

³²P-post-labelling analysis of DNA adducts formed by aristolochic acid in tissues from patients with Chinese herbs nephropathy

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Recently, we reported that aristolochic acid (AA) a naturally occurring nephrotoxin and carcinogen is implicated in a unique type of renal fibrosis, designated Chinese herbs nephropathy (CHN). Indeed, we identified the principal aristolochic acid-DNA adduct in the kidney of five such patients. We now extend these observations and demonstrate the presence of additional AA-DNA adducts by the ³²P-post-labelling method not only in the kidneys, but also in a ureter obtained after renal transplantation. Using the nuclease P1 version of the assay not only the major DNA adduct of aristolochic acid, 7-(deoxyadenosin-N⁶-yl)-aristolactam I (dA-AAI), but also the minor adducts, 7-(deoxyguanosin-N²-yl)-aristolactam I (dG-AAI) and 7-(deoxyadenosin-N⁶-yl)-aristolactam II (dA-AAII) were detected, and identified by cochromatographic analyses with TLC and HPLC. Quantitative analyses of six kidneys revealed relative adduct levels from 0.7 to 5.3/10⁷ for dA-AAI, from 0.02 to 0.12/10⁷ for dG-AAI and 0.06 to 0.24/10⁷ nucleotides for dA-AAII. The detection of the dA-AAII adduct is consistent with the occurrence of aristolochic acid II (AAII) in the herb powder imported under the name of *Stephania tetrandra* and confirms that the patients had indeed ingested the natural mixture of AAI and AAI. ³²P-post-labelling analyses of further biopsy samples of one patient showed the known adduct pattern of AA exposure not only in the kidney, but also in the ureter, whereas in skin and muscle tissue no adduct spots were detectable. In an attempt to explain the higher level of the dA-AAI adduct compared to the dG-AAI adduct level in renal tissue even 44 months after the end of regimen, the persistence of these two purine adducts was investigated in the kidney of rats given a single oral dose of pure AAI. In contrast to the dG-AAI adduct, the dA-AAI adduct exhibited a lifelong persistence in the kidney of rats. Our data demonstrate that AA forms DNA adducts in human tissue by the same activation mechanism(s) reported from animal studies. Thus, the carcinogenic/mutagenic activity of AA observed

***Abbreviations:** CHN, Chinese herbs nephropathy; AA, aristolochic acid; AAI, aristolochic acid I; AAI, aristolochic acid II; dA-AAI, 7-(deoxyadenosin-N⁶-yl)-aristolactam I; dG-AAI, 7-(deoxyguanosin-N²-yl)-aristolactam I; dA-AAII, 7-(deoxyadenosin-N⁶-yl)-aristolactam II; XO, xanthine oxidase; dAp, deoxyadenosine-3'-monophosphate; dGp, deoxyguanosine-3'-monophosphate; PEI-cellulose, polyethyleneimine-cellulose; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; RAL, relative adduct labelling.

in animals could also be responsible for the urothelial cancers observed in two of the CHN patients.

Introduction

Chinese herbs nephropathy (CHN*) is a recently described subacute interstitial disease associated with the prolonged use of Chinese herbs during a slimming cure. Over 80 patients have been identified, half of whom needing renal replacement therapy including renal transplantation (1–4). The absence in the delivered capsules of one of the prescribed Chinese herbs (*Stephania tetrandra*) taken together with the identification of aristolochic acid (AA), the major alkaloid of *Aristolochia* species, in 11 out of 12 different samples of herb powders imported in Belgium under the name of *Stephania tetrandra* (5) led to the suggestion that *Stephania tetrandra* had been replaced accidentally by *Aristolochia fangchi*, another Chinese herb. However, AA had not been identified initially in the capsules delivered to the patients probably as a result of an inadequate extraction procedure (5).

The active principle extracted from *Aristolochia* plants is aristolochic acid (AA). AA is a mixture consisting of structurally related nitrophenanthrene carboxylic acids, AAI and AAI being the major components. AA has been shown to stimulate various defense mechanisms and to exert antiviral, antibacterial and even anti-neoplastic properties in certain animal models (6–8). High doses of AA are nephrotoxic in animals (9) and in humans (10) prohibiting its use in cancer therapy (11). In 1982 the natural mixture AA was shown to be a strong carcinogen in rodents (12,13). Its carcinogenic and mutagenic effects (14,15) were found to be associated with the formation of AA DNA adducts. The main DNA adducts formed by AA *in vitro* were identified by us as the aristolactam structures bound to the exocyclic amino groups of deoxyguanosine and deoxyadenosine (16,17).

Recently, we demonstrated that AA had indeed been ingested by the CHN patients: we identified the presence of one AA-DNA adduct [7-(deoxyadenosin-N⁶-yl)-aristolactam I (dA-AAI)] in the kidneys of five patients by ³²P-post-labelling (18). We now extend these observations in eight kidneys from six patients by the identification of two additional AA-DNA adducts whose structure is elucidated. Furthermore, we demonstrate the presence of these three adducts in the ureter, but not in the muscle or skin of a patient with CHN. Finally, we conclude from an animal study that each AA adduct has its own kinetic characteristics accounting for the fact that only one adduct remains in tissues for an extensive period of time.

Material and methods

Nephroureterectomy specimens

Eight native kidneys were taken from six patients with documented CHN undergoing transplantation and treated as described (18). Corticomedullary, skin and muscle tissue samples, and the right ureter removed from one patient (case 6) were treated in the same manner. Similarly, corticomedullary samples

obtained from end-stage kidneys removed from six patients at the time of transplantation were used as controls as reported before (18).

Identification of AAI and AAI in herbal powders

Herbal powders (170 mg) delivered in Belgium under the name of *S.tetrandra* were extracted by treatment with 5 ml methanol. The methanolic extract was concentrated to 2.5 ml and aliquots (50 or 100 µl) were analysed by high performance liquid chromatography (HPLC). Separation was performed on a reversed phase column (Beckman Ultrasphere ODS RP18, 5 µm) using a linear gradient from 5 to 50% acetonitrile in 0.1 M triethylammoniumacetate, pH 7.0, within 45 min at a flow rate of 1 ml/min. Retention times of AAI (30.0 min) and AAI (28.2 min) were determined with aristolochic acid purchased from Sigma.

Animal experiments

Male Wistar rats (200–240 g) were given a single oral dose (5 mg/kg body weight) of AAI as the sodium salt in water. Control animals received only water. Animals (three rats per group) were killed 1 day and 1, 2, 4, 16 and 36 weeks after treatment. Organs were removed and stored at –80°C until DNA isolation.

³²P-post-labelling analysis

The method has been described elsewhere (19). DNA was isolated by phenol extraction. DNA samples (12.5 µg) were digested, enriched and analysed by ³²P-post-labelling as described (20). Adducts and normal nucleotides were detected by autoradiography and spots were excised from the chromatograms for Cerenkov counting. Count rates of adducted fractions were determined from triplicate maps after subtraction of count rates from adjacent blank areas. Excess [γ -³²P]ATP after the post-labelling reaction was confirmed. Adduct levels were calculated in units of relative adduct labelling (RAL) as reported before (19).

Preparation of reference compounds

dAp-AAI, dGp-AAI and dAp-AAII were prepared by incubations with the 3'-monophosphates deoxyadenosine-3'-monophosphate (dAp) or deoxyguanosine-3'-monophosphate (dGp) and AAI or AAI activated by xanthine oxidase as described (20). Twenty-microlitre aliquots of the incubations were directly used for the *n*-butanol extraction-mediated ³²P-post-labelling method described by Gupta and Earley (21). TLC and quantitation of adducts were performed as described (20).

Co-chromatography on PEI-cellulose

Adduct spots detected by the ³²P-post-labelling assay were excised from the thin layer plates, extracted and co-chromatographed with reference 3',5' bisphosphate adducts as reported previously (20).

HPLC analysis of ³²P-labelled 3',5'-deoxyribonucleoside bisphosphate adducts

HPLC analysis was performed essentially as described (20). Individual labelled adduct spots were excised and extracted. The dried extracts were redissolved in 100 µl methanol/phosphate buffer, pH 3.5, 1:1 (v/v). Aliquots (50 µl) were analysed on a phenyl-modified reversed-phase column (250×4.6 mm, 5 µm Zorbax Phenyl; Säulentechnik Dr Knauer, Berlin, Germany) with a linear gradient of methanol (from 40 to 80% in 45 min) in aqueous 0.5 M sodium phosphate and 0.5 M 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 HPLC radioactivity monitor (500 µl cell, dwell time 6 s).

Results

Detection of AAI and AAI in herbal powders

Three different samples of herb powders delivered in Belgium under the name of *S.tetrandra* were analysed for the presence of aristolochic acids (22). Similar batches have been used for the preparation of capsules (in reference 1 called 'formula 2 pills') prescribed during the slimming cure (18). Two of the three samples contained AAI and AAI in amounts of approximately 2 mg/g and 0.2 mg/g, respectively. Traces of the corresponding aristolactams were also detectable (data not shown).

DNA adduct detection

The renal samples from six patients with CHN revealed identical adduct patterns consisting of one major spot (spot 1)

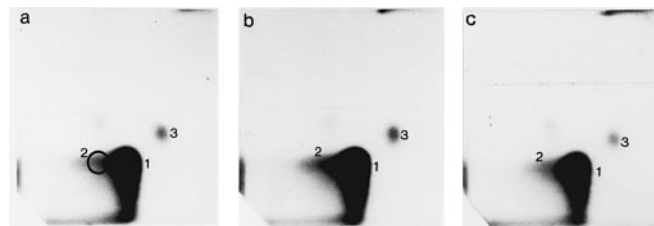


Fig. 1. Autoradiograms of ³²P-post-labelling analyses (nuclease P1-enrichment) of DNA adducts in renal cortical tissue from CHN patients: (a) case 4; (b) case 2, right kidney; (c) case 3, left kidney; from Table I. Spot 1 = dA-AAI; spot 2 = dG-AAI; spot 3 = dA-AAII. Film exposure 20 h at room temperature. Origins are in the bottom left-hand corner. Chromatographic conditions: D1, 1 M sodium phosphate, pH 6.8; D3, 3.5 M lithium formate, 8.5 M urea, pH 3.5; D4, 0.8 M LiCl, 0.5 M Tris-HCl, 8.5 M urea, pH 9.1; D5, 1.7 M NaH₂PO₄, pH 6.0.

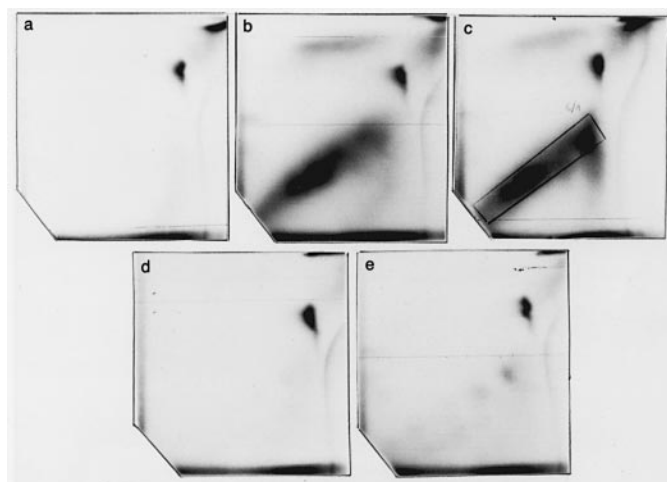


Fig. 2. Autoradiograms of ³²P-post-labelling analyses (nuclease P1-enrichment) of DNA adducts in renal corticomedullary tissue from patients with: (a) membranoproliferative glomerulonephritis 12 years/F; (b) chronic interstitial nephritis 27 years/F, smoking status not known, RAL: 1.22×10^{-7} ; (c) reflux nephropathy 41 years/F, smoker (1 pack per day for 15 years until June 1993, tissue taken in July 1994), RAL: 1.18×10^{-7} ; (d) cystic nephropathy 10 years/F; (e) nephrosclerosis 38 years/M. Film exposure 20 h at room temperature. Origins are in the bottom left-hand corner. Chromatographic conditions as in Figure 1. One case with lupus nephritis has been reported previously (18).

and two minor spots (spots 2 and 3) illustrated in Figure 1. Details on the major spot have been presented before for five patients (18). The two minor spots were detectable only after prolonged exposure time. The levels of the three DNA adducts ranged from 0.7 to 5.3 /10⁷ nucleotides for spot 1, from 0.02 to 0.12/10⁷ for spot 2 and from 0.06 to 0.24/10⁷ for spot 3. By contrast, DNA isolated from the kidneys of patients with several other renal diseases was virtually free of spots in the area where adducts associated with CHN were located (Figure 2). In two patients (Figure 2b and c) a diffuse band of radioactivity, the typical smoking-related adduct pattern (23), known as diagonal radioactive zone (DRZ) with total adduct levels of $\sim 1/10^7$ nucleotides was observed.

DNA adduct identification

It was clear from the cochromatographic analysis on polyethyleneimine (PEI) plates shown in Figure 3 that adduct spots 1, 2 and 3 obtained from human kidneys were chromatographically indistinguishable from reference adduct spots identified previously as AAI- and AAI-modified nucleosides. Thus, spot 1 was assigned as 3',5'-bisphospho-7-(deoxyadenosin-N⁶-yl)-

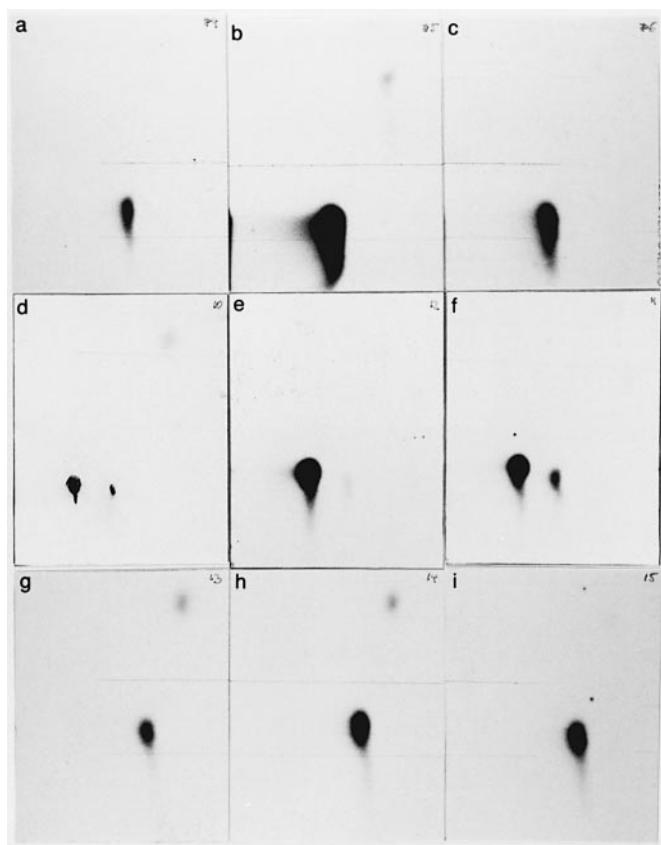


Fig. 3. Autoradiographs of PEI-cellulose TLC maps of eluted ^{32}P -postlabelled adducts obtained from digests of human kidney DNA and incubations of AAI or AAI with calf thymus DNA activated by xanthine oxidase. Adduct spots were excised and eluted from TLC plates and equal amounts of radioactivity were spotted and chromatographed in D3 and D4 directions. (a) Spot 1 from Figure 1a; (b) equal amounts of a and c; (c) dA-AAI standard; (d) spot 2 from Figure 1b; (e) equal amounts of d and f; (f) dG-AAI standard; (g) spot 3 from Figure 1b; (h) equal amounts of g and i; (i) dA-AAII standard. Film exposure was for 20 h at -80°C . Origins are in the bottom left-hand corner. Chromatographic conditions: D3, 3.5 M lithium formate, 8.5 M urea, pH 3.5; D4, 0.8 M LiCl, 0.5 M Tris-HCl, 8.5 M urea, pH 9.1.

aristolactam I (dA-AAI), spot 2 as 3',5'-bisphospho-7-(deoxyguanosin- N^2 -yl)-aristolactam I (dG-AAI) and spot 3 as 3',5'-bisphospho-7-(deoxyadenosin- N^6 -yl)-aristolactam II (dA-AAII).

As a second, independent chromatographic procedure to confirm identities of adduct spots we employed reversed-phase HPLC analysis. The results thus obtained confirmed findings produced by cochromatography on PEI plates. Results for spot 1 and the dA-AAI standard have been previously reported (18). Spot 3 eluted with a retention time of 18.3 min, identical to the dA-AAII standard (Figure 4a and b). When equal amounts of radioactivity from spot 3 and the reference compound dA-AAII were mixed prior to analysis a single spot was found (Figure 4c). Spot 2 eluted as three peaks one of them with the identical retention time of the dG-AAI standard (17.1 min) and one with the retention time of the dA-AAI standard (22.5 min). The compound eluting with a retention time of 13.5 min is unknown (Figure 4d,e). When the spot 2 sample was spiked with the dG-AAI standard only the peak at 17.1 min increased (Figure 4f).

Quantitative analysis of the three identified AA-DNA adducts in renal tissue from six CHN patients (Table I) revealed

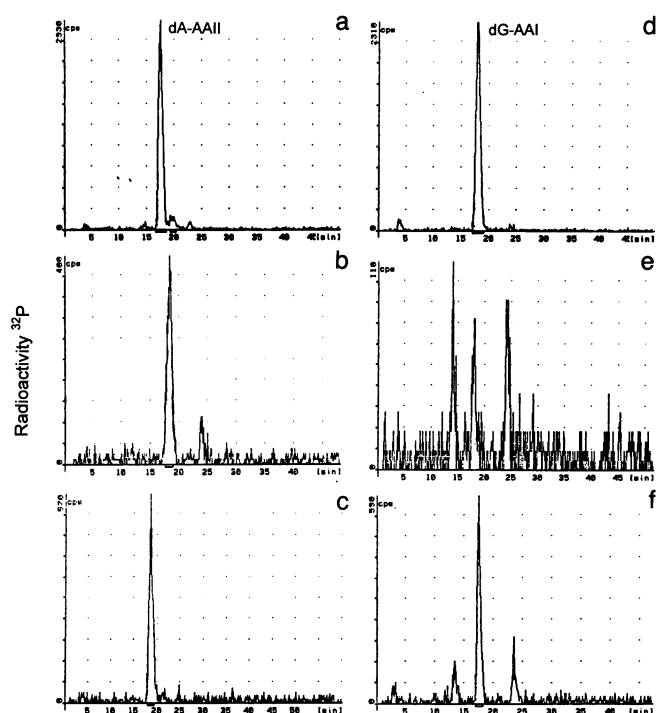


Fig. 4. Separation of ^{32}P -labelled nucleoside 3',5'-bisphosphate adducts on a phenyl-modified reversed-phase column. Chromatographic conditions are described in Materials and methods. Adduct spots were excised and extracted from PEI-plates, dissolved and injected. (a) dA-AAII standard from *in vitro* incubation of AAI, dAp and xanthine oxidase (XO); (b) spot 3 from Figure 1; (c) equal amounts of radioactivity of a and b mixed prior to analysis; (d) dG-AAI standard from *in vitro* incubation of AAI, dGp and XO; (e) spot 2 from Figure 1; (f) e spiked with dG-AAI standard.

that the previously described dA-AAI adduct had the highest concentration, followed by the dA-AAII adduct and the dG-AAI adduct. All three adducts were detectable in the kidneys up to 44 months after the interruption of the incriminated formula 2 pills.

Additional tissue samples of the ureter, muscle and skin, obtained in case 6 during transplantation, were analysed by ^{32}P -post-labelling. In the ureter the characteristic adduct pattern of AA was found (Figure 5), whereas muscle and skin samples exhibited no AA specific adduct spots (data not shown). RAL values for the adducts in the ureter were in the same range as for the kidneys with dA-AAI being the major adduct.

Persistence of dA-AAI in renal tissue of rats

The stability of dA-AAI and dG-AAI in rat kidney was examined up to 36 weeks after a single oral dose (5 mg/kg body weight) of pure AAI (Figure 6) as described before for other organs like forestomach, glandular stomach, lung, liver and urinary bladder (24). Both purine AAI-DNA adducts remained detectable in the kidney for up to 36 weeks. After a rapid decrease of dA-AAI and dG-AAI levels during the first 2 weeks (~50% of the adducts observed at day 1 are removed), dG-AAI adducts continued to disappear, whereas dA-AAI levels (25% of the adducts observed at day 1) remained practically unchanged between 4 and 36 weeks (Figure 6). Similar removal curves with a triphasic profile have been reported for the target organ forestomach and the non-target organs liver and lung (24).

Table I. Clinical data and quantitative analysis of AA-DNA adducts in kidney tissue of patients with CHN

Case	Age/ sex	Duration of formula 2 regimen [months]	Interval from regimen end to kidney removal [months]	RAL ^a in renal tissue ^b (mean \pm SD / 10^7 nucleotides) ^c		
				dA-AAI ^d	dG-AAI	dA-AAII
1	32/F	19	right, 9	1.7 \pm 0.9	0.050 ^e	0.24 \pm 0.12
2	28/F	13	left, 17	2.3 \pm 1.2	0.073 \pm 0.024	0.14 \pm 0.04
3 ^f	27/F	20	right, 20	5.3 \pm 3.2	0.120 \pm 0.053	0.17 \pm 0.09
			left, 15	4.1 \pm 2.7	0.090 \pm 0.063	0.13 \pm 0.08
4	42/F	21	right, 27	3.0 \pm 1.8	n.d.	0.16
			right, 18	2.5 \pm 2.1	0.032 \pm 0.020	0.06 \pm 0.03
5	42/F	23	right, 11	0.7 \pm 0.5	0.020 ^e	0.10 \pm 0.08
6	56/F	19	right, 44	0.7 ^g \pm 0.3	0.060 \pm 0.012	0.19 \pm 0.04

^aRelative adduct labelling; ^bcortical tissue; ^cmean of at least three determinations in separate experiments; ^ddA-AAI levels for cases 1–5 have been published before (18); ^eonly one determination; ^fcase with bladder transitional cell carcinoma; ^gcorticomedullary tissue; n.d., not determined. RAL values of the dG-AAI adduct are corrected to one-third of the radioactivity due to the contamination with dA-AAI adduct and an unknown compound found in all HPLC determinations (as shown in Figure 4e).

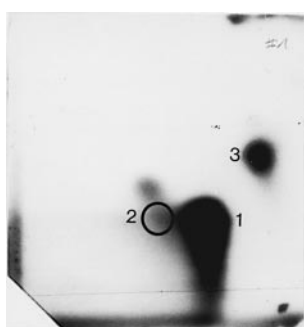


Fig. 5. ³²P-post-labelling analysis (nuclease P1-enrichment) of DNA adducts in the ureter of case 6 from Table I. Spot 1, dA-AAI, RAL: $0.71 \pm 0.45 \times 10^{-7}$; spot 2, dG-AAI, RAL: 0.07×10^{-7} ; spot 3, dA-AAII, RAL: $0.2 \pm 0.09 \times 10^{-7}$. Film exposure 20 h at room temperature. Origins are in the bottom left-hand corner. Chromatographic conditions: D1, 1 M sodium phosphate, pH 6.8; D3, 3.5 M lithium formate, 8.5 M urea, pH 3.5; D4, 0.8 M LiCl, 0.5 M Tris-HCl, 8.5 M urea, pH 9.1; D5, 1.7 M NaH₂PO₄, pH 6.0.

Discussion

Our results confirm that patients with CHN have ingested AA and demonstrate that this was under both forms of aristolochic acid, AAI and AAII. The compound provided was thus the natural mixture similar to that we have recognized in herbal powders. ³²P-post-labelling analyses of all six patients with CHN displayed identical adduct patterns, characteristic for AA exposure, in the kidneys and in one ureter. By contrast, ³²P-post-labelling analyses of renal tissues obtained from six patients with renal failure of other causes did not show such patterns. In two patients the complex pattern of adduct spots and areas of unresolved radioactivity, typical for smoking-related DNA adducts, was observed. At inquiry one patient (Figure 2c) was identified as a heavy smoker (1 pack per day for 15 years). It is worth mentioning that no smoking related adduct spots were detectable in samples of three CHN patients known to have been heavy smokers before renal transplantation (Figure 1c shows case 3; 15 cigarettes per day from 1986 to 1993).

The persistence of all three AA-DNA adducts in human tissues, even 44 months after the interruption of formula 2 pills, both in kidney and ureter is noteworthy. AA-DNA adducts are thus highly suitable for monitoring exposure to AA even years later. Highest adduct levels in human tissues were found for the dA-AAI adduct consistent with previous

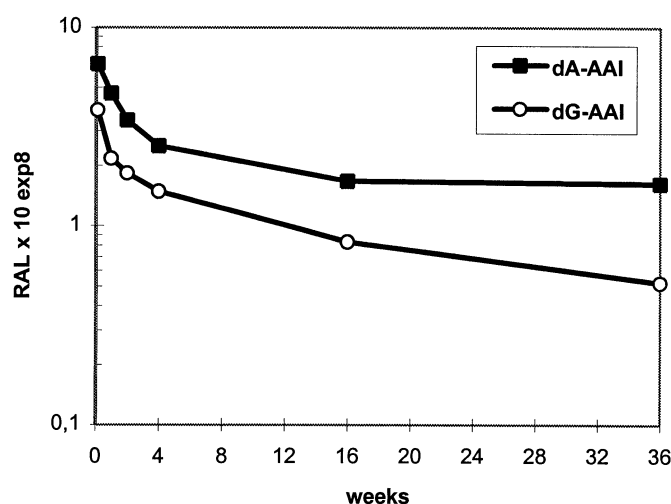


Fig. 6. Time course of dA-AAI (■) and dG-AAI (○) adduct levels in the kidney of the rat. Male Wistar rats were administered orally by gavage with a single dose of AAI (5 mg/kg body wt). Animals were killed 1 day and 1, 2, 4, 16 and 36 weeks post-treatment. ³²P-post-labelling analysis was performed using the nuclease P1-enrichment. Data are means of three triplicate DNA analyses. Standard deviation of the mean in all cases <10 %.

reports on rats given pure AAI or the natural mixture AA (20,25). Irrespective of the tissue analysed in those animal studies dA-AAI was always the predominant adduct.

The persistence of two purine DNA adducts of AAI in several rat organs has been previously investigated (24). Since the kidney was not included in this former study we here present the long-term persistence of the dA-AAI and dG-AAI adducts in this organ. The differential rates of disappearance of dG-AAI adducts versus dA-AAI adducts show that removal kinetics in the third phase (16–36 weeks) are not solely a function of cell turnover. Such triphasic decline characteristics have been reported for other carcinogens, including 7,12-dimethylbenz[*a*]anthracene (26), benzo[*a*]pyrene (27), aflatoxin B₁ (28), 2-acetylaminofluorene (29), N-hydroxy-2-acetylaminofluorene, N-hydroxy-4-acetylaminobiphenyl, N-hydroxy-2-acetylaminophenanthrene (30) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (31). An important, as yet open question is the apparent life-long persistence of specific adducts in particular organs. A possible explanation of this phenomenon is that these lesions reside in the genome of an essentially dormant subpopulation of tissue cells (32). This suggests that

carcinogen exposure itself may have caused mitosis inhibition and/or growth arrest of this subpopulation. Inhibition of DNA synthesis by carcinogens is a well-known concept (33,34). Kaden *et al.* (35) suggested that in the case of aflatoxin B₁-DNA adducts human cells have a short lived repair system for these lesions, which is induced and remains functioning only when the total number of DNA adducts rises above 1000 per cell. This suggested induction threshold lies for the AA-DNA adducts in rat kidney at ~100 adducts/cell assuming that 1 adduct per 10⁸ nucleotides corresponds to 60 adducts per cell (Figure 6). Both greater persistence and greater initial DNA binding probably contributed to the higher level of the dA-AAI adduct.

Ha-*ras* protooncogenes are activated with high frequency by a A→T transversion mutation in codon 61 from CAA to CTA in the DNA of AAI-induced carcinomas in rats (36). This suggests a relevant role of the dA-AAI adducts in AA-induced mutagenesis and carcinogenesis. Additionally, *in vitro* primer extension studies with site-specifically adducted oligonucleotides containing AA-DNA adducts suggested a higher mutagenic potential of adenine adducts than of guanine adducts (22). In conclusion, we demonstrate the presence of three specific AA-DNA adducts in the kidney and the ureter of patients with CHN, thus establishing that AA might be implicated in the renal fibrotic process as well as in the urothelial atypias and cancers observed in CHN (3,37,38).

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