# Carcinogenic and Nephrotoxic Alkaloids Aristolochic Acids upon Activation by NADPH : Cytochrome P450 Reductase Form Adducts Found in DNA of Patients with Chinese Herbs Nephropathy

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**Abstract.** Aristolochic acid (AA), a naturally occurring nephrotoxin and carcinogen, has been found to be implicated in an unique type of renal fibrosis, designated Chinese herbs nephropathy (CHN), and associated with the development of urothelial cancer in CHN patients Understanding, which enzymes are involved in AA activation and/or detoxication is important in the assessment of individual susceptibility of humans to this natural carcinogen Using the nuclease P1 version of the $^{32}$P-postlabeling assay we examined the ability of microsomal NADPH CYP reductase to activate AA to metabolites forming DNA adducts Renal and hepatic microsomes, containing NADPH CYP reductase, generated AA DNA adduct patterns reproducing those found in renal tissues in patients suffering from a renal fibrosis CHN and urothelial cancer 7-(Deoxyadenosin-N$^6$ yl)aristolactam I, 7-(deoxyguanosin-N$^2$-yl)aristolactam I and 7-(deoxyadenosin-N$^6$-yl)aristolactam II were identified as AA-DNA adducts formed by AAI Two AA-DNA adducts, 7 (deoxyguanosin-N$^2$-yl)aristolactam II and 7-(deoxyadenosin-N$^6$-yl)aristolactam II, were generated from AAII According to the structures of the DNA adducts iden tified, nitroreduction is the crucial pathway in the metabolic activation of AA The identity of NADPH CYP reductase as activating enzyme in microsomes has been proved with different cofactors and an enzyme inhibitor α-Lipoic acid, a selective inhibitor of NADPH CYP reductase, significantly decreased the amount of the adducts formed by microsomes Likewise, only a cofactor of the enzyme, NADPH, supported the DNA adduct formation of AAI and AAII, while NADH was inef fective These results demonstrate an involvement of NADPH CYP reductase in the activation pathway of AAI and AAII in the microsomal system Moreover, using the purified enzyme, the participation of this enzyme in the formation of AA-DNA adducts was confirmed The results presented here are the first report

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demonstrating a reductive activation of natural nitroaromatic compounds, AA, by NADPH CYP reductase

**Key words:** Aristolochic acid — NADPH cytochrome P450 reductase — Reductive activation — DNA adducts — $^{32}$P-postlabelling — Chinese herbs nephropathy

**Abbreviations:** AA, aristolochic acid, AAI, 8-methoxy 6-nitro phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid, AAII, 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5 carboxylic acid, $AA_M$, a natural mixture of AAI and AAII, AAs, aristolochic acid I and II, CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, CHN, Chinese herbs nephropathy, CYP, cytochrome P450, dAp, deoxyadenosine 3'-monophosphate, dGp, deoxyguanosine 3'-monophosphate, dA-AAI, 7-(deoxyadenosin-$N^6$-yl)aristolactam I, dA-AAII, 7-(deoxyadenosin-$N^6$-yl)aristolactam II, dG-AAI, 7-(deoxyguanosin-$N^2$-yl) aristolactam I, dG-AAII, 7-(deoxyguanosin $N^2$-yl) aristolactam II, HPLC, high performance liquid chromatography, PB, phenobarbital, PEI, polyethylenimine, RAL, relative adduct labeling, TLC, thin-layer chromatography, XO, xanthine oxidase

## Introduction

Some of the most potent carcinogens known are natural products (Woo et al 1988) Among those which have been identified in plants, including safrole, cycasin and pyrrolizidine alkaloids, aristolochic acids have recently attracted considerable attention We reported that aristolochic acid (AA) was implicated in an endemic renal fibrosis designated Chinese herbs nephropathy (CHN) (Vanherweghem et al 1993), in young Belgian women who had followed a slimming regimen including Chinese herbs (Schmeiser et al 1996, Bieler et al 1997, Stiborová et al 1999a, Nortier et al 2000) An inadvertent replacement of one of these herbs (*Stephania tetrandra*) by *Aristolochia fangchi* has been ascertained by the absence of tetrandrine and the detection of carcinogenic AA in the herbal powder used for the slimming pills We detected and identified three AA-specific DNA adducts, 7-(deoxyguanosin-$N^2$-yl) aristolactam I (dG-AAI), 7-(deoxyadenosin-$N^6$-yl)aristolactam I (dA-AAI) and 7 (deoxyadenosin-$N^6$-yl)aristolactam II (dA-AAII), in renal tissues and thereby demonstrated that all CHN patients had ingested the plant ingredient AA, the major alkaloid of aristolochia species AA is a mixture of structurally related nitrophenanthrene carboxylic acids, with 8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dio xolo 5-carboxylic acid (AAI) and 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAII), being the major components (Fig 1) To date over 100 patients suffering from CHN have been identified (Vanherweghem 1998) A third of the patients have already received a kidney transplant, another third are receiving dialysis, and the remainders suffer from slowly progressing renal failure (van Yperseele de Strihou 1998) The persistence of the three AA-DNA adducts in human tissues (kidney and ureter), several months or even years after cessation of the

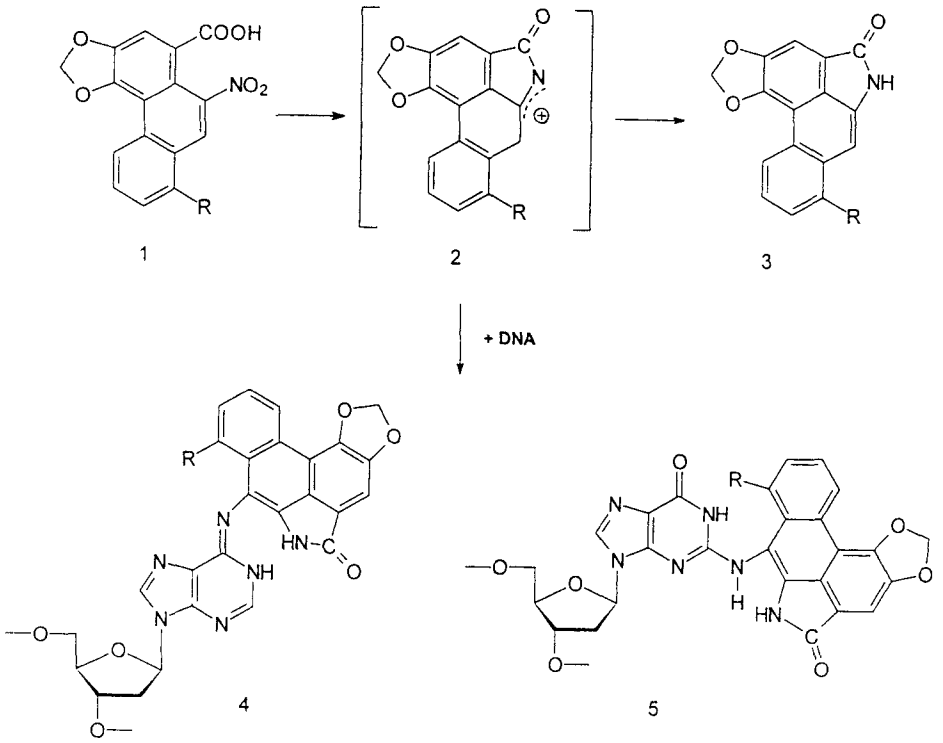


**Figure 1.** Metabolic activation and DNA adduct formation of AAI and AAII **1** R = $OCH_3$, AAI, R = H, AAII, **2** cyclic nitrenium ion of AAI or AAII, **3** R = $OCH_3$, aristolactam I, R = H, aristolactam II, **4** R = $OCH_3$, 7-(deoxyadenosin-$N^6$-yl)aristolactam I (dA-AAI), R = H, 7-(deoxyadenosin-$N^6$-yl)aristolactam II (dA-AAII), **5** R = $CH_3$, 7-(deoxyguanosin-$N^2$-yl) aristolactam I (dG-AAI), R = H, 7-(deoxyguanosin-$N^2$-yl) aristolactam II (dG-AAII)

regimen, is noteworthy (Schmeiser et al. 1996; Bieler et al. 1997; Stiborová et al 1999a, Nortier et al. 2000).

In human tissues the dA-AAI was the most prominent adduct (Schmeiser et al. 1996; Bieler et al. 1997; Nortier et al 2000) consistent with our previous reports on rats given pure AAI or the natural mixture $AA_M$ (Stiborová et al 1994) Both longer persistence and higher initial levels of dA-AAI probably contributed to the relative abundance of this adduct (Fernando et al 1993; Stiborová et al. 1994; Schmeiser et al. 1996). Moreover, H-*ras* protooncogenes are activated with high frequency by an A → T transversion mutation in codon 61 of DNA from AAI-induced carcinomas in rats (Schmeiser et al. 1990). This suggests a relevant role of the dA-AAI adduct not only in the AA-induced renal fibrotic process, but also in AA-induced mutagenesis and carcinogenesis. Indeed, an increasing num-

ber of urothelial carcinomas has been identified in patients with CHN, even after renal transplantation (Cosyns et al. 1994, 1999; Vanherweghem et al. 1995; Violon 1997). Therefore, patients with CHN should undergo regular follow-ups for urothelial cancer.

Recently, Cosyns and coworkers (1999) demonstrated that CHN-dependent carcinogenesis is associated with the overexpression of *p*53, which suggests a role for a mutation in the *p*53 gene. The relationship between AA-DNA adducts and *p*53 mutations in the kidneys and ureters remains to be explored.

Interestingly and luckily, to date only 2–3% of the patients treated with the slimming regimen have suffered from nephropathy. Taking into account that AA is toxic, should it not have affected more of the patients? One possible explanation for the different responses of patients may be differences in the individual activities of the enzymes catalyzing the biotransformation (detoxication and/or activation) of AA. Many enzymes metabolizing carcinogens or their genes are known to exist in variant forms or show polymorphism that have differing activities. These variations appear to be important determinants of cancer risk (Smith et al. 1995). Therefore, screening CHN patients as well as healthy women treated with the slimming regimen for variations in activities of enzymes involved in AA metabolism or in genes of them should help to find possible relationships between genotypes or phenotypes and nephropathy, AA-DNA adduct levels and urothelial cancer risk. Thus, the identification of the enzymes principally involved in the activation of AA in humans and detailed knowledge of their catalytic specificities is of major importance.

Recently we found that *in vitro* xanthine oxidase (XO), rat liver microsomal preparations and even peroxidases were competent in activating both AAI and AAII to form the same DNA adducts found *in vivo* in rodents (Fernando et al. 1993; Stiborová et al. 1994; Schmeiser et al. 1997) and in humans (Schmeiser et al. 1996; Bieler et al. 1997; Nortier et al. 2000). The multicomponent microsomal enzyme system consists of NADPH : CYP reductase (EC 1.6.2.4.) and one of many cytochrome P450 (CYP) isozymes (Rendic and DiCarlo 1997). Both types of enzymes are integral membrane proteins, and NADPH : CYP reductase is one of only two known mammalian enzymes containing both FMN and FAD as prosthetic groups, the other being various isoforms of nitric-oxide synthase (Wang et al. 1997) Other physiological electron acceptors of NADPH : CYP reductase include microsomal heme oxygenase and cytochrome $b_5$ and although nonphysiological, the reductase is capable of transferring reducing equivalents to cytochrome *c* and several exogenous low-molecular-weight substrates (i.e. azo dyes, quinones, nitroaromatics) (Wang et al. 1997). NADPH : CYP reductase is present in a membrane of endoplasmic reticulum of many human tissues including kidney, a target tissue for CHN. The efficiency of this enzyme to reduce studied natural nitro-aromatics, aristolochic acids, has not been demonstrated yet. Therefore, the present study was undertaken to determine the capability of the enzyme to activate these natural products, and to identify whether the enzyme is involved in AA-DNA adduct formation.

## Materials and Methods

### *Chemicals and enzymes*

Chemicals were obtained from the following sources NADH, NADPH, 3-[(3 cholamidopropyl) dimethylammonio]-1 propane sulfonate (CHAPS), nuclease P1, deoxyadenosine 3'-monophosphate (dAp), deoxyguanosine 3'-monophosphate (dGp), dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine and dilauroyl phosphatidylserine from Sigma Chemical Co (St Louis, MO, USA), bicinchoninic acid from Pierce, (Rockford, IL, USA) and calf thymus DNA from Roche Diagnostics Mannheim (Germany) The natural mixture consisting of 65% AAI and 34% AAII was a gift from Madaus, Cologne, Germany AAI and AAII were isolated from the mixture by preparative HPLC (Schmeiser et al 1984) All chemicals were of analytical purity or better Enzymes and chemicals for the $^{32}$P-postlabeling assay were obtained commercially from sources described previously (Stiborova et al 1994, Schmeiser et al 1997)

### *Animal experiments*

Gavage with AAI and a natural mixture of AA ($AA_M$) dissolved in 0 15 mmol $l^{-1}$ NaCl (10 mg/kg body weight) was administered to six male Wistar rats (100 150 g) once a day for four consecutive days Six control animals received an equal volume of 0 15 mmol $l^{-1}$ NaCl Rats were placed in cages in temperature- and humidity-controlled rooms Standardized diet and water were provided *ad libitum* Animals were killed 24 h after the last treatment by cervical dislocation (Stiborova et al 1999b) Liver and kidney of animals were excised immediately after sacrifice, quickly frozen in liquid nitrogen, and stored at −80°C until the isolation of microsomes

### *Preparation of microsomes, isolation of NADPH CYP reductase and assays*

Microsomes from livers and kidneys of control and pre-treated male Wistar rats were prepared as described previously (Stiborova et al 1990, 1995) Pre treatment of six male Wistar rats with phenobarbital (PB) and isolation of microsomes from livers of these rats were carried out using procedures described by Hodek et al (1988) Protein concentrations in all microsomal fractions were assessed using the bicinchoninic acid protein assay with serum albumin as a standard (Wiechelman et al 1988) Each microsomal preparation was analyzed for specific activities of NADPH CYP reductase The activity of NADPH CYP reductase was measured according to Sottocasa et al (1967) using cytochrome c as the substrate (i e as NADPH cytochrome *c* reductase) One unit of activity is defined as the amount of enzyme catalyzing the reduction of 1 $\mu$mol of cytochrome *c* ($a_m$ = 21 $mmol^{-1}$ l $cm^{-1}$) per minute The concentration of NADPH CYP reductase was estimated as described earlier (Vermilion and Coon 1978) NADPH CYP reductase was purified from rabbit livers as described (Yasukochi et al 1979)

*Incubations*

The deaerated and argon-purged incubation mixtures contained in a final volume of 0 75 ml 50 mmol $l^{-1}$ potassium phosphate buffer (pH 7 4), 1 mmol $l^{-1}$ NADPH 1 0 mg of microsomal protein, 0 5 mmol $l^{-1}$ AAI or AAII as sodium salts dissolved in water and 1 mg of calf thymus DNA (4 mmol $l^{-1}$) The reaction was initiated by adding NADPH Unless stated otherwise, incubations were carried out at 37 °C for 60 min The effect of α-lipoic acid (a selective inhibitor of NADPH CYP reductase) (Slepneva et al 1995) was tested using 1 6 5 0 mmol $l^{-1}$ α lipoic acid Control incubations were carried out either without activating system (microsomes) or with activating system, AAI and AAII but without DNA or with activating system and DNA but without AAs In incubations testing the activity of pure NADPH CYP reductase, the reaction mixtures were carried out with the enzyme (i) present in liposomes to mimic the conditions of microsomal membrane, and (ii) without liposome components Briefly, 5 μmol $l^{-1}$ NADPH CYP reductase, 0 5 μg/μl CHAPS, 0 1 μg/μl liposomes [dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, dilauroyl phosphatidylserine (1 1 1)], 3 mmol $l^{-1}$ reduced glutathione and 50 mmol $l^{-1}$ HEPES/KOH, pH 7 4 were mixed in the method (i) In addition, 5 μmol $l^{-1}$ NADPH CYP reductase and 50 mmol $l^{-1}$ HEPES/KOH, pH 7 4, without CHAPS, glutahione and liposomes were mixed and used for incubations in the method (ii) An aliquot containing appropriate amounts of NADPH CYP reductase (0 125 nmol) was added to incubation mixtures instead of microsomes and used for activation of AAs (see above) After incubation, (37 °C, 10 60 min) the mixtures were extracted twice with ethyl acetate (2×2 ml) DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described earlier (Stiborova et al 1990, 1995, Schmeiser et al 1997) The content of DNA was determined spectrophotometrically (Schmeiser et al 1997)

*$^{32}P$-Postlabeling analysis*

The nuclease P1 enrichment version (Reddy and Randerath 1986) and the 1-butanol extraction-mediated enrichment procedure (Gupta and Early 1988) were used DNA samples (12 5 μg) were digested with micrococcal nuclease (750 mU) and spleen phosphodiesterase (12 5 mU) in digestion buffer (20 mmol $l^{-1}$ sodium succinate, 8 mmol $l^{-1}$ $CaCl_2$, pH 6 0) for 3 h at 37 °C in a total volume of 12 5 μl 2 5 μl of the digests were removed and diluted 1 1500 to determine the amount of normal nucleotides In the nuclease P1 version, digests (10 μl) were enriched for adducts by incubation with 5 μg (5 U) of nuclease P1 in 3 μl of a buffer containing 0 8 mol $l^{-1}$ sodium acetate, pH 5 0 and 2 mmol $l^{-1}$ $ZnCl_2$ for 30 min at 37 °C The reaction was stopped by adding 3 μl of 427 mmol $l^{-1}$ tris base The extraction with 1-butanol to enrich the adducts was carried out as described earlier (Gupta and Early 1988) Four microlitres of labeling mix consisting of 400 mmol $l^{-1}$ bicine pH 9 5, 300 mmol $l^{-1}$ dithiothreitol, 200 mmol $l^{-1}$ $MgCl_2$, 10 mmol $l^{-1}$ spermidine, 100 μCi [$\gamma-^{32}P$]-ATP (15 pmol), 11 25 μmol $l^{-1}$ ATP and 10 U T4 polynucleotide kinase were added After incubation for 30 min at room temperature, 20 μl were

applied to a polyethylenimine (PEI)-coated cellulose TLC plate (Macherey-Nagel, Düren, Germany) and chromatographed as described (Schmeiser et al. 1988b) except that D3 and D4 were adjusted to pH 4.0 and 9.1 for better resolution. To determine the amount of normal nucleotides 5 $\mu$l of the 1 : 1500 dilution of digests were mixed with 2.5 $\mu$l of tris buffer (10 mmol·l$^{-1}$, pH 9.0) and 2.5 $\mu$l of labeling mixture (see above) and incubated for 30 min at room temperature. The labeling mixture was diluted by mixing 4 $\mu$l with 750 $\mu$l of 10 mmol·l$^{-1}$ tris buffer, pH 9.0. 5 $\mu$l of this solution were applied to a PEI-cellulose TLC plate and run in 0.28 mol·l$^{-1}$ $(NH_4)_2SO_4$, 50 mmol·l$^{-1}$ $NaH_2PO_4$, pH 6.5. Adducts and normal nucleotides were detected and quantitated by an Instant imager (Packard). Count rates of adducted fractions were determined from triplicate maps after subtraction of count rates from adjacent blank areas. Excess of [$\gamma$-$^{32}$P]ATP after the postlabeling reaction was confirmed. Adduct levels were calculated in units of relative adduct labeling (RAL) which is the ratio of cpm of adducted nucleotides to cpm of total nucleotides in the assay. Enzymatic synthesis of reference compounds, dAp-AAI, dGp-AAI, dAp-AAII and dGp-AAII and their $^{32}$P-postlabeling were carried out as described earlier (Stiborová et al. 1994).

*Co-chromatography on PEI-cellulose*

Adduct spots detected by the $^{32}$P-postlabeling assay were excised from the thin-layer plates, extracted and co-chromatographed with reference 3',5'bisphosphate adducts as reported previously (Stiborová et al. 1994).

*HPLC analysis of $^{32}$P-labeled 3',5'-deoxyribonucleoside bisphosphate adducts*

HPLC analysis was performed essentially as described previously (Pfau and Phillips 1991; Stiborová et al. 1994; Schmeiser et al. 1997). Individual spots detected by the $^{32}$P-postlabeling assay were excised from thin layer plates and extracted (Schmeiser et al. 1988a). The dried extracts were redissolved in 100 $\mu$l of methanol/phosphate buffer, pH 3.5, 1 : 1 (v/v). Aliquots (50 $\mu$l) were analysed on a phenyl-modified reversed-phase column (250 × 4.6 mm, 5 $\mu$m Zorbas Phenyl; Säulentechnik Dr. Knauer, Berlin, Germany) with a linear gradient of methanol (from 40 to 80 % in 45 min) in aqueous 0.5 mol·l$^{-1}$ sodium phosphate and 0.5 mol·l$^{-1}$ phosphoric acid, pH 3.5, at a flow rate of 0.9 ml/min. Radioactivity eluting from the column was measured by monitoring Cerenkov radiation with a Berthold LB 506 C-1 flow through radioactivity monitor (500 $\mu$l cell, dwell time 6 s).

## Results

We investigated the formation of adducts by AAI and AAII in calf thymus DNA in the presence of the rat microsomal enzyme systems. Microsomes isolated from kidneys, a target tissue for AA toxicity, and livers, the tissue rich in a majority of enzymes metabolizing xenobiotics, were used as model systems. The *in vitro* incubations were performed under standardized conditions of AAs (0.5 mmol·l$^{-1}$), dNp (4 mmol·l$^{-1}$) as calf thymus DNA, microsomal protein (1.0 mg) and NADPH

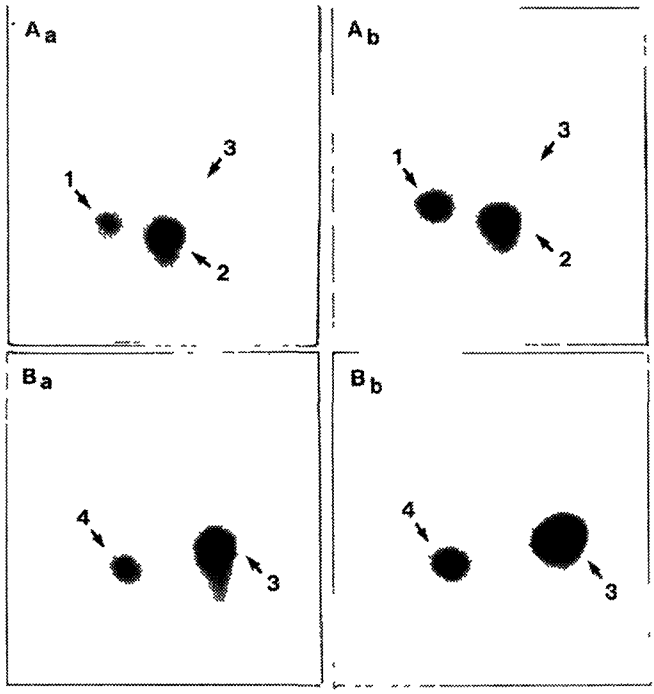


**Figure 2.** Autoradiographic profiles of AAI- (**A**) and AAII-DNA (**B**) adducts obtained from DNA after activation by kidney ($\mathbf{A}_a$, $\mathbf{B}_a$), and liver ($\mathbf{A}_b$ $\mathbf{B}_b$) microsomes. The nuclease P1-enrichment procedure was used for analysis. Origins, in the bottom left-hand corner were cut off before exposure. Screen enhanced autoradiography was at −80 °C from 2 to 5 h. Chromatographic conditions: D1, 1 mol $l^{-1}$ sodium phosphate, pH 6.8, D2 was omitted, D3, 3.5 mol $l^{-1}$ lithium formate, 8.5 mol $l^{-1}$ urea, pH 4.0, D4, 0.8 mol $l^{-1}$ LiCl, 0.5 mol $l^{-1}$ Tris-HCl, 8.5 mol $l^{-1}$ urea, pH 9.1, D5, 1.7 mol $l^{-1}$ $NaH_2PO_4$, pH 6.0. Spot 1, dG-AAI, spot 2, dA-AAI, spot 3, dA-AAII, spot 4, dG-AAII.

(1 mmol·$l^{-1}$) to ensure adduct levels easily detectable by the $^{32}$P-postlabeling method (RAL range 1 adduct in $10^6$–$10^9$ nucleotides for enzymatic reactions catalyzed by microsomes). Because enrichment of AA-DNA adducts by *n*-butanol extraction yielded a lower recovery (80–90%) as compared to the nuclease P1 version of the $^{32}$P-postlabeling assay, all further experiments were carried out with enrichment by nuclease P1.

Microsomes of both tissues (kidney, liver) were capable of activating both AAI and AAII to form DNA adducts. It is evident from Fig. 2 that AAI and AAII activated by microsomes from these two organs generated the same major DNA adduct spots as those obtained *in vivo* in rats and humans and as reported previously (Schmeiser et al. 1988b, 1996; Stiborová et al. 1994; Bieler et al. 1997; Nortier et al. 2000). Quantitative analysis (Table 1) revealed that the

**Table 1.** Quantitative analysis of adducts formed in DNA by AAI and AAII activated by liver and kidney microsomal samples

| AA-DNA adduct | RAL[a] | | | |
|---|---|---|---|---|
| | Liver microsomes | | Kidney microsomes | |
| *AAI-DNA adduct* | | | | |
| dG-AAI | 5 98 ± 1 04[b] | 28 48 ± 4 95[c] | 1 82 ± 0 19[b] | 30 33 ± 3 17[c] |
| dA-AAI | 10 64 ± 1 76[b] | 50 67 ± 8 38[c] | 3 17 ± 0 35[b] | 52 83 ± 5 83[c] |
| dA-AAII | 0 83 ± 0 05[b] | 3 95 ± 0 23[c] | 0 25 ± 0 03[b] | 4 33 ± 0 50[c] |
| Total | 17 45 ± 1 97[b] | 83 10 ± 9 59[c] | 5 25 ± 0 60[b] | 87 49 ± 9 53[c] |
| *AAII-DNA adduct* | | | | |
| dG-AAII | 0 50 ± 0 15[b] | 2 38 ± 0 71[c] | 0 17 ± 0 05[b] | 2 83 ± 0 83[c] |
| dA-AAII | 1 58 ± 0 18[b] | 7 52 ± 0 86[c] | 0 50 ± 0 10[b] | 8 33 ± 1 17[c] |
| Total | 2 08 ± 0 20[b] | 9 90 ± 0 95[c] | 0 67 ± 0 19[b] | 11 16 ± 1 83[c] |

Numbers are averages ± S E M ($n = 6$) of triplicate *in vitro* incubations, each DNA sample was determined by two post-labeled analyses [a]RAL, mean ± S E M /$10^7$ nucleotides, [b]RAL, mean ± S E M /$10^7$ nucleotides *per* mg protein, [c]RAL, mean ± S E M /$10^7$ nucleotides *per* unit of NADPH CYP reductase

extent of DNA binding by AAI was always higher than by AAII irrespectively of the type of microsomes used for activation In the organ comparison, microsomes from livers seemed to be more efficient as an activation system for both AAs than microsomes from kidneys (Table 1). However, when the levels of AA-DNA adducts were recalculated and expressed as amounts of adducts *per* activities of NADPH . CYP reductase, essentially the same specific levels of adducts were determined for microsomes of both organs (Table 1). Control incubations carried out in parallel either without microsomes, or without DNA, or without AAs were free of adduct spots in the region of interest even after prolonged exposure times. Adduct spots 1, 2 ,3 and 4 formed by AAs (Fig 2) cochromatographed on PEI-cellulose TLC plates (not shown) and by reversed-phase HPLC (Fig. 3) with those of synthetic standards (Stiborová et al 1994). Thus, spot 1 was assigned to 3',5'-bisphospho-7-(deoxyguanosin-$N^2$-yl)-aristolactam I (dG-AAI), spot 2 to 3',5'-bisphospho-7-(deoxyadenosin-$N^6$-yl)-aristolactam I (dA-AAI), spot 3 to 3',5'-bisphospho-7-(deoxyadenosin-$N^6$-yl)-aristolactam II (dA-AAII) and spot 4 to 3',5'-bisphospho-7-(deoxyguanosin-$N^2$-yl)-aristolactam II (dG-AAII). These adducts are known to be generated from AAs by nitro reduction (Pfau et al 1990, 1991, Stiborová et al. 1994) Therefore, the microsomes tested in this study contain enzymatic systems capable of catalyzing the reductive activation of AAs leading to formation of these DNA adducts. Reductases, NADPH : CYP reductase and NADH cytochrome $b_5$ reductase, are major candidates for the reductive activation of AAs by microsomes. To investigate these possibilities, the influences of an inhibitor of NADPH . CYP reductase and cofactors of both reductases upon AA-DNA adduct formation catalyzed by microsomes were examined. Rat hepatic microsomes were used as a model in these experiments.

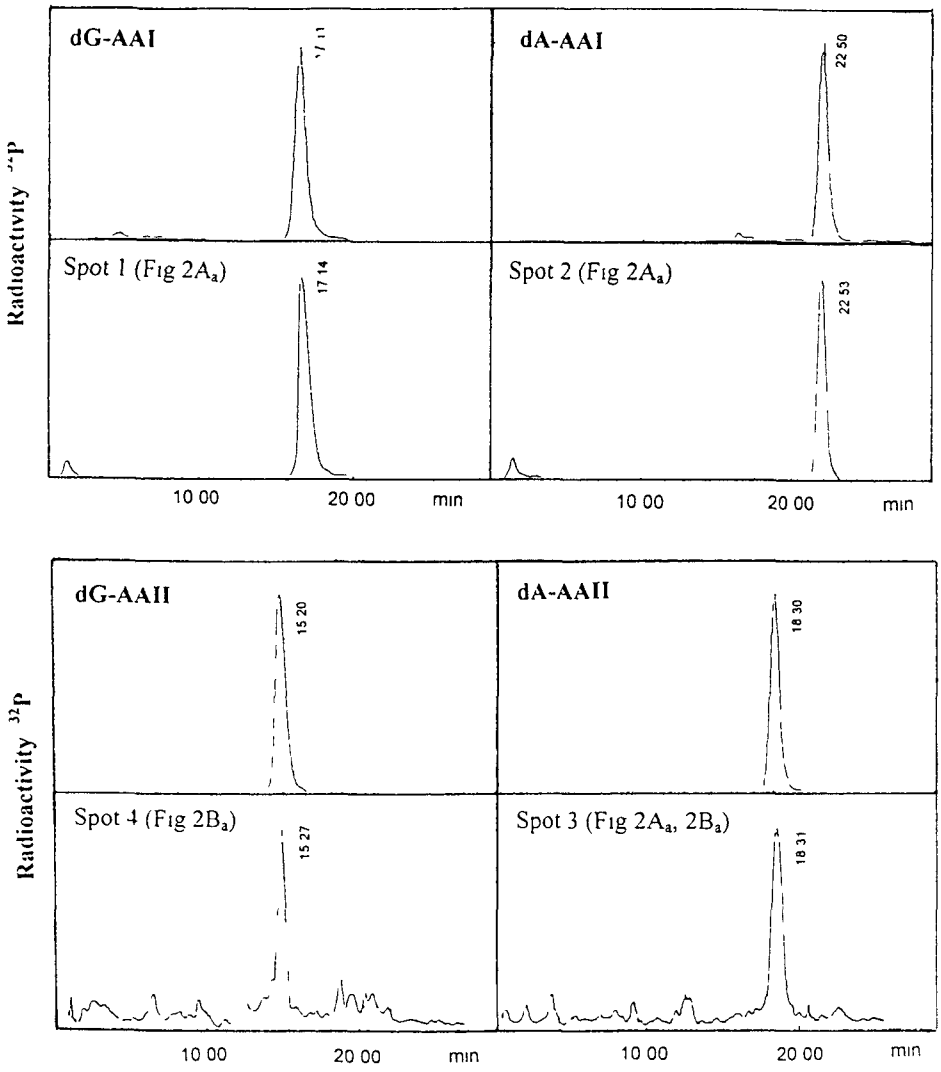


**Figure 3.** Separation of $^{32}$P-labelled deoxynucleoside 3',5'-bisphosphate adducts derived from AAI and AAII on a phenyl-modified reversed-phase column Chromatographic conditions are described in Materials and Methods Adduct spots were excised and extracted from PEI-plates, dissolved and injected Standards were obtained from *in vitro* incubations as described (Stiborová et al 1994) For clarity, HPLC profiles are shown in arbitrary units

As shown in Table 2 the formation of AA-DNA adducts had an absolute requirement for NADPH, a known cofactor of NADPH : CYP reductase. Adduct levels were negligible when NADPH was omitted from the incubation mixture

**Table 2.** The effect of enzyme cofactors on the AA-DNA adduct formation in rat hepatic microsomes

| Cofactor (1 mmol $l^{-1}$) | RAL (mean ± S E M /$10^7$ nucleotides) | | | | |
|---|---|---|---|---|---|
| | AAI | | | AAII | |
| | dG-AAI | dA-AAI | dA-AAII | dG-AAII | dA-AAII |
| None | 0 01 ± 0 001 (0 17%) | 0 04 ± 0 005 (0 37%) | 0 003 ± 0 001 (0 36%) | 0 001 ± 0 0005 (0 20%) | 0 005 ± 0 001 (0 31%) |
| NADPH | 5 98 ± 1 04 (100%) | 10 64 ± 1 76 (100%) | 0 83 ± 0 05 (100%) | 0 50 ± 0 15 (100%) | 1 58 ± 0 18 (100%) |
| NADH | 0 31 ± 0 04 (5 2%) | 1 01 ± 0 08 (9 5%) | 0 08 ± 0 01 (9 6%) | 0 06 ± 0 01 (12 0%) | 0 19 ± 0 02 (12 0%) |

Numbers are averages ± S E M ($n$ = 6) of triplicate *in vitro* incubations, each DNA sample was determined by two post-labeled analyses Percentages of RAL of adducts in incubations containing 1 mmol $l^{-1}$ NADPH are shown in brackets

**Table 3.** The effect of NADPH CYP reductase inhibitor, α-lipoic acid, on the AA-DNA adduct formation by rat hepatic microsomes

| AA-DNA adducts | RAL (mean ± S E M /$10^7$ nucleotides) | | | |
|---|---|---|---|---|
| | without α-lipoic acid | in the presence of α-lipoic acid (mmol $l^{-1}$) | | |
| | | 1 6 | 3 2 | 5 0 |
| AAI adducts | 17 45 ± 1 97 | 18 78 ± 1 90 | 14 75 ± 0 62 | 10 02 ± 0 51 |
| AAII adducts | 2 08 ± 0 20 | 2 95 ± 0 22 | 1 85 ± 0 20 | 1 02 ± 0 20 |
| Total | 19 53 ± 2 02 | 21 73 ± 1 99 | 16 60 ± 0 70 | 11 04 ± 0 53 |

Numbers are averages ± S E M ($n$ = 6) of triplicate *in vitro* incubations, each DNA sample was determined by two post-labeled analyses

NADH, a cofactor of the microsomal NADH : cytochrome $b_5$ reductase, was a much less efficient cofactor than NADPH. These results indicate a minor role of NADH : cytochrome $b_5$ reductase in AA activation, while NADPH : CYP reductase might be responsible for this activation. Indeed, an inhibitor of NADPH : CYP reductase, α-lipoic acid (Slepneva et al. 1995), inhibited AA-DNA adduct formation by microsomes in a dose dependent manner The significant inhibition was found when a 10-fold molar excess of this inhibitor over AAs was used (Table 3).

To confirm the role of NADPH : CYP reductase in the activation of AAs, this enzyme was purified and used in incubations The efficiency of the enzyme was studied using two different protocols. In the first one, the enzyme together with liposomes mimicking the conditions of a microsomal membrane, were added to the incubations. In the other protocol, NADPH : CYP reductase alone, without liposomes, was utilized. Fig. 4 shows that incubations of AAs with DNA, purified NADPH . CYP reductase together with liposomes and its cofactor, NADPH, re-

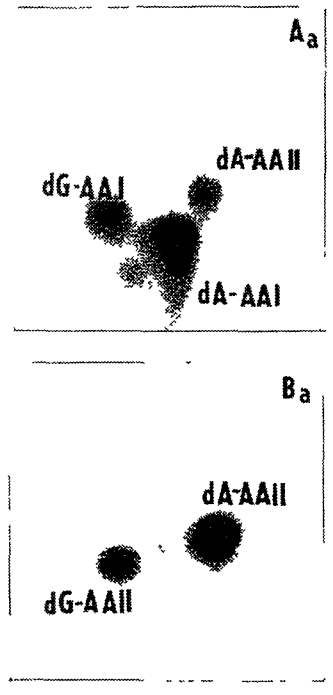


**Figure 4.** Autoradiographic profiles of AAI- ($\mathbf{A}_a$) and AAII-DNA ($\mathbf{B}_a$) adducts obtained from DNA after activation by purified NADPH CYP reductase The nuclease P1-enrichment procedure was used for analysis Origins, in the bottom left-hand corner were cut off before exposure Screen enhanced autoradiography was at −80 °C for 1 h Chromatographic conditions D1, 1 mol $l^{-1}$ sodium phosphate, pH 6 8, D2 was omitted, D3, 3 5 mol $l^{-1}$ lithium formate, 8 5 mol $l^{-1}$ urea, pH 4 0, D4, 0 8 mol $l^{-1}$ LiCl, 0 5 mol $l^{-1}$ Tris-HCl, 8 5 mol $l^{-1}$ urea, pH 9 1, D5, 1 7 mol $l^{-1}$ $NaH_2PO_4$, pH 6 0 Spot 1, dG-AAI, spot 2, dA-AAI, spot 3, dA-AAII, spot 4, dG-AAII

**Table 4.** The effect of the liposome components on AA-DNA adduct formation by 125 pmol purified NADPH CYP reductase

| Mixture | RAL (mean ± S E M /$10^7$ nucleotides) Total levels of DNA adducts of | |
|---|---|---|
| | AAI | AAII |
| Complete | 12 70 ± 0 99 | 3 07 ± 0 29 |
| without liposomes | 13 83 ± 1 18 | 3 42 ± 0 35 |
| without CHAPS | 11 75 ± 0 92 | 3 53 ± 0 31 |
| without glutathione | 13 76 ± 1 21 | 3 11 ± 0 33 |
| without liposomes, CHAPS and glutathione | 13 01 ± 1 32 | 3 37 ± 0 39 |

Numbers are averages ± S E M ($n$ = 6) of triplicate *in vitro* incubations, each DNA sample was determined by two post-labeled analyses Mixture used for testing the activity of NADPH CYP reductase to activate AA contained NADPH CYP reductase, liposomes, CHAPS and reduced glutathione in concentrations as described in Materials and Methods

sulted in the formation of the same pattern of DNA adducts as that determined in either microsomes (present paper) or *in vivo* (Stiborová et al 1994, 1999a, Schmeiser et al. 1996; Bieler et al. 1997; Nortier et al. 2000). Neither omitting

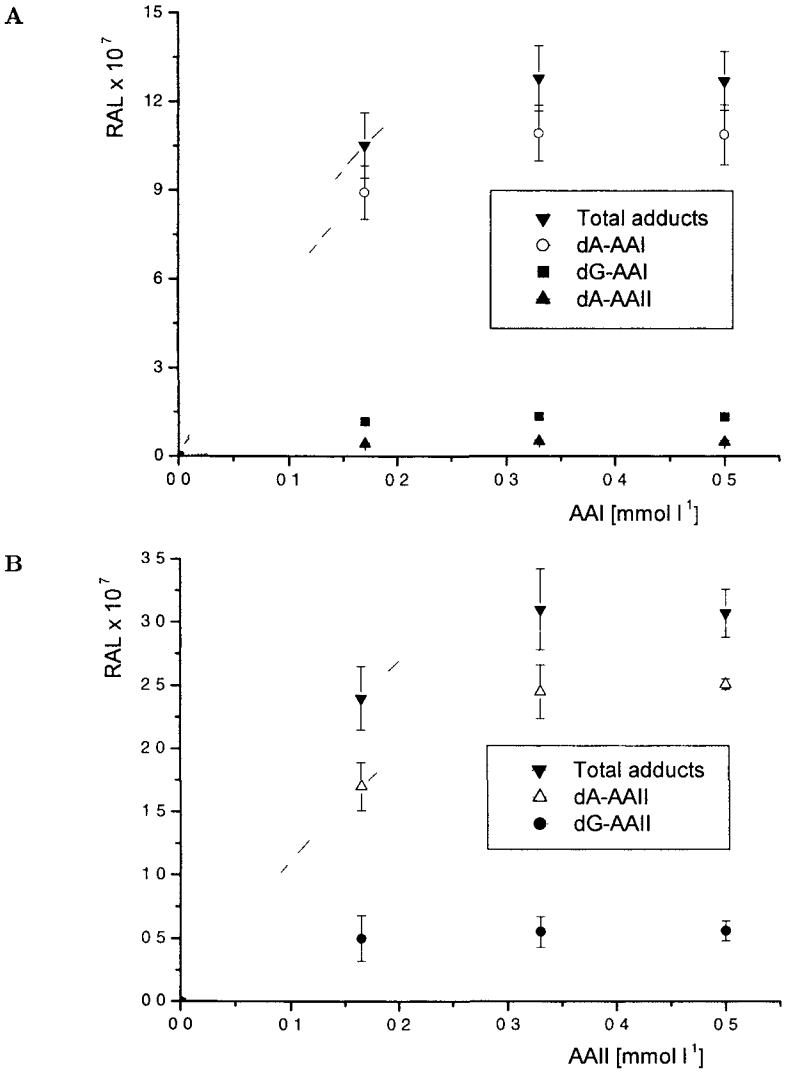


**Figure 5.** Dose-response curves for AAI (**A**) and AAII-DNA adduct (**B**) formation by NADPH CYP reductase dG-AAI (■), dA-AAI (○) dG-AAII (●), dA-AAII (△) and total adducts (▼) Experimental details are described in Materials and Methods

liposomes, nor other components of the liposome system (CHAPS, reduced glutathione) changed the efficiency of the enzyme to form AA-DNA adducts (Table 4) NADPH CYP reductase-mediated DNA adduct formation was shown to be dependent on incubation time, being linear up to 60 min of incubation (not shown), and on the concentration of AAI and AAII (Fig 5) The concentrations required for

**Table 5** Specific activities of NADPH CYP reductase in rat renal and hepatic microsomes

| Inducer | Specific activity of NADPH CYP reductase* in liver microsomes | kidney microsomes |
|---|---|---|
| None | 0 21 ± 0 01 | 0 060 ± 0 01 |
| AAI | 0 22 ± 0 01 | 0 057 ± 0 01 |
| $AA_M$ | 0 21 ± 0 01 | 0 052 ± 0 01 |
| Phenobarbital | 0 44 ± 0 05 | n d |

The activity of NADPH CYP reductase and the content of protein in cytosols of rats ($n$ = 6) were determined as described in Materials and Methods For details of pre treatment of rats see the Materials and Methods section Values are averages ± S E M of triplicate measurements of the enzyme activity in cytosols pooled from six rats *$\mu$mol reduced cytochrome $c$ $min^{-1}$ *per* mg protein n d , not determined

half-maximum DNA binding equal to 0 12 and 0 19 mmol $l^{-1}$ for AAI and AAII, respectively

Levels and activities of NADPH CYP reductase in several tissues in organisms including man are increased by some inducers (Wang et al 1997, Schuetz et al 2000) We investigated whether the tested AAs influenced the activity of the enzyme when rats were treated with AAI and the natural mixture of both AAs before isolation of microsomes from livers and kidneys While the treatment of rats with phenobarbital, a known NADPH CYP reductase inducer, led to an increase of the enzyme activity, the treatment of rats with the studied alkaloids had essentially no effect on the activity of the enzyme (Table 5)

## Discussion

The detection and quantitation of specific DNA adducts by the $^{32}$P-postlabeling procedure have proved to be a useful tool to monitor the exposure to the plant carcinogen AA *in vivo* (Fernando et al 1993, Stiborova et al 1994, 1999a, Schmeiser et al 1996, Bieler et al 1997, Nortier et al 2000) Based on the structures of the adducts identified, we reported that nitro reduction of AAI and AAII to the corresponding aristolactams I and II is the main activating pathway in animals (Stiborova et al 1994) and humans (Schmeiser et al 1996, Bieler et al 1997, Stiborova et al 1999a, Nortier et al 2000) As shown in Figure 1, an intermediate cyclic nitrenium ion with a delocalized positive charge was postulated by us as the ultimate electrophilic species binding to DNA *via* the C7 to the exocyclic amino groups of deoxyguanosine and deoxyadenosine

Recently, specific AA-DNA adducts were found to be associated with a unique nephropathy, CHN, and urothelial cancer in women who had followed a weight reducing treatment consisting of Chinese herbs containing AA Not all participants in the slimming procedure are affected by CHN Differences in carcinogen activation could be the reason for individual susceptibility

In mammalian tissues, both cytosol and microsomes contain enzymes catalyzing the reduction of nitro aromatic compounds (Fu 1990; Chae et al. 1999; Ritter et al. 2000). We have already identified microsomes from rat liver, which generate AAI- and AAII-DNA adduct profiles very similar or identical to the profiles found in the target tissue, as the most appropriate activation system *in vitro* (Schmeiser et al. 1997). The results of the present study clearly demonstrate that AAs are bioactivated also by microsomes from rat kidney forming deoxyguanosine and deoxyadenosine adducts identical to those found in humans exposed to AA (Schmeiser et al. 1996; Bieler et al. 1997). Herein, we show that the reductive formation of AA-DNA adducts is strongly dependent on the catalytic activities of NADPH : CYP reductase present in microsomes. Moreover, its efficiency in *in vitro* AA-DNA adduct formation was corroborated using purified NADPH : CYP reductase. The results presented in the paper indicate that the enzyme is capable of passing electrons to AA without anchoring its protein molecule to liposomes, which mimic a membrane of endoplasmic reticulum. This fact has already been observed with other non-physiological electron acceptors of the enzyme (e.g., cytochrome *c*) (Wang et al. 1997). These similarities suggest that the binding of AA to the enzyme surface seems to be situated in the binding site for non-physiological substrates which is located close to the NADPH binding site at the opposite side of the N-terminus of the NADPH : CYP reductase molecule (Wang et al. 1997).

Soluble enzymes with reducing activity such as buttermilk XO are known to be effective nitroreductases activating several nitro-aromatics including AAs (Fu 1990; Pfau et al. 1990; Schmeiser et al. 1997; Ritter et al. 2000). Other cytosolic soluble reductases (D,T-diaphorase, aldehyde oxidase) are also enzymes metabolizing various nitro-aromatics (Fu 1990; Ritter et al. 2000). While their contributions to the nitroreductive metabolism of AA have not been examined, the present study strongly suggests a significant role of microsomal NADPH : CYP reductase in the nitroreduction of AA. Although we did not detect any increase in the activity of this reductase in kidneys and livers of rats pre-treated with AAI or the natural mixture of both AAs (see Table 5), its activity might be affected by some components of the slimming regimen, other than AA, which were ingested by CHN patients in whose kidneys or ureters AA-DNA adducts were found [i.e. meprobamate (Violon 1997) and other barbiturates (see Table 5) as NADPH : CYP reductase inducers or other herbal drugs with yet unknown effects]. Another factor causing variability of the activities and levels of NADPH : CYP reductase is a variation in hormonal levels in organisms including human (Schuetz et al. 2000). Taken together, the variability of NADPH : CYP reductase levels and its activity might play a role in determining risk to the carcinogenicity of AA for participants in the slimming cure.

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