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Metabolic activation of carcinogenic aristolochic acid, a risk factor for Balkan endemic nephropathy*

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Abstract

Aristolochic acid (AA), a naturally occurring nephrotoxin and carcinogen, is associated with tumor development in patients suffering from Chinese herbs nephropathy (now termed Aristolochic acid nephropathy, AAN) and may also be a cause for the development of a similar type of nephropathy, the Balkan endemic nephropathy (BEN). Major DNA adducts [7-(deoxyadenosin-N⁶yl)-aristolactam and 7-(deoxyguanosin-N²-yl)aristolactam] formed from AA after reductive metabolic activation were found in renal tissues of patients with both diseases. Understanding which human enzymes are involved in AA activation and/or detoxication is important in the assessment of an individual's susceptibility to this plant carcinogen. This paper reviews major hepatic and renal enzymes responsible for AA-DNA adduct formation in humans. Phase I biotransformation enzymes play a crucial role in the metabolic activation of AA to species forming DNA adducts, while a role of phase II enzymes in this process is questionable. Most of the activation of AA in human hepatic microsomes is mediated by cytochrome P450 (CYP) 1A2 and, to a lower extent, by CYP1A1; NADPH:CYP reductase plays a minor role. In human renal microsomes NADPH:CYP reductase is more effective in AA activation. Prostaglandin H synthase (cyclooxygenase, COX) is another enzyme activating AA in human renal microsomes. Among the cytosolic reductases, NAD(P)H:quinone oxidoreductase (NQO1) is the most efficient in the activation of AA in human liver and kidney. Studies with purified enzymes confirmed the importance of CYPs, NADPH:CYP reductase, COX and NQO1 in the AA activation. The orientation of AA in the active sites of human CYP1A1, -1A2 and NQO1 was predicted from molecular modeling and explains the strong reductive potential of these enzymes for AA detected experimentally. We hypothesized that inter-individual variations in expressions and activities of enzymes activating AA may be one of the causes responsible for the different susceptibilities to this carcinogen reflected in the development of AA-induced nephropathies and associated urothelial cancer.

Key words: Aristolochic acid; Aristolochic acid nephropathy; Balkan endemic nephropathy;

Reductive activation; DNA adducts.

Contents

- 1. Introduction
- 2. Aristolochic acid-mediated carcinogenesis
- 3. Biotransformation of aristolochic acid
- 4. Enzymatic activation of aristolochic acid and DNA adduct formation
 - 4.1. Enzymes in human hepatic and renal microsomes activating aristolochic acid
 - 4.2. Enzymes in human hepatic and renal cytosol activating aristolochic acid
 - 4.3. Contribution of microsomal and cytosolic enzymes to AA activation
- 5. Is the endowment with activating and/or detoxicating enzymes of AA a risk factor for AANand/or BEN-associated urothelial cancer?
- 6. Conclusions

Acknowledgement

References

1. Introduction

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 (AAI), being the major components (Fig. 1) [1,2]. Herbal drugs derived from *Aristolochia* species have been known since antiquity and were used in obstetrics and in the treatment of snake bites [1]. Contempory medicine has used *Aristolochia* plant extracts for the therapy of arthritis, gout, rheumatism and festering wounds [3-5]. The anti-inflammatory properties of AA encouraged the development of pharmaceutical preparations in Germany [6-8] until Mengs and coworkers observed that AA is a strong carcinogen in rats [9,10]. Moreover, AA was shown to be a genotoxic mutagen [11-16] and nephrotoxic to rodents [17-19]. Therefore all pharmaceutical preparations containing AA have been withdrawn from the market in Germany and in many other countries [20]. However, *Aristolochia* plants and their extracts have been further used in traditional medicine in some parts of the world [1,5,21,22].

Recently AA was proven to be the cause of so-called Chinese herbs nephropathy (CHN), a unique type of rapidly progressive renal fibrosis associated with the prolonged intake of Chinese herbal remedies during a slimming regimen, observed for the first time in Belgium in 1991 [23,24]. About 100 CHN cases have been identified so far in Belgium, half of which needed renal replacement therapy, mostly including renal transplantation [25-27]. The observed nephrotoxicity has been traced to the ingestion of herbal preparation *Aristolochia fangchi* containing nephrotoxic AA inadvertently included in slimming pills [24]. CHN patients, who were exposed to *Aristolochia* species containing AA and had no relationship with the Belgian slimming clinic, have been identified in other European countries, in Asia and in the USA (about 170 cases) [28]. Therefore, this disease is now called aristolochic acid nephropathy (AAN) [29,30]. Recently, a high prevalence of urothelial cancer was found in the cohort of AAN patients in Belgium [31,32] and cases of urothelial cancer have also been described in other countries [33-35]. These findings highlight the

carcinogenic potential of AA to humans. Indeed, AA is among the most potent 2% of known carcinogens [33]. As a consequence, 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].

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) [36-40], which is widely found in certain areas of Romania, Croatia, Bosnia, Serbia and Bulgaria along the Danube river basin [36,37,41]. 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 50 years ago the etiology of BEN remains unclear and is a matter of debate [37,41]. For the last years evidence has accumulated that BEN is an environmental disease. Recent experimental data shows that AA might be one of the most important etiologic factors in BEN and associated urothelial cancer [38,41,42]. AA exposure is associated with chronic dietary uptake of seeds of *Aristolochia clematitis* by the population living in BEN regions [28,36,43].

Since the demonstration that AA forms covalent DNA adducts in rodents [44-46] as well as in AAN patients (Fig. 2A) [28,32,47-50], AA-DNA adducts have been used as biomarkers of exposure to AA and to investigate the mutagenic and carcinogenic potential of AA. The major AA-DNA adducts found in rodents exposed to AA and in patients suffering from AAN were identified 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) (Figs. 1 and 2), 7-(deoxyguanosin- N^2 -yl)aristolactam II (dG-AAII) and dA-AAII were detected as the major adducts in animals treated with AAII [45,46,51-55]. One of the AA-DNA adducts, dA-AAI, has also been found in two out of three renal tissues collected randomly from farmers with end-stage renal failure and upper urinary tract malignancy living in areas endemic for BEN (Fig. 2B), although these patients have not been

classified as clearly suffering from BEN [38]. More recently Grollman et al. [42] found dA-AAI and dG-AAI adducts in DNA of renal cortex of four Croatian patients with BEN. These results underline the importance of AA as a risk factor for BEN and BEN-associated urothelial cancer. Nevertheless, the specific role of AA in the development of BEN still awaits further investigation.

2. Aristolochic acid-mediated carcinogenesis

The predominant AA-DNA adduct *in vivo*, 7-(deoxyadenosin-N⁶-yl)aristolactam I (dA-AAI) (Figs. 1 and 2), which is the most persistent of the adducts in the target tissue, is a mutagenic lesion leading to $A \rightarrow T$ transversions [56,57]. This transversion mutation is found at high frequency in codon 61 of the H-ras oncogene in tumors of rodents induced by AAI, suggesting that dA-AAI might be the critical lesion in the carcinogenic process in rodents. DNA binding studies confirmed that both AAs bind to the adenines of codon 61 in the mouse H-ras gene [56,57] and preferentially to purines in the human p53 gene [27,28,58]. In DNA isolated from an urothelial tumor of one AAN patient the dA-AAI adduct and an AAG to TAG mutation in codon 139 (Lys→Stop) of exon 5 in the human p53 gene was detected [59]. In a recent report examining p53 mutations in urothelial tumours of BEN patients in Croatia (N=11) mutations at A:T pairs accounted for 89% (17/19) of all mutations, with the majority of these (15/17) being A \rightarrow T transversions, representing 78% of all base substitutions detected in the p53 gene [42]. Interestingly, in two cases A \rightarrow T transversions were found in human p53 (codon 209 and 280) in immortalized cells derived from primary Hupki embryonic fibroblasts [derived from a human p53 knock-in (Hupki) mouse] exposed to AAI [60,61], All these findings indicate a link between urothelial tumours, p53 mutations and exposure to AA, as we suggested recently [62]. With respect to AA as a risk factor for BEN-associated urothelial tumours observed outside Croatia we predict that many of these tumours carry characteristic A \rightarrow T transversion mutations in *p53* [62].

3. Biotransformation of aristolochic acid

The metabolism of AA has been widely studied in different species including man and has shown that the corresponding aristolactams (Alacs) [63] are major metabolites found free or as conjugates in urine and faeces (Fig. 3) [64,65]. Thus, AAs are predominantly reduced to *N*-hydroxy-Alacs [66], which could be either further reduced to Alacs or rearranged to 7-hydroxyaristolactams [66] (Figs. 1 and 3). The principle metabolite of AAI was aristolactam Ia (AlacIa) produced by two metabolic pathways, one pathway runs via aristolactam I (AlacI) and the other via demethylation of AAI to aristolochic acid Ia (AAIa) (Fig. 3). This interpretation is supported by the results of Schmeiser et al. [67], who showed that AlacI and aristolactam II (AlacII) are produced *in vitro* under anaerobic conditions from AAI and AAII, respectively, with rat liver S9 mix, whereas under aerobic conditions the metabolite formed from AAI is AAIa while AAII remains unaltered. Thus AlacIa, though the principal metabolite *in vivo* has not been detected *in vitro* [64,65]. *In vivo* the oxygen concentration of tissues may affect the relative extents of nitroreduction and *O*-dealkylation of AAI, whereas for AAII only nitroreduction might be influenced by oxygen concentration [68]. Minor AA metabolites *in vivo* are products of AA denitrosation [64] and decarboxylation [66].

The phase II-metabolism of both AAs, studied by Krumbigel et al. [64] and Chan et al. [65,66], indicated that large amounts of AA metabolites in the urine and faeces in rodents were present in conjugated form, either as glucuronides or as sulfate or acetate esters [64-66]. Recently, Chan et al. [65,66] identified three phase II metabolites of AAIa, namely the *O*-glucuronide, the *O*-acetate and the *O*-sulfate esters [66] and three conjugates of Alacs, the *N*- and *O*-glucuronides of AlacIa (with prevalence of the *N*-glucuronide) as well as the *N*-glucuronide of AlacII [65], in the urine of rats treated with AA (Fig. 3).

Simple nitro reduction is the major pathway responsible for the carcinogenic potential of AAI and AAII because during such reactions reactive metabolites binding to DNA *in vitro* and *in vivo*

are generated (Fig. 1) [28,46,51,52]. While, Alacs are the final products of the reduction of the nitro group of both AAs, they are not the direct DNA binding species. This view is supported by Ames assay results demonstrating that Alacs are not mutagenic themselves, but require activation by rat liver S9 mix [67]. Whereas AAI and AAII were direct mutagens in the most commonly used *Salmonella* strains TA100 and TA1537 [11,69] the mutagenic potency of the corresponding Alacs in TA100 is about one-half of that of the parent compounds [67]. This result is consistent with the observation reported by Dong and co-workers [55], who found that 50 times lower amounts of dA-AAI and dG-AAI adducts, with the highest levels in a target tissue, renal pelvis, were generated in Wistar rats treated with AlacI than with AAI and AAII [55]. No such DNA adducts (dA-AA and dG-AA adducts) were however found for AlacI and AlacII in the presence of rat liver S9 mix [44] or rat hepatic microsomes containing cytochrome P450 (CYP) enzymes [49]. In contrast to this finding, formation of dA-AA and dG-AA adducts by both Alacs was observed after *in-vitro* activation with different peroxidases [49,70] of which several, such as COX-1 and/or COX-2, are expressed at high levels in renal tissue [71,72].

4. Enzymatic activation of aristolochic acid and DNA adduct formation

One of the common features of AAN and BEN is that not all individuals exposed to AA (AAN and/or BEN patients) suffer from nephropathy and tumor development. To date only 5% of the patients treated with the slimming regimen in Belgium are suffering from AAN [28]. One cause for these different responses may be individual differences in the activities of the enzymes catalyzing the biotransformation (detoxication and/or activation) of AA. Many genes of enzymes metabolizing 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 [73]. Indeed, the combination of polymorphic genes with various environmental factors such as AA that may result in an increased risk for BEN has been proposed by Toncheva,

Atanasova et al. [74-78]. Thus, the identification of the enzymes principally involved in the metabolism (detoxication and/or activation) of AA in humans and a detailed knowledge of their catalytic specificities is of major importance.

A powerful tool to determine the activation of AAs is to characterize and quantify the DNA adducts they form, and to determine which factors either enhance or inhibit adduct formation. The detection of specific AA-DNA adducts by ³²P-postlabeling [44-49,53] has allowed us to use DNA binding as a probe for metabolic activation of AA in *in-vitro* systems. The same AA-DNA adducts found in rodents and patients suffering from AAN, namely dA-AAI, dG-AAI, dA-AAII and dG-AAII [44-50,53], are generated in *in-vitro* systems [44,49,70,79-86]. This indicates that a cyclic *N*-acylnitrenium ion with a delocalized positive charge as the ultimate carcinogenic species binds preferentially to the exocyclic amino groups of purine nucleotides in DNA or is converted to the corresponding 7-hydroxyaristolactam (Fig. 1) [51,52,66]. It is known that in the activation of carcinogenic nitroaromatics and aromatic amines acetylation of the amino or hydroxyamino group plays a key role. Therefore the activation of AA is a unique example of an intra-molecular acylation which leads to the ultimate carcinogen.

The first enzymatic study evaluating the activation of AAI and AAII to species forming DNA adducts *in vitro* utilized rat liver S9 mix as the enzymatic system [44]. Whereas for AAI the same DNA adducts were observed under aerobic and anaerobic conditions, AAII gave rise to adduct formation only under anaerobic conditions in these studies. Both microsomal and cytosolic reductases are present in S9 mix and might be responsible for AA-DNA adduct formation. Therefore, we evaluated the contribution of individual human microsomal and cytosolic reductases to AA-DNA adduct formation. Enzymes of two organs, liver and kidney, were investigated; the liver as a tissue rich in biotransformation enzymes and thus predominantly responsible for carcinogen metabolism and the kidney as the target for AA-derived nephrotoxicity and carcinogenesis.

4.1. Enzymes in human hepatic and renal microsomes activating aristolochic acid

Human hepatic and renal microsomes were capable of reductive activation of AAs to species generating the same AA-DNA adducts as found *in vivo* [81,86]. Using extensive enzymatic studies we identified the enzymes, which are predominantly responsible for AAI-DNA adduct formation. We demonstrated that most of the activation of AAI in human hepatic microsomes is mediated by CYP1A2 and/or -1A1. Using microsomes from baculovirus-transfected insect cells (SupersomesTM) containing recombinantly expressed human CYPs (-1A1, -1A2, -1B1, -2A6, -2B6, -2C9, -2D6, -2E1 or -3A4) and/or human NADPH:CYP reductase this finding was corroborated. AAI activation by SupersomesTM containing individual CYP species and NADPH:CYP reductase showed clearly that human CYP1A1 and -1A2 were the most active (Fig. 4). Because of a relatively low content of CYP1A1 in human liver [87,88], its contribution to AAI activation in this tissue has to be much lower than that of CYP1A2.

CYP1A1 and -1A2 homology modeling followed by docking of AAI to the active centers of CYP1A1 and -1A2 was utilized to explain the potential of these enzymes to reduce AAI. The *in-silico* docking of AAI to the active sites of CYP1A1 and -1A2 indicates that AAI binds as an axial ligand of the heme iron with the nitro group is in close vicinity to the heme iron of CYP1A2 in an orientation allowing the efficient reduction of this group observed experimentally (Fig. 5B). The orientation of AAI in the active centre of CYP1A1, however, leads to cause an interaction of the heme iron with both the nitro- and the carboxylic groups of AAI (Fig. 5A). This observation explains the lower reductive potential of CYP1A1 for AAI than CYP1A2, detected experimentally (Fig. 4).

It is noteworthy that the efficacy of microsomes from a human kidney to activate AAI was comparable to that of microsomes of human livers, even though the 7-ethoxyresorufin *O*-deethylase activity, a marker for CYP1A, was more than one order of magnitude lower than in liver

microsomes analyzed in our study [86]. CYP1A expression levels in this human kidney specimen were low (<0.005 pmol CYP1A1/mg protein and no CYP1A2), in agreement with CYP levels published for human kidney [88]. Therefore, the relevance of these CYP enzymes in AA activation in kidney seems to be low. In kidney microsomes NADPH:CYP reductase and COX, a peroxidase abundant in kidney [71,72], were found to be the principal enzymes reductively activating AAI [86]. Indeed, purified COX-1 was found to efficiently catalyze DNA adduct formation of AAI [80].

4.2. Enzymes in human hepatic and renal cytosol activating aristolochic acid

In addition to human microsomes, cytosolic samples from human livers and kidney are capable of activating AAI leading to the same DNA adduct pattern as formed in humans exposed to AA [84]. In these subcellular systems formation of AAI-DNA adducts was found to be principally catalyzed by NAD(P)H:quinone oxidoreductase (NQO1) [84]. Using human recombinant NQO1 the efficiency of this enzyme to activate AAI was corroborated [89]. Molecular modeling whereby the AAI molecule was docked to the active site of human NQO1 suggests that AAI binds in the same orientation as other NQO1 substrates in the X-ray structures, with the planar aromatic AAI rings parallel to the flavin ring (Fig. 6). This allows for an efficient electron transfer during the reductive activation of AAI.

In comparison to NQO1, xanthine oxidase (XO), another cytosolic reductase in human hepatic and renal cytosols, had only a minor impact on the activation of AAI to form DNA adducts [84]. In contrast to this finding we observed that the isolated buttermilk XO was an effective activator of this compound [79,84], but the high enzyme levels needed are not physiological. Another reason for the observed discrepancies might be the different substrate specificities of human cytosolic and buttermilk XO.

Besides cytosolic reductive enzymes, conjugation enzymes such as *N*-acetyltransferases (NATs) and sulfotransferases (SULTs) are involved in the metabolic activation of several nitro-

aromatics [90,91]. Their participation in AA activation is, however, still a matter of debate. Recently, Meinl et al. [92] demonstrated that expression of some humans SULTs, particularly SULT1A1, in bacterial and mammalian target cells enhances the mutagenic activity of AA. Moreover, an increase in AAI-induced mutagenicity was correlated with higher AA-DNA adduct levels in fibroblastic V79 cells transfected with human SULT1A1 [Arlt et al. unpublished data]. However, neither in human hepatic and renal cytosols, to which the SULT cofactor, 3'phosphoadenosine-5'-phosphosulfate (PAPS) was added, nor in an *in-vitro* system consisting of human NQO1 and SULT1A1, an increase in AA-DNA adduct levels was found [Stiborová et al. unpublished data]. Thus, the exact role of conjugation enzymes in AA activation awaits further investigation.

4.3. Contribution of microsomal and cytosolic enzymes to AA activation

When comparing the efficiency of microsomal and cytosolic enzymes, NQO1 is more effective in reduction of AAI to form DNA adducts than CYP1A1, -1A2, NADPH:CYP reductase or COX-1. The concentrations of AAI required for half-maximum DNA binding was 17 μ M for reductive activation by human NQO1 [89], while 38, 65, 126 and 153 μ M AAI for its activation by human CYP1A2, -1A1, NADPH:CYP reductase and COX-1, respectively [80,85]. In addition, the comparison of AA-DNA adduct levels formed by human hepatic microsomes and cytosols revealed that the cytosolic enzyme systems are more efficient than microsomes. In the presence of the cofactor NADPH the levels of AAI-derived DNA adducts expressed as relative adduct labelings (RALs) per mg protein were more than 2-fold higher in cytosols than in microsomes [84,86]. Because the content of cytosolic protein per gram of human liver tissue is about one order of magnitude higher than that of microsomal protein [Stiborová et al. unpublished data], the importance of cytosolic enzymes in AAI activation in the intact organ will be even greater. Nevertheless, in the *in-vitro* experiments we could not evaluate exactly the significance of the phase I enzymes in microsomal and cytosolic fractions of human tissues, because the effect of cytosolic conjugation enzymes on AAI-DNA adduct formation could not be quantified. SULT1A1 is known to be expressed in human liver and kidney [92]. To determine the contribution of cytosolic and microsomal nitroreductases to AA activation, we plan to study the *in vivo* situation. Mice carrying a deletion in the hepatic NADPH:CYP reductase gene [93,94], and thus lacking NADPH:CYP reductase and NADPH:CYP reductase-mediated CYP activity in hepatocytes will be used.

5. Is the endowment with activating and/or detoxicating enzymes of AA a risk factor for AANand/or BEN-associated urothelial cancer?

As shown, the most important human enzymes activating AA by simple nitroreduction leading to DNA binding species are hepatic and renal cytosolic NQO1, hepatic microsomal CYP1A2 and renal microsomal NADPH:CYP reductase, in addition to COX [80,86], which is highly expressed in urothelial tissue [71,72].

The levels of two forms of human COX (COX-1 and -2) appear to be induced in response to a number of hormonal and membrane active agents [71,72]. Their levels in individuals can, therefore, differ significantly. This is also true for other enzymes activating AA. Expression levels and activities of NQO1, CYP1A1/2 and NADPH:CYP reductase in humans are influenced by several factors (smoking, drugs, environmental chemicals and genetic polymorphisms) and differ considerably among individuals [88,95-97]. Glucocorticoid levels also influence the activity and levels of NADPH:CYP reductase [98].

One of the most efficient AA activating enzymes is NQO1, ubiquitously present in all tissue types [99-102]. Expression levels and activities of NQO1 differ considerably among individuals [99,103,104]. Biochemical studies have already demonstrated that NQO1 activity is induced by a wide range of chemicals [99,105-107]. Two distinct regulatory elements in the 5' flanking region of the *NQO1* gene, the antioxidant response element (ARE) and the xenobiotic response element

(XRE), involving the liganded aromatic hydrocarbon (Ah) receptor, have been shown to regulate NQO1 expression in many cellular systems. Moreover, the antiestrogens tamoxifen and hydroxytamoxifen stimulate the expression of NQO1 by activating the estrogen receptor, which is different from the *Ah* locus [108,109]. ARE-mediated *NQO1* gene expression is increased by a variety of phenolic antioxidants, tumor promoters, and hydrogen peroxide [99,105,107]. The XRE of *NQO1* shares significant homology with the XRE of *CYP1A1* [110]. Both *NQO1* and *CYP1A1* genes can be induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polycyclic aromatic hydrocarbons, Sudan I, and β -naphthoflavone [99]. Because NQO1 activity is increased in rats treated with AA [111], it might also be induced in AAN and/or BEN patients.

So far two polymorphisms in the human *NQO1* gene have been found in the general population. One of them the 609 C to T variant, designated *NQO1*2*, has profound phenotypic consequences and has been associated with an increased risk of urothelial tumors [103], therapy-related acute myeloid leukemia [112], cutaneous basal cell carcinoma [113] and pediatric leukemia [114]. The frequency of homozygous *NQO1*2* mutation varies across ethnic groups and was reported to be approximately 5% in Caucasians [99].

Collectively, these data suggest that variations of NQO1 and regulatory proteins controlling expression of this enzyme as well as of another enzyme activating AA, CYP1A1 (Ah receptor, or its associated transcription factor, the Ah receptor nuclear translocator or Arnt protein) [110], might play a role in the risk of cancer by AAs. Therefore, AAN and BEN patients as well as healthy persons exposed to AA by herbal remedies or inhabitants living in BEN regions should be screened for genetic polymorphisms of *NQO1, CYP1A1* and genes controlling their expression. The role of genetic polymorphisms in several genes of phase I biotransformation enzymes such as NQO1, CYP2D6, 3A4, 3A5 as well as in those of the conjugation enzymes NAT1, NAT2, GSTT1 and GSTM1, relevant for detoxication of xenobiotics has already been investigated in BEN patients [41,74,76-78]. Even though NQO1-polymorphism is not as strongly related to BEN as to urinary

tract tumours [41,76], the genotype NQO1*2/*2 predisposed BEN patients to the development of urothelial malignancy of the upper urinary tract (OR=13.75, 95%CI 1.17-166.21) [78]. This finding together with the demonstration of the importance of NQO1 in AA activation could be an explanation for the development of BEN or AAN and to cancer induction by AA in some patients suffering from either nephropathy.

While the enzymes catalyzing the reductive activation of AA have already been investigated, those participating in its oxidation to AAIa, suggested to be mainly a detoxication pathway, have not been extensively studied so far. Our preliminary studies indicated that CYP enzymes can generate this oxidative metabolite [Stiborová et al. unpublished data]. A large-scale investigation in BEN patients on the role of genetic polymorphisms in genes of some phase I detoxication CYP enzymes [41,77,78] revealed a higher risk for BEN (OR 2.41) in individuals carrying *CYP3A5*1* allele G6989 [41,77,78]. We do not know, however, if this CYP species is involved in AA detoxication or activation. The evaluation of the oxidative detoxication of AA by this and other CYP enzymes is our next goal.

6. Conclusions

The present article summarizes our knowledge on the enzymes, which are responsible for metabolic activation of AA to species forming AA-DNA adducts found in patients suffering from AAN and BEN. While the enzymes catalyzing the reductive activation of AA have already been established, those participating in detoxication remain to be investigated. The most important human enzymes activating AA by simple nitroreduction are hepatic and renal cytosolic NQO1, hepatic microsomal CYP1A2 and renal microsomal NADPH:CYP reductase, besides COX, which is highly expressed in urothelial tissue. Expression levels and activities of these enzymes in humans are influenced by several factors (smoking, drugs, environmental chemicals and genetic polymorphisms) and differ considerably among individuals. This feature might be one of the

reasons for different individual susceptibilities to AA and subsequent development of urothelial cancer seen in both patient groups. Therefore, the evaluation of inter-individual variations in the enzymes involved in AA activation and detoxication, including their genetic polymorphisms, remains one of the challenges to explain an individual's susceptibility to AA and to predict cancer risk among the AAN and BEN patients.

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References

- [1] International Agency for Research on Cancer. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monogr. Eval. Carcinog. Risks Hum. 82 (2002).
- [2] V. Kumar, D. Ponam, A.K. Prasad, V.S.Parmar, Naturally occurring aristolactams, aristolochic acids and diaxoapophines and their biological activities, Nat. Prod. Rep. 20, (2003) 565-583.
- [3] G. Rücker, B.S. Chung, Aristolochic acids from *Aristolochia manshuriensis.*, Planta Med. 27 (1975) 68-71.
- [4] G. Hahn, Die Osterluzei Aristolochia clematits eine alte Medizinal-Pflanze, Dr. Med. 8
 (1979) 41-43. Verlag Dr. Med., Fachverlag GmbH, A-3002 Purkesdorf.
- [5] H.A. Priestap, Minor aristolochic acids from *Aristolochia argentina* and mass spectral analysis of aristolochic acids, Phytochemistry 26 (1987) 519-529.
- [6] J.R. Möse, Weitere Untersuchungen über die Wirkung der Aristolochiasäure, Drug. Res. 16 (1966) 118-122.
- [7] J.R. Möse, Weitere Studien über Aristolochiasäure, Drug Res. 24 (1974) 151-153.
- [8] R. Kluthe, A. Vogt, S. Batsford, Doppelblindstudie zur Beeinflussung der Phagocytosefähigkeit von Granulocyten durch Aristolochiasäure, Drug Res. 32 (1982) 443-445.
- [9] U. Mengs, W. Lang, J.A. Poch, The carcinogenic action of aristolochic acid in rats, Arch. Toxicol. 107 (1982) 107-119.
- [10] U. Mengs, On the histopathogenesis of rat forestomach carcinoma caused by aristolochic acid, Arch. Toxicol. 52 (1983) 209-220.
- [11] Robisch G, Schimmer O, Göggelmann W. Aristolochic acid is a direct mutagen in Salmonella typhimurium, Mutat. Res. 105 (1982) 201-204.
- [12] G. Abel, O. Schimmer, Induction of structural chromosome abberations and sister chromatid exchanges in human lymphocytes *in-vitro* by aristolochic acid, Hum. Genet. 64 (1983) 131-133.

- [13] H. Frei, F.E. Würgler, H. Juon, C.B. Hall, U. Graf, Aristolochic acid is mutagenic and recombinogenic in *Drosophila* genotoxicity tests, Arch. Toxicol. 56 (1985) 158-166.
- [14] J.M. Pezutto, S.M. Swanson, M. Woongchon, C. Che, G.A. Cordell, H.H.S. Fong, Evaluation of the mutagenic and cytostatic potential of aristolochic acid (3,4-methylenedioxy-8-methoxy-10-nitrophenanthrene-1-carboxylic acid) and several of its derivatives, Mutat. Res. 206 (1988) 447-454.
- [15] S. Kevekordes, C.M. Burghaus, J. Spielberger, V. Mersch-Sundermann, H.H. Schmeiser, V.M. Arlt, H. Dunkelberg, SOS induction of selected naturally occuring substances in Escherichia coli (SOS chromotest), Mutat. Res. 445 (1999) 81-91.
- [16] S. Kevekordes, J. Spielberger, C.M. Burghaus, P. Birkenkamp, B. Zietz, P. Paufler, M. Diez, C. Bolten, H. Dunkelberg, Micronucleus formation in human lymphocytes and in the metabolically competent human hepatoma cell line Hep-G2: results with 15 naturally occurring substances, Anticancer Res. 21 (2001) 461-470.
- [17] U. Mengs, Acute toxicity of aristolochic acid in rodents, Arch. Toxicol. 59 (1987) 328-331.
- [18] U. Mengs, Tumor induction in mice following exposure to aristolochic acid, Arch. Toxicol. 61 (1988) 504-505.
- [19] U. Mengs, C.D. Stotzem, Renal toxicity of aristolochic acid in rats as an example of nephrotoxicity testing in routine toxicology, Arch. Toxicol. 87 (1993) 307-311.
- [20] K.F.F. Reynolds (Ed.), Martindale, The Extra Pharmacopeia. Pharmaceutical Press, London.1986, p. 130.
- [21] P.J. Houghton, M. Ogutveren, Aristolochic acids and aristolactams from Aristolochia auricularia, Phytochemistry 30 (1991) 253-254.
- [22] B.S. Vishwanath, T.V. Gowda, Interaction of phospholipase A₂ from *Vipera russelli* with aristolochic acid, Toxicon 25 (1987) 939-946.
- [23] J.L. Vanherweghem, M. Depierreux, C. Tielemans, D. Abramowicz,, M. Dratwa, M. Jadoul,

C. Richard, D. Valdervelde, D. Verbeelen, B. Vanhaelen-Fastre, M. Vanhaelen, Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs, Lancet 341 (1993) 387-391.

- [24] M. Vanhaelen, B. Vanhaelen-Fastre, P. But, J.L. Vanherweghem, Identification of aristolochic acid in Chinese herbs, Lancet 343 (1994) 174.
- [25] M. Vanhaelen, J.J. Cuykens, P.H. Vandenberg, K.P. Bouman, Y. Hagens, Valvular heart disease and Chinese-herbs nephropathy, Lancet 351 (1998) 991.
- [26] M.C. Martinez, J. Nortier, P. Vereerstaeten, J.L. Vanherweghem, Progression rate of Chinese herb nephropathy: impact of *Aristolochia fangchi* ingested dose, Nephrol. Dial. Transplant. 17 (2002) 408-412.
- [27] J.-P. Cosyns, Aristolochic acid and "Chinese herbs nephropathy": a review of the evidence to date, Drug Safety 26 (2003) 33-48.
- [28] V.M. Arlt, M. Stiborova, H.H. Schmeiser, Aristolochic acid as a probable human cancer hazard in herbal remedies: a review. Mutagenesis 17 (2002) 265-277.
- [29] G. Gillerot, M. Jadoul, V.M. Arlt,C. van Ypersele de Strihou, H.H. Schmeiser, P.H.H. But, C.A. Bieler, J.-P. Cosyns, Aristolochic acid nephropathy in a Chinese patient: time to abandon the term "Chinese herbs nephropathy"? Am. J. Kidney Dis. 38 (2001) E26.
- [30] K. Solez, J. Daugirdes, M.C. Gregory, P.P. Frohnert, D.M. Blownik, V. Iha, J.-P. Cosyns, Is "Chinese Herbs Nephropathy" a prejudical term? Am. J. Kidney Dis. 38 (2001) 1141-1142.
- [31] J.-P. Cosyns, M. Jadoul, J.P. Squifflet, F.X. Wese, C. van Ypersele de Strihou C, Urothelial lesions in Chinese-herb nephropathy, Am. J. Kidney Dis. 33 (1999) 1011-1017.
- [32] J.L. Nortier, M.C. Martinez, H.H. Schmeiser, V.M. Arlt, C.A. Bieler, M. Petein, M.F. Depierreux, L. De Pauw, D. Abramowicz, P. Vereerstraeten, J.L. Vanherweghen, Urothelial carcinoma associated with the use of a Chinese herb (Aristolochia fangchi), N. Engl. J. Med. 342 (2000) 1686-1692.

- [33] G.M. Lord, T. Cook, V.M. Arlt, H.H. Schmeiser, G. Williams, C.D. Pusey, Urothelial malignant disease and Chinese herbal nephropathy, Lancet 358 (2001) 1515-1516.
- [34] V.M. Arlt, V. Alunni-Perret, G. Quatrehomme, P. Ohayon, L. Albano, H. Gaid, J.F. Michiels,
 A. Meyrier, E. Cassuto, M. Wiessler, H.H. Schmeiser, J.-P. Cosyns, Aristolochic acid (AA)DNA adduct as marker of AA exposure and risk factor for AA nephropathy-associated cancer,
 Int. J. Cancer. 111 (2004) 977-980.
- [35] S.H. Lo, K.S. Wong, V.M. Arlt, D.H. Phillips, C.K. Lai, W.T. Poon, C.K. Chan, K.L. Mo, K.W. Chan, A. Chan, Detection of Herba Aristolochia Mollissemae in a patient with unexplained nephropathy, Am. J. Kidney Dis. 45 (2005) 407-410.
- [36] M. Ivic, Etiology of endemic nephropathy, Lijec Vjesn. 91 (1969) 1273-1281.
- [37] C.A. Tatu, W.H. Oren, R.B. Finkelman, G.L. Feder, The etiology of Balkan endemic nephropathy: still more questions than answers, Environ. Health. Perspect. 106 (1998) 689-700.
- [38] V.M. Arlt, D. Ferluga, M. Stiborova, A. Pfohl-Leszkowicz, M. Vukelic, S. Ceovic, H.H. Schmeiser, J.-P. Cosyns, Is aristolochic acid a risk factor for Balkan endemic nephropathy-associated urothelial cancer? Int. J. Cancer. 101 (2002) 500-502.
- [39] A. Pfohl-Leszkowicz, T. Petkova-Bocharova, I.N. Chernozemsky, M. Castegnaro, Balkan endemic nephropathy and associated urinary tract tumours: a review on aetiological causes and the potential role of mycotoxins, Food Addit. Contam. 19 (2002) 282-302.
- [40] M. Stiborová, J. Patočka, E. Frei, H.H. Schmeiser, Biochemistry and toxicological aspects of etiology of Balkan endemic nephropathy [in Czech], Chem. Listy 99 (2005) 782-788.
- [41] V. Stefanovic, D. Toncheva, S. Atanasova, M. Polenakovic, Etiology of Balkan endemic nephropathy and associated urothelial cancer, Am. J. Nephrol. 26 (2006) 1-11.
- [42] A.P. Grollman, S Shibutani, M. Moriya, F. Miller, L. Wu, U. Moll, N. Suzuki, A. Fernandes,T. Rosenquist, Z. Medverec, K. Jakovina, B. Brdar, N. Slade, R. Turesky, A. K. Goodenough,

R. Rieger, M. Vukelic, B. Jelakovic, Aristolochic acid and the etiology of endemic (Balkan) nephropathy, Proc. Natl. Acad Sci. USA 104 (2007) 12129-12134.

- [43] T. Hranjec, A. Kovac, J. Kos, W. Mao, J.J. Chen, A.P. Grollman, B. Jelakovic, Endemic nephropathy: the case for chronic poisoning by aristolochia, Croat. Med. J. 46 (2005) 116-125.
- [44] H.H. Schmeiser, K.-B. Schoepe, M. Wiessler, DNA adduct formation of aristolochic acid I and II *in vitro* and *in vivo*, Carcinogenesis 9 (1988) 297-303.
- [45] W. Pfau, H.H. Schmeiser, M. Wiessler, ³²P-postlabelling analysis of the DNA adducts formed by aristolochic acid I and II, Carcinogenesis 11 (1990) 1627-1633.
- [46] M. Stiborová, R.C. Fernando, H.H. Schmeiser, E. Frei, W. Pfau, M. Wiessler, Characterization of DNA adducts formed by aristolochic acids in the target organ (forestomach) of rats by ³²Ppostlabelling analysis using different chromatographic procedures, Carcinogenesis 15 (1994) 1187-1192.
- [47] H.H. Schmeiser, C.A. Bieler, M. Wiessler, C. van Ypersele de Strihou, J.-P. Cosyns, Detection of DNA adducts formed by aristolochic acid in renal tissue from patients with Chinese herbs nephropathy,. Cancer Res. 56 (1996) 2025-2028.
- [48] C.A. Bieler, M. Stiborová, M. Wiessler, J.-P. Cosyns, C. van Ypersele de Strihou, H.H. Schmeiser, ³²P-post-labelling analysis of DNA adducts formed by aristolochic acid in tissues from patients with Chinese herbs nephropathy, Carcinogenesis 18 (1997) 1063-1067.
- [49] M. Stiborová, E. Frei, A. Breuer, C.A. Bieler, H.H. Schmeiser, Aristolactam I a metabolite of aristolochic acid I upon activation forms an adduct found in DNA of patients with Chinese herbs nephropathy, Exp. Toxic. Pathol. 51 (1999) 421-427.
- [50] V.M. Arlt, A. Pfohl-Leszkowicz, J.-P. Cosyns, H.H. Schmeiser, Analyses of DNA adducts formed by ochratoxin A and aristolochic acid in patients with Chinese herbs nephropathy, Mutat. Res. 494 (2001) 143-150.

- [51] W. Pfau, H.H. Schmeiser, M. Wiessler, Aristolochic acid binds covalently to the exocyclic amino group of purine nucleotides in DNA, Carcinogenesis 11 (1990) 313-319.
- [52] W. Pfau, H.H. Schmeiser, M. Wiessler, N⁶-Adenyl arylation of DNA by aristolochic acid II and a synthetic model for the putative proximate carcinogen, Chem. Res. Toxicol. 4 (1991) 581-586.
- [53] R.C. Fernando, H.H. Schmeiser, H.R. Scherf, M. Wiessler, Formation and persistence of specific purine DNA adducts by ³²P-postlabelling in target and non-target organs of rats treated with aristolochic acid I, IARC Sci. Publ. 124 (1993) 167-171.
- [54] J.-P. Cosyns, R.-M. Goebbels, V. Liberton, H.H. Schmeiser, C.A. Bieler, A.M. Bernard, Chinese herbs nephropathy-associated slimming regimen induces tumors in the forestomach but no interstitial nephropathy in rats, Arch. Toxicol. 72 (1998) 738-743.
- [55] H. Dong, N. Suzuki, M.C. Torres, R.R. Bonala, F. Johnson, A.P. Grollman, S. Shibutani, Quantitative determination of aristolochic acid-derived DNA adducts in rats using ³²Ppostlabeling/polyacrylamide gel electrophoresis analysis, Drug. Metab. Dispos. 34 (2006) 1122-1127.
- [56] H.H. Schmeiser, W. Janssen, J. Lyons, H.R. Scherf, W. Pfau, A. Buchmann, C.R. Bartram, M. Wiessler, Aristolochic acid activates *ras* genes in rat tumors at deoxyadenosine residues, Cancer Res. 50 (1990) 5464-5469.
- [57] V.M. Arlt, M. Wiessler, H.H. Schmeiser, Using polymerase arrest to detect DNA binding specificity of aristolochic acid in the mouse H-*ras* gene, Carcinogenesis 21 (2000) 235-242.
- [58] V.M. Arlt, H.H. Schmeiser, G.P. Pfeifer, Sequence-specific detection of aristolochic acid-DNA adducts in the human p53 gene by terminal transferase-dependent PCR, Carcinogenesis 22 (2001) 133-140.
- [59] G.M. Lord, M. Hollstein, V.M. Arlt, C. Roufosse, C.D. Pusey, T. Cook, H.H. Schmeiser, DNA adducts and p53 mutations in a patient with aristolochic acid-associated nephropathy, Am. J.

Kidney Dis. 43 (2004) e11-17.

- [60] Z. Liu, M. Hergenhahn, H.H. Schmeiser, G.N. Wogan, A. Hong, M. Hollstein, Human tumor p53 mutations are selected for in mouse embryonic fibroblasts harboring a humanized p53 gene, Proc. Natl. Acad. Sci. USA 101 (2004) 2963-2968.
- [61] N. Feldmeyer, H.H. Schmeiser, K.R. Muehlbauer, D. Belharazem, Y. Knyazev, T. Nedelko, M. Hollstein, Further studies with a cell immortalization assay to investigate the mutation signature of aristolochic acid in human p53 sequences, Mutat. Res. 608 (2006) 163-168.
- [62] V. M. Arlt, M. Stiborová, J. vom Brocke, M. L. Simões, G. M. Lord, J. L. Nortier, M. Hollstein, D. H. Phillips, H. H. Schmeiser, Aristolochic acid mutagenesis: molecular clues to the aetiology of Balkan endemic nephropathy-associated urothelial cancer, Carcinogenesis 2007 Apr 13; [Epub ahead of print].
- [63] D.B. Mix, H. Guinaudeau, M. Shamma, The aristolochic acids and aristolactams, J. Nat. Products 45 (1982) 657-666.
- [64] G. Krumbiegel, J. Hallensleben, W.H. Mennicke, N. Rittmann, Studies on the metabolism of aristolochic acids I and II, Xenobiotica 17 (1987) 981-991.
- [65] W. Chan, L. Cu, G. Xu, Z. Cai, Study of the phase I and phase II metabolism of nephrotoxin aristolochic acid by liquid chromatography/tandem mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 1755-1760.
- [66] W. Chan, H.-B. Luo, Y. Zheng, Y.-K. Cheng, Z. Cai, Investigation of the metabolism and reductive activation of carcinogenic aristolochic acid in rats, Drug Metab. Dispos. 35 (2007) 866-874.
- [67] H.H. Schmeiser, B.L. Pool, M. Wiessler, Identification and mutagenicity of metabolites of aristolochic acid formed by rat liver, Carcinogenesis 7 (1986) 59-63.
- [68] P. Maier, H. Schawalder, B. Weibel, Low oxygen tension as found in tissues in vivo, alters the mutagenic activity of aristolochic acid I and II, Environ. Mol. Mutagen. 10 (1987) 275-284.

- [69] H.H. Schmeiser, B.L. Pool, M. Wiessler, Mutagenicity of the two main components of commercially available carcinogenic aristolochic acid in *Salmonella typhimurium*, Cancer Lett. 23 (1984) 97-98.
- [70] M. Stiborová, E. Frei, H.H. Schmeiser, M. Wiessler, Cytochrome P-450 and peroxidase oxidize detoxication products of carcinogeneic aristolochic acids (aristolactams) to reactive metabolites binding to DNA in vitro, Collect. Czech. Chem. Commun. 60 (1995) 2189-2199.
- [71] T.E. Eling, D.C. Thompson, G.L. Foureman, J.F. Curtis, M.F. Hughes, Prostaglandin H synthase and xenobiotic oxidation, Annu. Rev. Pharmacol. Toxicol. 30 (1990) 1-45.
- [72] T.E. Eling, J.F. Curtis, Xenobiotic metabolism by prostaglandin H synthase, Pharm. Ther. 53 (1992) 261-273.
- [73] G. Smith, L.A. Stanley, E. Sim, R.C. Strange, R. Wolf, Metabolic polymorphism and cancer susceptibility, Cancer Surveys 25 (1995) 27-65.
- [74] D. Toncheva, T. Dimitrov, S. Stojanova, Etiology of Balkan endemic nephropathy: a multifactorial disease? Eur. J. Epidemiol. 14 (1998) 389-394.
- [75] S. Atanasova, N. von Ahsen, T. Dimitrov, V. Amstrong, M. Oellerich, D. Toncheva, MDR1 haplotypes modify BEN disease risk: a study in Bulgarian patients with balkan endemic nephropathy compared to healthy controls, Nephron Exp. Nephrol. 96 (2004) 7-13.
- [76] D. I. Toncheva, N. von Ahsen, S. Y. Atanasova, T. G. Dimitrov, V. M. Amstrong, M. Oellerich, Identification of NQO1 and GSTs genotype frequencies in Bulgarian patients with Blakan endemic nephropathy, J. Nephrol. 17 (2004) 384-389.
- [77] S. Y. Atanasova, N. von Ahsen, D. I. Toncheva, T. G. Dimitrov, M. Oellerich, V. M. Amstrong, Genetic polymorphism of cytochrome P450 among patients with Balkan endemic nephropathy (BEN), Clin. Biochem. 38 (2005) 223-228.

- [78] D. Toncheva, Genetic studies in BEN and associated urothelial cancers, Coll. Antropol. 30 Suppl 1 (2006) 34.
- [79] H.H. Schmeiser, E. Frei, M. Wiessler, M. Stiborová, Comparison of DNA adduct formation by aristolochic acids in various *in vitro* activation systems by ³²P-post-labeling: evidence for reductive activation by peroxidases, Carcinogenesis 18 (1997) 1055-1062.
- [80] M. Stiborová, E. Frei, A. Breuer, M. Wiessler, H.H. Schmeiser, Evidence for reductive activation of carcinogenic aristolochic acids by prostaglandin H synthase - ³²P-postlabeling analysis of DNA adduct formation, Mutat. Res. 493 (2001) 153-164.
- [81] M. Stiborová, E. Frei, M. Wiessler, H.H. Schmeiser, Human enzymes involved in the metabolic activation of carcinogenic aristolochic acids: evidence for reductive activation by cytochrome P450 1A1 and 1A2, Chem. Res. Toxicol. 14 (2001) 1128-1137.
- [82] M. Stiborová, E. Frei, M. Hájek, H.H. Schmeiser, Carcinogenic and nephrotoxic aristolochic acids upon activation by NADPH:cytochrome P450 reductase form adducts found in DNA of patients with Chinese herbs nephropathy, Gen. Physiol. Biophys. 20 (2001) 375-392.
- [83] M. Stiborová, E. Frei, B. Sopko, M. Wiessler, H.H. Schmeiser, Carcinogenic aristolochic acids upon activation by DT-diaphorase form adducts found in DNA of patients with Chinese herbs nephropathy, Carcinogenesis 23 (2002) 617-625.
- [84] M. Stiborová, E. Frei, B. Sopko, K. Sopková, V. Marková, M. Laňková, T. Kumstýřová, M. Wiessler, H.H. Schmeiser, Human cytosolic enzymes involved in the metabolic activation of carcinogenic aristolochic acid: evidence for reductive activation by human NAD(P)H:quinone oxidoreductase, Carcinogenesis 24 (2003) 1695-1703.
- [85] M. Stiborová, B. Sopko, P. Hodek, E. Frei, H.H. Schmeiser, J. Hudecek, The binding of aristolochic acid I to the active site of human cytochromes P450 1A1 and 1A2 explains their potential to reductively activate this human carcinogen, Cancer Lett. 229 (2005) 193-204.
- [86] M. Stiborová, E. Frei, P. Hodek, M. Wiessler, H.H. Schmeiser, Human hepatic and renal

microsomes, cytochromes P450 1A1/2, NADPH:cytochrome P450 reductase and prostaglandin H synthase mediate the formation of aristolochic acid-DNA adducts found in patients with urothelial cancer, Int. J. Cancer 113 (2005) 189-197.

- [87] M. Stiborová, V. Martínek, H. Rýdlová, P. Hodek, E. Frei, Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes, Cancer Res. 62 (2002) 5678-5684.
- [88] S. Rendic, F.J. DiCarlo, Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors, Drug Metab. Rev. 29 (1997) 413-480.
- [89] M. Stiborová, E. Frei, H.H. Schmeiser, Metabolic activation of aristolochic acid, Coll. Antropol. 30 Suppl 1 (2006) 25.
- [90] D.W. Hein, M.A. Doll, A.J. Fretland, M.A. Leff, S.J. Webb, G.H. Xiao, U.S. Devanoboyina, C.A. Nangiu, Y. Feng, Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms, Cancer Epidemiol. Biomarkers Prev. 9 (2000) 29-42
- [91] H. Glatt, H. Boeing, C.E.H. Engelke, L. Ma, A. Kuhlow, U. Pabel, D. Pomplun, W. Teubner, W. Meinl, Human cytosolic sulphotransferases: genetics, characteristics, toxicological aspects, Mutat. Res. 482 (2001) 27-40.
- [92] W. Meinl, U. Pabel, H. Osterloh-Quitroz, J.G. Hengstler, H. Glatt, Human sulphotransferases are involved in the activation of aristolochic acids and are expressed in renal target tissue, Int. J. Cancer 118 (2006) 1090-1097
- [93] C.J. Henderson, D.M.E. Otto, D. Carrie, M.A. Magnuson, A.W. McLaren, I. Rosewell, C.R. Wolf, Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase, J. Biol. Chem. 278 (2003) 13480-13486
- [94] C.J. Henderson, D.M. Otto, A.W. McLaren, D. Carrie, C.R. Wolf, Knockout mice in xenobiotic metabolism, Drug Metab. Rev. 35 (2003) 385-392.
- [95] F.P. Perera, Environment and cancer: who are susceptible? Science 278 (1997) 1068-73.

- [96] M.T. Landi, R. Sinha, N.P. Lang, F.F. Kadlubar, Human cytochrome P4501A2. in: P. Vineis, N. Malats, M. Lang, A. d'Errico, N. Caporaso, J. Cuzick, and P. Boffetta, (Eds.), Metabolic Polymorphisms and Susceptibility to Cancer, International Agency for Research on Cancer, IARC Sci. Publ. Lyon, 1999, No. 148, pp. 173-195.
- [97] F.P. Guengerich, A. Parikh, R.I. Turesky, P.D. Josephy, Inter-individual differences in the metabolism of environmental toxicants: cytochrome P450 1A2 as a prototype, Mutat. Res. 428 (1999) 115-124.
- [98] E.G. Schuetz, W. Schmid, G. Schurt, C. Brimer, K. Yasuda, T. Kamataki, L. Borheim, K. Myles, T.J. Cole, The glucocorticoid receptor is essential for drug or steroid induction of CYP3A or P-450 reductase in mouse liver, Drug Metab. Dispos. 28 (2000) 268-278.
- [99] D. Ross, J.K. Kepa, S.L. Winski, H.D. Beall, A. Anwar, D. Siegel, NAD(P)H:quinone oxidoreductase (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms, Chem.-Biol. Interact. 129 (2000) 77-97.
- [100] L. Ernster, DT-diaphorase: A historical review, Chem.. Scr. 27A (1987) 1-13.
- [101] R.J. Riley, P. Workman, DT-diaphorase and cancer chemotherapy, Biochem. Pharmacol. 43 (1992) 1657-1669.
- [102] D.J. Long II, A.K. Jaiswal, NRH:Quinone oxidoreductase2 (NQO2), Chem.-Biol. Interact. 129 (2000) 99-112.
- [103] W.A. Schulz, A. Krummeck, I. Rosinger, P. Eickelman, C. Neuhaus, T. Ebert, B.J. Schmitz-Drager, H. Sies, Increased frequency of a null-allele for NAD(P)H - quinone oxidoreductase in patients with urological malignancies, Pharmacogenetics 7 (1997) 235-239.
- [104] H.W. Chen, A. Luni, A. Seifried, L.R. Wilkens, L. Le Marchand, Association of the NAD(P)H-quinone oxidoreductase C-609-T polymorphism with a decreased lung-cancer risk, Cancer Res. 59 (1999) 3045-3048.
- [105] S. Dhakshinamoorthy, D.J. Long II, A.K. Jaiswal, Antioxidant regulation of genes enconding

enzymes that detoxify xenobiotics and carcinogens, Curr. Top. Cellul. Regul. 36 (2000) 201-216.

- [106] P. Joseph, A.K. Jaiswal, C.C. Stobbe, J.D. Chapman, The role of specific reductases in the intracellular activation and binding of 2-nitroimidazoles, Int. J. Radiat. Oncol. Biol. Phys. 29 (1994) 351-355.
- [107] P. Talalay, H.J. Prochaska, Mechanism of induction of NAD(P)H:quinone reductase, Chem. Scr. 27A (1987) 61-66.
- [108] M.M. Montano, B.S. Katzenellenbogen, The quinone reductase gene a unique receptorregulated gene that is activated by antiestrogens, Proc. Natl. Acad. Sci. USA 94 (1997) 2581-2586.
- [109] M.M. Montano, A.K. Jaiswal, B.S. Katzenellenbogen, Transcriptional regulation of the human quinone reductase gene by antiestrogen-liganded estrogen receptor-alpha and estrogen receptor-beta, J. Biol. Chem. 273 (1998) 25443-25449.
- [110] D.W. Nebert, J.E. Jones, Regulation of the mammalian cytochrome P1-450 (CYP1A1) gene, Int. J. Biochem. 3 (1989) 243-252.
- [111] M. Stiborová, M. Hájek, H. Vošmiková, E. Frei, H.H. Schmeiser, Isolation of DT-diaphorase [NAD(P)H dehydrogenase (quinone)] from rat liver cytosol: identification of new enzyme substrates, carcinogenic aristolochic acids, Collect. Czech. Chem. Commun. 66 (2001) 959-972.
- [112] R.A. Larson, Y. Wang, M. Banerjee, J. Wiemels, C. Hartford, M.M. Beau, M.T. Smith, Prevalence of the inactivating 609C→T polymorphism in the NAD(P)H:quinone oxidoreductase (NQO1) gene in patients with primary and therapy-related myeloid leukaemia, Blood 94 (1999) 803-807.
- [113] A. Clairmont, H. Sies, S. Ramachandran, J.T. Lear, A.G. Smith, B. Bowers, P.W. Jones, A.A. Fryer, R.C. Strange, Association of NAD(P)H:quinone oxidoreductase (NQO1) null with numbers of basal cell carcinomas use of a multivariate model to rank the relative importance of this polymophism and those at other relevant loci, Carcinogenesis 20 (1999) 1235-1240.

[114] J. Wiemels, A. Pagnamenta, G.M. Taylor, O.B. Eden, F.E. Alexander, M.F. Greaves, A lack of functional NAD(P)H:quinone oxidoreductase allele is selectively associated with pediatric leukemias that have MLL fusions. United Kingdom Childhood Cancer Study Investigators, Cancer Res. 59 (1999) 4095-4099.

Figure Legends

Figure 1. Metabolic activation and DNA adduct formation of AAI and 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).

Figure 2. Autoradiographic profiles of DNA adducts obtained from renal DNA of a patient with aristolochic acid nephropathy (AAN) (**A**) and from a patient living in an area endemic for BEN area (**B**) using the nuclease P1 enrichment version of the ³²P-postlabelling assay (adapted from reference [38]). **Insert:** Separation of the ³²P-labeled nucleoside 3',5'-bisphosphate dA-AAI adduct from Figure **2B** (top) and a dA-AAI standard (bottom). The dA-AAI standard was obtained from *in-vitro* incubations as described [79]. For clarity, HPLC profiles are shown in arbitrary units.

Figure 3. Proposed pathway for biotransformation of AAI and AAII in rodents and humans

Figure 4. DNA binding of AAI after activation with Supersomes containing different human recombinant CYPs (50 pmol) and NADPH:P450 reductase (light columns) or NADPH:CYP reductase alone (control, a dark column) (adapted from reference [86]). The nuclease P1-enrichment procedure was used for analysis. Values represent mean \pm S.E.M. (n = 4) of two separate incubations each determined by two post-labeled analyses. RAL, relative adduct labeling.

Figure 5. AAI is shown docked to the active sites of human CYP1A1 (**A**) and CYP1A2 (**B**) indicating the position of the AAI molecule to the heme prosthetic group (adapted from reference [85]).

Figure 6. AAI is shown docked to the active site of human NQO1 where several key amino acid residues position the AAI substrate parallel to a flavin prosthetic group [(adapted from reference [76]).



Figure 1



Figure 2



33



Figure 4



Figure 5A



Figure 5B



Figure 6