Aristolochic acid as a probable human cancer hazard in herbal remedies: a review

Volker M.Arlt1, Marie Stiborova1 and Heinz H.Schmeiser2

Section of Molecular Carcinogenesis, Institute of Cancer Research, Cotswold Road, Sutton, Surrey SM2 5NG, UK. 1Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 12840 Prague 2, The Czech Republic and 2Division of Molecular Toxicology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

The old herbal drug aristolochic acid (AA), derived from Aristolochia spp., has been associated with the development of a novel nephropathy, designated aristolochic acid nephropathy (AAN), and urothelial cancer in AAN patients. There is clear evidence that the major components of the plant extract AA, aristolochic acid I (AAI) and aristolochic acid II (AAII), both nitrophenanthrene carboxylic acids, are genotoxic mutagens forming DNA adducts after metabolic activation through simple reduction of the nitro group. Several mammalian enzymes have been shown to be capable of activating both AAI and AAII in vitro and in cells. The activating metabolism has been elucidated and is consistent with the formation of a cyclic nitrenium ion with delocalized charge leading to the preferential formation of purine adducts bound to the exocyclic amino groups of deoxyadenosine and deoxyguanosine. The predominant DNA adduct in vivo, 7-(deoxyadenosin-β-N6-yl)aristolactam I (dA–AAI), which is the most persistent of the adducts in target tissue, is a mutagenic lesion leading to AT→TA transversions in vitro. This transversion mutation is found at high frequency in codon 61 of the H-ras oncogene in tumours 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 H-ras mouse gene and preferentially to purines in the human p53 gene. In contrast, the molecular mechanism of renal interstitial fibrosis in humans after chronic administration of AA remains to be explored. However, preliminary findings suggest that DNA damage by AA is not only responsible for the tumour development but also for the destructive fibrotic process in the kidney. It is concluded that there is significant evidence that AA is a powerful nephrotoxic and carcinogenic substance with an extremely short latency period, not only in animals but also in humans. In particular, the highly similar metabolic pathway of activation and resultant DNA adducts of AA allows the extrapolation of carcinogenesis data from laboratory animals to the human situation. Therefore, all products containing botanicals known to or suspected of containing AA should be banned from the market world wide.

Introduction

Aristolochic acid (AA), the plant extract of Aristolochia spp. (e.g. Aristolochia clematitis, Aristolochia fangchi and Aristolochia manshuriensis), is a mixture of structurally related nitrophenanthrene carboxylic acids, mainly aristolochic acid I (AAI) and aristolochic acid II (AAII) (Figure 1) (Pailer et al., 1955). AA is found primarily in the genus Aristolochia, but may be present in other botanicals. Herbal drugs derived from Aristolochia spp. have been known since antiquity and were used in obstetrics and in the treatment of snake bites (Rosenmund and Reichstein, 1943). Contemporary medicine has used Aristolochia plant extracts for the therapy of arthritis, gout, rheumatism and festering wounds (Rucker and Chung, 1975; Hahn, 1979; Priestap, 1987). The anti-inflammatory properties of AA encouraged the development of pharmaceutical preparations in Germany (Mose, 1966; Mose and Porta, 1974; Kluete et al., 1982) until Mengs and co-workers observed that AA is a strong carcinogen in rats (Mengs et al., 1982; Mengs, 1983). Subsequently, AA was shown to be a genotoxic mutagen in several short-term tests (Table I). Therefore, all pharmaceutical preparations containing AA have been withdrawn from the market in Germany and in many other countries. However, Aristolochia plants and their extracts have been further used in traditional medicine in some parts of the world (Priestap, 1987; Vishwanath and Gowda, 1987; Houghton and Ogtuveren, 1991). Recently the FDA advised consumers to immediately discontinue use of any botanical products containing AA and has published a list of botanical products that have been shown to contain AA (Schwert, 2001).

So-called Chinese herbs nephropathy (CHN), a unique type of rapidly progressive renal fibrosis associated with the prolonged intake of Chinese herbs during a slimming regimen, was observed for the first time in Belgium in 1991 (Vanherweghem et al., 1993). About 100 CHN cases have been identified so far in Belgium (Table II), half of which needed renal replacement therapy, mostly including renal transplantation (Vanherweghem, 1998). The observed nephrotoxicity has been traced to the ingestion of A.fangchi containing AA inadvertently included in slimming pills (Vanhaelen et al., 2001). So-called CHN has been described in patients in other European and in Asian countries and in the USA (about 170 cases) (Table II), who were exposed to Aristolochia spp. containing AA and had no relationship with the Belgian slimming clinic. Therefore, it has been proposed to designate the interstitial nephropathy in which the unequivocal role of AA has been fully documented as aristolochic acid nephropathy (AAN) (Gillerot et al., 2001; Solez et al., 2001). Recently, a high prevalence of urothelial cancer was found in a large cohort of AAN patients in Belgium (Cosyns et al., 1999; Nortier et al., 2000) and a case with urothelial cancer has also...
The natural mixture AA is a strong carcinogen in rats (Mengs et al., 1982; Mengs, 1983). In Wistar rats treated orally with 0.1, 1.0 or 10 mg AA/kg body wt/day for 3 months multiple tumours were found after a short induction time (3 months). AA showed mainly a high incidence of tumours in the forestomach at the two high doses, but primary tumours were also found in the renal cortex, renal pelvis and urinary bladder. In a few cases formation of metastases was observed in the regional lymph nodes. At the lowest dose tumours in the forestomach occurred only 12 months after treatment and no urogenital tract tumours were found. However, the observed hyperplasia in the renal pelvis suggests that neoplastic growth might have ensued if the period of observation had been prolonged. Although no carcinogenic activity of AA was initially reported in the liver, a single non-necrogenic dose of AA (10 mg/kg body wt, i.p. injection) given 18 h after two-thirds partial hepatectomy initiated liver cell carcinogenesis (formation of hepatic foci and nodules) (Rossiello et al., 1993). AA is also a potent carcinogen in mice (Mengs, 1988). Oral treatment with 5 mg AA/kg body wt/day for 3 weeks resulted in subsequent tumour formation in the forestomach, lungs, uterus and lymphoid organs. Apart from these carcinogenic effects, acute and subchronic studies in rats and mice showed acute tubular necrosis and renal failure after oral administration of AA (Mengs, 1987; Mengs and Stotzem, 1993). Chronic interstitial fibrosis was observed in rats after i.p. injection of AA (Zheng et al., 2001; Debelle et al., 2002).

Metabolism of AA

The metabolism of AA has been studied in different species including man and has shown that the products of nitrreduction, the corresponding aristolactams (Mix et al., 1982), are the major metabolites found in urine and faeces (Figure 2) (Krumbiegel et al., 1987). The principal metabolite of AAI was aristolactam Ia, produced by two metabolic pathways, one via aristolactam I and the other via AAII (Figure 2). This interpretation is supported by the results of Schmeiser et al. (1986), which showed that aristolactam I and aristolactam II are also produced in vitro by anaerobic incubation of AAI and AAII with rat liver S9 mix. Under aerobic incubation conditions

![Fig. 1. Chemical structures of aristolochic acid I (AAI) and II (AAII).](image)
### Table II. Reports of CHN/AAN patients in Belgium and other parts of the world in the literature

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>Country</th>
<th>Published year(s)</th>
<th>Material causing CHN/AAN</th>
<th>Clinical and Histological picture of CHN/AAN</th>
<th>Detection of AA in herbal preparation by DCHPLC</th>
<th>Detection of AA–DNA adducts by 32P-post-labelling</th>
<th>Detection of urothelial tumours</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>18b</td>
<td>Belgium</td>
<td>1994–2001</td>
<td>Chinese herbal remedy containing <em>Aristolochia fangchi</em> prescribed for weight loss</td>
<td>+</td>
<td>+</td>
<td>+b</td>
<td>+b</td>
<td>Cosyns et al. (1994a,b, 1999), Schmeiser et al. (1996), Bieler et al. (1997), Reginster et al. (1997), Arlt et al. (2001a)</td>
</tr>
<tr>
<td>1</td>
<td>Spain</td>
<td>1996</td>
<td>Herbal tea containing <em>Aristolochia pistolochia</em></td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>–</td>
<td>Pena et al. (1996)</td>
</tr>
<tr>
<td>1</td>
<td>Japan</td>
<td>1997</td>
<td>Chinese health food</td>
<td>+</td>
<td>+</td>
<td>+d</td>
<td>–</td>
<td>Tanaka et al. (1997a)</td>
</tr>
<tr>
<td>1</td>
<td>Japan</td>
<td>1997</td>
<td>Chinese herbal remedy</td>
<td>+</td>
<td>+</td>
<td>+d</td>
<td>–</td>
<td>Tanaka et al. (1997b)</td>
</tr>
<tr>
<td>2</td>
<td>UK</td>
<td>1999</td>
<td>Chinese herbal tea containing <em>Aristolochia manshurienesis</em> prescribed against eczema</td>
<td>+</td>
<td>+</td>
<td>+d</td>
<td>–</td>
<td>Lord et al. (1999, 2001)</td>
</tr>
<tr>
<td>1</td>
<td>Japan</td>
<td>1999</td>
<td>Chinese herbal remedy to 'promote health'</td>
<td>+</td>
<td>+</td>
<td>+d</td>
<td>–</td>
<td>Ubara et al. (1999)</td>
</tr>
<tr>
<td>12</td>
<td>Taiwan</td>
<td>2000</td>
<td>Chinese herbal remedies prescribed for weight control or as a nutritional supplement</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>–</td>
<td>Yang et al. (2000)</td>
</tr>
<tr>
<td>1</td>
<td>USA</td>
<td>2000</td>
<td>Chinese herbal remedy prescribed for pain relief</td>
<td>+</td>
<td>+</td>
<td>+d</td>
<td>–</td>
<td>Meyer et al. (2000)</td>
</tr>
<tr>
<td>1</td>
<td>Germany</td>
<td>2001</td>
<td>Chinese herbal remedy distributed as 'herbal food combination'</td>
<td>+</td>
<td>+</td>
<td>+d</td>
<td>–</td>
<td>Krumme et al. (2001)</td>
</tr>
<tr>
<td>13</td>
<td>Japan</td>
<td>2001</td>
<td>Chinese herbal medicine or health food</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>–</td>
<td>Tanaka et al. (2001) and references therein</td>
</tr>
<tr>
<td>1</td>
<td>China</td>
<td>2001</td>
<td>Chinese herbal remedy prescribed for 'waste discharging and youth keeping' purposes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>Gilleret et al. (2001)</td>
</tr>
<tr>
<td>1</td>
<td>Japan</td>
<td>2001</td>
<td>Chinese herbal remedy prescribed against edema</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>–</td>
<td>Nishimagi et al. (2001)</td>
</tr>
<tr>
<td>58</td>
<td>China</td>
<td>2001</td>
<td>Chinese traditional drugs</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>–</td>
<td>Chen et al. (2001)</td>
</tr>
<tr>
<td>51</td>
<td>China</td>
<td>2001</td>
<td>Chinese herbal drugs containing <em>Aristolochia manshurienesis</em></td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>–</td>
<td>Li et al. (2001)</td>
</tr>
<tr>
<td>20</td>
<td>Taiwan</td>
<td>2001</td>
<td>Chinese herbal remedies prescribed for weight reduction or as a nutritional supplement</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+f</td>
<td>Chang et al. (2001)</td>
</tr>
<tr>
<td>1</td>
<td>UK</td>
<td>2002</td>
<td>Chinese herbal remedy to treat hepatitis B</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>–</td>
<td>Cronin et al. (2002)</td>
</tr>
<tr>
<td>1</td>
<td>Taiwan</td>
<td>2002</td>
<td>Chinese herbal remedy for leg edema</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>–</td>
<td>Yang et al. (2002)</td>
</tr>
</tbody>
</table>

aAAN patients treated in the Hopital Erasme, Brussels, until January 2002. Fifty patients in end-stage renal failure (transplanted or dialysed), 28 patients in chronic renal failure (moderate to severe), six deceased patients (three from invasive urothelial carcinoma); 39 patients running the risk of developing renal failure (J.L. Nortier and J.-L. Vanherweghem, personal communication); AA–DNA adducts in urothelial tissue were detected in all 38 patients analysed; urothelial carcinoma were found in 18 patients, one patient had a papillary bladder tumour.

bAAN patients treated in the Cliniques St Luc, Brussels, until January 2002. Sixteen patients in end-stage renal failure (transplanted or dialysed), two patients in chronic renal failure; five patients running the risk of developing renal failure; urothelial carcinoma were found in seven patients (M. Jadoul and J.-P. Cosyns, personal communication); AA–DNA adducts in urothelial tissue were detected in all eight patients analysed.

cn.d., not determined.

dAA–DNA adducts were detected in urothelial tissue of one patient analysed and were associated with urothelial carcinoma.

fOne bladder carcinoma and one bladder transitional cell carcinoma were found.

One bladder transitional cell carcinoma was found.
Fig. 2. Metabolism of aristolochic acid I and II (from Krumbiegel et al., 1987).

The major metabolite formed by AAI is AAIa, while AAII remains unaltered. Thus, aristolactam Ia, the major metabolite found in vivo, has not been detected in vitro. The oxygen concentration of tissues in vivo may affect the relative extents of nitroreduction and O-dealkylation for AAI, whereas for AAII only nitroreduction might be influenced by oxygen concentration (Maier et al., 1987). The phase II metabolism of both AAs has not been extensively studied so far, however, large amounts of AA metabolites in the urine and faeces of rodents were present in conjugated form and suggested to be either glucuronides or sulfate esters (Krumbiegel et al., 1987).

Enzymatic activation of AA and DNA adduct formation
Aristolactams represent the final state of reduction of the nitro group of both AAs, but not the DNA-binding species. Aristolactams are not mutagenic themselves and require metabolic activation by an exogenous metabolic system (Table I). Whereas AAI and AAII are direct mutagens in Salmonella strains TA100 and TA1537 (Table I), the mutagenic potency of the corresponding aristolactams in TA100 activated by rat liver S9 mix is about half of that of the parent compounds (Schmeiser et al., 1986). In contrast, both AAs were only weakly mutagenic in strain TA100NR lacking the classical bacterial nitroreductase, indicating that nitroreduction is a crucial step in the pathway of metabolic activation of AA to their ultimate mutagenic species (Schmeiser et al., 1984). Using genetically engineered YG strains, Götzl and Schimmer (1993) confirmed that only the nitro group is important for the mutagenic activity of AA in Salmonella. Nevertheless, both AAs are only weak mutagens in the Ames assay (<1 revertant/nmol) when compared with other nitroaromatic compounds (Purohit and Basu, 2000).

A powerful tool for elucidating the pathway of activation of carcinogens is to characterize and quantify the DNA adducts it forms and to determine what factors either enhance or inhibit adduct formation. The most commonly used method to detect DNA adducts is the highly sensitive $^{32}$P-post-labelling assay.
and detection of DNA adduct formation by AA in vitro and in vivo has been by this assay almost exclusively (Stiborova et al., 1998). Both AAI and AAII form DNA adducts in vitro using rat liver S9 mix, resulting in two major adduct spots for AAI and AAII (Schmeiser et al., 1988). In addition, a minor adduct was formed in incubations with AAI, which is one of the major adducts formed in incubations with AAII. Whereas for AAI the same DNA adducts were observed under aerobic and anaerobic conditions, AAII gave rise to adduct formation only anaerobically. In contrast, no DNA adducts were found for aristolactam I and aristolactam II in the presence of rat liver S9 mix (Schmeiser et al., 1988). The structures of the major AA–DNA adducts were elucidated spectroscopically as 7-(deoxyadenosin-N6-yl)aristolactam I (dA–AAI), 7-(deoxyguanosin-N7-yl)aristolactam I (dG–AAI) and 7-(deoxyadenosin-N6-yl)aristolactam II (dA–AAII) (Pfau et al., 1990b, 1991). It was also shown that the dA–AAII adduct is formed from AAI through a demethoxyl-ation reaction of AAI (Stiborova et al., 1994). A second major guanosine adduct formed by reaction of AAII with deoxyguanosine 3′-monophosphate and DNA was tentatively determined as 7-(deoxyguanosin-N7-yl)aristolactam II (dG–AAII) (Stiborova et al., 1994). These chemical structures indicate 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 hydrolysed to the corresponding 7-hydroxyaristolactam (Figure 3). This preference for reaction with the exocyclic amino group is unusual for nitroaromatic compounds since their major target site in DNA is the C-8 atom of deoxyguanosine. However, this fits in with the concept introduced by Dipple (1995) that polycyclic arylaminating and polycyclic aralkylating agents that delocalize charge and are substantially distorted from planarity react extensively at the amino groups of both deoxyguanosine and deoxyadenosine. It is known that in the activation of carcinogenic nitroaromatics and aromatic amines acetylation of the amino or hydroxymino group plays a key role. Therefore, the activation of AA is a
unique example of intramolecular acylation, which leads to the ultimate carcinogen.

Enzymatic activation of both AAs by buttermilk xanthine oxidase and rat DT-diaphorase, cytosolic nitroreductases, produced a similar adduct pattern to that obtained by rat liver S9 mix-mediated metabolism (Schmeiser et al., 1988; Stiborova et al., 2001a, 2002), confirming that nitroreduction is the crucial step in the pathway of metabolic activation of AAs to their ultimate DNA binding species. It was also demonstrated that both AAs could be activated by rat liver microsomes via simple nitroreduction (Schmeiser et al., 1997). This hepatic microsomal activation of AA was attributed to cytochrome P450 (CYP) 1A1 and CYP1A2 and, although to a minor extent, to NADPH:CYP reductase using specific CYP/NADPH:CYP reductase inhibitors and purified enzymes (Stiborova et al., 2001b,c).

All four purine AA–DNA adducts were identified by 32P-post-labelling in vivo in different organs of rats treated orally with five daily doses (10 mg/kg body wt) of AAI and AAII (Pfau et al., 1990a). The adduct patterns in DNA from forestomach and kidney, target tissues of AA-mediated carcinogenesis, and from non-target tissues such as stomach, liver and lung were similar, indicating that adduct formation is not directly correlated with initiation of the carcinogenic process and subsequent tumour formation in target tissues in rats. In this in vivo study DNA binding by AAII was in general 10 times higher compared with AAI. For AAI total relative adduct labelling was highest in forestomach DNA, with ~3 adducts/10^6 nt. In the bladder (also a target tissue) DNA binding by AAII was much greater than for AAI (relative adduct labelling was ~0.4 adducts/10^6 nt for AAI, compared with 0.8 adducts/10^6 nt for AAII) (Pfau et al., 1990a). This difference in organotropism activity could be related to different phase II metabolic pathways for AAII and AAI. Whereas AAIa may be excreted as an O-glucuronide, AAII, unlike AAI, is metabolized to the corresponding lactam, which can only form an N-glucuronide, which could be hydrolysed in the bladder due to the acidic nature of urine and form DNA-reactive species. These combined data indicate that AAI may be responsible for the induction of carcinoma in the gastrointestinal tract while AAII could give rise to neoplastic changes and to toxic effects in the urinary tract. This suggestion is further supported by the fact that in rats treated with pure AAI a high incidence of tumours of the forestomach was observed but no neoplastic changes were found in the urinary tract (Schmeiser et al., 1990).

Oncogene activation by AA

Protooncogenes have been identified as genetic targets that are involved in chemical carcinogenesis (Balmain and Brown, 1988). In rodents many chemical carcinogens activate the ras protooncogene by a single point mutation, resulting in the alteration of amino acid residue 12, 13 or 61. Likewise, AA-initiated carcinogenesis in rodents is associated with a distinct molecular characteristic, activation of H-ras by a specific AT→TA transversion mutation in codon 61 (CAA). This mutation occurs exclusively at the first adenine of codon 61 in all forestomach and ear duct tumours of rats treated with AAI (Schmeiser et al., 1990) and was confirmed in tumours of the forestomach and lung of mice treated with the plant extract AA (Schmeiser et al., 1991). The mutagenic activity of AA was also investigated in different organs of the λlacZ transgenic mouse (Muta™Mouse) after intragastric treatment with 15 mg AA/kg body wt once a week for 4 weeks (Kohara et al., 2002). Increased mutation frequencies in the lacZ and cII genes were observed in the target organs (forestomach, kidney and bladder) compared with non-target organs (e.g. glandular stomach and liver). Moreover, mainly AT→TA transversion mutations were found by sequence analysis of cII mutants in the target organs. This selectivity of AAI for mutations at adenine residues is consistent with the extensive formation of dA→AAI adducts in the target organs in rats (Pfau et al., 1990a; Stiborova et al., 1994). Moreover, an apparently life-long persistence of dA→AAI adducts in forestomach DNA was found, whereas dG→AAI adducts were continuously removed from the same DNA over a 36 week period in rats treated with a single dose of AAI (Fernando et al., 1993). As suggested by others, it is possible that persistent DNA adducts may occupy specific genomic sites that are not amenable to repair and that these DNA adducts may be converted into the mutations found in target genes of carcinogenesis, e.g. cellular oncogenes (Randerath et al., 1985).

Mutagenic activity of AA–DNA adducts and DNA binding specificity of AA

Oligonucleotides containing defined DNA adducts placed at specific sites are useful tools for investigating how individual chemical lesions formed in DNA by carcinogens are converted into mutations (Singer and Essigmann, 1991). To examine the mutagenic activity of AA–DNA adducts, mono-adducted oligonucleotides containing the major AA–DNA adducts located at a defined site have been used in primed DNA replication reactions with phage T7 DNA polymerase (Broschard et al., 1994) and human DNA polymerase α (Broschard et al., 1995). It was found that dAMP and dTMP were incorporated equally well opposite the adenine adducts (dA→AAI and dA→AAII), whereas the guanine adducts (dG→AAI and dG→AAII) led to preferential incorporation of dCMP. The translesional bypass past adenine adducts of AA indicates a mutagenic potential resulting from dAMP incorporation by DNA polymerase, suggesting that an AT→TA transversion mutation would be the mutagenic consequence. Incorporation of dTMP opposite the adenine adducts or dCMP opposite the guanine adducts results in a non-mutagenic event. Therefore, the adenine adducts have a higher mutagenic potential compared with the guanine adducts, which may explain the apparent selectivity for mutations found at adenine residues in codon 61 of the H-ras gene in AA-induced rodent tumours (Schmeiser et al., 1990, 1991) and the preferential induction of AT→TA transversion mutations in the cII gene in target organs of the AA-treated Muta™Mouse (Kohara et al., 2002).

Moreover, this assay showed that, regardless of the type of AA–DNA adduct examined, DNA synthesis was blocked predominantly (80–90%) at the nucleotide 3′ of each adduct (Broschard et al., 1994). Thus, DNA polymerase arrest due to the presence of bulky AA–DNA adducts can be used to examine sequence-specific DNA binding by AA in genes involved in the carcinogenic process. To a certain degree, it is possible to relate the DNA binding specificity of a carcinogen to specific mutations found in a target gene for tumour formation (Denissenko et al., 1996). Using an adduct-specific polymerase arrest assay it was demonstrated that both adenines in codon 61 of the H-ras gene in a plasmid are AA–DNA binding sites (Arlt et al., 2000), indicating that the mutations observed in AA-treated rodents may originate from adduct formation in this codon, thereby triggering tumorigenesis.
Aristolochic acid nephropathy and urothelial cancer

**Fig. 4.** Postulated mechanism for the carcinogenicity of aristolochic acid in rodents and humans. AAI, aristolochic acid I; dA–AAI, 7-(deoxyadenosin-N6-yl)aristolactam I; dA, deoxyadenosine.

Our postulated mechanism for the carcinogenicity of AA in rodents is summarized in Figure 4.

**Nephrotoxic and carcinogenic mechanism of AA in humans**

*Chinese herbs nephropathy and AA nephropathy*

So-called CHN is a unique type of rapidly progressive interstitial nephritis, which was associated with the intake of Chinese herbs during a slimming regimen in Belgium (Vanherweghem *et al.*, 1993). CHN is characterized by normal blood pressure, aseptic leukocyturia and early and severe anemia on clinical grounds and morphologically by extensive hypocellular interstitial sclerosis, tubular atrophy, global sclerosis of glomeruli, cellular atypia and malignant transformation of the urothelium (Cosyns *et al.*, 1994b; Deperrieux *et al.*, 1994; Reginster *et al.*, 1997; Nortier *et al.*, 2000). All patients had been treated, in the same private clinic in Brussels, with slimming pills consisting mainly of a preparation containing acetazolamide, diethylpropion, fenfluramine and powdered extracts of Chinese herbs, nominally *Stephania tetrandra* and *Magnolia officinalis*. However, it was suspected that one prescribed Chinese herb, *S.tetrandra*, was inadvertently replaced by *A.fangchi* because both plants are used in Chinese folk medicine under the same name, *Fangji* (Vanhaelen *et al.*, 1994). Indeed, the major alkaloid of *A.fangchi* (AA) was found in the herbal batches delivered to Belgium instead of tetrandrine, the major alkaloid of *S.tetrandra* (Vanhaelen *et al.*, 1994). AA had already been shown to be nephrotoxic in humans given at high doses (Jackson *et al.*, 1964). The rationale for this study in the 1960s was that AA exhibited antineoplastic properties in certain animal models (Kupchan and Doskotch, 1962) and had been tested in cancer therapy.
Nevertheless, the role of the Chinese herbs (specifically *A. fangchi*) as the cause of the renal failure was still a matter of debate, since promoters of Chinese herbs have claimed that the renal disease originated from a ‘hidden’ serotonin-like substance at the time of concomitant mesotherapy [s.c. injections of artichoke extracts, theophylline or both, given to the patients every 2 weeks (Violon, 1997)] (Malak, 1998; McIntyre, 1998; Shum, 2000). Indeed, Colson et al. (1999) found ischaemic renal lesions in serotonin-treated rats that could progress to renal fibrosis. Others argued that analgesic nephropathy is a frequent type of renal disease in Belgium and could thus be misdiagnosed as CHN (De Broe and Elseviers, 1998). Nevertheless renal failure was observed in CHN patients who had not received mesotherapy and who were not regular users of analgesics (Nortier et al., 2000). Moreover, statistical analysis showed a relationship between the cumulative dose of *A. fangchi* ingested by the CHN patients and the renal failure progression rate, confirming that regular ingestion of *Aristolochia* spp. remedies is causally involved in the onset of interstitial nephropathy leading to end-stage renal failure (Martinez et al., 2002). Using the 32P-post-labelling method specific AA–DNA adducts were detected in all urothelial tissues of CHN patients, which showed unambiguously that all CHN patients analysed so far had indeed ingested AA (Schmeiser et al., 1996; Bieler et al., 1997; Nortier et al., 2000; Arlt et al., 2001a).

More and more cases with similar clinical presentations have now been described in other parts of the world and related to exposure to *Aristolochia* spp. containing AA (Table II). Therefore, it has been proposed to designate so-called CHN aristolochic acid nephropathy (AAN) when the unequivocal role of AA has been fully documented (Gillerot et al., 2001; Solez et al., 2001).

**AA nephropathy and urothelial cancer**

As early as 1994 two cases of urothelial cancer had been reported in Belgian AAN patients (Cosyns et al., 1994a; Vanherweghem et al., 1995). Recently an increasing number of urothelial carcinomas was reported in this cohort, suggesting that AA also plays a role in formation of these tumours (Cosyns et al., 1999; Nortier et al., 2000). Statistical analysis predicted that the cumulative dose of *A. fangchi* and therefore the dose of AA was associated with a significantly higher risk of developing urothelial cancer (Nortier et al., 2000). Patients with a mean intake of 200 g Chinese herbs had a 50% higher risk of developing urothelial cancer. This clearly indicates that AA is not only a strong rodent carcinogen but also a potent human carcinogen. On the other hand no difference was found between the levels of AA–DNA adducts in AAN patients with urothelial cancer and tumour-free AAN patients (Nortier et al., 2000). This might be due to the fact that adduct formation is not linear with dose at the high amounts of AA that AAN patients had ingested. Moreover, it has to be considered that many tumour-free patients in this study already had urothelial atypia or preneoplastic lesions.

The possible influence of the concomitantly administered medication on the development of urothelial carcinoma in Belgian AAN patients has also been investigated (specifically acetazolamide, fenfluramine and dexfenfluramine ingestion, use of analgesics and cigarette smoking) and compared for AAN patients with urothelial cancer and tumour-free AAN patients (Nortier et al., 2000). Although no other significant risk factor was identified, since most of the patients were treated with appetite suppressants as well as acetazolamide, it cannot be excluded that the former drugs, with vasoconstrictive properties (De Broe, 1999), or the latter agents, which alkalize the urine (Violon, 1997), enhance the toxicity of AA. Indeed, aortic insufficiency was reported in a few Belgian AAN patients (Reginster et al., 1997) and it was suggested that this valvular heart disease was more likely caused by the concomitant intake of appetite suppressants such as fenfluramine (Vanherweghem, 1997; van Ypersele de Strihou, 1998).

In the meantime, urothelial carcinoma associated with high levels of AA–DNA adducts in the urothelial tissue have been reported outside the Belgian cohort, pointing to the direct carcinogenic potential of AA in AAN patients (Lord et al., 2001). Moreover, the recent demonstration that in rabbits and in rats AA given as a single drug causes similar renal interstitial fibrosis as well as urothelial tumours as observed in AAN patients removed any doubt as to the causal role of AA in AAN and AAN-associated urothelial malignancy (Cosyns et al., 2001; Debelle et al., 2002).

The potential role of AA–DNA adducts in AAN-associated urothelial cancer

Many studies on the mutagenic and carcinogenic properties of AA in rodents have been done that can now be used as a model for the human situation. Not only are AA–DNA adducts a suitable biomarker for exposure to AA, but they also seem to play a critical role in the carcinogenic process of AA. In renal and ureteral tissue of AAN patients three AA-specific DNA adducts, one major (dA–AAI) and two minor (dG–AAI and dA–AAII), were identified (Schmeiser et al., 1996; Bieler et al., 1997; Nortier et al., 2000; Arlt et al., 2001a). These are the same AA–DNA adducts detected in rats exposed to AA (Pfau et al., 1990a; Stiborova et al., 1994).

The highest levels of AA–DNA adducts were found in urothelial tissue of AAN patients, ranging from ~0.1 to 50 adducts/10^8 nt. The persistence of AA–DNA adducts in human tissue even many years after cessation of the slimming regimen is noteworthy (Nortier et al., 2000). The most prominent adduct found in all AAN patients analysed so far is the dA–AAI adduct. Irrespective of the tissue analysed in rats the dA–AAI adduct is also always the predominant one (Pfau et al., 1990a; Stiborova et al., 1994; Arlt et al., 2001a). This suggests that each AA–DNA adduct has its own kinetic characteristics in AAN patients accounting for the fact that only the dA–AAI adduct remains in urothelial tissues for an extensive period of time. This is consistent with high levels of the dA–AAI adduct in the target tissue, forestomach, in rats (Stiborova et al., 1994) and its life-long persistence in forestomach DNA (Fernando et al., 1993). Furthermore, the dA–AAI adduct was also highly persistent in rat kidney (Bieler et al., 1997). Both the longer persistence and higher initial levels of the dA–AAI adduct in urothelial tissue of AAN patients probably contributed to the relative abundance of this adduct. Since the H-ras gene is activated at high frequency by an AT→TA transversion mutation in codon 61 of DNA from AAI-induced tumours in rats (Schmeiser et al., 1990) and since both adenines in codon 61 (CAA) were shown to be AA–DNA binding sites (Arlt et al., 2000), this also suggests a relevant role of dA–AAAI adducts in AAN-related urothelial cancer.

As the presumed guardian of the genome, p53 is one of the most commonly mutated genes observed in human tumours and is mutated in over 50% of all human cancers (Greenblatt...
et al., 1994). In AAN patients, urothelial carcinomas as well as urothelial atypia were associated with overexpression of P53 protein (Cosyns et al., 1999), suggesting that the p53 gene is also mutated in AAN-associated urothelial cancer. In many cancers the distribution of mutations along p53 is tumour specific and characterized by several mutational hot-spots (Hussain and Harris, 1998). Using an adduct-specific polymerase arrest assay combined with terminal transferase-dependent PCR the distribution of AA–DNA adducts along exons 5–8 on the non-transcribed strand of p53 was examined in human DNA modified in vitro by AA1 and AAII (Arlt et al., 2001b). Polymerase arrest spectra thus obtained showed a preference for reaction with purine bases in human p53 for both AAs. Moreover, adduct distribution was not random, indicating that adduct formation by AA is sequence specific. No pattern of polymerase arrest was found that predicts AA-specific mutational hot-spots in urothelial tumours of the p53 database (Arlt et al., 2001b). Thus, AA is not a likely cause of non-AAN-related urothelial tumours. However, all AAN patients have been exposed to high amounts of AA very specifically, so a comparison of the AA–DNA binding spectrum in the p53 gene with the p53 mutational spectrum of tumours from AAN patients may provide a link between specific adduct formation and possible mutations induced by AA in p53. These mutations could trigger tumorigenesis in humans in the same way as mutations in codon 61 of H-ras trigger tumorigenesis by AA in rodents (Figure 4). Therefore, urothelial tumours of AAN patients should be screened for p53 gene mutations.

Potential role of AA and AA–DNA adducts in the renal fibrotic process

One of the earliest signs of AAN is urinary excretion of low molecular weight proteins, consisting chiefly of β2-microglobulin and albumin, increasing with the degree of renal failure (Kabanda et al., 1995), occasionally associated with glycosuria (Reginster et al., 1997) and neutral endopeptidase enzymuria (Nortier et al., 1997). This indicates that proximal tubular cells are the primary target in AAN. Moreover, this tubular proteinuria suggests that impairment of proximal tubule functions might be an early manifestation of AA toxicity in the kidney. The induction of acute and selective proximal tubule lesions after administration of high doses of AA to rats as well as renal biopsies from AAN patients, which showed regenerative tubular epithelia mainly along the proximal tubulus, support this hypothesis (Mengs, 1987; Cosyns et al., 1994b; Deperrieux et al., 1994). It was suggested that AA–DNA adducts somehow trigger a fibrotic process that progressively destroys the kidney (van Ypersele de Strihou and Vanherweghem, 1998). Using opossum kidney cells, a classical model for the study of protein reabsorption occurring in the proximal tubule, Lebeau and co-workers investigated the effects of AA on proximal tubule functions, including the reabsorption of low molecular weight proteins such as β2-microglobulin and albumin (Lebeau et al., 2001). Receptor-mediated endocytosis of both proteins was significantly impaired by AA and led to a persistent inhibition of receptor-mediated endocytosis even after its removal. The persistence of the toxicity by AA in opossum kidney cells was associated with a time- and dose-dependent formation of AA–DNA adducts, suggesting a causal relationship between specific DNA damage due to AA and cell-specific alterations at the transcription level of proteins involved in receptor-mediated endocytosis. It was shown that megalin expression was decreased, which accounts, at least in part, for the inhibition of luminal protein reabsorption by proximal tubulus cells (Lebeau et al., 2001). The inhibition of protein reabsorption observed on opossum kidney cells leads to a phenotype that is quite similar to one of the earliest and most consistent manifestations encountered in patients suffering from AAN. It is therefore tempting to speculate that the impaired proximal tubule protein reabsorption induced by AA might be of primary importance in explaining the rapidly progressive nature of AAN.

Metabolic activation of AA in humans

The exact number of patients exposed to the herbal slimming regimen containing AA in Belgium is not known, but it was calculated that around 1500–2000 patients may have been treated in the slimming clinic (Vanherweghem, 1998). Therefore, the identified AAN cases in Belgium (~100) thus represent ~5% of the exposed population. Besides differences in the cumulative dose of AA and the duration of AA intake, differences in carcinogen activation could be the reason for this individual susceptibility. Many genes of enzymes metabolizing carcinogens are known to exist in variant forms or polymorphisms, which appear to be important determinants of cancer risk (Perera, 1997). Thus, the identification of enzymes principally involved in the activation of AA in humans and a detailed knowledge of their catalytic specificities is of major importance. As for other nitroaromatics, cytotoxic nitroreductases (xanthine oxidase and DT-diaphorase) seem to play the major role in the metabolic activation of AA (Schmeiser et al., 1988; Stiborova et al., 2001a, 2002). Besides this, AA is activated by a variety of other enzymes, different peroxidases (Schmeiser et al., 1997; Stiborova et al., 2001d) and human liver microsomes (Stiborova et al., 2001b), leading to the same DNA adducts as found in AAN patients.

Using human recombinant enzymes (Supersomes™) and/or specific CYP/NADPH: CYP reductase inhibitors in vitro, most of the hepatic microsomal activation of AA was attributed to human CYP1A1 and CYP1A2 and a minor, but measurable, activating capacity to human NADPH:CYP reductase (Stiborova et al., 2001b,c). Levels of expression and activities of both these CYPs and DT-diaphorase in humans are influenced by several factors (nutrition, smoking, drugs, environmental chemicals and genetic polymorphisms) and differ considerably among individuals (Joseph et al., 1994; Puga et al., 1997). Because in rats DT-diaphorase activity is increased by AA treatment (Stiborova et al., 2001a, 2002), the activity of this enzyme might also be induced in AAN patients. The activities of both CYPs, xanthine oxidase and DT-diaphorase might also be affected by some components included in the slimming regimen, other than AA, which were ingested by AAN patients, e.g. meprobamate, a CYP inducer, or other herbal drugs, with as yet unknown effects (Violon, 1997). Therefore, AAN patients and other participants in the slimming regimen should be screened for genetic polymorphisms of genes involved in xenobiotic metabolism.

Because human urothelial tissue is rich in peroxidases, whether AAs are substrates for peroxidases was also investigated. Schmeiser et al. (1997) found that lactoperoxidase and horseradish peroxidase catalysed the activation of both AAs, leading to the formation of some of the AA–DNA adducts observed in vivo. The corresponding aristolactams were also activated by these peroxidases, forming the AA–DNA adducts that are observed in AAN patients (Stiborova et al., 1995,
1999). Moreover, both AAs were activated by prostaglandin
H synthase, the most abundant peroxidase present in the kidney
and ureter, using rat seminal vesical microsomes (Stiborova et al., 2001d). Therefore, prostaglandin H synthase could be
one of the most important activators of AA to toxic and
carcinogenic metabolites in the target tissues of AAN patients.
In all activation systems mediated by peroxidases the da–AAI
duct was one of the major adducts formed.

AA nephropathy and its relationship to Balkan endemic
nephropathy
On both clinical and morphological grounds, AAN is very
similar to another fibrosing nephropathy, Balkan endemic
nephropathy, including the association of both with urothelial
tumours (Cosyns et al., 1994b; Tatu et al., 1998). Evidence
has accumulated that Balkan endemic nephropathy is an
environmentally induced disease strongly associated with the
oral intake of food of plant origin contaminated with the fungal
mycotoxin ochratoxin A (Tatu et al., 1998). The hypothesis
that ochratoxin A is implicated in Balkan endemic nephropathy
has been further supported by the detection of ochratoxin
A-related DNA adducts in urinary tract tumours of Bulgarian
patients suffering from Balkan endemic nephropathy (Pföhl-
Leszkowicz et al., 1993). As a consequence, ochratoxin A has
also been under suspicion as a fungal contaminant in the
batches of Chinese herbs prescribed to the AAN patients in
Belgium (Vanherweghem et al., 1993). However, no trace of
ochratoxin A was detected in several batches of Chinese herbs
imported into Belgium (Vanhaelen et al., 1994; Vanherweghem
et al., 1998). Further, only a small number of renal tissue
samples of Belgian AAN patients exhibited ochratoxin
A-related DNA adducts and adduct levels were close to the
detection limit of the 32P-post-labelling assay (Nortier et al.,
2000; Arlt et al., 2001a). Furthermore, in rats that were treated
with the slimming regimen in the same way as the Belgian
AAN patients high levels of AA–DNA adducts were found in
renal tissues but DNA adducts derived from ochratoxin A
were not observed (Arlt et al., 2001a). The presence of AA–
DNA adducts in the renal tissue of these rats was in line with
the observed induction of tumours in the forestomach (Cosyns
et al., 1998). Collectively, these results demonstrate that
ochratoxin A-related DNA adducts do not play a key role in
AAN or AAN-associated urothelial cancer. The most likely
reason for low levels of ochratoxin A-related DNA adducts
found in a few renal tissues from Belgian AAN patients is
that ochratoxin A is a widespread natural contaminant in
animal feed and food (Tatu et al., 1998).

In this context it is noteworthy that AA consumption
was considered a possible causal factor in Balkan endemic
nephropathy in 1970 (Ivac, 1970). Therefore, the epidemiology
of AAN might provide a clue to Balkan endemic nephropathy.
The respective role of AA in this entity could be further
assessed by evaluation of AA–DNA adducts in urothelial
tissue from patients with Balkan endemic nephropathy.

Conclusions
There is increasing evidence that the plant extract AA plays a
causal role not only in AAN but also and even more importantly
in the development of AAN-associated urothelial cancer. Since
more and more AAN cases besides those reported in Belgium
have been described worldwide and all are related to exposure
to AA (when determined), we are concerned that this form of
nephropathy and associated malignancies may occur more
commonly in the future due to the widespread availability of
herbal medicines containing AA. Whether patients exposed to
AA but who have not developed AAN are also at risk is
currently unknown. We therefore strongly believe that herbal
substances should be subjected to the same stringent scrutiny
and controls as common drugs before their release onto the
market. Owing to the fact that AA is both a powerful
nephrotoxic and carcinogenic substance all products containing
botanicals known to or suspected of containing AA should be
banned from the market world wide.

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