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THE ROLE OF BIOTRANSFORMATION ENZYMES IN THE DEVELOPMENT OF RENAL INJURY AND UROTHELIAL CANCER CAUSED BY ARISTOLOCHIC ACID: URGENT QUESTIONS AND DIFFICULT ANSWERS

Marie Stiborova[a]*, Eva Frei[b], Volker M. Arlt[c], Heinz H. Schmeiser[b]

[a] *Department of Biochemistry, Faculty of Sciences, Charles University, Albertov 2030, 128 40 Prague , Czech Republic*
[b] *Division of Molecular Toxicology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*
[c] *Section of Molecular Carcinogenesis, Institute of Cancer Research, Sutton, Surrey, SM25NG, United Kingdom*

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Background: Ingestion of aristolochic acid (AA) is associated with the development of aristolochic acid nephropathy (AAN), which is characterized by chronic renal failure, tubulointerstitial fibrosis and urothelial cancer. AA may also cause another type of kidney fibrosis with malignant transformation of the urothelium, called Balkan Endemic Nephropathy (BEN). The compound predominantly responsible for the nephropathy and urothelial cancer of AA, is aristolochic acid I (AAI) which is a genotoxic mutagen after metabolic activation The activation pathway involves reduction of the nitro group to a cyclic *N*-acylnitrenium ion that can form covalent DNA adducts. These specific DNA adducts have been detected in experimental animals exposed to AAI, and in urothelial tissues from AAN patients. In rodent tumours induced by AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I was the most abundant DNA adduct formed and associated with activation of *ras* oncogenes through a characteristic transversion mutation. Such A:T→T:A mutations have been identified in *TP53* of urothelial tumour DNA of an AAN patient and in several patients suffering from BEN along with specific AA-DNA adducts. Understanding which enzymes are involved in AAI activation to species forming DNA adducts and/or detoxification to its *O*-demethylated metabolite aristolochic acid Ia (AAIa) is important in order to assess susceptibility to this carcinogen.

Methods and Results: A literature search.

Conclusions: The most important human enzymes activating AAI by simple nitroreduction *in vitro* are hepatic and renal cytosolic NAD(P)H:quinone oxidoreductase, hepatic microsomal cytochrome P450 (CYP) 1A2 and renal microsomal NADPH:CYP reductase as well as cyclooxygenase which is highly expressed in urothelial tissue. However, the contribution of most of these enzymes to the development of AAN and BEN diseases is still unclear. Hepatic CYP enzymes were found to detoxify AAI to AAIa in mice, and thereby protect the kidney from injury. CYP enzymes of the 1A subfamily seem to play a major role in this process in mouse liver. Likewise, among human CYP enzymes, CYP1A1 and 1A2 were found to be the most efficient enzymes participating in AAI oxidation to AAIa *in vitro*. Nevertheless, which CYPs are the most important in this process in both animal models and in humans have not been entirely resolved as yet. In addition, the relative contribution of enzymes found to activate AAI to species responsible for induction of urothelial cancer in humans remains still to be resolved.

INTRODUCTION

Aristolochic Acid

Aristolochic acid (AA), the plant extract of *Aristolochia* species, is a mixture of structurally related nitrophenanthrene carboxylic acids, with 8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAI) and 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAII), being the major components[1] (Fig. 1). AA is proven to be the cause of so-called Chinese herb nephropathy (CHN), a unique type of rapidly progressive renal fibrosis associated with the prolonged intake of Chinese herbal remedies for slimming, and observed for the first time in Belgium in 1991[2-4]. Over 100 CHN cases have been identified so far in Belgium, half of which needed renal replacement therapy, mostly including renal transplantation[5]. The nephrotoxicity has been traced to the ingestion of the herbal preparation *Aristolochia fangchi* containing nephrotoxic AA inadvertently included in slimming pills[3]. CHN patients, who were exposed to *Aristolochia* species containing AA and had no relationship with the Belgian slimming clinic in question, have been identified in other European countries, in Asia and in the USA[5,6]. Therefore, this disease is now called aristolochic acid nephropathy (AAN) [5-8]. A high prevalence of urothelial cancer was found in the cohort of AAN patients in Belgium[9] and cases of urothelial cancer have also been described in other countries[10,11]. These findings highlight the carcinogenic potential of AA to humans. Indeed, AA is among the most potent 2 % of known carcinogens[1,5,12]. As a con-

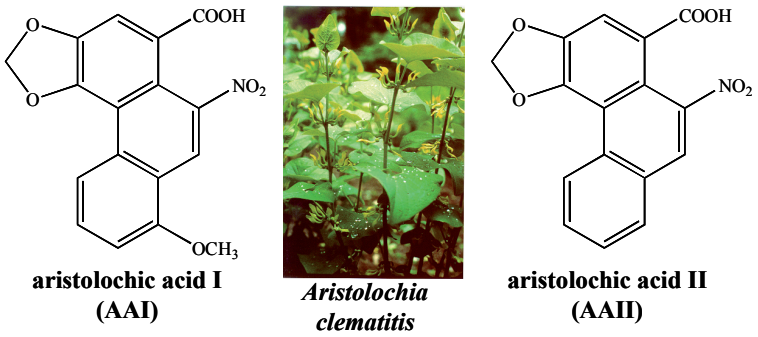

Fig. 1. Chemical structures of aristolochic acid I (AAI) and II (AAII)

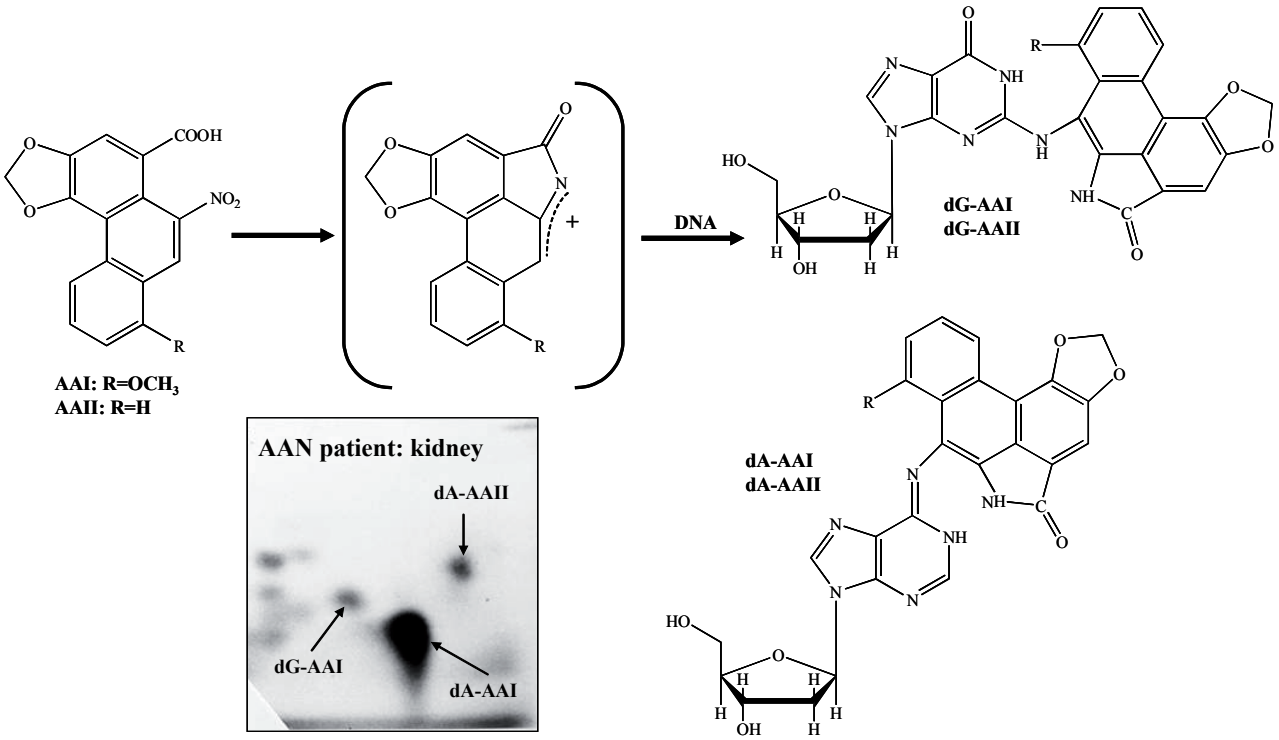

Fig. 2. Metabolic activation and DNA adduct formation of aristolochic acid I (AAI) and II (AAII); 7-(deoxyadenosin-N^6-yl)aristolactam I or II (dA-AAI or dA-AAII), 7-(deoxyguanosin-N^2-yl)aristolactam I or II (dG-AAI or dG-AAII). Insert: Autoradiographic profile of DNA adducts detected by ^{32}P-postlabelling in renal DNA of a patient with aristolochic acid nephropathy (AAN).

sequence, herbal remedies containing species of the genus *Aristolochia* were recently classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC)[1,13].

It is also noteworthy that AA consumption may be a cause for the development of a similar type of kidney fibrosis with malignant transformation of the urothelium, the Balkan Endemic Nephropathy (BEN)[14-17], which is widely found in certain areas of Romania, Croatia, Bosnia, Serbia and Bulgaria along the Danube river basin[15,17,18]. At least 25,000 individuals suffer from BEN or are suspected of having the disease, while the total number of people at risk in these countries may exceed 100,000. Although first described more than 50 years ago, the etiology of BEN remains unclear and is a matter of debate[15,18]. Over the last few years, evidence has accumulated that BEN is an environmental disease. Recent experimental data show that AA might be one of the most important etiologic factors in BEN and associated urothelial cancer[16,18,19,20]. AA exposure is associated with chronic dietary uptake of seeds of *Aristolochia clematitis* by the population living in BEN regions[14,16,21].

Aristolochic Acid-mediated Renal Injury and Carcinogenesis

The molecular mechanisms of AA-mediated renal injury and its role in urothelial-specific tumor development, are still a matter of debate and require further investigation. In this context, it is noteworthy that a case reported of AA-induced tumor development without renal injury[22] suggests dissociation between AA-mediated nephrotoxicity and carcinogenicity. One recent study has indicated that AA seems to directly cause renal injury through activating mitochondrial permeability transition which was

found recently in human renal tubular epithelial cells[23]. This suggestion, however, needs to be confirmed by further studies. Others suggest that metabolic activation of AA to species forming DNA adducts is not the only important step for AA-induced malignant transformation[24,25] but that specific DNA damage also leads to cell-specific alterations at the protein transcriptional level and this might impair physiological processes[26-29]. In contrast to the suggestion that AA might be the direct cause of the interstitial nephropathy, metabolic activation of AA to species forming DNA adducts is an important step in AA-induced malignant transformation[24,25]. Indeed, the molecular mechanism of AA-induced carcinogenesis demonstrates strong association between DNA adduct formation, mutation pattern and tumor development[24,30,31]. The DNA adduct 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) (Fig. 2), which is the most persistent AA-adduct in target tissue, is a premutagenic lesion leading to A:T→A:T transversions in *TP53* in DNA from urothelial tumours of AAN and BEN patients[19,24,30,32].

Metabolism of Aristolochic Acid and Biotransformation Enzymes

A feature common to AAN and BEN is that not all individuals exposed to AA develop nephropathy or tumors. We have suggested earlier that one cause for these variable response may be individual differences in the activities of the enzymes catalyzing the biotransformation (detoxification and/or activation) of AA (for a summary see[25]). A large number of genes for the enzymes that metabolize toxins and carcinogens are known to exist in variant forms or show polymorphisms resulting in differing activities of the gene products. These genetic variations appear to be important determinants of cancer risk and other toxic effects of xenobiotics[25].

The proposed activation and detoxification pathways of AAI are shown in Figure 3. AAI is activated by nitroreduction to *N*-hydroxyaristolactam I, a metabolite recently detected in the urine of AA-treated rats which confirms its formation during metabolism *in vivo*[33]. *N*-Hydroxyaristolactam I can form a cyclic *N*-acylnitrenium ion as the ultimate carcinogen binding to DNA. This leads to dA-AAI, the most persistent adduct in initiating car-

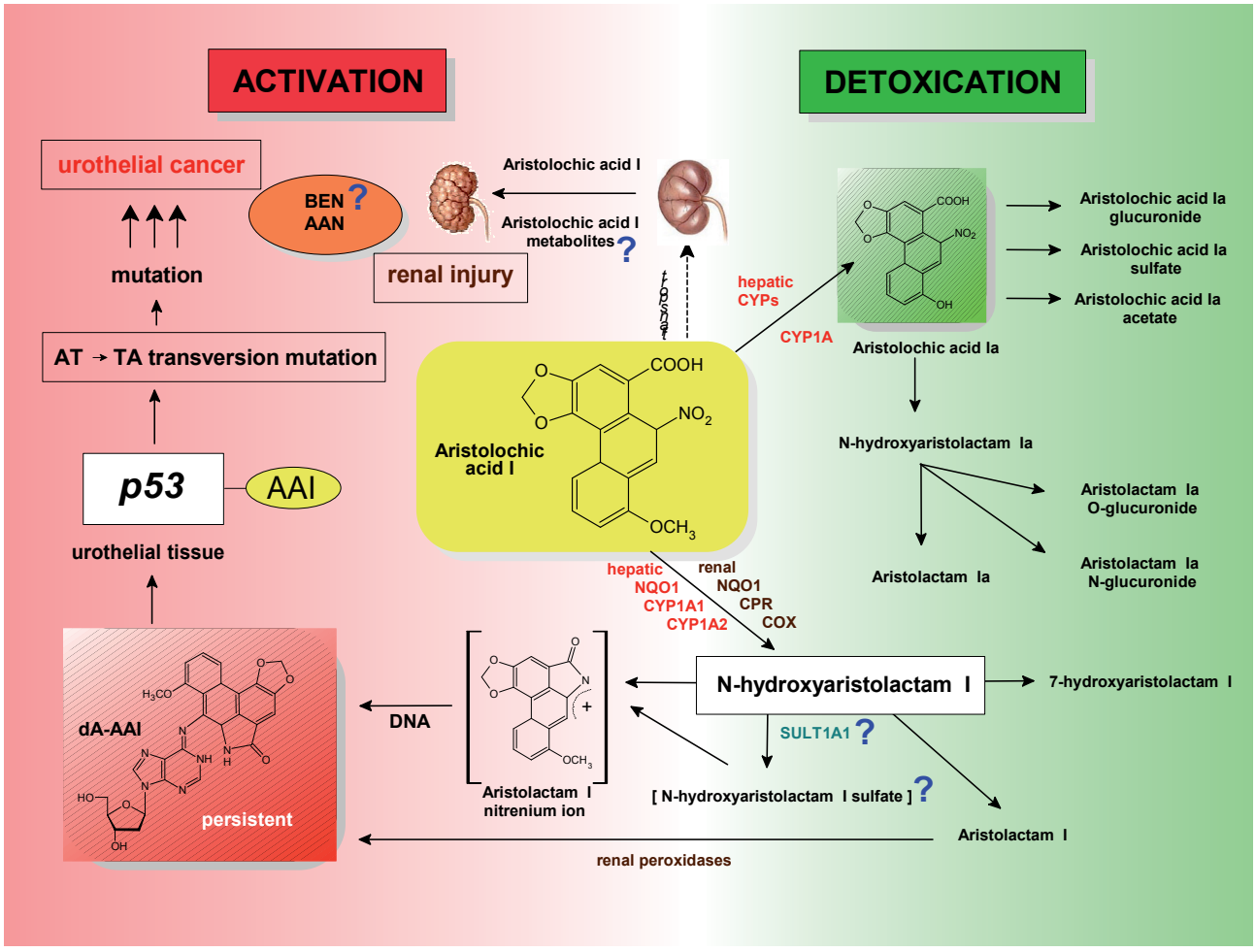

Fig. 3. Proposed pathway for metabolic activation and detoxication of aristolochic acid I (AAI), leading to renal injury and urothelial cancer. Aristolochic acid nephropathy (AAN); Balkan endemic nephropathy (BEN); 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI); cytochrome P450 (CYP); NADPH:CYP reductase (CPR); cyclooxygenase (COX); sulfotransferase (SULT) (adapted from reference[54]).

cinogenesis. The preference of AAI for reaction with the exocyclic amino group of DNA purine bases is unusual for nitroaromatic compounds since their ultimate carcinogenic species is a nitrenium ion whose major target site in DNA is the C-8 atom of guanine.

Enzymes activating AAI to species binding to DNA *in vitro* have been studied in detail[25]. Several *in-vitro* studies report that the most important human and rat enzyme to activate AAI *in vitro* in hepatic or renal cytosolic subcellular fractions, is NAD(P)H:quinone oxidoreductase (NQO1)[34-36] followed by cytochrome P450 (CYP) 1A1/2 in hepatic microsomes[37,38] and NADPH:CYP reductase (POR) in renal microsomes[36,39]. In addition, prostaglandin H synthase (cyclooxygenase, COX) is able to bioactivate AAI[36,40], which is highly expressed in urothelial tissue. It still remains to be confirmed however that isolated (purified) human NQO1 is actually able to activate AAI. Preliminary data shown in Table 1 indicate that human NQO1 is capable of activating AAI, much more efficiently than other enzymes (Stiborová *et al.,* unpublished results). Such proof is of great importance as NQO1 polymorphism (the genotype *NQO1*2/*2*) seems to predispose patients suffering from BEN to the development of urothelial malignancy (OR=13.75, 95 %CI 1.17-166.21)[41]. Therefore, future work aims to confirm the predisposition of AAN patients to the development of cancer by NQO1-polymorphism. Such studies might answer the question why AAI-induced cancer develops in some but not all BEN and/or AAN patients.

While most enzymes catalyzing the reductive activation of AAI *in vitro* have already been identified, it remains to be answered which of them actually participate in bioactivation *in vivo*. Additional factors such as route of administration, absorption, renal clearance and tissue-specific enzyme expression make it difficult to extrapolate from *in vitro* data to *in-vivo* situations[25]. Therefore such studies are required in the future.

The competing conversion of *N*-hydroxyaristolactam I to the corresponding 7-hydroxyaristolactam or its further reduction to aristolactam I should be considered a detoxification pathway as both metabolites have been found to be excreted. However, even though aristolactam I is not a direct DNA binding species, low levels of dA-AAI adduct, with highest levels in one of the target tissues (*e.g.* renal pelvis) were generated in rats treated with aristolactam I[42]. This is consistent with the formation of dA-AAI adduct by aristolactam I observed after *in-vitro* activation with different peroxidases such as COX-1 and/or COX-2 that are highly expressed in urothelial tissue[43].

It is well known that conjugation reactions like acetylation and sulfonation catalyzed by phase II enzymes are important in the metabolic activation of carcinogenic nitroaromatics and aromatic amines. However, phase II conjugation reactions do not seem to play a role in AAI activation. In reality, it is still questionable whether enzymes capable of conjugating the proximate carcinogenic metabolite of AAI, *N*-hydroxyaristolactam I, are involved in AAI activation. While Meinl *et al.*[31] demonstrated that expression of some human sulfotransferases (SULTs), particularly SULT1A1, in bacterial and mammalian target cells enhances the mutagenic activity of AAI, the preliminary data from our laboratory produced ambiguous findings: on the one hand, we found that an increase in AAI-induced mutagenicity correlated with higher AAI-DNA adduct levels in V79 cells transfected with human SULT1A1 (Glatt *et al*., unpublished data). On the other hand, further results suggested that SULTs in human hepatic and renal cytosols do not participate in AAI-DNA adduct formation in these subcellular systems (Stiborová *et al.*, unpublished data). It seems more likely that instead, conjugation of *N*-hydroxyaristolactam I catalyzed by SULTs, the carboxy group in the peri position to the nitro group represents a unique example of an intra-molecular conjugation (acylation) leading to the formation of a cyclic hydroxamic acid (*N*-hydroxylactam). In sum, the exact role of conjugation reactions and phase II enzymes in AAI activation awaits further investigations.

Table 1. Contribution of NQO1, CYP1A1/2, NADPH:CYP reductase and COX-1 enzymes to AAI activation.

Enzyme	$K_{0.5}$ for AAI-DNA binding[a] (μM)
NQO1	17 ± 0.8
CYP1A1	65 ± 3.1
CYP1A2	38 ± 1.8
NADPH:CYP reductase	126 ± 6.2
COX-1	153 ± 4.1

$K_{0.5}$ – concentrations of AAI producing half of the maximal AAI binding to DNA

[a] Data shown are means ± S.E.M. of three independent experiments. Data shown are based on results of our former studies for CYP1A1, 1A2, NADPH:CYP reductase and COX-1[37-40], while on unpublished results for NQO1.

The oxidation of AAI to aristolochic acid Ia (AAIa) has been suggested to be a detoxification pathway of AAI[5,25] (Fig. 3) as AAIa or its conjugates, the *O*-glucuronide, the *O*-acetate and the *O*-sulfate esters, are excreted in the urine[33,44]. AAIa is also reduced to *N*-hydroxyaristolactam Ia forming aristolactam Ia which together with its conjugates, the *N* – and *O*-glucuronides, is excreted[33,44]. Enzymatic reactions leading to aristolactam Ia and its metabolites seem to be solely a detoxification pathway because DNA adducts containing aristolactam Ia structure have, as yet, never been found.

In contrast to the enzymes activating AAI *in vitro*, those participating in AAI oxidation to AAIa both *in vitro* and *in vivo* have not been extensively studied so far. Recent studies in our laboratory have shown that human, rat and mouse hepatic CYP enzymes oxidize AAI into this detoxification metabolite AAIa[25,45]. Large-scale investigations in BEN patients on the role of genetic polymorphisms in some genes of phase I detoxification CYP enzymes revealed a possible risk for BEN (OR 2.41) in individuals

carrying *CYP3A5*1* allele G6989[41,46]. Although we found that this CYP did not activate AAI to form DNA adducts (Stiborová *et al.*, unpublished data), we do not know, whether this CYP species is involved in AAI detoxication.

Participation of mouse hepatic CYP enzymes in AAI oxidation to AAIa was recently confirmed by Xiao *et al.*[47], who published novel data on the enzymes detoxifying AAI. In this study, the authors used the HRN (Hepatic Cytochrome P450 Reductase Null) mouse model, previously shown to be suitable for examining hepatic *versus* extrahepatic xenobiotic metabolism *in vivo*[48-51]. For this reason , we have advocated this animal model for elucidating AA metabolism[25]. Xiao et al found that mouse hepatic CYPs detoxify AAI through demethylation to AAIa and in this way protect the kidney from AAI-induced injury[47]. The question, which of the mouse CYP enzymes is responsible for formation of AAIa requires further study. *In-vitro* experiments using hepatic mouse microsomes show that CYP1A enzymes generate AAIa[47]. but the mouse model used to evaluate the role of CYP1A in the formation of AAIa *in vivo* by inducing CYP1A via 3-methycholanthrene (MC), produced ambiguous results[47,52], MC induces other enzymes beside CYP1A. Although treatment of mice with MC leads to decrease in AAI concentrations in the liver and kidney, no increase in AAIa concentrations was found in the liver, only in the kidney of mice treated with the higher dose of AAI (20 mg/kg body weight). An increase in AAIa excretion due to its conjugation with glucuronide, caused by induction of UDP-glucuronosyltransferase with MC, could occur. However as CYP1A enzymes also activate AAI to species forming DNA adducts[25,36-38], the decrease in AAI in liver and kidney might also result from this reaction. Also, NQO1 which is also efficiently induced by MC, could contribute to decreased AAI levels in MC-treated mice.

More recently, we have increased our knowledge of the role of human hepatic CYP enzymes in AAI detoxification[45]. The role of human hepatic CYP enzymes in AAI oxidation was investigated by modulation of this reaction by selective inhibitors of individual CYPs. We found most of the activation of AAI in human hepatic microsomes was attributable to CYP1A. In addition, human recombinant CYP1A1 and 1A2 were the most efficient CYPs for oxidizing AAI. Other CYPs such as CYP1B1, 2C8, 3A4 as well as CYP2B6 with cytochrome b_5, can also oxidize AAI but with efficiencies more than one order of magnitude lower than CYP1A[45].

However, we found that under the anaerobic conditions, human CYP1A enzymes also reductively activate AAI to species forming DNA adducts[37,38]. Therefore, the oxygen concentration of tissues may affect the relative extent of AAI activation by nitroreduction and its detoxication by *O*-demethylation. In addition, orientation and moving of the AAI molecule to the active sites of CYP1A1 and 1A2 enzymes should also influence the pathways of AAI metabolism, namely, *O*-demethylation of AAI *versus* reduction of the nitro group of AAI. We plan to use the *in-silico* docking of AAI to the active sites of CYP1A1 and 1A2 using soft-soft (flexible) docking procedure[53] in further studies to elucidate the molecular mechanisms of oxidation and reduction of AAI by human CYP1A1 and 1A2.

The literature on the role of CYP1A in AAI detoxication[45,47] combined with the reports found for CYP1A – and NQO1-mediated AAI activation[25,34,35,37,38], strongly support the hypothesis[25] that a key factor determining the carcinogenic and nephrotoxic effects of AAI, is the balance of reductase activity such as NQO1, catalyzing the AAI-DNA adduct formation and enzymes such as CYPs which can both activate and detoxify AAI. Taking into account all the currently known data, we propose that the pathways of AAI metabolism are determined by: the binding affinity of AAI to CYP1A or NQO1 enzymes and their enzymatic turnover, as well as the balance between the efficiency of CYP1A in oxidizing and reducing AAI. To test this assumption and to complement earlier studies[25,45,47,52,54], we are currently investigating the formation of AAI-DNA adducts in HRN and *CYP1A* gene knock-out mouse models.

CONCLUSIONS

Although hepatic CYP enzymes have been shown to detoxify AAI *in vitro* and *in vivo* in mice, thus reducing AA1 nephrotoxicity, individual enzymes that may take part in the metabolisation (activation and/or detoxification) of AAI *in vivo* have not been fully explored as yet. Their impact on AAI-mediated nephrotoxicity and carcinogenicity by evaluating inter-individual variations including genetic polymorphisms may be important to explaining individual susceptibility to AAI and to predict cancer risk among AAN and/or BEN patients. These are the subject of our ongoing investigations.

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