively, free acids), AAI (sodium salt), OTA, Sudan I [1-(phenylazo)-2-hydroxynaphthalene], menadione (2-methyl-1,4-naphthoquinone) and calf thymus DNA were from Sigma Chemical Co (St Louis, MO, USA). 7-Ethoxyresorufin and 7-methoxyresorufin were from Fluka Chemie AG (Buchs, Switzerland). Enzymes and chemicals for the ^{32}P-postlabeling assay were from sources described (Stiborová et al. 2005).

Animal experiments and sample preparation

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Animals were acclimatized for 5 days and maintained at 22 °C with a 12-h light/dark period. Standardized diet and water were provided ad libitum. Groups of five-week old male Wistar rats (~150 g, $n = 3$/group) were treated *i.p.* daily for five consecutive days with (i) 10 mg/kg body weight (bw) of the natural plant extract AA (33 % AAI + 64 % AAII mixture), (ii) 2 mg/kg bw OTA and with (iii) both AA and OTA at doses mentioned above. The plant extract AA was dissolved in 0.15 M NaCl with addition of dimethylsulfoxide (DMSO; 5 %) at a concentration of 4 mg/ml, and OTA was dissolved in 0.1 M $NaHCO_3$ at a concentration of 0.8 mg/ml. Animals in the control groups received vehicle only. Animals were killed 24 h after the final treatment. Livers and kidneys were removed after killing, frozen in liquid nitrogen and stored at −80 °C until analysis. DNA from livers and kidneys was isolated by a standard phenol/chloroform extraction method (Schmeiser et al. 1996). Microsomes and cytosols were isolated from the rat tissues by a procedure described previously (Stiborová et al. 2003, 2005). Protein concentration in the microsomal fraction was measured using bicinchoninic acid protein assay (Wiechelman et al. 1988) with bovine serum albumin as a standard. Pooled microsomal and cytosolic samples ($n = 3$ rats/group) were used for the analyses.

DNA adduct analysis by ^{32}P-postlabeling

DNA samples were analyzed for the presence of AA–DNA adducts (Schmeiser et al. 1996; Bieler et al. 1997) and OTA-related DNA adducts (Arlt et al. 2001; Pfohl-Leszkowicz et al. 1993) by the nuclease P1 version of ^{32}P-postlabeling. Chromatographic conditions used for the detection of AA–DNA adducts were as described previously (Bieler et al. 1997): D1: 1 M sodium phosphate, pH 6.8; D3: 3.5 M lithium formiate, 8.5 M urea, pH 4.0; D4: 0.8 M LiCl, 0.5 M Tris–HCl, 8.5 M urea, pH 9.0; D5: 1.7 M NaH_2PO_4, pH 6.0. Chromatographic conditions used for the detection of OTA-related DNA adducts were as described previously (Pfohl-Leszkowicz et al. 1993): D1: 2.3 M sodium phosphate pH 5.7; D3: 4.77 M lithium formiate, 7.65 M urea, pH 3.5; D4: 0.6 M NaH_2PO_4, 5.95 M urea, pH 6.4; D5: 1.7 M NaH_2PO_4, pH 6.0. DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Schmeiser et al. 1996, 2013). AA–DNA adducts were identified using reference standards as described (Schmeiser et al. 1996). For OTA-related DNA adducts, kidney DNA from OTA-treated Wistar rats (Arlt et al. 2001; Pfohl-Leszkowicz et al. 1993) served as reference.

Preparation of antibodies and estimation of CYP1A1, 1A2 and NQO1 protein content in microsomal and cytosolic fractions isolated from rat liver and kidney

The chicken anti-rat CYP1A1, anti-rabbit CYP1A2 and anti-rat NQO1 antibodies were prepared as described previously (Stiborová et al. 2002b, 2006). Immunoquantification of microsomal CYP1A1 and 1A2 and cytosolic NQO1 was performed using Western blotting (Stiborová et al. 2006). Rat CYP1A1, rabbit CYP1A2 and human NQO1 (Sigma) were used to identify the CYP1A1, 1A2 and NQO1 bands, respectively. The antigen–antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium as dye and bands are expressed as arbitrary units (AU)/mg protein (Stiborová et al. 2002b, 2006). Glyceraldehyde phosphate dehydrogenase was used as loading control and detected by its antibody (1:750, Millipore; MA, USA).

NQO1, CYP1A1/2 and 2C11 enzyme activity assays

In hepatic and renal cytosols, NQO1 activity was measured using menadione (2-methyl-1,4-naphthoquinone) as a substrate; the assay was improved by the addition of cytochrome *c* and NQO1 activity expressed as nmol cytochrome *c* reduced (Levová et al. 2011, 2012). Microsomal samples were characterized for specific CYP1A1 and 1A2 activities: ethoxyresorufin *O*-deethylation (EROD) (CYP1A1/2) and methoxyresorufin *O*-demethylation (MROD) (CYP1A2) (Burke et al. 1994). CYP1A1 enzyme activity was also monitored by Sudan I hydroxylation to 4′-hydroxy-, 6-hydroxy- and 4′,6-dihydroxy-Sudan I (Stiborová et al. 2002b). Hepatic microsomal samples were also characterized for specific CYP2C11 activity with its marker substrate determining testosterone 16α-hydroxylation (Yamazaki et al. 2006). In hepatic and renal microsomes activity of NADPH:cytochrome, P450 oxidoreductase (POR) was analyzed using cytochrome *c* as a substrate (Stiborová et al. 2012).

Cytosolic and microsomal formation of AA–DNA adducts

The de-aerated and nitrogen-purged incubation mixtures, in which cytosols were used to activate AA, contained 50 mM Tris–HCl buffer (pH 7.4), 0.2 % Tween 20, 1 mM NADPH, 1 mg rat hepatic or renal cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AA (dissolved in 6 µl of DMSO) in a final volume of 750 µl. Incubations with cytosols were performed at 37 °C for 60 min; AA-derived DNA adduct formation was found to be linear up to 2 h (Stiborová et al. 2003). Control incubations were

performed either (i) without cytosol, (ii) without NADPH, (iii) without DNA or (iv) without AA. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Stiborová et al. 2005) and analyzed for AA–DNA adducts as described above.

The de-aerated and nitrogen-purged microsomal incubation mixtures used to activate AA ex vivo contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg of hepatic or renal microsomal protein, 0.5 mg of calf thymus DNA (2 mM dNp) and 0.5 mM AA in a final volume of 750 µl. Microsomal incubations were carried out at 37 °C for 60 min; AA–DNA adduct formation was found to be linear up to 2 h in microsomes (Stiborová et al. 2005). Control incubations were carried out either (i) without microsomes, (ii) without NADPH, (iii) without DNA or (iv) without AA. After extraction with ethyl acetate, DNA was isolated from the residual water phase and analyzed for AA–DNA adducts as described above.

Microsomal incubations to study AA and AAI oxidation to AAIa

Incubation mixtures contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 1 mg rat hepatic or renal microsomal protein and 10 µM AA (dissolved in 2.5 µl DMSO) or AAI (dissolved in distilled water) in a final volume of 250 µl and were incubated at 37 °C for 20 min; AAI *O*-demethylation to AAIa was determined to be linear up to 25 min (Levová et al. 2011; Sistkova et al. 2008). Control incubations were carried out either (i) without microsomes, (ii) without NADPH or (iii) without AA or AAI. AAI, AAII and AAIa were separated by reverse-phase HPLC, identified by mass spectrometry and quantified as described previously (Levová et al. 2011). Briefly, HPLC was carried out with an Nucleosil 100-5 C_{18}, 25 × 4.0 mm, 5 mm (Macherey–Nagel) column, using a linear gradient of acetonitrile (20–60 % acetonitrile in 55 min) in 100 mM triethylammonium acetate with a flow rate of 0.6 ml/min. A Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector set at 254 nm was used. Peaks were integrated with CHROMELEON™ 6.01 integrator. A peak eluting at retention time (r.t.) ~22 min was identified as AAIa using mass-spectroscopy analysis (Levová et al. 2011). Typical HPLC chromatograms of AAIa formed from AA or AAI are shown as a supplementary Figure 1.

Statistical analyses

For statistical data analysis, we used Student's *t* test. All *P* values are two-tailed and considered significant at the 0.001 level.

Results

DNA adduct formation in rats treated with OTA and the plant extract AA compared to adduct formation in rats treated with AA alone

Formation of AA–DNA adducts and OTA-related DNA adducts was determined by ^{32}P-postlabeling in liver and kidney of rats treated with a total *i.p.* dose of 50 mg/kg bw of the plant extract AA (natural mixture of AAI and AAII), with a total *i.p.* dose of 10 mg/kg bw of OTA and with both these agents together.

It is noteworthy that modifications of the nuclease P1 version of ^{32}P-postlabeling assay were made in order to detect and quantify the AA–DNA adducts (Bieler et al. 1997; Schmeiser et al. 1996, 1997) and OTA-related DNA adducts (Arlt et al. 2001; Pfohl-Leszkowicz et al. 1993), respectively. It was necessary to use different chromatographic solvent conditions as outlined in the "Materials and methods."

Using the nuclease P1 version of ^{32}P-postlabeling assay routinely used to detect AA–DNA adducts, all liver and kidney samples from Wistar rats treated with AA showed an adduct pattern similar to that found in kidney tissue from BEN and AAN patients (Arlt et al. 2002a, Nortier et al. 2000; Schmeiser et al. 1996, 1997, 2012). As shown in Fig. 2a, b, the adduct pattern consisted of three adduct spots (spots 1–3) with spot 3 being the major one. These spots have been identified as 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI, spot 1), 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI; spot 2) and 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII; spot 3). We have shown previously that the dA-AAII adduct can also be generated from AAI, probably via a demethoxylation reaction of AAI or dA-AAI (Schmeiser et al. 1997; Stiborová et al. 1994). Therefore, deoxyadenosine is the major target for DNA modifications by AA, pointing to the general importance of deoxyadenosine adducts in the carcinogenic process of AA. In contrast, no adducts were found in DNA of control rats treated with vehicle only (data not shown).

When labeled DNA samples were chromatographed under conditions suitable for the detection of OTA-related adducts (Arlt et al. 2001; Pfohl-Leszkowicz et al. 1993), all three purine AA–DNA adducts were detectable (Fig. 2d) and none of the AA–DNA adducts migrated in the area of the thin-layer chromatography plate where OTA-related DNA adduct spots would be located [compare the Fig. 2d, f shown in the work of Arlt et al. (2001)]. Both chromatographic procedures yielded similar AA–DNA adduct levels (see Supplementary Table 1). However, in contrast, no OTA-related DNA adducts were detectable in rats treated with OTA alone (Fig. 2c). Likewise, no OTA-related DNA

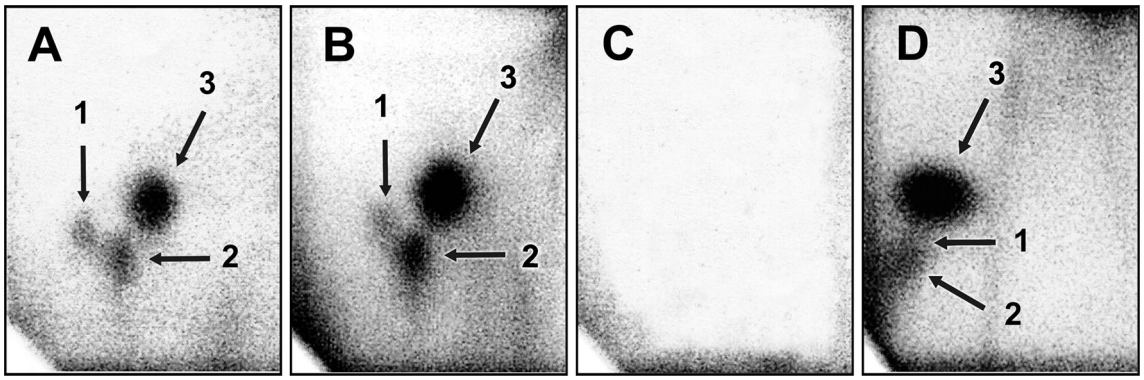


Fig. 2 Autoradiographic pattern of DNA adducts found in (**a**) liver tissue of rats treated with AA (33 % AAI and 64 % AAII), **b** with AA combined with OTA, **c** with OTA and **d** with AA combined with OTA by the nuclease P1 enrichment version of the ^{32}P-postlabeling method. Chromatographic conditions used for the detection of AA–DNA adducts in (**a**, **b**) were as follows: D1: 1 M sodium phosphate, pH 6.8; D3: 3.5 M lithium formiate, 8.5 M urea, pH 4.0; D4: 0.8 M LiCl, 0.5 M Tris–HCl, 8.5 M urea, pH 9.0; D5: 1.7 M NaH_2PO_4, pH 6.0. Chromatographic conditions used for the detection of OTA-related DNA adducts in (**c**, **d**) were as follows: D1: 2.3 M sodium phosphate pH 5.7; D3: 4.77 M lithium formiate, 7.65 M urea, pH 3.5; D4: 0.6 M NaH_2PO_4, 5.95 M urea, pH 6.4; D5: 1.7 M NaH_2PO_4, pH 6.0. Origins in the *bottom left corner* were cut off before exposure. PEI-cellulose TLC plates from Macherey and Nagel (Düren, Germany) have been used. Spot 1, dG-AAI; spot 2, dA-AAI; and spot 3, dA-AAII

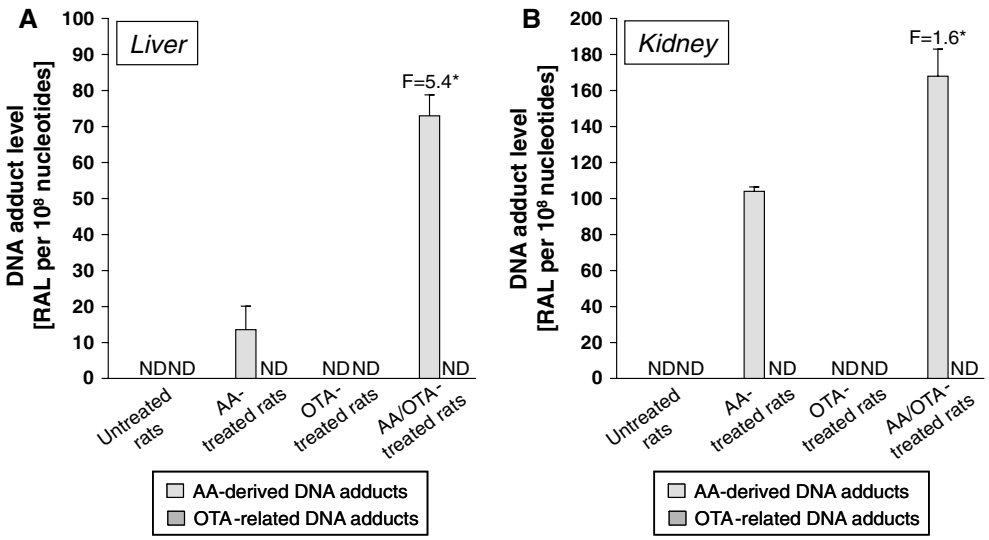


Fig. 3 Quantitative TLC ^{32}P-postlabeling analysis of AA–DNA adduct levels in organs of rats treated with AA, OTA and AA combined with OTA or in those of untreated (control) rats (see 'Materials and methods' for details). Numbers above columns ("*F*") indicate fold changes in DNA adduct levels in animals treated with AA combined with OTA compared to animals treated with AA alone. 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; $^*P < 0.001$, different from animals treated with AA alone. *ND* not detected

adducts were found in liver and kidney of rats treated with AA together with OTA, whereas AA–DNA adducts were clearly detectable in these samples and identified as described above (Fig. 2d).

Generally, AA–DNA adduct levels were higher in kidney, the target organ of AA genotoxicity, than in liver (Fig. 3a, b). In kidney, the levels of AA–DNA adducts increased 1.6-fold when AA treatment was combined with the OTA exposure, in liver 5.4-fold. ($P < 0.001$) (see also Supplementary Table 1). Therefore, OTA, when administered to rats together with AA, seems to induce pathways which lead to a higher bioactivation of AA in both organs. Since NQO1 and CYP1A1/2 contribute to the metabolic activation of AA (for a review see Stiborová et al. 2008a, b, 2013b, 2014a, b), their protein levels and enzyme activities were investigated.

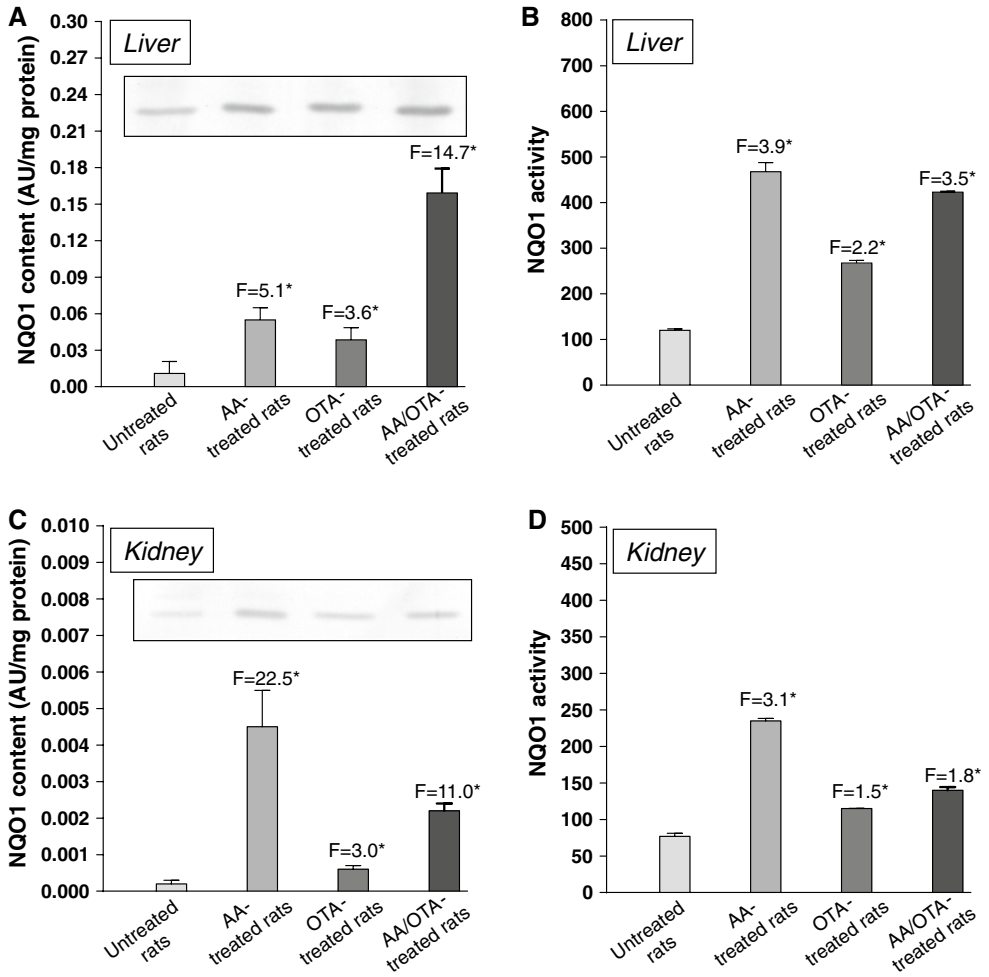


Fig. 4 NQO1 protein levels (**a**, **c**) and NQO1 enzyme activity (**b**, **d**) in rat cytosols isolated from untreated (control) animals and animals treated with AA, OTA or AA combined with OTA. Cytosol isolated from liver (**a**) or kidney (**c**) was analyzed by Western blotting in the same blot (insert) and, therefore, can be compared directly. Human recombinant NQO1 (Sigma) was used to identify the rat NQO1 band in rat cytosol (data not shown). NQO1 activity in hepatic (**b**) and renal cytosols (**d**) was determined using menadione and cytochrome *c* as substrate (expressed as nanomoles cytochrome *c* reduced per minute per milligram protein). Numbers above columns ("*F*") indicate fold changes in protein level or enzyme activity compared to control. Values are given as the means ± SD ($n = 3$). Comparison was performed by *t* test analysis; $^*P < 0.001$, different from control

The effect of AA treatment with or without OTA upon NQO1 and CYP1A1/2 protein levels and their enzymatic activities in rat liver and kidney

Multiple treatment of rats with AA led to a significant induction (up to 22-fold) of NQO1 protein levels in cytosolic liver and kidney samples (Fig. 4a, c). NQO1 enzyme activities increased 3.9- and 3.1-fold in liver and kidney, respectively ($P < 0.001$; Fig. 4b, d). Likewise, administration of OTA to rats induced NQO1 protein levels in liver and kidney 3–4 times which also resulted in an increase in NQO1 enzyme activity (Fig. 4). These findings indicate that both compounds are potent NQO1 inducers in rats.

When amounts of NQO1 protein expression found in rats treated with AA combined with OTA were compared with those treated with AA alone, a ~3-fold increased expression was produced in the liver, but this was not paralleled by NQO1 enzyme activity in this organ (Fig. 4a, b). In contrast to the liver, no increase in NQO1 protein expression was found in kidney after the combined treatment compared with AA alone. Only half of the NQO1 protein level was observed after the combined treatment, and this decrease corresponded to a lower NQO1 enzyme activity in this tissue (Fig. 4c, d).

The effect of exposure to AA and OTA on the protein levels of CYP1A1 and 1A2 was examined in hepatic and renal microsomes as both enzymes participate in AA metabolism. A dual role of these CYPs has been demonstrated (Stiborová et al. 2013b, 2014a) where under anaerobic (i.e., reductive) conditions, AA is activated to species

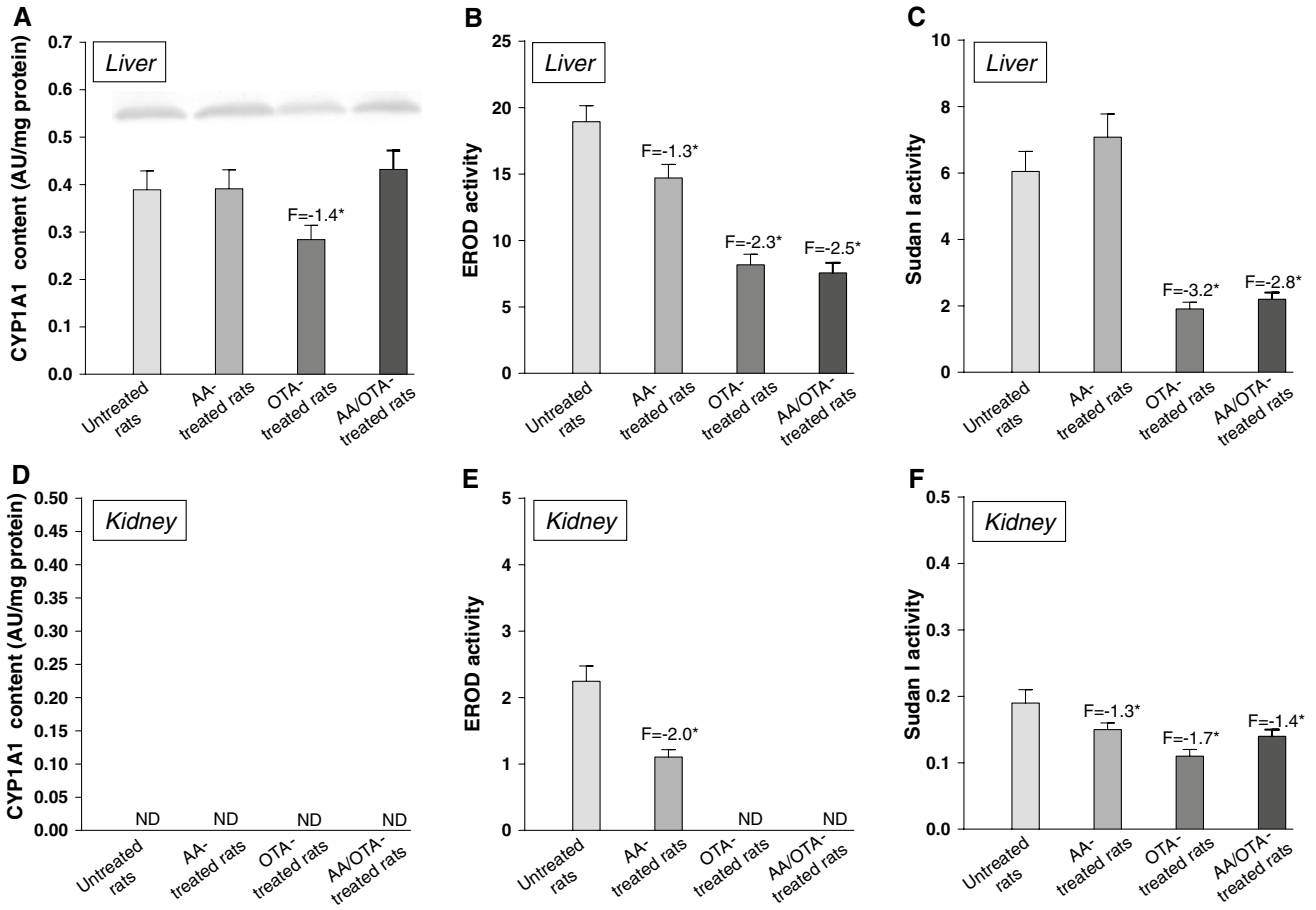


Fig. 5 CYP1A1 (**a**, **d**) protein levels in rat microsomes isolated from untreated (control) animals and animals treated with AA, OTA or AA combined with OTA. Microsomes isolated from liver (**a**) and kidney (**d**) were analyzed by Western blotting. Values are given as the means of arbitrary units (AU) ± SD ($n = 3$). Levels of CYP1A1 protein in kidney microsomes were close to the detection limit and therefore not quantified. CYP1A enzyme activity as measured by EROD (picomoles resorufin per minute per milligram protein) (**b**, **e**) or Sudan I oxidation (nanomoles total C-hydroxylated metabolites per minute per milligram protein) (**c**, **f**). Numbers above columns ("*F*") indicate fold changes in protein level or enzyme activity compared to control. *ND* not detected. Comparison was performed by *t* test analysis; $^*P < 0.001$, different from control

forming DNA adducts, whereas under aerobic (i.e., oxidative) conditions, AAI is detoxified to AAIa (Fig. 1). In the present study, we found that CYP1A1 protein levels were higher in hepatic than in renal microsomes (Fig. 5a, d). Because levels of CYP1A1 protein in the kidney determined by Western blotting were close to the detection limit, they were not quantified (Fig. 5d). Marker activities of the CYP1A1/2 enzymes were detectable in both studied organs; EROD activity for CYP1A1/2 is shown in Fig. 5b, e, and Sudan I oxidation, a marker for CYP1A1 enzyme activity, is shown in Fig. 5c, f. However, only very low activities of these enzymes were measurable in kidney.

CYP1A2 was expressed only in rat liver, and not in kidney (Fig. 6a, c) confirming that CYP1A2 is almost exclusively a hepatic enzyme (Rendic and DiCarlo 1997). In concordance, MROD activity, a marker reaction of CYP1A2, was found in liver (Fig. 6b), with very low activity in kidney (Fig. 6d).

As shown in Fig. 5, AA and OTA treatment alone or their combined administration essentially had no effect on CYP1A1 protein expression in the liver. OTA treatment seems to result in a slight decrease in CYP1A1 expression (Fig. 5a). However, a decrease in EROD activity and Sudan I oxidation was found particularly after OTA exposure (Fig. 5), suggesting that the CYP1A1 signals detected by Western blotting probably do not truly reflect the protein levels of the active enzyme and that the measurement of CYP1A enzyme activity provides a more accurate assessment of enzyme expression. CYP1A2 protein level in liver and MROD activity remained essentially unchanged by the treatment with both agents. Again, as seen for CYP1A1 protein, OTA treatment resulted in a 2.7-fold decrease in CYPA2 expression (Fig. 6).

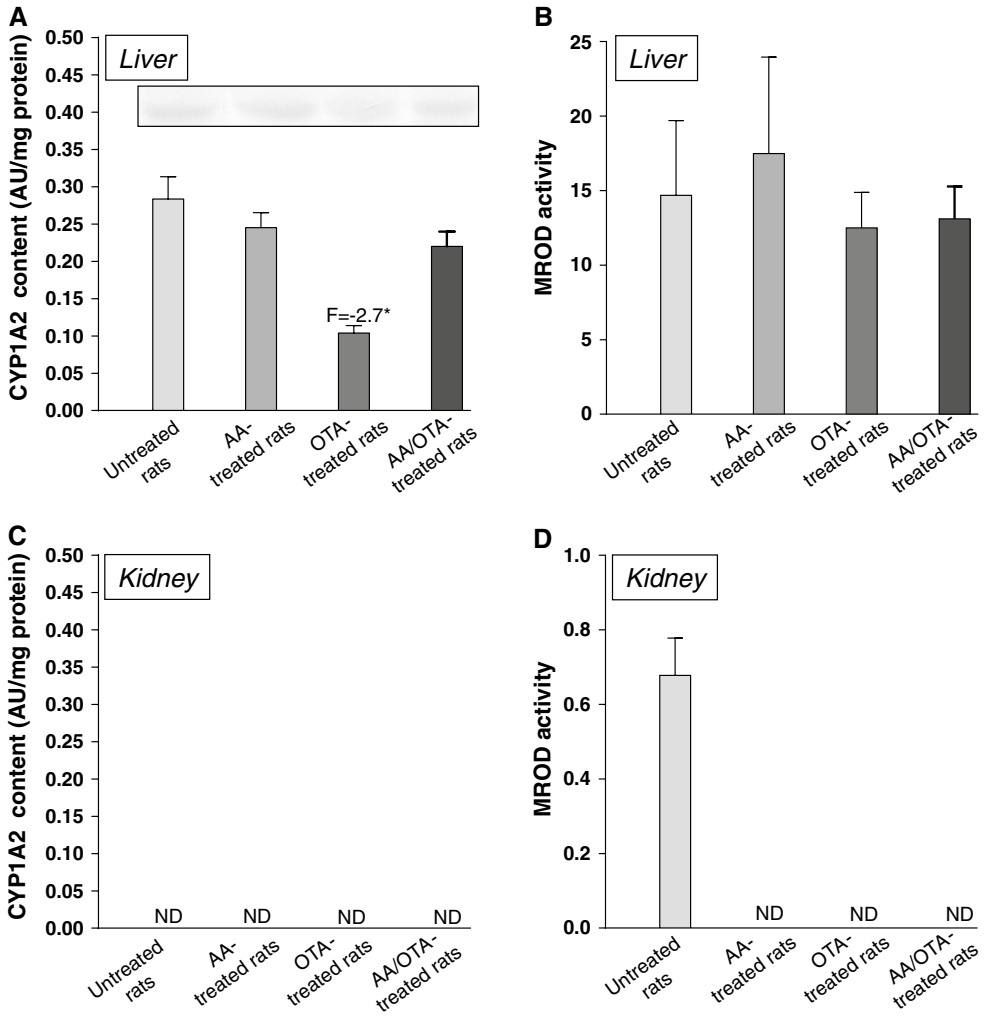


Fig. 6 CYP1A2 (**a**, **c**) protein levels in rat microsomes isolated from untreated (control) animals and animals treated with AA, OTA or AA combined with OTA. MROD activity (picomoles resorufin per minute per milligram protein) is shown in panels (**b**, **d**). All values are given as the means ± SD ($n = 3$). Numbers above columns ("*F*") indicate fold changes in protein level or enzyme activity compared to control. *ND* not detected. Comparison was performed by *t* test analysis; $^{*}P < 0.001$, different from control

The activity of POR was decreased in rat liver and kidney by treatment with OTA alone or in combination with AA whereas AA treatment alone had no effect in liver (Fig. 7). POR not only acts as an electron donor in catalytic functions of CYPs (Laursen et al. 2011; Pudney et al. 2011), but is also able to activate AA to some extent (Stiborová et al. 2005).

Cytosolic versus microsomal activation of AA

In further experiments, AA–DNA adduct formation catalyzed by cytosols isolated from liver and kidney of rats from all treatment groups was investigated ex vivo. Cytosols were incubated with AA, calf thymus DNA and NADPH, the cofactor of NQO1, and analyzed for DNA adduct formation by ^{32}P-postlabeling. AA was activated by hepatic and renal cytosols as evidenced by specific AA–DNA adduct formation (Fig. 8a, c). The observed adduct pattern was the same as that found in rat liver in vivo (see Fig. 2). Interestingly, in incubations with renal cytosols, dA-AAII was the only adduct formed, while dA-AAI and dG-AAI were not detectable (see Supplementary Table 2). No DNA adducts were observed in control incubations carried out in parallel (data not shown). Liver cytosols from rats treated with AA produced AA–DNA adduct levels 1.5-fold higher relative to cytosols isolated from untreated animals (controls), which corresponded to higher NQO1 activity in this cytosol (compare Figs. 4b, 8a). Renal cytosols isolated from AA-treated rats and rats exposed to AA with OTA led to 1.4- and 1.7-fold higher adduct levels in calf thymus DNA, respectively, relative to cytosol from control animals ($P < 0.001$). Again, observed adduct levels

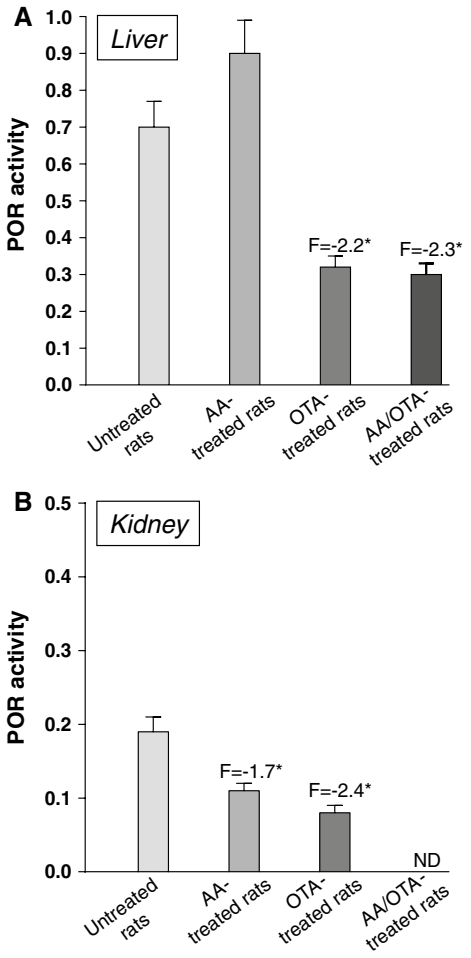


Fig. 7 POR enzymatic activity in rat microsomes isolated from untreated (control) animals and animals treated with AA, OTA or AA combined with OTA. POR was measured using cytochrome *c* as substrate (nanomoles cytochrome *c* reduced per minute per milligram protein). All values are given as the means ± SD ($n = 3$). Numbers above columns ("*F*") indicate changes in enzyme activity compared to control. Comparison was performed by *t* test analysis; $*P < 0.001$, different from control

corresponded to NQO1 enzyme activity (compare Figs. 4d, 8c) while cytosols isolated from OTA-treated rats were as active as control (untreated) cytosols.

When AA–DNA adduct levels formed in ex vivo incubation using cytosols isolated from rats treated with AA combined with OTA were compared with those treated with AA alone, essentially no difference was found both in hepatic and renal cytosols, more or less parallel to the NQO1 enzyme activity in these organs (Figs. 4, 8a, c).

Because microsomal CYP1A1/2 are also able to activate AA by nitroreduction to species forming DNA adducts (Arlt et al. 2011a; Levová et al. 2011; Stiborová et al. 2001a, 2005, 2012, 2014b), AA–DNA adduct formation was analyzed in ex vivo incubations using hepatic and renal microsomes isolated from control (untreated) and treated rats. AA was reductively activated by hepatic and renal microsomes from all treatment groups (Fig. 8b, d). The adduct pattern generated was the same as that found in vivo (see Fig. 2). No adducts were observed in control incubations carried out in parallel (data not shown). The only significant increase in AA–DNA adduct levels catalyzed by hepatic microsomes was seen in rats exposed to OTA where AA–DNA adduct formation was 2.8-fold higher relative to controls (Fig. 8b). The reason for this finding remains to be explained, because no increase in CYP1A or POR activities that could be responsible for AA activation in this sample were found (compare Figs. 5, 6, 7, 8). In ex vivo incubations with renal microsomes, lower levels of AA–DNA adducts were found in all treatment groups relative to controls (Fig. 8d). When AA–DNA adduct levels produced by microsomes isolated from rats treated with AA were compared to those from rats treated with AA together with OTA, AA–DNA adduct formation was the same (Fig. 8b, d).

The effect of treatment of rats with AA, OTA and both agents in combination on oxidation of AA and AAI to AAIa by rat hepatic and renal microsomes

Since microsomal CYP1A1/2 also detoxifies AAI to its *O*-demethylated metabolite AAIa (Arlt et al. 2011a; Levová et al. 2011; Rosenquist et al. 2010; Sistkova et al. 2008; Stiborová et al. 2012, 2013b, 2014a, b), AAIa formation from AA by hepatic and renal microsomes of all groups were investigated ex vivo and AAIa was formed in all incubation (Fig. 9; Supplementary Fig. 1). AAIa formation decreased to 80, 77 and 50 % in hepatic microsomes isolated from rats treated with AA, OTA and AA combined with OTA, respectively, relative to controls (Fig. 9a). Likewise, AAIa formation decreased up to ~70 % in renal microsomes of all groups of treated rats relative to controls (Fig. 9b). Similar results were found when pure AAI was used as substrate (Fig. 9c, d). The amounts of AAIa generated from AAI were lower in hepatic and renal microsomes of all treated rat groups, and the lowest amounts of AAIa were formed in microsomes of rats treated with AA combined with OTA. Overall, the decreases in AAIa formation corresponded to the measured CYP1A1/2 and POR enzyme activities in the microsomes (see Figs. 5, 6, 7). In addition to the CYP1A1/2, the CYPs of the 2C subfamily, which are highly expressed in livers of male rats (Nedelcheva and Gut 1994; Večeřa et al. 2011; Zachařová et al. 2012) and known to oxidize AAI to AAIa (Levová et al. 2011; Stiborová et al. 2014b), might also contribute to a decrease in *O*-demethylation of AAI to AAIa in hepatic microsomes. Indeed, the marker activity of CYP2C11,

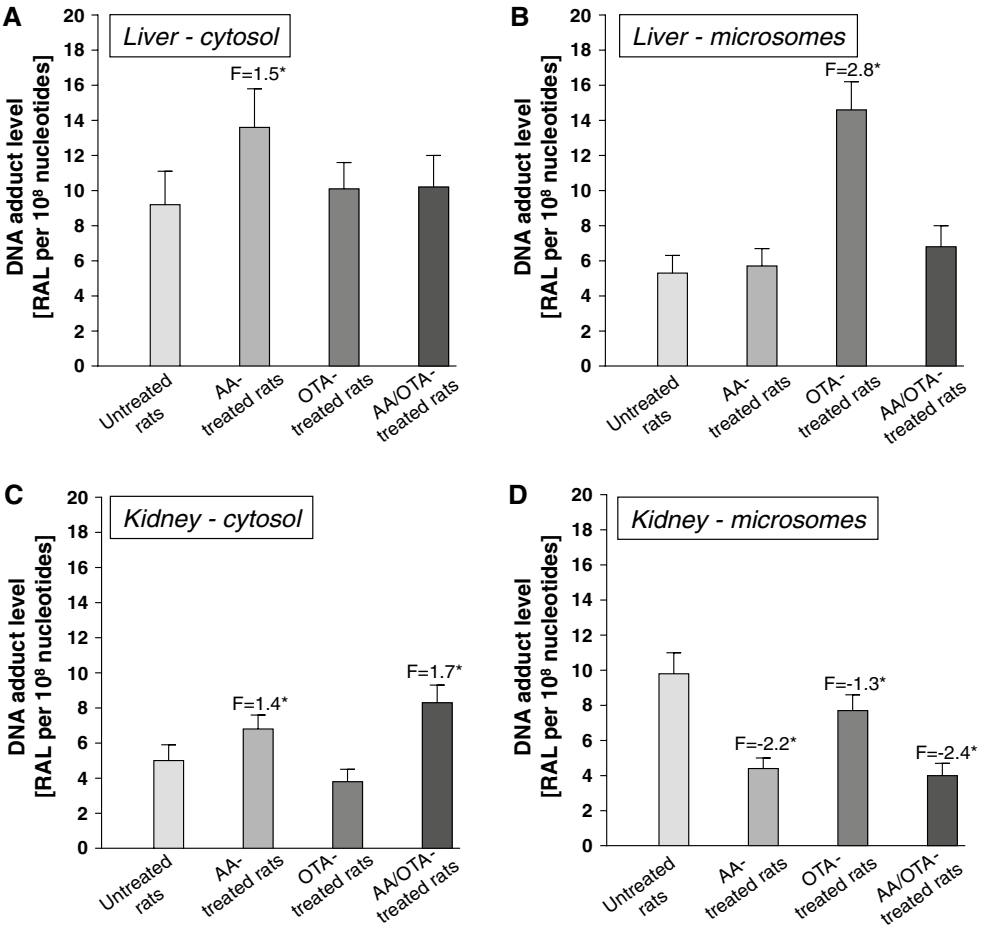


Fig. 8 DNA adduct formation ex vivo by AA in rat cytosols (**a**, **c**) and microsomes (**b**, **d**) isolated from liver (**a**, **b**) and kidney (**c**, **d**) of untreated (control) animals and animals treated with AA, OTA or AA combined with OTA and incubated with DNA, AA and NADPH. AA–DNA adduct formation was determined by ^{32}P-postlabeling. Values are given as the means ± SD ($n = 3$); each DNA sample was determined by two postlabeling analyses. RAL, relative adduct labeling. Numbers above columns ("*F*") indicate fold changes in DNA adduct levels compared to control. Comparison was performed by *t* test analysis; **P* < 0.001, different from control

testosterone 16α-hydroxylation (Yamazaki et al. 2006), was decreased by treating rats with OTA and AA together with OTA (Fig. 10), and this was paralleled by a decrease in AAIa formation.

Because the metabolism of AAII has not been investigated as yet, we evaluated whether AAII is oxidized to AAIa and/or to other metabolites by rat microsomes. However, neither AAIa nor any other AAII metabolites were detectable by HPLC analysis in hepatic and renal microsomal incubations with AAII under the experimental conditions used (Supplementary Fig. 1).

Discussion

The etiology of BEN and BEN-associated UUC has been the subject of many studies since the first description of this disease nearly 60 years ago (Bamias and Boletis 2008; Grollman et al. 2007; Jelakovic et al. 2012, 2013; Long and Voice 2007; Olivier et al 2012; Pfohl-Leszkowicz 2009; Radovanovic 2002; Stefanovic 1983; Tatu et al. 1998; Voice et al. 2006). It has long been suspected that BEN is an environmental disease and the role of the mycotoxin and nephrotoxin OTA has been investigated extensively for decades (Djukanovic et al. 2003; Pfohl-Leszkowicz 2009; Pfohl-Leszkowicz et al. 2007; Stefanovic 1983; Toncheva et al. 1998). Nevertheless, the hypothesis that OTA plays a causative role in the etiology of BEN/UUC has been rejected by the EU Committee on Food Safety (EFSA 2006). In contrast, AA has been shown to be the major risk factor for BEN/UUC (Arlt et al. 2002b, 2007; Chen et al. 2012; Gökmen et al. 2013; Grollman et al. 2007; Hranjec et al. 2005; Jelakovic et al. 2012, 2013; Schmeiser et al. 2009, 2012). However, information is still lacking whether exposure to OTA among residents living in endemic areas influences the development of AA-induced BEN/UUC. Therefore, the aim of the present study was to evaluate the impact of OTA exposure on the genotoxicity of AA in rats because they have been found to be a suitable model to study AAN in experimental animals (Arlt et al. 2001; Cosyns et al. 1998; Debelle et al. 2002, 2003; Lebeau et al. 2005; Pfohl-Leszkowicz 2009; Stiborová et al. 2014b; Vettorazzi et al. 2011).

Previous studies in rats have shown the formation of AA–DNA adducts in liver and kidney 24 h after

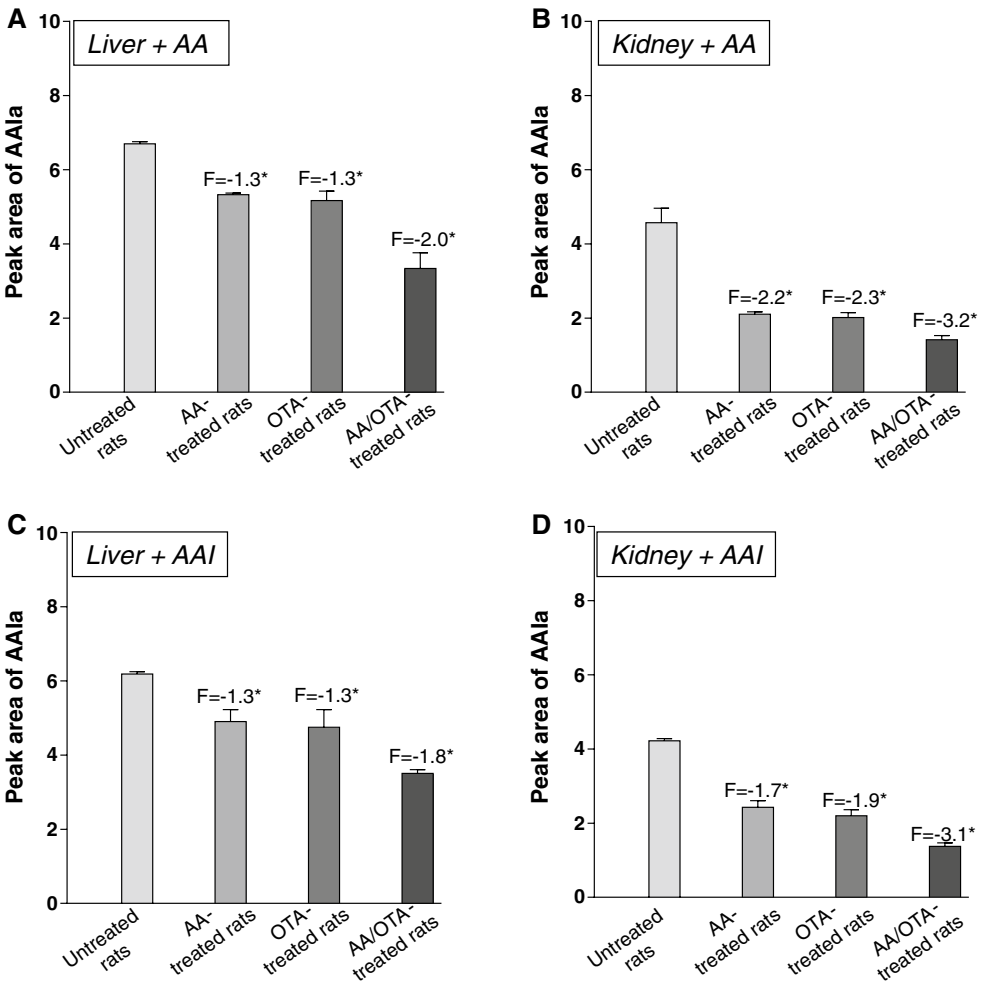


Fig. 9 Formation of AAIa (*peak area* per minute per milligram protein) in rat microsomes isolated from untreated (control) animals and animals treated with AA, OTA or AA combined with OTA with AA (**a**, **b**) or AAI (**c**, **d**) as substrates. All values are given as the means ± SD ($n = 3$). Numbers above columns ("*F*") indicate fold changes in AAIa levels compared to control. Comparison was performed by *t* test analysis; $*P < 0.001$, different from control

administration (Arlt et al. 2002a; Pfau et al. 1990; Stiborová et al. 1994; 2014b). Therefore, we used this treatment duration to study DNA adduct formation in vivo in our study. We demonstrated that exposure of Wistar rats to OTA in combination with AA affected DNA adduct formation induced by AA. Levels of AA–DNA adducts in liver and kidney of these animals were higher than the levels formed after exposure to AA alone. In contrast, while AA generated covalent DNA adducts in vivo, no OTA-related DNA adducts were detectable by the ^{32}P-postlabeling method. These results are fully in concordance with previous conclusions indicating that exposure to AA seems to be the crucial factor responsible for the development of UUC in patients suffering from BEN/AAN (Arlt et al. 2002a, b, 2007; Chen et al. 2012; Gökmen et al. 2013; Grollman et al. 2007; Hranjec et al. 2005; Jelakovic et al. 2012, 2013; Schmeiser et al. 2009, 2012). On the other hand, our results suggest that interactions between OTA and AA during exposure might influence the development of BEN/UUC. Several reasons might be considered to be responsible for the obtained results including effects of OTA on the bioavailability of AA in different organs, its absorption and renal clearance as well as on the expression of enzymes catalyzing the activation and detoxification of AA.

Therefore, one of these possible features was further investigated. We examined whether the increase in AA–DNA adduct formation in vivo in liver and kidney after the combined exposure of rats to AA and OTA is linked to alterations in expression and activity of the AA metabolizing enzymes NQO1, CYP1A and/or CYP2C. Twenty-four hour after repeated treatment, rat liver and kidney NQO1 protein levels were induced both by OTA and AA, and this induction corresponded to higher NQO1 enzyme activity. The inducing effect of AA on NQO1 found in this work confirmed previous studies, where NQO1 protein levels and its enzyme activity were induced by AA in kidney and/or liver of rats and mice (Arlt et al. 2011b; Bárta et al. 2014; Levová et al. 2011, 2012; Stiborová et al. 2001b, 2002a,

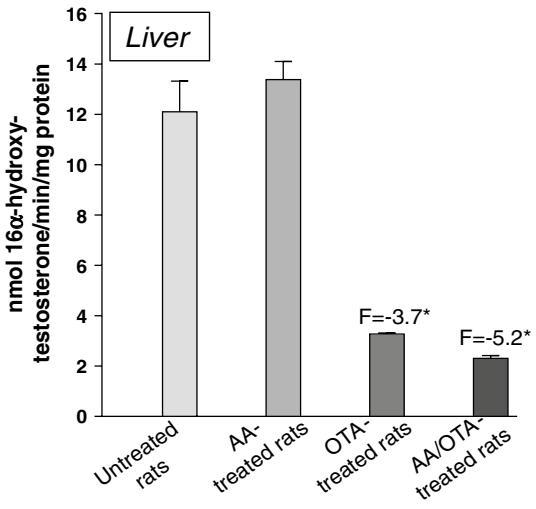


Fig. 10 CYP2C11 enzyme activities in rat hepatic microsomes isolated from untreated (control) animals and animals treated with AA, OTA or AA combined with OTA. CYP2C11 was measured as testosterone 16α-hydroxylation (nanomoles 16α-hydroxytestosterone per minute per milligram protein). All values are given as the means ± SD ($n = 3$). Numbers above columns ("*F*") indicate fold changes in enzyme activities compared to control. Comparison was performed by *t* test analysis; *$P < 0.001$, different from control

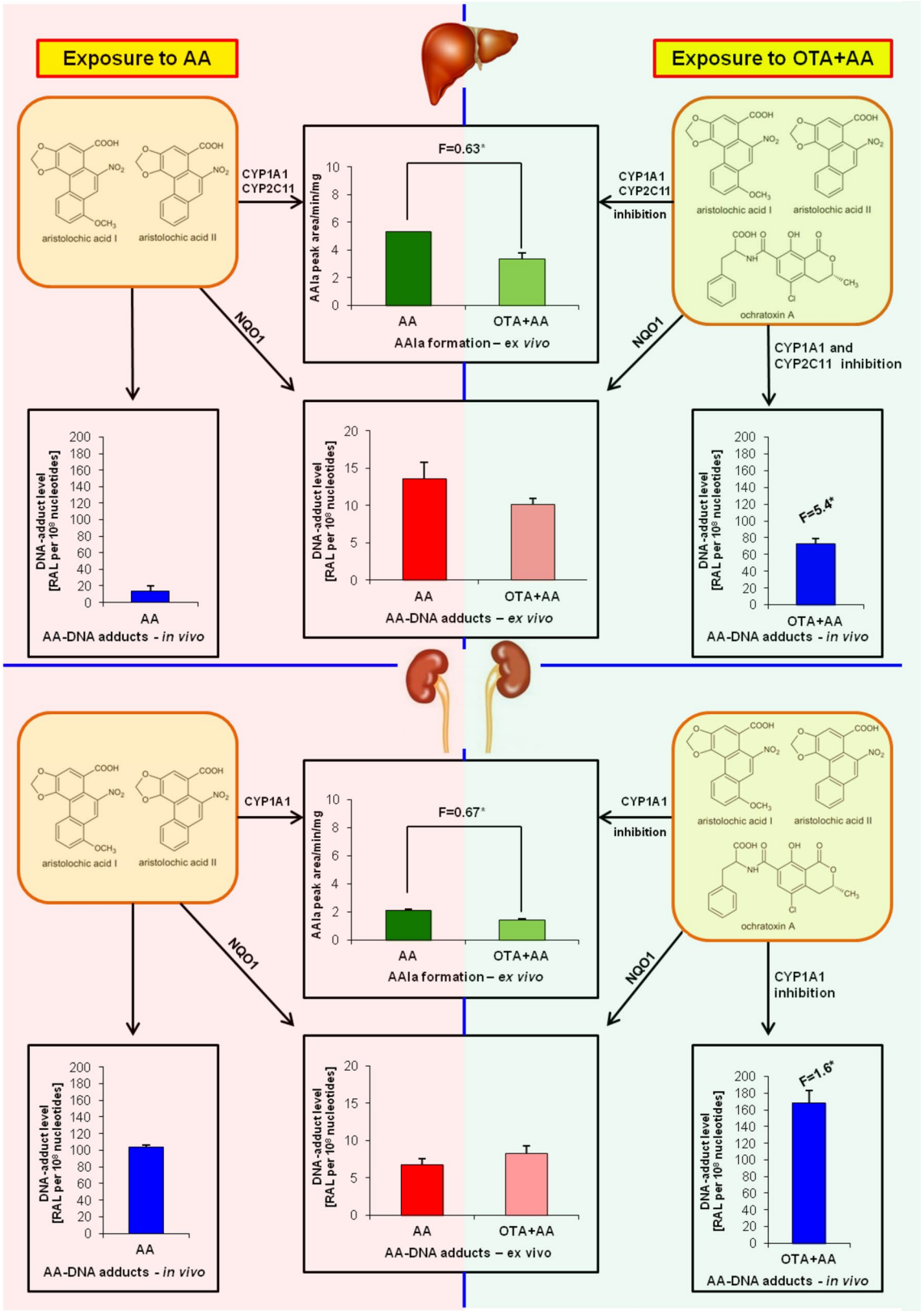
2014a, b). This increase in NQO1 protein expression and its enzyme activity also led to elevated levels of AA–DNA adducts formed in ex vivo incubations of AA with DNA and hepatic and renal cytosols of these rats. Thus, it is possible that NQO1 might be induced in patients suffering from BEN/AAN, and this feature could contribute to an elevated cancer risk. However, in this study, we also found that OTA induces NQO1 protein and its enzyme activity in rat liver and kidney. Although we showed that both AA and OTA increased NQO1 protein levels and activity, the mechanism(s) of induction are not yet clear. NQO1 induction has been widely investigated in a variety of studies; protein levels of NQO1 are induced by several chemicals including 2,3,7,8-tetrachlorodibenzo[1,4]dioxine, polycyclic aromatic hydrocarbons, azo dyes Sudan I and Sudan III, butylated hydroquinone, butylated hydroxyanisole, tumor promoters and hydrogen peroxide and often by pathways generating reactive oxygen species [ROS; reviewed in (Dinkova-Kostova et al. 2004; Dinkova-Kostova and Talalay 2010; Jaiswal 2000; Ross 2004; Stiborová et al. 2013a; Talalay and Prochaska 1998)]. *NQO1* gene expression is primarily regulated by the KEAP1/NRF [NF-E2-related factor 2 (Nrf-2)] pathway, which controls redox homeostasis and facilitates the adaptation of most cells to oxidative stress (Dinkova-Kostova et al. 2004; Dinkova-Kostova and Talalay 2010; Jaiswal 2000; Ross 2004). Because ROS formation has been found to be generated by AAI in some human cell lines (Yu et al. 2011; Zhu et al. 2012) as well as by OTA in rat and human liver and kidney cells (Hadjeba-Medjdoub et al. 2012; Ramyaa et al. 2014; Schaaf et al. 2002), ROS formation caused by OTA and/or AA might contribute to NQO1 induction.

OTA, however, had no additive effect on NQO1 expression induced by AA, particularly the activity levels stayed the same as after exposure of the rats to AA alone (see Figs. 4, 8, 11). Therefore, the higher AA–DNA adduct formation found in vivo in liver and kidney of rats treated with AA together with OTA seems not to be mediated by elevated enzymatic activity of NQO1. Likewise, the higher levels of AA–DNA adducts in vivo were also not caused by increased activation of AA due to CYP1A1/2. On the contrary, enzymatic marker activities of CYP1A, EROD and Sudan I oxidation were even lower in hepatic and renal microsomes of treated rats.

Since CYP1A1/2 is also able to oxidize AAI present in the plant extract AA to AAIa, the decrease in their enzyme activities resulted in lower AAIa formation in microsomes, predominantly in hepatic and renal microsomes of rats treated with both AA and OTA (see Figs. 5, 9). In addition to CYP1A, a decrease in the activity of another CYP enzyme, namely CYP2C11, was found in liver microsomes of rats exposed to OTA and to OTA with AA. As CYP2C11 is also capable of detoxifying AAI to AAIa (Levová et al. 2011; Stiborová et al. 2014b), it likely contributes to the detoxification of the plant extract AA.

Interestingly, although AAI is oxidized by microsomal CYP enzymes to AAIa, no oxidation of AAII, which is the other major component of the plant extract AA, was detectable by this enzymatic system under the conditions used. Thus, AAII seems to be metabolized only by nitroreduction to *N*-hydroxyaristolactam II which either reacts with DNA or is further reduced to aristolactam II (Schmeiser et al. 1986; Fig. 1).

In summary, the results of the present study indicate that higher levels of AA–DNA adducts found in the liver and kidney of rats exposed to AA together with OTA in vivo might be caused by a decrease in AAI detoxification to AAIa as a result of the inhibition of CYP1A1 and/or CYP2C11 enzymes by the combined treatment. Consequently, higher levels of AAI are available to the tissues for its reductive activation which subsequently leads to higher AA–DNA adduct formation in this animal model in vivo (Fig. 11). Chronic OTA intake is common in many populations ranging up to 25 ng/kg bw and day, e.g., in Tunisia (Zaied et al. 2011), and OTA is detectable in plasma in BEN patients at levels up to 3.9 ng/ml (Yordanova et al. 2011) but also in healthy controls. The doses to which humans are


Exposure to AA
Exposure to OTA+AA
aristolochic acid I
aristolochic acid II
ochratoxin A
CYP1A1
CYP2C11
inhibition
NQO1
CYP1A1 and CYP2C11 inhibition
CYP1A1 inhibition
AAIa peak area/min/mg
F=0.63*
AA
OTA+AA
AAIa formation – ex vivo
DNA-adduct level [RAL per 10⁸ nucleotides]
AA-DNA adducts – ex vivo
F=5.4*
AA-DNA adducts - in vivo
F=0.67*
F=1.6*

◀ **Fig. 11** Schematic summary showing the effects of OTA treatment of rats on AA metabolism. Inhibition of CYP1A1 and CYP2C11 in hepatic microsomes and that of CYP1A1 in renal microsomes by treatment of rats with AA combined with OTA led to a decrease in AAIa formation in microsomes of both organs (see panel*s* showing AA oxidation to AAIa formed ex vivo). Higher levels of AAI caused by this inhibition are available for its reductive activation to form AA–DNA adducts in vivo (see panel*s* showing AA–DNA adduct levels formed in vivo). No significant changes in AA–DNA adduct formation catalyzed by rat hepatic and renal cytosols in vitro were found (see panel*s* showing AA–DNA adduct levels formed ex vivo). "*F*" indicates fold increase in levels of AA–DNA adducts formed in vivo, in rats treated with AA combined with OTA compared to those found in animals treated with AA alone or fold decrease in formation of AAIa from AA in rat hepatic and renal microsomes in vitro. Comparison was performed by *t* test analysis; $*P < 0.001$. AA, aristolochic acid; OTA, ochratoxin A; CYP, cytochrome P450; NQO1, NAD(P)H:quinone oxidoreductase 1

exposed are orders of magnitude lower than the OTA dose administered to rats in this study, and drug–drug interactions between AA and OTA at lower but chronic and lifelong doses may be different. Collectively these results, however, indicate for the first time that exposure of OTA together with AA may enhance the development of AA-induced UUC in BEN patients.

Acknowledgments This work was supported by Grant Agency of the Czech Republic (grant P301/12/G163) and Charles University in Prague (grant 570513). Work at King's College London is supported by Cancer Research UK.

Conflict of interest The authors declare no conflict of interest.

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Neuroendocrinology Letters Volume 36 Suppl. 1 2015
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
Web of Knowledge / Web of Science: Neuroendocrinol Lett
Pub Med / Medline: Neuro Endocrinol Lett

The effects of heavy metal ions, phthalates and ochratoxin A on oxidation of carcinogenic aristolochic acid I causing Balkan endemic nephropathy

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Submitted: 2015-07-18 Accepted: 2015-09-09 Published online: 2015-10-15

Key words: **aristolochic acid; oxidation; cytochrome P450; heavy metals; phthalates; ochratoxin A**

Neuroendocrinol Lett 2015; **36**(Suppl. 1):13–21 **PMID:** 26757129 NEL360915A01

Abstract

OBJECTIVES: Balkan endemic nephropathy (BEN) is a chronic progressive fibrosis associated with upper urothelial carcinoma (UUC). Aetiology of BEN is still not fully explained. Although carcinogenic aristolochic acid I (AAI) was proven as the major cause of BEN/UUC, this nephropathy is considered to be multifactorial. Hence, we investigated whether other factors considered as potential causes of BEN [a mycotoxin ochratoxin A (OTA), Cd, Pb, Se and As ions and organic compounds (i.e. phthalates) released from lignite deposits in BEN areas] can influence detoxication of AAI, whose concentrations are crucial for BEN development.

METHODS: Oxidation of AAI to 8-hydroxyaristolochic acid I (AAIa) in the presence of Cd, Pb, Se, As ions, dibutylphthalate (DBP), butylbenzylphthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP) and OTA by rat liver microsomes was determined by HPLC.

RESULTS: Only OTA, cadmium and selenium ions, and BBP inhibited AAI oxidation by rat liver microsomes. These compounds also inhibited activities of CYP1A1 and/or CYP2C6/11 catalysing AAI demethylation in rat livers. Therefore, these CYP inhibitions can be responsible for a decrease in AAIa formation. When the combined effects of these compounds were investigated, the most efficient inhibition was caused by OTA combined with BBP and selenium ions.

CONCLUSION: The results show low effects of BBP, cadmium and selenium ions, and/or their combinations on AAI detoxication. No effects were produced by the other metal ions (Pb, As) and phthalates DBP and DEHP. This finding suggests that they do not influence AAI-mediated BEN development. In contrast, OTA might influence this process, by inhibition of AAI detoxication.

ORIGINAL ARTICLE

Abbreviations

AA	- aristolochic acid
AAI	- 8-methoxy-6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid
AAIa	- 8-hydroxyaristolochic acid I
AAII	- 6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid
AAN	- aristolochic acid nephropathy
BBP	- butylbenzylphthalate
BEN	- Balkan endemic nephropathy
CYP	- cytochrome P450
dA-AAI	- 7-(deoxyadenosin-N6-yl)aristolactam I
DBP	- dibutylphthalate
DEHP	- bis(2-ethylhexyl)pthalate
dG-AAI	- 7-(deoxyguanosin-N2-yl) aristolactam I
EROD	- 7-ethoxyresorufin-O-deethylase
HPLC	- high performance liquid chromatography
IARC	- International Agency for Research on Cancer
NADP+	- nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
NQO1	- NAD(P)H:quinone oxidoreductase
OTA	- ochratoxin A
$Pb(Ac)_2$	- lead acetate
r.t.	- retention time
TP53	- tumour suppressor gene
UUC	- upper urothelial carcinoma

INTRODUCTION

Balkan endemic nephropathy (BEN) is a chronic progressive renal fibrosis affecting rural population in Bulgaria, Bosnia and Herzegovina, Romania, Croatia and Serbia along Danube river basin (Stefanovic 1983; Radanovic 2002). The unique feature of this disease is that BEN seems to be a familial, but not inherited occurring in several endemic areas (Toncheva *et al.* 1998; Radanovic 2002; Grollman 2013). Moreover, this serious disease is closely associated with upper urothelial carcinomas (UUC) of the renal pelvis and ureter (Stefanovic 1983; Jankovic *et al.* 1988; Nikolic *et al.* 2002). Although BEN has been studied for more than 50 years, aetiology of this nephropathy is still a matter of debate. There are several hypotheses suggesting that BEN/UUC is the multifactorial disease which might be caused by environmental compounds such as: (*i*) aristolochic acid (AA) (Ivic 1969; Hranjec *et al.* 2005; Arlt *et al.* 2002a, 2007; Grollman *et al.* 2007), (*ii*) mycotoxins [i.e. ochratoxin A (OTA), citrinine] (Radic *et al.* 1997; Pfohl-Leszkowicz 2009), (*iii*) heavy metal ions (Nichifor *et al.* 1985; Long *et al.* 2001; Karmaus *et al.* 2008) and (*iv*) organic compounds released from lignite deposits in the endemic areas (Feder *et al.* 1991; Tatu *et al.* 1998).

During the last decade, AA was identified as the main cause of this environmental disease (Ivic 1969; Arlt *et al.* 2002a, 2007; Grollman *et al.* 2007; Stiborova *et al.* 2008). The AA was suggested as possible cause of BEN/UUC for the first time in late 1960s (Ivic 1969). This plant alkaloid found in *Aristolochia* species was found in wheat used for home-prepared bread (Ivic 1969; Jelakovic *et al.* 2012; Gokmen *et al.* 2013). The plant extract of AA is a mixture of structurally related nitrophenanthrene carboxylic acids whose major components are aristolochic acid I (AAI) and aristolochic acid II (AAII). AAI is supposed to be the predominant compound responsible for BEN development. AA and herbal products derived from genera *Aristolochia* have been classified by International Agency for Research on Cancer (IARC) as carcinogenic to human (Group 1) (Grosse *et al.* 2009). Furthermore, this nephrotoxic and carcinogenic agent was found to cause also another disease similar to BEN, aristolochic acid nephropathy (AAN) (Cosyns *et al.* 1994; Arlt *et al.* 2002b, 2007; Debelle *et al.* 2008; Schmeiser *et al.* 1996, 2009; Gokmen 2013).

In contrast to the finding that AAI might directly cause interstitial nephropathy, metabolic activation of this alkaloid to species forming DNA adducts is a necessary step for AAI-induced malignant transformation (Cosyns *et al.* 1994; Arlt *et al.* 2002b; Schmeiser *et al.* 1996, 2009; Chen *et al.* 2012). In organisms, AAI can be either reductively activated to *N*-acylnitrenium ion leading to AAI-DNA adduct formation or oxidatively detoxified to an *O*-demethylated product, 8-hydroxyaristolochic acid (aristolochic acid Ia, AAIa; Figure 1) (Arlt *et al.* 2002b, 2007; Grollman *et al.* 2007; Stiborova *et al.* 2014a, 2014b). The AA-DNA adducts formed from activated AAI with adenosine and guanosine residues in DNA were found in BEN and AAN patients (Schmeiser *et al.* 1996; Bieler *et al.* 1997; Arlt *et al.* 2002a). The most persistent DNA adduct, 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI), is proposed to cause a characteristic AT→TA transversion mutations which have been detected in the *TP53* tumour suppressor gene in tissues of patients from the endemic areas. Such AT→TA transversions are responsible for tumour development in patients suffering from BEN and AAN (Arlt *et al.* 2007; Grollman *et al.* 2007; Stiborova *et al.* 2008; Hollstein *et al.* 2013). These findings indicate that the concentration of AAI in organisms is essential for both renal injury and induction of UUC initiated by activated AAI. The effective concentration of AAI in organism is dictated by its metabolism. Since AAI can be both bio-activated to reactive species forming AAI-DNA adducts resulting in cancer development and detoxified to AAIa, these reactions might significantly modulate the AAI toxic/genotoxic potential (Stiborova *et al.* 2008, 2011a, 2012, 2013b; Arlt *et al.* 2011).

A common feature of BEN/UUC is that not all individuals exposed to AAI suffer from these diseases (Arlt *et al.* 2002b, 2011; Stiborova *et al.* 2008, 2012, 2013b, 2015; Jelakovic *et al.* 2012). This phenomenon might be explained by different efficiencies of enzymes participating in metabolism of AAI and by genetic sensitivity of individuals (Stiborova *et al.* 2001, 2003, 2008, 2013b; Toncheva *et al.* 2004; Grollman 2013). Therefore, detailed understanding of enzymes involved in AAI

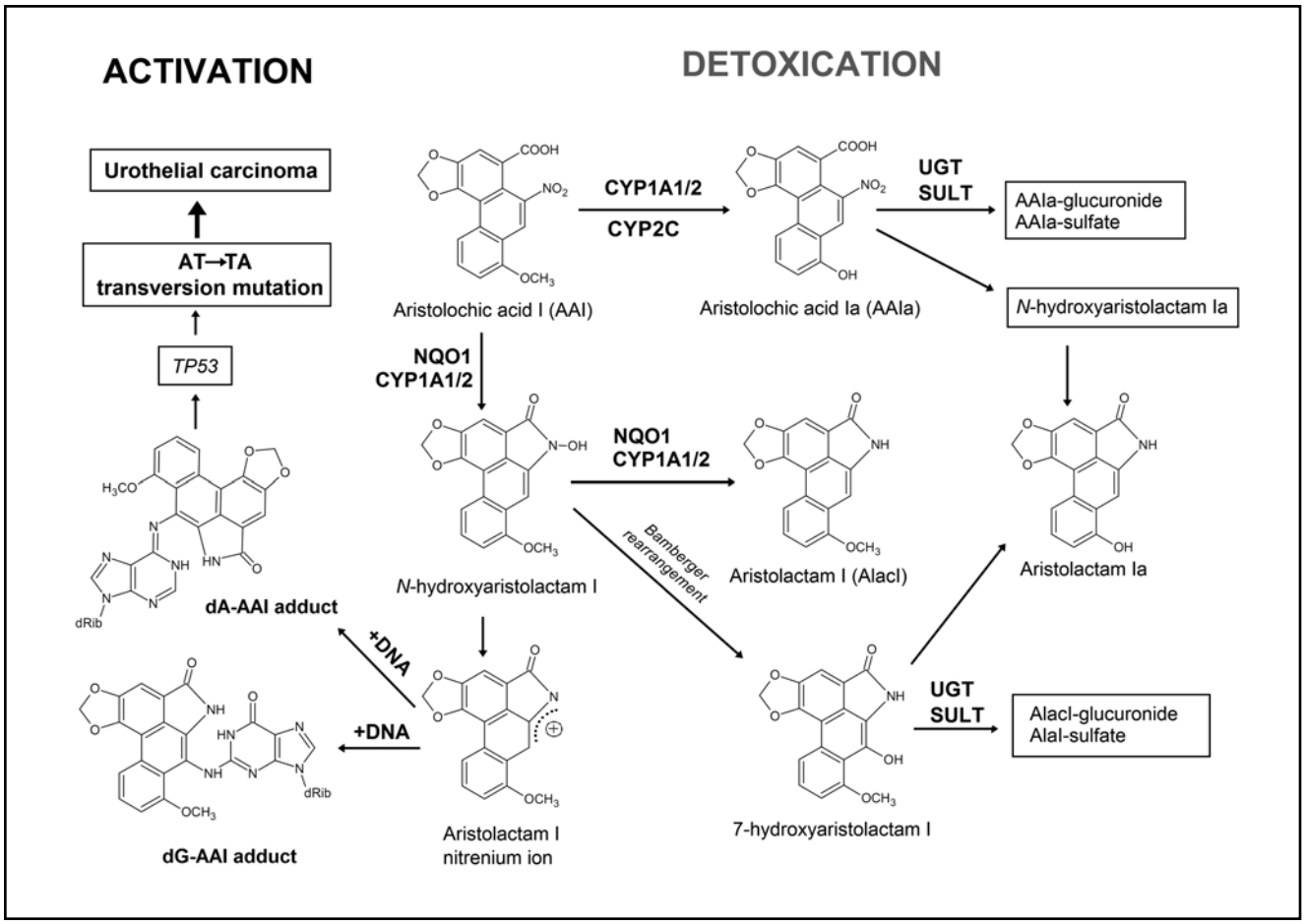

Fig. 1. Scheme of detoxication and bio-activation of AAI in organisms. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; UGT, UDP glucuronosyl transferase; SULT, sulfotransferase.

metabolism (activation and/or reduction) is crucial for risk assessment of AA exposure.

The major enzymes involved in reductive bio-activation of AAI are NAD(P)H:quinone oxidoreductase (NQO1) and cytochromes P450 (CYP) of the 1A subfamily in humans and mouse or rat animal models (Stiborova *et al.* 2008, 2011a, 2012, 2013b, 2014a, 2014b; Arlt *et al.* 2011; Barta *et al.* 2014). The most efficient enzymes participating in AAI detoxication are human, mouse and rat CYP1A1/2 and rat and human CYP2C (Arlt *et al.* 2011; Stiborova *et al.* 2011b, 2012, 2013b).

However, it is still not exactly known whether the other compounds suggested to be responsible for BEN development might influence the AAI-induced BEN. Hence, in this work, we investigated whether OTA, heavy metal ions and organic chemicals released from lignite deposits in the endemic areas can influence the detoxication of AAI, whose concentrations are crucial for BEN development. Heavy metal ions and organic compounds were selected according to data from epidemiologic studies (Radic *et al.* 1997; Karmaus *et al.* 2008; Pfohl-Leszkowicz 2009; Yordanova *et al.* 2010; Maharaj *et al.* 2014).

MATERIALS AND METHODS

Chemicals

AAI sodium salt, $CdCl_2$, $Pb(CH_3COO)_2$, Na_2SeO_3, $Na_2HASO_4{\cdot}7H_2O$, dibutylphthalate (DBP), butylbenzylphthalate (BBP) and bis(2-ethyhexyl)phthalate (DEHP) as well as other chemicals were purchased from Sigma Chemical Co. (St. Louis MO, USA). All chemical were of 97% purity or better.

Preparation of rat hepatic microsomal fraction

Microsomes were prepared from liver of untreated Wistar rats by differential centrifugation as described previously (Stiborova *et al.* 2013a; Indra *et al.* 2014).

AAIa formation in the presence of heavy metal ions/phthalates/OTA

Incubation mixtures, in a final volume 500 µL, consisted of 100 mmol.L^{-1} potassium buffer (pH 7.4), NADPH-generation system (1 mmol.L^{-1} $NADP^+$, 10 mmol.L^{-1} $MgCl_2{\cdot}6H_2O$, 1 U/mL glucose-6-phosphate dehydrogenase), 1–100 µmol.L^{-1} of heavy metal ions/phthalates/OTA, 0.25 mg rat hepatic micro-

somes and 10 μmol.L^{-1} AAI. $CdCl_2$, $Pb(CH_3COO)_2$, Na_2SeO_3, $Na_2HASO_4{\cdot}7H_2O$ were dissolved in distilled water and OTA was dissolved in 0.1 mol.L^{-1} $NaHCO_3$ (pH 7) whereas phthalates were prepared in acetonitrile. Incubations with microsomes were carried out at 37 °C for 10 min and AAI oxidation to AAIa was linear up to 25 min (Levova *et al.* 2011; Stiborova *et al.* 2012). Control incubations were carried out (*i*) without microsomes, (*ii*) without NADPH-generating system or (*iii*) without AAI. AAI and AAIa were analysed by high performace liquid chromatography (HPLC) as described (Sistkova *et al.* 2008; Levova *et al.* 2011; Stiborova *et al.* 2012).

HPLC analysis of AAIa formation

AAI and its *O*-demethylated metabolite (AAIa) were extracted from incubations with ethyl acetate (2 × 1 mL), the extracts were evaporated to dryness and the residues redissolved 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 mmol.L^{-1} triethylamonium acetate with a flow rate of 0.5 mL.min^{-1}. A Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector was set at 250 nm and CHROMELEON™ 6.01 integrator was used for integration of peaks. AAIa and AAI eluted with retention times (r.t.) of 24.5 and 37.7 min, respectively. The product eluting at 24.5 min was identified as AAIa by mass spectrometry previously (Sistkova *et al.* 2008; Levova *et al.* 2011; Stiborova *et al.* 2011b).

Determination of CYP1A1/2 and CYP2C6/11 enzyme activities

In rat hepatic microsomes, CYP1A1/2 was determined by ethoxyresorufine-*O*-deethylation (EROD) (Burke *et al.* 1994). Enzyme activity of CYP1A1 was measured as capability of Sudan I oxidising (Stiborova *et al.* 2002). CYP2C6/11 activities in rat microsomes were characterised as well: CYP2C6 was measured with diclofenac as a marker substrate (Kaphalia *et al.* 2006) and CYP2C11 activity was determined as testosterone 16α-hydroxylation (Yamazaki *et al.* 2006). The effect of heavy metal ions/phthalates/OTA on the above mentioned enzyme activities was carried out by addition of compounds tested to incubation mixtures in a final concentration of 100 μmol.L^{-1}.

Statistical analyses

For statistical data analysis we used Student's *t*-test. All *p*-values are two-tailed and considered significant at the 0.001 level.

RESULTS AND DISCUSSION

Oxidation of AAI to AAIa in the presence of the heavy metal ions, phthalates and ochratoxin A

In rat microsomes, AAI was oxidised to one metabolite eluted by HPLC at r.t. of 24.5 minutes (Figure 2). This metabolite was previously identified by positive MALDI-TOF-TOF analysis as AAIa (Levova *et al.* 2011; Stiborova *et al.* 2011b). Because of a low toxicity, AAIa was considered to be the detoxication metabolite of AAI (Shibutani *et al.* 2010). The effects of compounds, which were suggested that might contribute to development of BEN/UUC, namely the heavy metal ions, phthalates and OTA, on detoxication of AAI to AAIa catalysed by rat liver microsomes are shown in Figures 3–5. Cadmium and selenium ions inhibited AAIa formation (Figures 3A,C) whereas no such effect was found in the presence of Pb^{2+} and arsenate ions (Figures 3B,D). However, the 10-times higher concentration of cadmium and selenium ions than the concentration of AAI was necessary for the significant decrease in AAI oxidation. Of phthalates examined, only butylbenzylphthalate (BBP) inhibited oxidation of AAI to AAIa

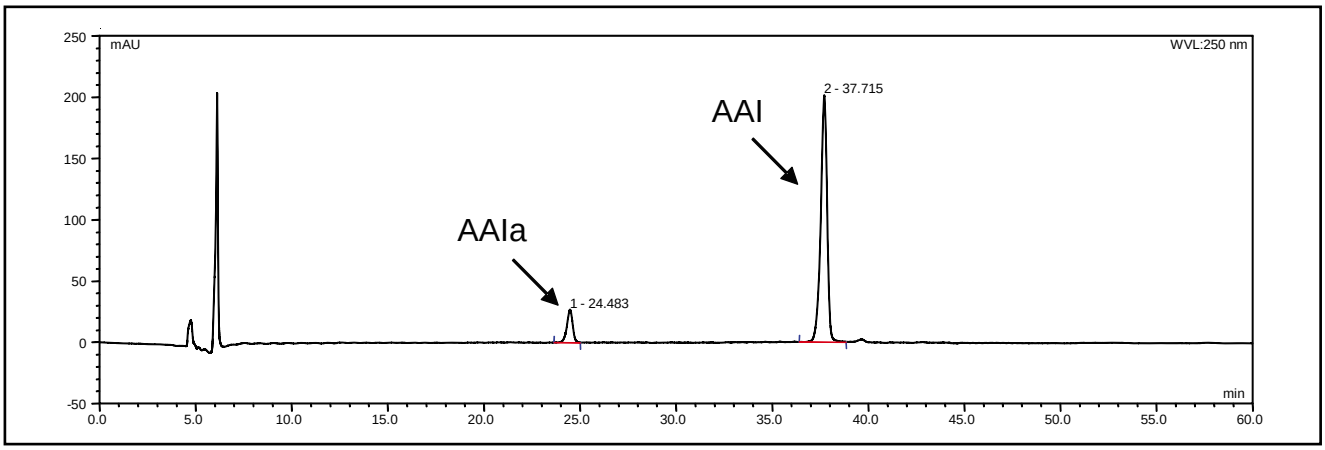

Fig. 2. HPLC of AAI (peak r.t. at 37.7 min) and AAIa metabolite (peak r.t. at 24.5 min) produced by hepatic microsomes of control (untreated) rats incubated with AAI and the NADPH-generating system. The peaks with the characterised metabolite AAIa and the parent AAI are indicated in the chromatograms.

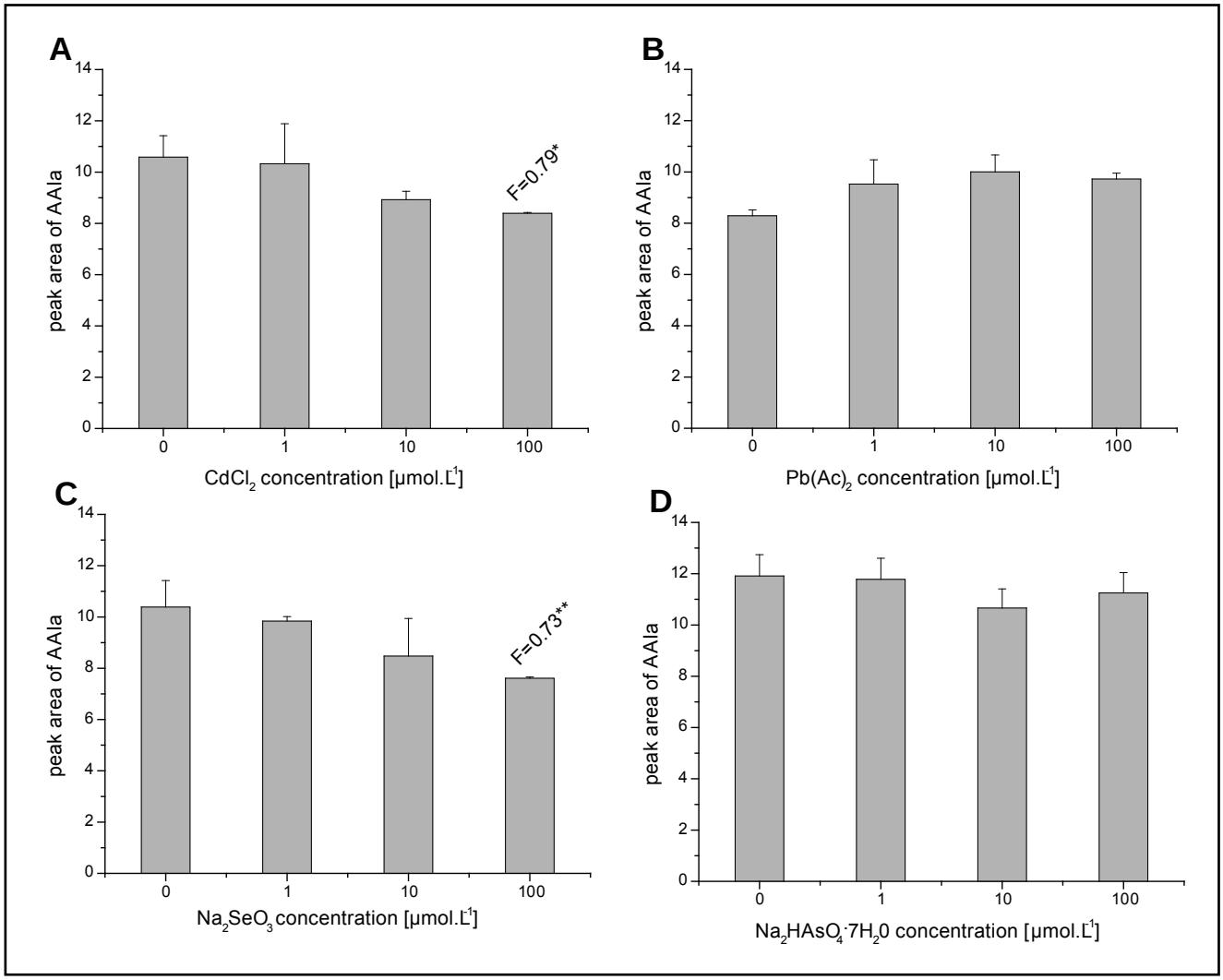

Fig. 3. AAI oxidation to AAIa catalysed by rat hepatic microsomes in the presence of heavy metal ions present in incubation mixtures, $CdCl_2$ (A), $Pb(CH_3COO)_2$ [$Pb(Ac)_2$; B), Na_2SeO_3 (C), $Na_2HASO_4{\cdot}7H_2O$ (D). Values are given as means ± standard deviations of three experiments (n = 3). Values significantly different from incubation with buffer only: *p<0.1, **p<0.01 (Student's t-test). The incubation mixtures contained 0.5 mg.mL^{-1} microsomal protein, 1–100 μmol.L^{-1} heavy metal ions dissolved in distilled water, the NADPH-generating system containing 1 mmol.L^{-1} $NADP^+$, and 10 μmol.L^{-1} AAI dissolved in distilled water (see Materials and Methods). Numbers above the columns ("F") indicate fold changes in amounts of AAIa compared to incubations without heavy metal ions.

(Figure 4B). The other two studied phthalates (DBP and DEHP) that are known as important toxic environmental pollutants (Ferguson *et al.* 2014; Yan *et al.* 2015) were without this effect (Figures 4A,C). In the case of OTA, the significant inhibition of AAI demethylation by this mycotoxin was found. The 10 and 100 μmol.L^{-1} OTA led to a 23% and 42% decrease in AAIa formation, respectively (Figure 5). These findings are consistent with the results found in our former study which demonstrated that OTA is capable of inhibiting AAI oxidation to AAIa *in vivo* (Stiborova *et al.* 2015).

Because human population living in the endemic areas might be exposed not only to each of these compounds individually but also to their combination, in the next step of this study we investigated a combined effect of the substances that inhibited AAI oxidation, namely, cadmium and selenium ions, BBP and OTA (Figure 6). Interestingly, although oxidation of AAI to AAIa was not influenced by a combination of BBP and OTA, all four contaminants (cadmium and selenium ions, BBP and OTA) added to incubation mixtures led to the significant inhibition of AAIa formation, by 34% (Figure 6B). In addition, the most efficient inhibition of AAI demethylation was caused by OTA combined with BBP and selenuim ions, by 37% (Figure 6B). Using the other combinations, no additive effects of these combinations compared to inhibition caused by individual compounds were found. This phenomenon is now difficult to be explained. One can speculate that compounds might compete against each other, thereby decreasing the inhibition of enzymes involved in AAI oxidation or inter-molecular interactions between the

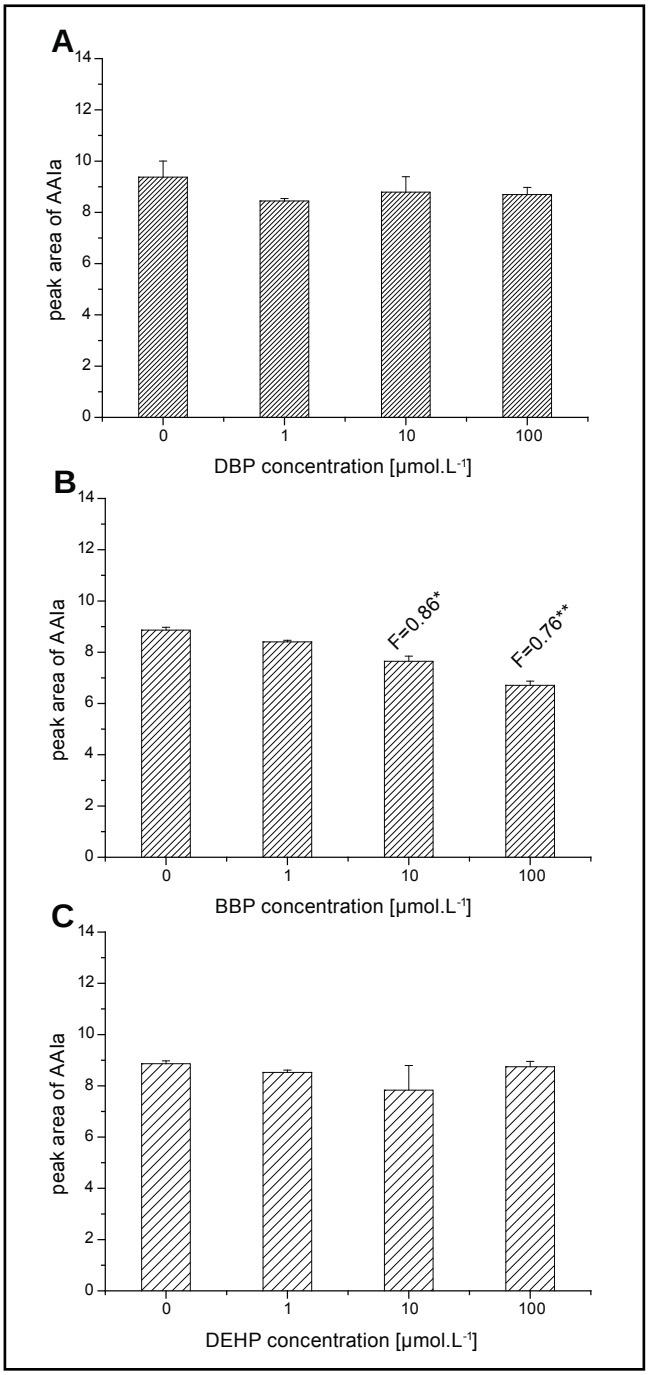

Fig. 4. AAI oxidation to AAIa catalysed by rat hepatic microsomes in the presence of phthalates. In incubation mixtures, dibutylphthalate (DBP, A), butylbenzylphthalate (BBP, B) and bis(2-ethyhexyl)phthalate (DEHP, C) all dissolved in acetonitrile were used. Values are given as means ± standard deviations of three experiments ($n = 3$). Values significantly different from incubation with acetonitrile only: *$p<0.1$, **$p<0.01$ (Student's t-test). The incubation mixtures contained 0.5 mg.mL^{-1} microsomal protein, 1–100 μmol.L^{-1} phthalates, the NADPH-generating system containing 1 mmol.L^{-1} NADP$^+$, and 10 μmol.L^{-1} AAI dissolved in distilled water (see Materials and Methods). Numbers above the columns ("F") indicate fold changes in amounts of AAIa compared to incubations without phthalates.

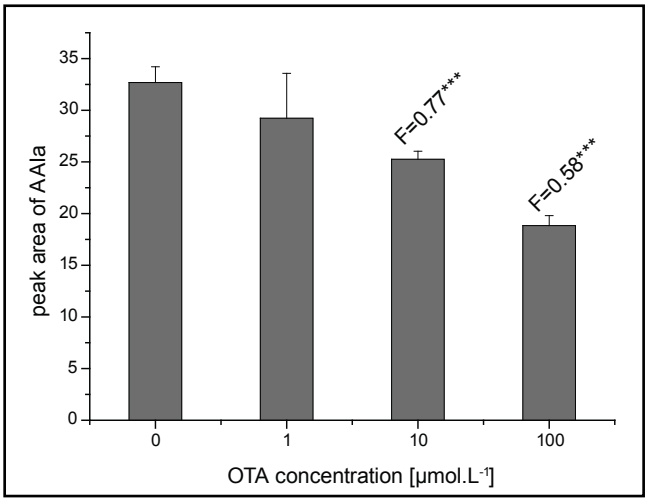

Fig. 5. AAI oxidation to AAIa catalysed by rat hepatic microsomes in the presence of OTA dissolved in 0.1 mol.L^{-1} $NaHCO_3$ (pH 7). Values are given as means ± standard deviations of three experiments (n = 3). Values significantly different from incubation with 0.1 mol.L^{-1} $NaHCO_3$ (pH 7) only: ***$p<0.001$ (Student's t-test). The incubation mixtures contained 0.5 mg.mL^{-1} microsomal protein, 1–100 μmol.L^{-1} OTA, the NADPH-generating system containing 1 mmol.L^{-1} NADP$^+$, and 10 μmol.L^{-1} AAI dissolved in distilled water (see Materials and Methods).

tested compounds might decrease their actual concentrations. These suggestions need, however, to be explored in further studies.

Effect of the heavy metal ions, butylbenzylphthalate and ochratoxin A on enzymatic activities of cytochromes P450.

In order to evaluate the mechanisms of inhibition of AAI oxidation in rat microsomes, the effects of the compounds found to inhibit this reaction (Figures 3–5) on activities of the major enzymes participating in AAI detoxication were analysed. Namely, the effects of cadmium and selenium ions, BBP and OTA on activities of CYP1A and 2C6/11 enzymes were tested. The enzyme activities were determined utilising a marker substrates (see the Material and Methods section). The data shown in Table 1 demonstrate that CYP1A1 activity was inhibited mainly by Cd^{2+} and BBP; a 54 and 75% decrease in a CYP1A1 marker activity (Sudan I oxidation) was found, respectively, whereas the other tested compounds did not influence CYP1A activity. In the case of CYP2C, BBP significantly inhibited activity of CYP2C6, whereas OTA decreased activity of CYP2C11, the CYP enzyme that is predominantly expressed in rat liver (Zachařová *et al.* 2012). Based on these results, the decreased oxidation of AAI to AAIa caused by tested

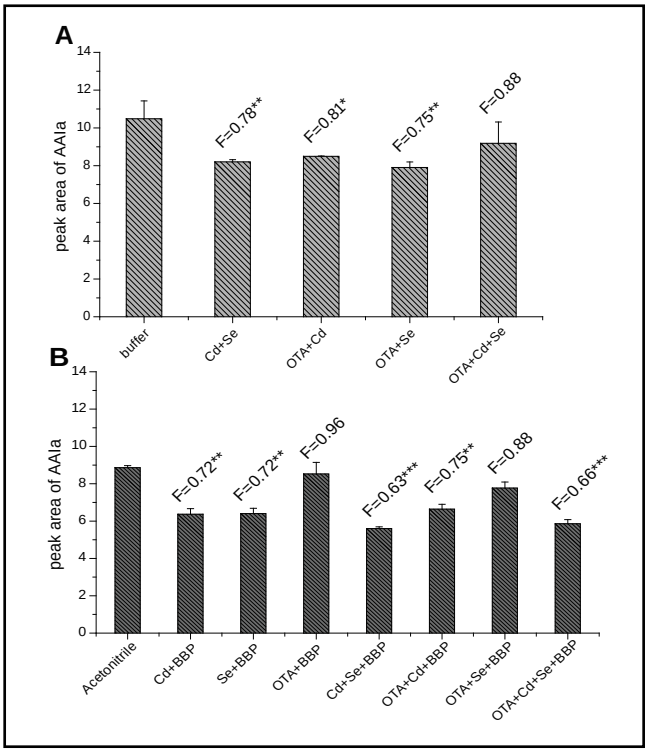

Fig. 6. Combined effect of cadmium and selenium ions, BBP and OTA on AAI detoxication to AAIa. Incubations with $CdCl_2$, Na_2SeO_3 and OTA (A) and in combination of these compounds with BBP (B) were carried out. Values are given as means ± standard deviations of three experiments (n = 3). Values significantly different from incubation with buffer/acetonitrile only: *p<0.1, **p<0.01, ***p<0.001 (Student's t-test). The incubation mixtures contained 0.5 mg.mL^{-1} microsomal protein, 100 µmol.L^{-1} $CdCl_2$, Na_2SeO_3, dissolved in distilled water, OTA dissolved in 0.1 mol.L^{-1} $NaHCO_3$ (pH 7) or BBP dissolved in acetonitrile, the NADPH-generating system containing 1 mmol.L^{-1} $NADP^+$, and 10 µmol.L^{-1} AAI dissolved in distilled water (see Materials and Methods). Numbers above the columns ("F") indicate fold changes in amounts of AAIa compared to incubations without the above mentioned compounds.

contaminants might be caused by decreased enzyme activities of these CYPs.

Several studies suggested that BEN and AAN may be the same diseases which differ slightly (Grollman *et al.* 2009). One of the differences between BEN and AAN is that whereas BEN is characterised by an insidious onset and slow gradual progression (Grollman *et al.* 2007; Stiborova *et al.* 2008; Jelakovic *et al.* 2012.), AAN is defined as rapidly progressive interstitial renal fibrosis (Vanherweghem *et al.* 1993). One of the reasons responsible for this difference seems to be a different exposure schedule of patients; chronic exposure to low concentrations of AA of population living in BEN areas, whereas exposure to high doses of individuals in which AAN was developed (Gokmen *et al.* 2013). However, based on the results found in the present and our former (Stiborova *et al.* 2015) studies, this difference might also follow from inhibitions of AAI detoxication by the compounds such as heavy metal ions (cadmium and selenium), phthalates (BBP) and OTA, the substances to which BEN/UUC patients are exposed. Although BBP and cadmium and selenium ions were shown to be present in water and lignite samples in the BEN areas (Karmaus *et al.* 2008; Maharaj *et al.* 2014), there is still not enough information on their exact concentrations in these samples. Therefore, we cannot evaluate whether their concentrations found in this study to inhibit AAI detoxication are valid for the BEN development. Nevertheless, even though there is a study which has demonstrated that a role of heavy metal ions in BEN/UUC development is negligible (Karmaus *et al.* 2008), because of inhibition of AAI detoxication by cadmium and selenium ions found in this work, the participation of the chronic exposure to these ions in the AAI-mediated BEN development cannot be excluded. Chronic OTA intake is common in many populations ranging up to 25 ng.kg^{-1} body weight and day, and OTA is detectable in plasma in BEN patients at levels up to 3.9 ng.mL^{-1} (Yordanova *et al.* 2010) but also in healthy controls. The doses to which humans are exposed are lower than the OTA concentrations found in this study to inhibit AAI detoxication. However, drug-drug interactions between AAI and OTA at lower but chronic and life-long doses of human exposure in BEN areas may be different. Therefore, OTA, because of its potency to

Tab. 1. The effects of cadmium and selenium ions, BBP, and OTA on enzyme activity of rat CYP1A and 2C.

Pollutant	EROD activity	Sudan I oxidation	Diclofenac 4′-hydroxylation	Testosterone 16α-hydroxylation
	(CYP1A)[a]	(CYP1A1)	(CYP2C6)	(CYP2C11)
$CdCl_2$	83±0.09*	46±4.27***	89±0.44*	100±6.43
Na_2SeO_3	95±0.83	100±2.62	94±5.59	82±1.01**
OTA	100±7.50	99±4.93	NE[b]	18±1.24***
BBP	83±1.24*	25±1.27***	7±0.26***	68±12.48**

Data are expressed as % of control without pollutants. Values in the table are averages ± standard deviations of three experiments (n = 3). The incubation mixtures contained 0.5 mg.mL^{-1} microsomal protein, 10 µmol.L^{-1} AAI (dissolved in distilled water), the NADPH-generating system containing 1 mmol.L^{-1} $NADP^+$, and 100 µmol.L^{-1} pollutant [heavy metal ions dissolved in distilled water, OTA dissolved in 0.1 mol.L^{-1} $NaHCO_3$ (pH 7) or BBP dissolved in acetonitrile] (see Materials and Methods). Values significantly different from control incubations without pollutants; *P<0.1, **P<0.01, ***P<0.001 (Student's t-test). [a]Isoforms of CYP whose enzyme activity is measured are shown in brackets. [b]NE, no effect.

increase actual concentrations of AAI by inhibition of its detoxication, may enhance the development of AAI-induced UUC in BEN patients.

CONCLUSIONS

The results found in this study demonstrate that AAI detoxication to AAIa is inhibited by OTA, phthalate BBP and partially also by cadmium and selenium ions. Such inhibition is caused by inhibition of the CYP1A1 and 2C enzymes that catalyse this reaction. Even though these inhibitions are not fatal, their contributions to the AAI-mediated development of BEN/UUC, considering mainly the chronic exposure of population living in the endemic areas to these pollutants, cannot be ruled out.

ACKNOWLEDGEMENTS

The authors thank Grant Agency of Czech Republic (grant P301/12/G163) and Charles University in Prague (grant GAUK 570513).

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