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REVIEW

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

Volker M.Arlt[3], Marie Stiborova[1] and Heinz H.Schmeiser[2]

Section of Molecular Carcinogenesis, Institute of Cancer Research, Cotswold Road, Sutton, Surrey SM2 5NG, UK, [1]Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, The Czech Republic and [2]Division 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-N^6-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 (Rücker and Chung, 1975; Hahn, 1979; Priestap, 1987). The anti-inflammatory properties of AA encouraged the development of pharmaceutical preparations in Germany (Möse, 1966; Möse and Porta, 1974; Kluthe *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 Ogutveren, 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 (Schwetz, 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.*, 1994). 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

[3]To whom correspondence should be addressed. Tel: +44 208 643 8901; Fax: +44 208 770 7290; Email: v.arlt@icr.ac.uk

Table I. Genetic and related effects of aristolochic acid

Test system	Without exogenous metabolic system	With exogenous metabolic system	Compound	Reference
Ames test, *Salmonella typhimurium* TA 1537	+	+	AA mixture	Robisch *et al.* (1982)
Ames test, *Salmonella typhimurium* TA 100	+	+	AA mixture	Robisch *et al.* (1982)
Ames test, *Salmonella typhimurium* TA 1537	+	+	AAI, AAII	Schmeiser *et al.* (1984)
Ames test, *Salmonella typhimurium* TA 100	+	+	AAI, AAII	Schmeiser *et al.* (1984)
Ames test, *Salmonella typhimurium* TA 1537	–	+	AlacI, AlacII	Schmeiser *et al.* (1986)
Ames test, *Salmonella typhimurium* TA 100	–	+	AlacI, AlacII	Schmeiser *et al.* (1986)
Chromosomal abberation, human lymphocytes *in vitro*	+	n.d.[a]	AA mixture	Abel and Schimmer (1983)
Sister chromatid exchange, human lymphocytes *in vitro*	+	n.d.	AA mixture	Abel and Schimmer (1983)
Unscheduled DNA synthesis, rat stomach mucosa *in vitro*	–	n.d.	AA mixture	Furihata *et al.* (1984)
Drosophila melanogaster, somatic mutations, recombinations	+	n.d.	AA mixture	Frei *et al.* (1985)
Gene mutation, rat subcutaneous granuloma tissue, *hprt* locus *in vivo*	+	n.d.	AA mixture	Maier *et al.* (1985)
Gene mutation, rat subcutaneous granuloma tissue, *hprt* locus *in vivo* and *in vitro*	+	n.d.	AAI, AAII	Maier *et al.* (1987)
Gene mutation, *Salmonella typhimurium* TM677, *hprt* locus *in vitro*	+	n.d.	AAI	Pezutto *et al.* (1988)
Gene mutation, Chinese hamster ovary cells (AA8), *hprt* locus *in vitro*	+	n.d.	AAI	Pezutto *et al.* (1988)
Micronucleus test, mouse bone marrow cells *in vivo*	+	n.d.	AA mixture	Mengs and Klein (1988)
Micronucleus test, human lymphocytes *in vitro*	+	+	AA mixture	Kevekordes *et al.* (2001)
Micronucleus test, human hepatoma cells (HepG2) *in vitro*	+	+	AA mixture	Kevekordes *et al.* (2001)
SOS chromotest, *Escherichia coli* PQ37	+	+	AA mixture	Kevekordes *et al.* (1999)
SOS chromotest, *Escherichia coli* PQ37	+	+	AAI, AAII	Kevekordes *et al.* (1999)
Mutation frequency, *lambda/lacZ transgenic mouse* (Muta™Mouse), various tissues, lacZ and *cII* locus *in vivo*	+	n.d.	AA mixture	Kohara *et al.* (2002)
Gene mutation, *lambda/lacZ transgenic mouse* (Muta™Mouse), forestomach, kidney and bladder tissue, *cII* locus *in vivo*	+	n.d.	AA mixture	Kohara *et al.* (2002)

[a] n.d., not determined.

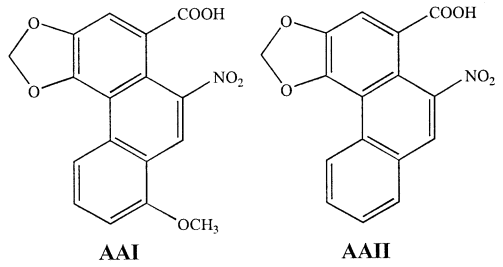

Fig. 1. Chemical structures of aristolochic acid I (AAI) and II (AAII).

been described in the UK (Lord *et al*., 2001). This highlights the carcinogenic potential of AA in human beings.

Since the demonstration that AA forms covalent DNA adducts in rodents (Schmeiser *et al.*, 1988; Pfau *et al.*, 1990a; Stiborova *et al.*, 1994) as well as in AAN patients (Table II), AA–DNA adducts have been used as a biomarker of exposure and to investigate the mutagenic and carcinogenic potential of AA. The intention of this article is to summarize data on the genotoxic mechanism for AA carcinogenicity in rodents and to speculate on the mechanism of AA nephrotoxicity and carcinogenicity in humans.

Genotoxic and carcinogenic mechanism of AA in rodents

Carcinogenic and nephrotoxic effects of AA in rodents

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 nitro-reduction, 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 AAIa (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

Table II. Reports of CHN/AAN patients in Belgium and other parts of the world in the literature

No. of cases	Country	Published year(s)	Material causing CHN/AAN	Clinical and Histological picture of CHN/AAN	Detection of AA in herbal preparation by DC/HPLC	Detection of AA–DNA adducts by ^{32}P-post-labelling	Detection of urothelial tumours	References
84[a]	Belgium	1993–2002	Chinese herbal remedy containing *Aristolochia fangchi* prescribed for weight loss	+	+	+[a]	+[a]	Vanherweghem *et al.* (1993, 1995, 1996, 1998), Depierreux *et al.* (1994), Vanhaelen *et al.* (1994), Nortier *et al.* (2000), Martinez *et al.* (2002)
18[b]	Belgium	1994–2001	Chinese herbal remedy containing *Aristolochia fangchi* prescribed for weight loss	+	+	+[b]	+[b]	Cosyns *et al.* (1994a,b, 1999), Schmeiser *et al.* (1996), Bieler *et al.* (1997), Reginster *et al.* (1997), Arlt *et al.* (2001a)
2	France	1994	Chinese herbal remedy prescribed for weight loss	+	n.d.[c]	n.d.	–	Pourrat *et al.* (1994)
1	Spain	1996	Herbal tea containing *Aristolochia pistolochia*	+	n.d.	n.d.	–	Pena *et al.* (1996)
1	Japan	1997	Chinese health food	+	+	n.d.	–	Tanaka *et al.* (1997a)
1	Japan	1997	Chinese herbal remedy	+	+	n.d.	–	Tanaka *et al.* (1997b)
2	France	1998	Chinese herbal remedy	+	n.d.	n.d.	–	Stengel and Jones (1998)
2	UK	1999	Chinese herbal tea containing *Aristolochia manshuriensis* prescribed against eczema	+	+	+[d]	+[d]	Lord *et al.* (1999, 2001)
1	Japan	1999	Chinese herbal remedy to 'promote health'	+	+	n.d.	–	Ubara *et al.* (1999)
12	Taiwan	2000	Chinese herbal remedies prescribed for weight control or as a nutritional supplement	+	n.d.	n.d.	+[e]	Yang *et al.* (2000)
2	Japan	2000	Chinese herbal medicine	+	+	n.d.	–	Tanaka *et al.* (2000)
1	USA	2000	Chinese herbal remedy prescribed for pain relief	+	+	n.d.	–	Meyer *et al.* (2000)
1	Germany	2001	Chinese herbal remedy distributed as 'herbal food combination'	+	+	n.d.	–	Krumme *et al.* (2001)
13	Japan	2001	Chinese herbal medicine or health food	+	+	n.d.	–	Tanaka *et al.* (2001) and references therein
1	China	2001	Chinese herbal remedy prescribed for 'waste discharging and youth keeping' purposes	+	+	+	–	Gillerot *et al.* (2001)
1	Japan	2001	Chinese herbal remedy prescribed against edema	+	+	n.d.	–	Nishimagi *et al.* (2001)
58	China	2001	Chinese traditional drugs	+	+	n.d.	–	Chen *et al.* (2001)
51	China	2001	Chinese herbal drugs containing *Aristolochia manshuriensis*	+	+	n.d.	–	Li *et al.* (2001)
20	Taiwan	2001	Chinese herbal remedies prescribed for weight reduction or as a nutritional supplement	+	n.d.	n.d.	+[f]	Chang *et al.* (2001)
1	UK	2002	Chinese herbal remedy to treat hepatitis B	+	+	n.d.	–	Cronin *et al.* (2002)
1	Taiwan	2002	Chinese herbal remedy for leg edema	+	+	n.d.	–	Yang *et al.* (2002)

[a]AAN 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, personnal 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.
[b]AAN 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, personnal communication); AA–DNA adducts in urothelial tissue were detected in all eight patients analysed.
[c]n.d., not determined.
[d]AA–DNA adducts were detected in urothelial tissue of one patient analysed and were associated with urothelial carcinoma.
[e]One bladder carcinoma and one bladder transitional cell carcinoma were found.
[f]One bladder transitional cell carcinoma was found.

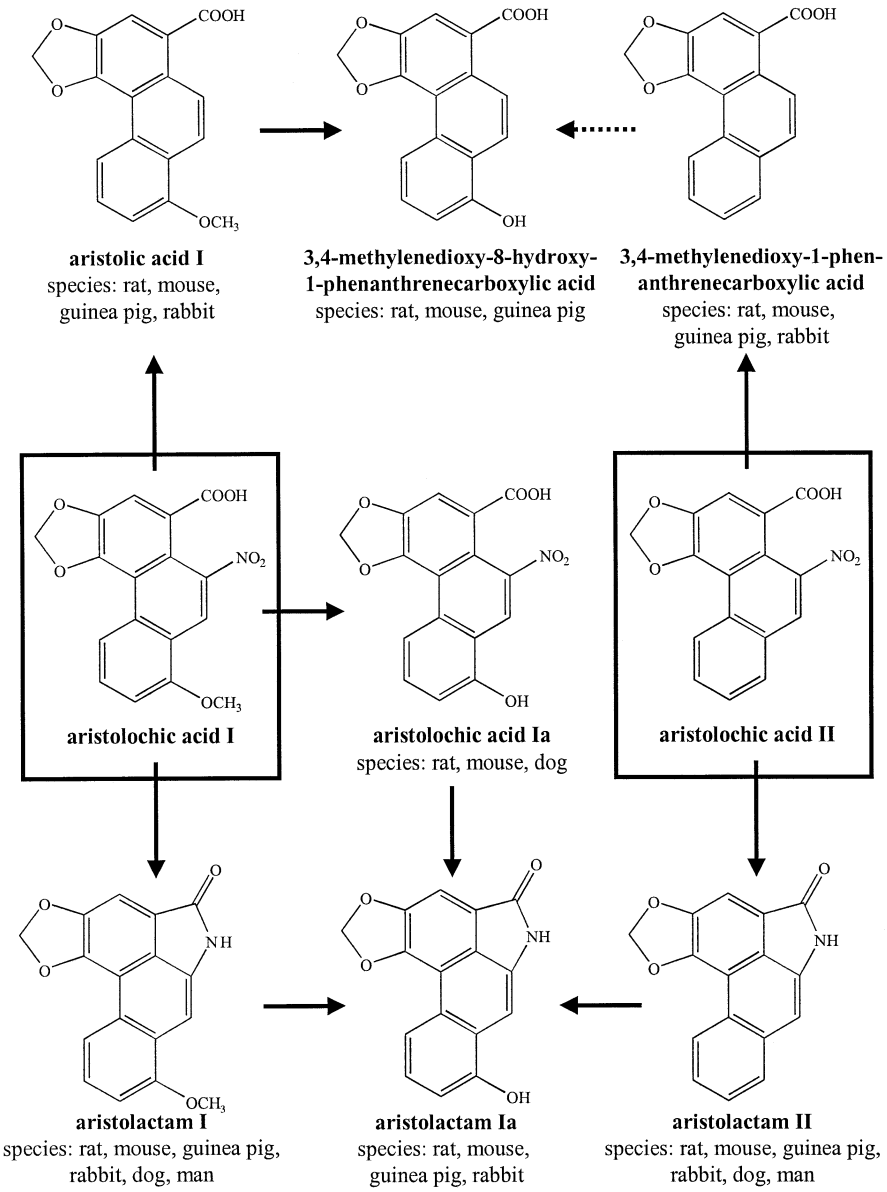

Fig. 2. Metabolism of aristolochic acid I and II (from Krumbiegel *et al.*, 1987).

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

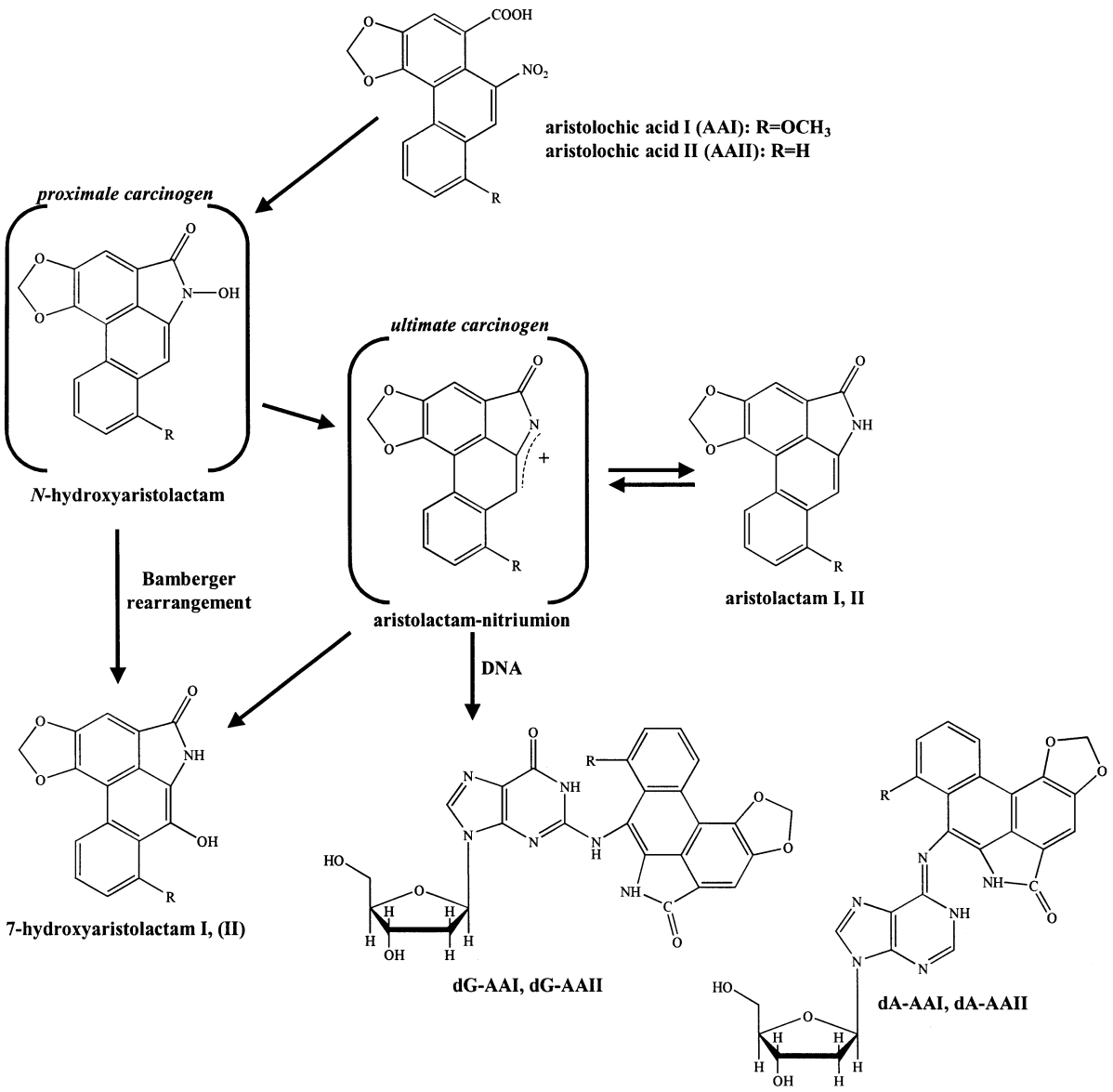

Fig. 3. Metabolic activation and DNA adduct formation of aristolochic acid I (AAI, R = OCH_3) and II (AAII, R = H); 7-(deoxyadenosin-N^6-yl)aristolactam I or II (dA–AAI or dA–AAII), 7-(deoxyguanosin-N^2-yl)aristolactam I or II (dG–AAI or dG–AAII).

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-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) (Figure 3) (Pfau *et al.*, 1990b, 1991). It was also shown that the dA–AAII adduct is formed from AAI through a demethoxylation 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-N^2-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 hydroxyamino 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 ^{32}P-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 like 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 AAI was in general 10 times higher compared with AAII. 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 organotropic activity could be related to different phase II metabolic pathways for AAI and AAII. 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 λ/*lacZ* 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 bindings 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.

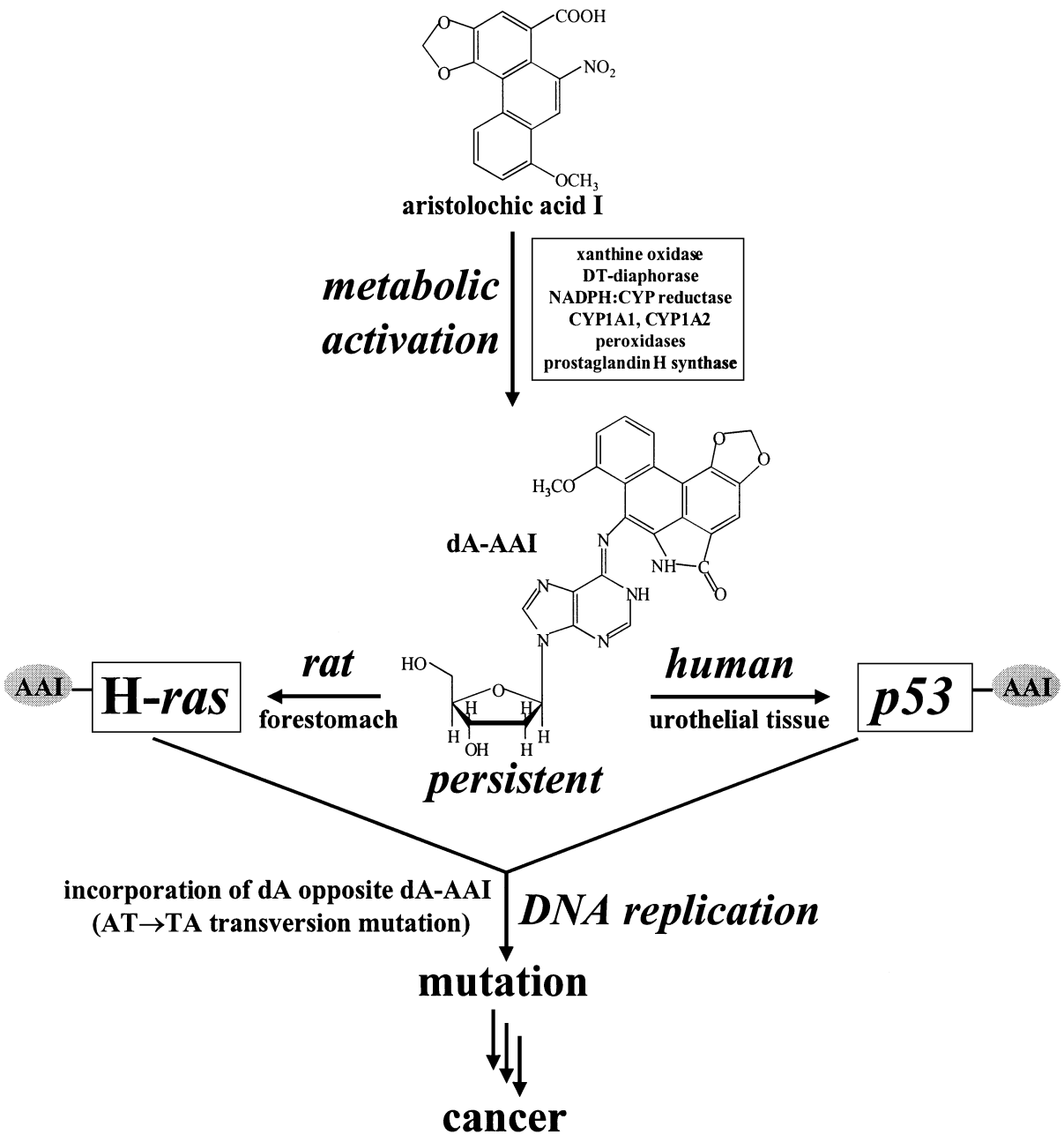

Fig. 4. Postulated mechanism for the carcinogenicity of aristolochic acid in rodents and humans. AAI, aristolochic acid I; dA–AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dA, deoxyadenosine.

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; McIntrye, 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 ^{32}P-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; Gillerot *et al.*, 2001; Lord *et al.*, 2001). 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 AAI 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 β_2-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 β_2-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, cytosolic 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 ram 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 adduct 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 (Pfohl-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 ^{32}P-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 (Ivic, 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 world wide 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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