

## QUANTITATIVE DETERMINATION OF ARISTOLOCHIC ACID-DERIVED DNA ADDUCTS IN RATS USING $^{32}\text{P}$ -POSTLABELING/POLYACRYLAMIDE GEL ELECTROPHORESIS ANALYSIS

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### ABSTRACT:

Aristolochic acids (AA) are nephrotoxic and carcinogenic nitroaromatic compounds produced by the *Aristolochiaceae* family of plants. Ingestion of these phytotoxins by humans results in a syndrome known as AA nephropathy, characterized by renal tubulointerstitial fibrosis and upper urothelial cancer. After activation by cellular enzymes, AA I and II react with DNA to form covalent adducts and as such represent potential biomarkers for studies of AA toxicity. Using site-specifically modified oligodeoxynucleotides as standards, we have developed a method for quantifying 7-(deoxyadenosin- $N^6$ -yl) aristolactam-DNA or 7-(deoxyguanosin- $N^2$ -yl) aristolactam-DNA adducts in tissues of Wistar rats using an assay in which  $^{32}\text{P}$ -postlabeling techniques are coupled with nondenat-

uring polyacrylamide gel electrophoresis. The limit of detection with this technique is five adducts in  $10^9$  nucleotides for a 5- $\mu\text{g}$  DNA sample. In contrast to previous reports, we find that the levels of AA adducts in renal tissues of Wistar rats treated p.o. with AA for 1 week with 5 mg/kg/day of AA I or AA II were much higher than that in the forestomach. Highest adduct levels were observed in rats treated with AA II, suggesting that this compound may be more genotoxic than AA I. Treatment of rats with aristolactam I, an end-product of AA I metabolism, resulted in a much lower level of adduction. This study establishes the feasibility of using AA-DNA adducts as intermediate biomarkers of exposure in studies of AA nephropathy and its associated urothelial cancer.

The nephrotoxic and carcinogenic effects of aristolochic acids (AA) in animals have been established (Pohl, 1892; Dumić, 1954; Mengs et al., 1982; Mengs, 1987; IARC, 2002). Similar toxicities were observed in humans when a cluster of cases of chronic renal failure reported in Belgium was traced to the ingestion of an herbal preparation containing *Aristolochia fangchi* (Depierreux et al., 1994; Cosyns et al., 1999; Nortier et al., 2000; Arlt et al., 2002; Cosyns, 2003; Li and Wang, 2004). This syndrome, initially referred to as Chinese herb nephropathy and more recently as AA nephropathy, soon was recognized as a global health problem (IARC, 2002; Cosyns, 2003). The persistence of AA-DNA adducts in renal tissues of the Belgian women cohort is consistent with the postulated role of AA in urothelial cancer (Nortier et al., 2000).

AA includes a mixture of structurally related nitrophenanthrene carboxylic acids produced by the *Aristolochiaceae* family, with 8-methoxy-6-nitrophenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AA I) and its 8-desmethoxylated form (AA II) representing the major constituents of these plants (Kumar et al., 2003). The biotransforma-

tion of AA has been investigated in extracts prepared from human and rodent cells (Stiborova et al., 1999, 2001, 2002, 2003, 2005) and, to a limited degree, in humans and experimental animals (Krumbiegel et al., 1987). As shown in Fig. 1, AA I is metabolized along two major pathways: 1) demethylation to form AA Ia, a substrate for phase II conjugation reactions, and 2) reduction of the nitro group to form aristolactam I (L I), which in turn can be converted to L Ia and subsequently esterified (Krumbiegel et al., 1987; Arlt et al., 2002). The *N*-hydroxy intermediate formed during the reduction process is expected to form an aristolactam nitrenium ion, which in turn can give rise to an isomeric carbonium ion that reacts covalently with DNA. Microsomal enzymes, including CYP1A1 and CYP1A2 (Stiborova et al., 2001), and cytosolic enzymes, including nitroreductases, xanthine oxidase (Schmeiser et al., 1988), and NAD(P)H:quinone oxidoreductase (Stiborova et al., 2002), are believed to be involved in these reactions. The primary route of AA II metabolism appears to be the nitro reduction pathway to form L II (Arlt et al., 2002).

Treatment of animals and humans with AA results in the formation of covalent DNA adducts (Fig. 2), identified chromatographically as 7-(deoxyadenosin- $N^6$ -yl) aristolactam I (dA-AA I), 7-(deoxyguanosin- $N^2$ -yl) aristolactam I (dG-AA I), and 7-(deoxyadenosin- $N^6$ -yl) aristolactam II (dA-AA II) (Pfau et al., 1990a,b). After p.o. ingestion of AA, extensive formation of AA-DNA adducts was observed in the forestomach, accompanied by development of tumors (Fernando et

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**ABBREVIATIONS:** AA, aristolochic acid(s); AA I, aristolochic acid I; AA II, aristolochic acid II; L I, aristolactam I; dA-AA I, 7-(deoxyadenosin- $N^6$ -yl) aristolactam I; dG-AA I, 7-(deoxyguanosin- $N^2$ -yl) aristolactam I; dA-AA II, 7-(deoxyadenosin- $N^6$ -yl) aristolactam II; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight.

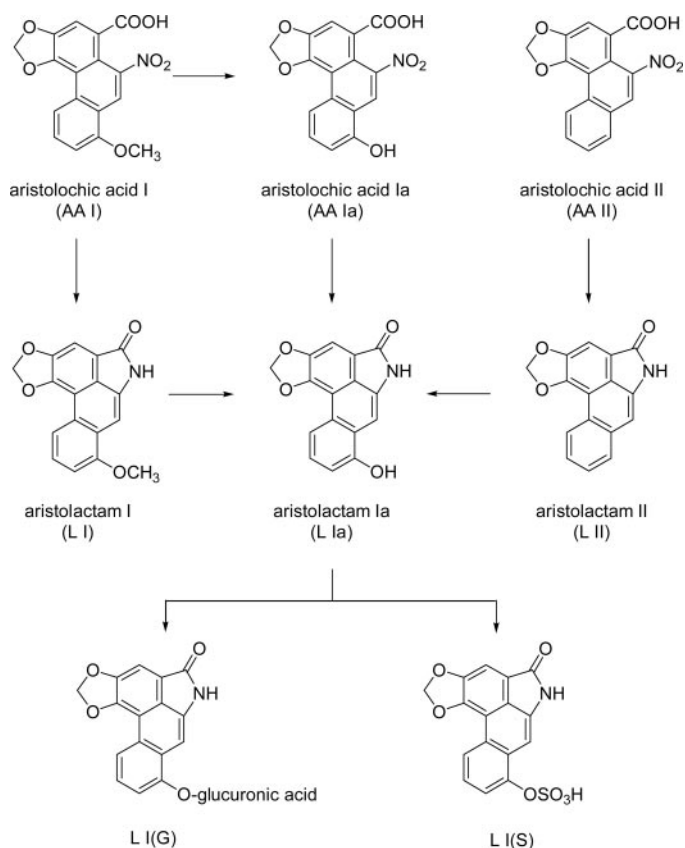


FIG. 1. Proposed pathway for biotransformation of AA I and AA II in rodents and humans.

al., 1993; Stiborova et al., 1994; Cosyns et al., 1998). The s.c. injection of rats with AA (10 mg/kg/day for 35 days) induced proximal tubule necrosis, elevated serum creatinine levels, microproteinuria, and glycosuria, resulting in interstitial renal fibrosis (Debelle et al., 2002). Acute nephrotoxicity also was observed in mice treated with AA (Sato et al., 2004). It remains unclear whether AA I and/or AA II are responsible for the nephrotoxicity and/or carcinogenicity

observed when rodents or humans ingest the naturally occurring mixture of these isomers.

AA-DNA adducts block transcription and/or DNA replication, promoting cell cycle arrest and apoptosis, and are potential substrates for nucleotide excision repair. AA-induced cancers in rats are associated with activation of *H-ras* via an A:T→T:A transversion mutation of the first adenine of codon 61 (Schmeiser et al., 1991). The observation in rats that AA induces a mutation spectrum dominated by A→T transversions (Schmeiser et al., 1990) is consistent with the preferential formation of dA-AA I adducts at adenine bases in DNA (Pfau et al., 1990b; Stiborova et al., 1994). An A:T→T:A mutation also was detected in the p53 gene of a urothelial tumor of a patient with AA nephropathy (Lord et al., 2004).

AA-DNA adducts potentially could serve as intermediate biomarkers of exposure for investigations of the mutagenic and/or carcinogenic potential of AA. Toward this end, we have developed a quantitative <sup>32</sup>P-postlabeling/nondenaturing polyacrylamide gel electrophoresis (PAGE) method and used it to quantify the level of AA-DNA adducts in tissues of Wistar rats treated with AA I and AA II. In contrast to previous reports using the same species (Pfau et al., 1990b), AA-DNA adducts are concentrated in renal tissues rather than forestomach of rats treated with AA I, and the level of DNA adducts derived from AA II in almost all the tissues was higher than those derived from AA I. Relatively few adducts were found in tissues of rats treated with L I.

#### Materials and Methods

**Materials.** Potato apyrase was purchased from Sigma-Aldrich (St. Louis, MO); micrococcal nuclease and spleen phosphodiesterase were from Worthington Biochemical Corp. (Lakewood, NJ); 3'-phosphatase-free T4 PNK was from Roche Applied Science (Indianapolis, IN); nuclease P1 was from United States Biological (Swampscott, MA); and [<sup>32</sup>P]ATP (specific activity, >6000 Ci/mmol) was from GE Healthcare Bio-Science Corp. (Piscataway, NJ).

**Preparation of AA I and AA II.** A 40:60 mixture of AA I and II was purchased from Fisher Scientific (Fairlawn, NJ); the two acids were separated by preparative reverse-phase high-performance liquid chromatography (HPLC) on a Varian automated ProStar System (Palo Alto, CA). An X-Terra MS C-18 (5 μm with 19 × 50 mm) column (Waters, Milford, MA) was used in this procedure and eluted at a flow rate of 15 ml/min with acetonitrile in 0.1

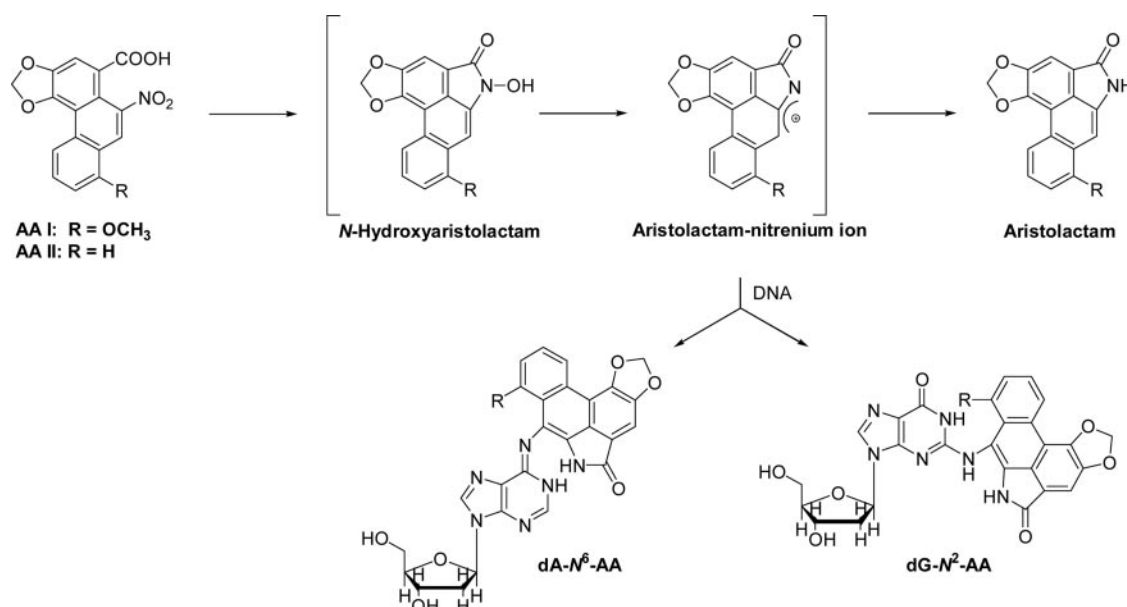


FIG. 2. Proposed pathway for the formation of AA-derived DNA adducts.

M triethylamine/acetic acid buffer, pH 7.5. A gradient of 16 to 25% acetonitrile in 15 min was used, with a short wash of 50% acetonitrile after each run. To improve sample solubility, the mixture (15–20 mg) was injected as the triethylammonium salt. The two acids were fully resolved by this system, and the desired fractions were dried, acidified, and rinsed with water several times. Purity of AA I and AA II (>98%) was confirmed by HPLC analysis.

L I was prepared by chemical reduction of AA I as follows: AA I (100 mg, 95% pure) was dissolved in approximately 20 ml of tetrahydrofuran and, after adding 20 mg of Pd-C catalyst, the mixture was hydrogenated on a Parr hydrogenator at 50 psi. After stirring for 3 h, an aliquot was examined by HPLC to determine the degree of reduction. At this point, the sample contained ~20% of the corresponding *N*-hydroxylactam. To carry the reaction to completion, the mixture was filtered, and another 20 mg of fresh Pd-C catalyst was added to the reaction. The mixture was hydrogenated for an additional 3 h, filtered, and evaporated to dryness. The crude product was dissolved in a small amount of tetrahydrofuran, and the crystallized solid was recovered by filtration. The overall yield of L I (50 mg) was 58%. AA I was not detected by HPLC analysis.

**Preparation of Oligodeoxynucleotides Containing a Single AA-Derived DNA Adduct.** A 10-mer oligodeoxynucleotide (200  $\mu$ g; 5'-TTTTXTTTT, where X is A or G) was incubated overnight with AA I (0.5 mg) and zinc dust (3 mg) under neutral pH conditions, as described by Schmeiser et al. (1997). The reaction mixture was then centrifuged, and the modified oligomers were recovered from the supernatant. Ten mers containing a single dA-AA I or dG-AA I were isolated on a reverse-phase  $\mu$ Bondapak C<sub>18</sub> column (0.39  $\times$  30 cm, Waters) eluted over 60 min at a flow rate of 1.0 ml/min with a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10 to 30% acetonitrile. HPLC analysis was performed using a Waters 515 HPLC pump, 996 photodiode array detector, and pump control module. Based on the extinction coefficient at 260 nm, the concentrations of the 10-mer containing a single dA-AA I or dG-AA I were found to be 30.3  $\mu$ g (9.24 nmol)/ml = 1.0

OD<sub>260 nm</sub> and 31.4  $\mu$ g (9.53 nmol)/ml = 1.0 OD<sub>260 nm</sub>, respectively. These modified oligomers then were used to prepare standards for <sup>32</sup>P-postlabeling analyses.

**Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectroscopy.** The molecular weight of the modified oligomers was measured using a Voyager-DE STR matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer system. The mass scale (*m/z* 1000–10,000) was calibrated in the positive ion mode with a mixture of standard oligodeoxynucleotides; approximately 100 laser bursts were used to produce each spectrum.

**Animal Studies.** Wistar rats (male, 6 weeks old) were purchased from Taconic (Germantown, NY). All the procedures were conducted in compliance with guidelines established by the National Institutes of Health Office of Laboratory Animal Welfare. Animals were acclimatized in temperature (22  $\pm$  2°C)- and humidity (55  $\pm$  5%)-controlled rooms with a 12-h light/dark cycle for at least 1 week before the experiment. Regular laboratory chow and tap water were allowed ad libitum. Three Wistar rats were treated p.o. with AA I, AA II, or L I using a dose of 5 mg/kg/day for 7 days. Control rats were treated with an identical volume of corn oil. Rats were euthanized by CO<sub>2</sub> asphyxiation 5 h after the final treatment. After thoracotomy, tissues were removed quickly, immersed in liquid nitrogen, and stored at -80°C.

**DNA Digestion.** DNA was extracted using a DNase tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The concentration of DNA was determined by UV spectroscopy with 50  $\mu$ g/ml = O.D.<sub>260 nm</sub> 1.0. The DNA sample (5.0  $\mu$ g) was enzymatically digested at 37°C for 16 h in 100  $\mu$ l of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM CaCl<sub>2</sub> using micrococcal nuclease (30 units) and spleen phosphodiesterase (0.15 unit) (Terashima et al., 2002). The reaction mixture was incubated for an additional hour with nuclease P1 (1 unit). After incubation, 200  $\mu$ l of water was added, and the reaction mixtures were extracted twice with 200  $\mu$ l of butanol. Butanol

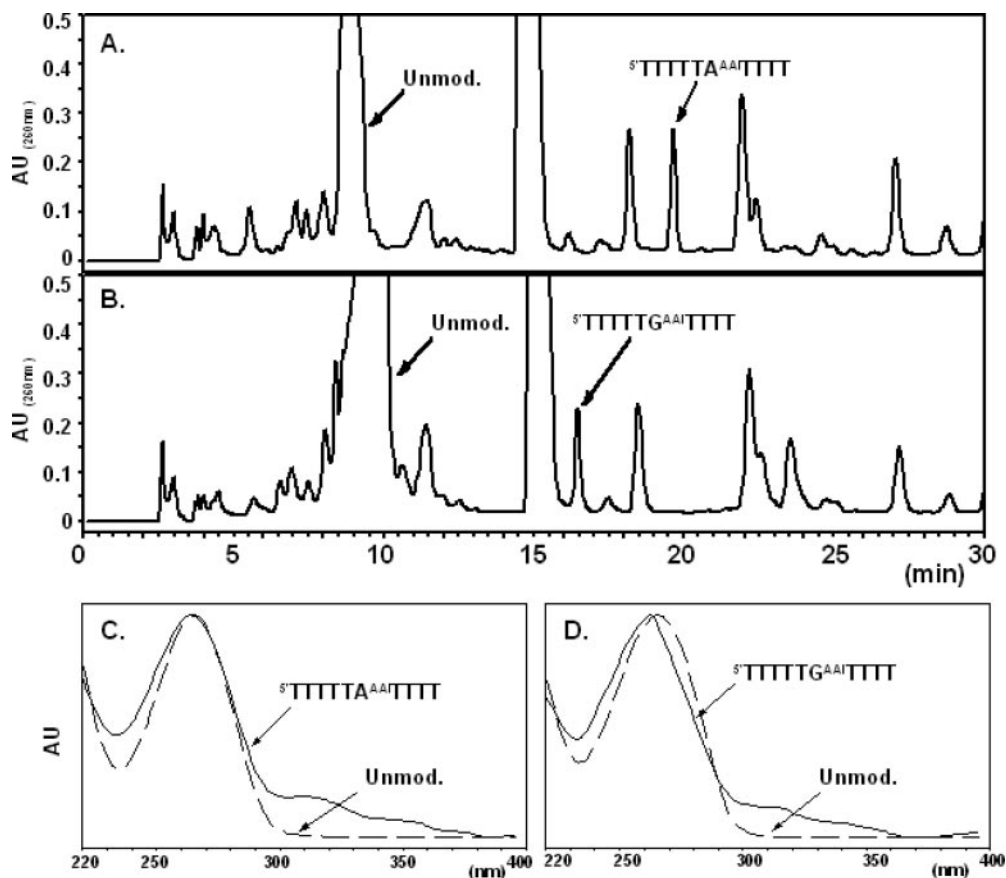


Fig. 3. HPLC resolution of 10-mer oligodeoxynucleotides containing a single dA-AA I or dG-AA I. A, HPLC separation of an oligodeoxynucleotide containing a single dA-AA I adduct. B, HPLC separation of the oligodeoxynucleotide containing a single dG-AA I adduct. C, UV absorbance spectra of dA-AA I in modified and unmodified oligodeoxynucleotides. D, UV absorbance spectra of dG-AA I in modified and unmodified oligodeoxynucleotides.

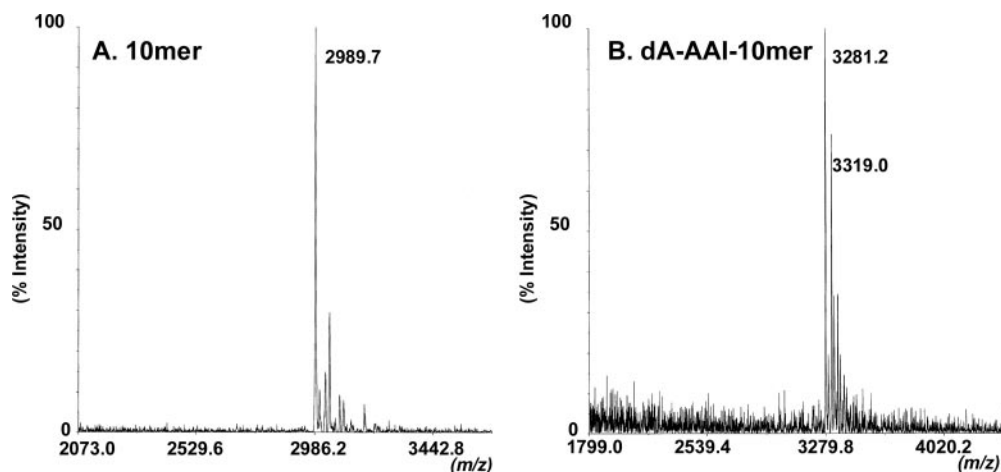


FIG. 4. MALDI-TOF mass spectroscopy. A, unmodified 10-mer. B, dA-AA I-modified 10-mer oligodeoxynucleotide.

fractions were combined, back-extracted with 50  $\mu$ l of distilled water, and evaporated to dryness.

**$^{32}$ P-Postlabeling/PAGE Analysis.** DNA digests were incubated at 37°C for 40 min with 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP and 3'-phosphatase-free T4 PNK (10 units), and then incubated with apyrase (50 mU) for an additional 30 min, as described previously (Terashima et al., 2002). Known amounts (0.152, 0.0152, 0.00152, or 0.000152 pmol) of dA-AA I- or dG-AA I-modified oligodeoxynucleotides, prepared as described above, were mixed with 5  $\mu$ g of calf thymus DNA (15,200 pmol). These mixtures [characterized as 1 adduct/ $10^5$  nucleotides (dNs), 1 adduct/ $10^6$  dNs, 1 adduct/ $10^7$  dNs, or 1 adduct/ $10^8$  dNs] served as standards for analytic purposes. The  $^{32}$ P-labeled sample was subjected to electrophoresis for 4 to 5 h on a nondenaturing 30% polyacrylamide gel (35  $\times$  42  $\times$  0.04 cm) at 1500 to 1800 V/20 to 40 mA. The position of  $^{32}$ P-labeled adducts was established by  $\beta$ -phosphorimager analysis (Molecular Dynamics Inc.). To quantify the level of  $^{32}$ P-labeled products, integrated values, measured with a  $\beta$ -phosphorimager, were compared with the standards. The detection limit for 5  $\mu$ g of DNA was approximately 5 adducts/ $10^9$  nucleotides.

## Results

**Characterization of Oligodeoxynucleotides Containing a Single AA-Derived DNA Adduct.** Oligonucleotide (5'-TTTTTGTTTT or 5'-TTTTTATTTT) 10-mers containing a single dA-AA I [retention time ( $t_R$ ) = 19.6 min] or dG-AA I ( $t_R$  = 16.5 min) were isolated by HPLC (Fig. 3, A and B); yields of dA-AA I- and dG-AA I-modified oligomers were 2.6% and 2.0%, respectively. Modified oligomers can be distinguished from the unmodified ones by their UV absorbance spectra (Fig. 3, C and D).

The presence of dA-AA I and dG-AA I adducts in the modified oligomers was confirmed by MALDI-TOF mass spectroscopy (Fig. 4). The parent ion of the unmodified dA-10-mer appeared at  $m/z$  2990, identifying the molecular mass as 2989 Da (Fig. 4A). The parent ion of the dA-AA I-modified 10-mer showed  $m/z$  3281, confirming the molecular mass of 3280 Da (Fig. 4B). Because potassium ion forms a complex with the deprotonated phosphate, peaks representing  $[M+K]^+$  also were detected. The molecular masses of dG-10-mer and dG-AA I-modified 10-mer were 3005 Da and 3296 Da, respectively (data not shown). Based on these data, reaction products were identified as 10-mers containing a single dA-AA I or dG-AA I.

**Quantitative Analysis of AA-Derived DNA Adducts by  $^{32}$ P-Postlabeling Analysis.** An oligodeoxynucleotide containing a single adduct (5'-TTTTXTTTT, where X is dA-AA I or dG-AA I) was used as an internal standard to quantify AA-derived adducts in tissues by  $^{32}$ P-postlabeling/PAGE techniques. By mixing 0.152, 1.52, 15.2, or 152 fmol of this oligomer with 5  $\mu$ g of purified calf thymus DNA

( $1.52 \times 10^7$  fmol dNs), levels of AA-derived adducts in the mixture were established as 1 adduct/ $10^8$  dNs, 1 adduct/ $10^7$  dNs, 1 adduct/ $10^6$  dNs, and 1 adduct/ $10^5$  dNs, respectively. The amount of dA-AA I or dG-AA I adducts increased proportionally to the amount of oligodeoxynucleotide added (Fig. 5). Based on the standard curve obtained from the experimental and calculated frequencies of AA-derived adducts, recoveries of dA-AA I and dG-AA I adducts were found to

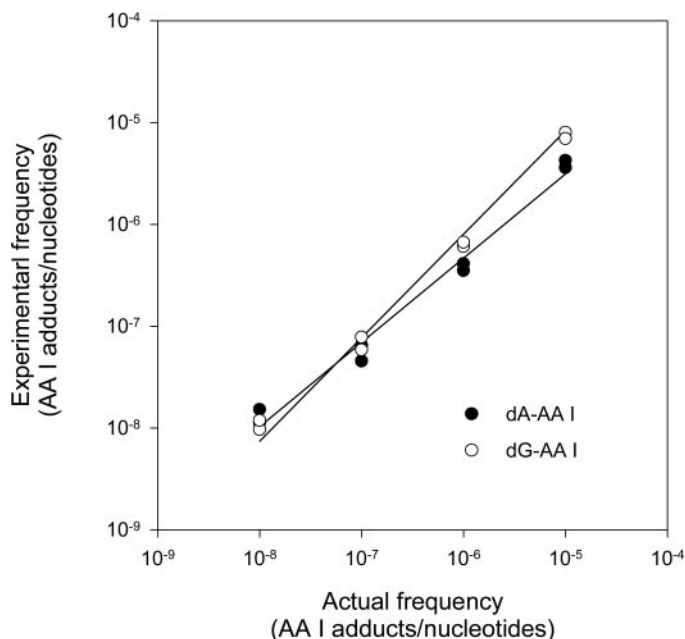


FIG. 5. Comparison of experimental and calculated frequencies of AA-derived DNA adducts. Variable amounts (152, 15.2, 1.52, and 0.152 fmol) of 10-mer oligodeoxynucleotides containing a single dA-AA I or dG-AA I (5'-TTTTXTTTT, where X is dA-AA I or dG-AA I) were mixed with 5  $\mu$ g of purified calf thymus DNA (equivalent to 15,200 pmol dNs) to prepare a DNA standard mixture containing 1 AA-DNA adduct/ $10^5$  dNs, 1 adduct/ $10^6$  dNs, 1 adduct/ $10^7$  dNs, and 1 adduct/ $10^8$  dNs, respectively. The DNA standards were digested using the nuclease P1-enrichment method, followed by butanol extraction as described under *Materials and Methods*. The digested DNA then was labeled with  $^{32}$ P using 3'-phosphatase-free T4 PNK (30 units) and [ $\gamma$ - $^{32}$ P]ATP (30  $\mu$ Ci), and then incubated with apyrase (50 mU). The  $^{32}$ P-labeled samples were electrophoresed for 4 to 5 h on a nondenaturing 30% polyacrylamide gel (35  $\times$  42  $\times$  0.04 cm). To compare the radioactivity of standard [ $\gamma$ - $^{32}$ P]ATP (0.0167 pmol), the level of AA adducts was determined by  $\beta$ -phosphorimager analysis. Data from two or three samples are plotted for each analysis.



TABLE 1

Formation of AA-DNA adducts in rats treated p.o. with AA I, AA II, or L I

Data are expressed as mean  $\pm$  S.D. from analyses of three rats.

	Adducts/ $10^6$ Nucleotides					
	AA I		AA II		L I	
	dA-AA I	dG-AA I	dA-AA II	dG-AA II	dA-AA I	dG-AA I
Kidney-pelvis	4.01 $\pm$ 2.51	0.44 $\pm$ 0.27	14.1 $\pm$ 4.25	2.94 $\pm$ 1.78	0.24 $\pm$ 0.12	0.08 $\pm$ 0.06
Kidney-cortex	4.85 $\pm$ 1.14	0.54 $\pm$ 0.09	19.7 $\pm$ 6.02	5.06 $\pm$ 1.38	0.01 $\pm$ 0.02	0.01 $\pm$ 0.01
Bladder	1.20 $\pm$ 0.50	0.15 $\pm$ 0.07	13.8 $\pm$ 4.38	1.85 $\pm$ 0.55	0.06 $\pm$ 0.02	0.03 $\pm$ 0.01
Forestomach	2.76 $\pm$ 1.80	0.44 $\pm$ 0.29	4.84 $\pm$ 3.50	0.72 $\pm$ 0.47	0.09 $\pm$ 0.11	0.04 $\pm$ 0.02
Glandular stomach	2.50 $\pm$ 0.71	0.39 $\pm$ 0.15	2.39 $\pm$ 0.28	0.33 $\pm$ 0.09	0.05 $\pm$ 0.05	0.02 $\pm$ 0.03
Intestine	6.86 $\pm$ 2.63	1.15 $\pm$ 0.36	8.11 $\pm$ 2.53	1.06 $\pm$ 0.37	0.22 $\pm$ 0.20	0.04 $\pm$ 0.05
Liver	4.11 $\pm$ 1.89	0.43 $\pm$ 0.20	3.33 $\pm$ 1.62	1.27 $\pm$ 0.45	0.03 $\pm$ 0.05	0.01 $\pm$ 0.01
Spleen	0.47 $\pm$ 0.45	0.07 $\pm$ 0.05	1.02 $\pm$ 0.29	0.15 $\pm$ 0.06	0.05 $\pm$ 0.08	0.03 $\pm$ 0.03
Lung	2.03 $\pm$ 0.65	0.27 $\pm$ 0.12	2.37 $\pm$ 0.33	0.44 $\pm$ 0.07	0.04 $\pm$ 0.03	0.02 $\pm$ 0.01

be 84% and 73%, respectively. In the following studies, we use this system for all the quantitative analyses of AA-DNA adducts.

**Formation of AA-Derived DNA Adducts in Rats Treated with AA I, AA II, or L I.** Male Wistar rats were treated p.o. for 7 days with AA I, AA II, or L I (5 mg/kg/day); controls received only the corn oil vehicle. The dA-AA and dG-AA adducts were detected in all the organs collected (Table 1 and Fig. 6). In rats treated with AA I, the level of AA-DNA adducts ranged from 0.54 to 8.0 adducts/ $10^6$  dNs, with the highest levels being in the intestine, kidney, and liver. The level of AA-DNA adducts detected in renal pelvis and cortex (urothelial tissue) was 4.5 adducts/ $10^6$  dNs and 5.4 adducts/ $10^6$  dNs, 1.4 to 1.7 and 1.5 to 1.9 times greater than levels in forestomach and glandular stomach, respectively. In rats treated with AA II, the distribution of AA-DNA adducts in various organs ranged from 1.2 to 24.8 adducts/ $10^6$  dNs (Table 1). Again, highest adduct levels were detected in the kidney, intestine, and bladder, with the level of AA-DNA adducts in the renal pelvis and cortex being 3.1 to 4.5 and 6.3 to 9.1 times higher than that in the forestomach and glandular stomach, respectively. The total amount of DNA adducts in kidneys of AA II-treated rats was approximately 4 to 5 times higher than that observed in AA I-treated rats, whereas the relative number of DNA adducts formed by AA II and AA I in the forestomach and glandular

stomach did not differ significantly. In both AA I- and AA II-treated rats, the level of dA-AA adducts was 5 to 10 times greater than that of dG-AA adducts in other organs (Fig. 6 and Table 1). Administration of L I promoted a much lower level of DNA adduct formation, ranging from 0.02 to 0.32 adducts/ $10^6$  dNs. Thus, formation of AA-DNA adducts in renal pelvis and cortex was 14 to 53 and 270 to 1200 times less, respectively, than that observed in rats treated with AA I or AA II. Thus, it appears that AA II potentially is more genotoxic than AA I and that L I represents a biologically inactive end-product of AA metabolism.

## Discussion

The  $^{32}\text{P}$ -postlabeling technique is a powerful tool for detecting DNA damage in animals and humans exposed to carcinogens and mutagens (Poirer et al., 2000). Using  $^{32}\text{P}$ -postlabeling/polyethyleneimine-cellulose thin layer chromatographic analysis, several groups have reported high levels of AA-DNA adducts in the forestomach of rats treated with AA, suggesting that this organ is the primary target organ for these toxins (Pfau et al., 1990b; Fernando et al., 1993; Bieler et al., 1997). Internal standards were not used in these studies, and the relative AA-DNA adduct level was estimated by comparison with  $^{32}\text{P}$ -labeled normal nucleotides (Reddy and Randerath, 1986). Such assays may seriously underestimate adduct levels because of incomplete DNA digestion, inefficiency of adduct labeling by polynucleotide kinase, and/or loss of adducts during the enrichment procedure (Terashima et al., 2002). In fact, omission of the butanol extraction during the adduct enrichment procedure reduces the level of detection 3- to 9-fold (Fernando et al., 1992). For these reasons, the  $^{32}\text{P}$ -postlabeling method described in the present study is preferable for determining accurately the level of AA-DNA adducts in animal tissues.

The major advantages of the  $^{32}\text{P}$ -postlabeling/PAGE technique used in this study (Terashima et al., 2002) are the ability to load several DNA samples and standard markers on the gel simultaneously and to measure the amount of DNA adducts present in a relatively short period. We included a butanol extraction step in the procedure to enrich the AA-derived nucleotides and DNA containing a defined amount of dA-AA- and dG-AA-modified oligonucleotides as standards. We showed that the number of dA-AA I and dG-AA I adducts increases linearly with the amount of oligomer added and that the overall recovery of adducts was approximately 80% (Fig. 5), thereby validating the  $^{32}\text{P}$ -postlabeling/PAGE system for the quantitative analysis of AA-DNA adducts in rodent tissues.

To establish the organ distribution of AA-DNA adducts, we administered AA I, AA II, or L I p.o. to Wistar rats at a dose of 5 mg/kg/day for 7 days. The level of AA-DNA adduct formation was

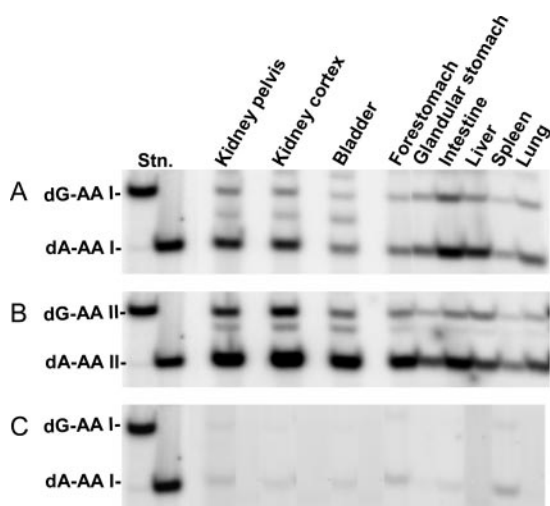


FIG. 6. Detection of AA-DNA adducts in rats using  $^{32}\text{P}$ -postlabeling/PAGE. Wistar rats were treated p.o. with AA I (A), AA II (B), or L I (C) with 5 mg/kg/day for 7 days. Rats were euthanized, tissues were removed, and the DNA was extracted. DNA adduct levels of each tissue were determined using  $^{32}\text{P}$ -postlabeling/PAGE technique. Standard (Stn.) represents 5  $\mu\text{g}$  of calf thymus DNA ( $1.52 \times 10^7$  fmol dNs) containing a known amount ( $1.52 \times 10^2$  fmol dNs) of dA-AA I- or dG-AA I-modified oligomer, representing 1 adduct/ $10^5$  dNs.

determined using the quantitative  $^{32}\text{P}$ -postlabeling/PAGE assay. Distribution of AA-DNA adducts varied according to the compound tested (Table 1). A high level of AA-DNA adducts was found in kidneys of rats treated with AA I or AA II. This result is consistent with the observation that intake of AA results in severe tubulointerstitial nephritis and upper urothelial carcinoma in humans (Nortier et al., 2000; Debelle et al., 2002; Cosyns, 2003). However, our results differ from those reported for the same strain of rats treated p.o. with AA I (10 mg/kg/day) for 5 days (Pfau et al., 1990b) or with a single dose (5 mg/kg/day) of AA I (Fernando et al., 1993; Bieler et al., 1997), in which the highest concentration of adducts was reported to be the forestomach. This apparent discrepancy may reflect the use of a qualitative  $^{32}\text{P}$ -postlabeling analysis and omission of the butanol extraction procedure.

In addition to the high rate of AA-DNA adduct formation in the kidney, we found a high level of adduction in the intestine (Table 1). This may result from metabolic activation of AA in this tissue and the higher concentrations achieved when the toxin is administered p.o.

AA II generates higher level amounts of AA-DNA adducts than does AA I in most of the tissues analyzed (Table 1 and Fig. 6). These results also differ significantly from the previous report (Pfau et al., 1990b), in which the authors claimed that the extent of DNA modification by AA II was 10-fold lower than that for AA I. We found that the formation of AA II-derived adducts in the kidney pelvis and cortex was 3.8 and 4.6 times higher than that observed for AA I. In bladder tissue, AA II promoted approximately 12 times greater numbers of AA-DNA adducts than did AA I. Thus, AA II may have a higher order of genotoxicity than does AA I. If so, this could reflect different repair efficiencies of DNA adducts derived from AA I or AA II and/or different activation rates for the nitroreductase(s) that convert these compounds to their respective aristolactam.

We further observed that L I, one of the major reduction products of AA I, exhibited low genotoxicity in comparison with AA I and AA II. In fact, DNA adduct formation after administration of L I was more than 50 times lower than that observed in rats treated with AA I or AA II (Table 1 and Fig. 6). In vitro, L I is reported to be activated by cytochrome P450 and peroxidase to form dA-AA I and dG-AA I adducts (Stiborova et al., 1999). In vivo, however, it appears that only a minor amount of L I is converted to a form that can react with DNA to form AA-DNA adducts.

In conclusion, we have used an ultrasensitive, quantitative  $^{32}\text{P}$ -postlabeling/PAGE technique to show that AA I- and AA II-derived DNA adducts accumulate preferentially in renal tissues of the Wistar rat, whereas L I displayed only limited potential genotoxicity. This study establishes the feasibility of using AA-DNA adducts as a biomarker to explore AA nephropathy and its associated urothelial cancer in experimental animals and humans.

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