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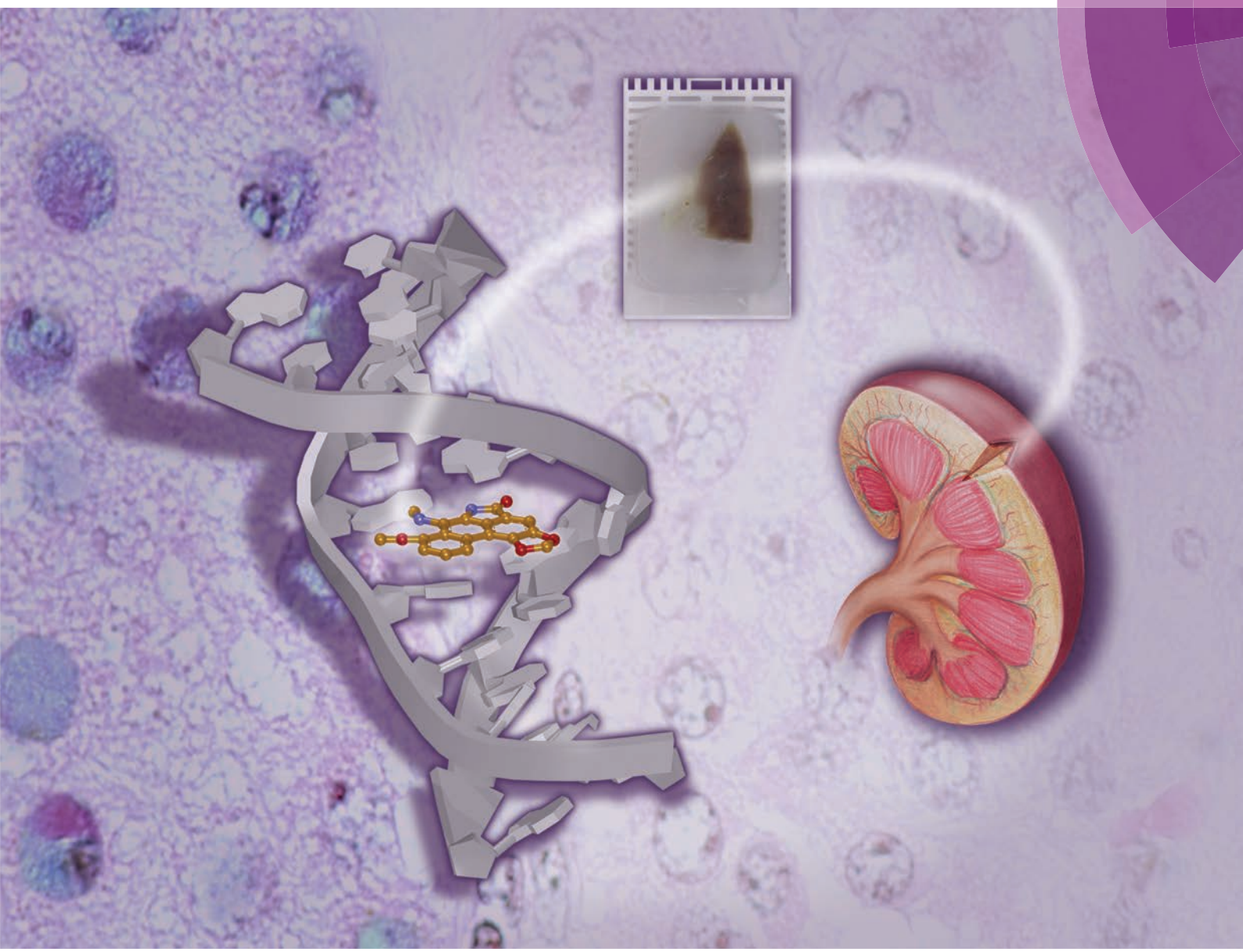

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REVIEW

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New approaches for biomonitoring exposure to the human carcinogen aristolochic acid

Byeong Hwa Yun,[a] Viktoriya S. Sidorenko,[b] Thomas A. Rosenquist,[b] Kathleen G. Dickman,[b,c] Arthur P. Grollman*[b,c] and Robert J. Turesky*[a]

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Aristolochic acids (AA) are found in all *Aristolochia* herbaceous plants, many of which have been used worldwide for medicinal purposes for centuries. AA are causal agents of the chronic kidney disease entity termed aristolochic acid nephropathy (AAN), and potent upper urinary tract carcinogens in humans. AAN and upper urinary tract cancers are endemic in rural areas of Croatia and other Balkan countries where exposure to AA occurs through the ingestion of home-baked bread contaminated with *Aristolochia* seeds. In Asia, exposure to AA occurs through usage of traditional Chinese medicinal herbs containing *Aristolochia*. Despite warnings from regulatory agencies, traditional Chinese herbs containing AA continue to be used world-wide. In this review, we highlight novel approaches to quantify exposure to AA, by analysis of aristolactam (AL) DNA adducts, employing ultraperformance liquid chromatography–electrospray ionization/multistage mass spectrometry (UPLC-ESI/MS^n). DNA adducts are a measure of internal exposure to AA and serve as an important end point for cross-species extrapolation of toxicity data and human risk assessment. The level of sensitivity of UPLC-ESI/MS^3 surpasses the limits of detection of AL-DNA adducts obtained by ^{32}P-postlabeling techniques, the most widely employed methods for detecting putative DNA adducts in humans. AL-DNA adducts can be measured by UPLC-ESI/MS^3, not only in fresh frozen renal tissue, but also in formalin-fixed, paraffin-embedded (FFPE) samples, an underutilized biospecimen for assessing chemical exposures, and in exfoliated urinary cells, a non-invasive approach. The frequent detection of AL DNA adducts in renal tissues, combined with the characteristic mutational spectrum induced by AA in *TP53* and other genes provides compelling data for a role of AA in upper urothelial tract cancer.

Introduction

Genotoxic chemicals or their reactive metabolites induce chemical modifications of DNA and form covalent DNA adducts,[1] some of which lead to mutations during cell division and initiate malignant transformation.[2] DNA adducts are regarded as one important factor in the carcinogenic potential of genotoxicants and have been used for cross-species extrapolation of toxicity data for human risk assessment.[3,4] The quantitative measurement of DNA adducts of carcinogens has been a long sought goal for risk assessment and molecular epidemiological studies. However, the lack of specific and sensitive methods to quantitate DNA adducts have hampered the use of DNA adducts as biomarkers in human studies. Fortunately, with recent improvements in the sensitivity of mass spectrometry (MS) instrumentation, it is now possible to implement quantitative MS-based methods to measure DNA adducts in human biospecimens.[5,6]

Aristolochic acids (AA) are potent human renal toxicants and upper urinary tract carcinogens. Over the past 20 years, many of the biochemical mechanisms involved in toxicities associated with AA exposure and in AA-induced upper urothelial carcinomas (UTUC) have been elucidated.[7–15] Despite the acquisition of compelling data supporting the role of AA in human cancer, specific and quantitative mass spectrometry (MS) based-methods to assess exposure to AA and its attendant DNA damage in humans have been lacking. In this review, we highlight recent approaches to biomonitor aristolochic acids (AA) in high-risk human populations exposed to these potent carcinogens, by MS-based measurement of aristolactam (AL)-DNA adducts. Quantitative methods employing ultraperformance liquid chromatography–electrospray ionization/multistage mass spectrometry (UPLC-ESI/MS^n) with a linear quadrupole ion trap mass spectrometer were established to measure AL-DNA adducts in freshly frozen tissues, and for the first time in archived formalin-fixed, paraffin-embedded

[a]*Masonic Cancer Center and Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, USA. E-mail: Rturesky@umn.edu*
[b]*Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY 11794, USA. E-mail: arthur.grollman@stonybrook.edu*
[c]*Department of Medicine, Stony Brook University, Stony Brook, NY 11794, USA*

Byeong Hwa Yun, Viktoriya S. Sidorenko, Thomas A. Rosenquist, Kathleen G. Dickman, Arthur P. Grollman and Robert J. Turesky

Dr Byeong Hwa Yun received his PhD in Chemistry from New York University, followed by a postdoctoral fellowship and research associate position in Dr Turesky's laboratory. His research interests are focused on mechanisms of oxidative DNA damage and developing liquid chromatography-mass spectrometry techniques to measure carcinogen DNA adducts. His current work involves the biomonitoring of DNA adducts of aristolochic acids in human cohorts. He has developed a long sought mass spectrometric method to measure DNA adducts in formalin-fixed paraffin embedded tissues, a largely untapped biospecimen for DNA adduct biomarker research.

Dr Viktoriya Sidorenko received her M.S and PhD degrees in molecular biology and biochemistry from Novosibirsk State University. During her graduate studies at the Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia, she conducted research on DNA damage and repair. She joined the Laboratory of Chemical Biology at Stony Brook University as a Postdoctoral Research Associate in 2009 and was appointed Assistant Professor in the Department of Pharmacological Sciences at SBU in 2014. Viktoriya has been working on research related to the bioactivation and nephrotoxic effects of aristolochic acid and on biomarkers of exposure to this human carcinogen.

Dr Thomas Rosenquist is a Research Associate Professor in the Department of Pharmacological Sciences, Stony Brook University School of Medicine. He received his PhD from the University of Wisconsin at Madison, Department of Biochemistry and did his post-doctoral training at the University of California, San Francisco. He is currently active in investigating the genetic basis of susceptibility to renal toxins, such as aristolochic acid, and also in deciphering the mutagenic signatures found in tumor genomes that reveal exposure to specific environmental agents.

Dr Kate Dickman received a BA in Biology, and a PhD in Physiology, from the University of Connecticut, followed by postdoctoral training in the Department of Cell Biology at Duke University. She holds a joint appointment as Associate Professor in the Departments of Pharmacological Sciences and Medicine/Nephrology at Stony Brook University. As a classically trained physiologist, her research interests have focused on the kidney, particularly on the roles of membrane transport and energy metabolism in renal disease. Her current research is centered on the pathophysiology of kidney and urinary tract diseases caused by the genotoxic nephrotoxin aristolochic acid.

Arthur P. Grollman, M.D. is Distinguished Professor of Pharmacological Sciences, Glick Professor of Medicine and Director of the Zickler Laboratory of Chemical Biology at Stony Brook University. His research interests focus on the biological consequences of DNA damage as they relate to mechanisms of DNA replication, mutagenesis and DNA repair. Currently, he is engaged in an interdisciplinary research program involving aristolochic acid nephropathy and its associated urothelial cancer. The goal of this research is to document human exposure to aristolochic acid and its relationship to kidney disease and cancer in countries where Aristolochia *has been widely used for medicinal purposes.*

Dr Robert Turesky received his PhD in the Department of Nutrition and Food Science from MIT. He is a Professor in the Department of Medicinal Chemistry, and Director of the Masonic Cancer Center's Analytical Biochemistry shared resource, a mass spectrometry facility devoted to the cancer and chemoprevention programs at the University of Minnesota. His research is focused on the biochemical toxicology of dietary and environmental genotoxicants, employing novel mass spectrometry techniques to measure biomarkers, including urinary metabolites, and protein and DNA adducts, which are implemented in molecular epidemiological studies to understand the role of chemical exposures in carcinogenesis.

(FFPE) tissues and exfoliated urinary cells. FFPE tissue and exfoliated urinary cells are largely untapped specimens for biomonitoring DNA adducts. The screening of FFPE tissue for AL-DNA adducts can be used to retrospectively estimate exposure of AA, which may occur 20–30 years prior to the development of urothelial cancer. Exfoliated urinary cells serve a dual purpose by providing a non-invasive approach to screen for AL-DNA adducts and detect mutations.

Aristolochic acid exposure and renal toxicity

Aristolochia herbs have been used for medicinal purposes throughout the world for centuries; however, the recognition that AA can cause nephropathy and urothelial cancer is relatively recent.[16,17] The public became aware of the health risk of traditional Chinese herbs twenty years ago when a group of otherwise healthy young Belgian women took weight-reducing pills containing Chinese herbs and subsequently developed renal failure and upper tract urothelial carcinoma.[14,16] It was suspected that one of the Chinese herbs in the pills, *Stephania tetrandra*, was inadvertently replaced by *Aristolochia fangchi*, a nephrotoxic herb containing AA. Until that time, the public generally viewed herbal products as naturally occurring safe products. The International Agency for Research on Cancer (IARC) classified herbal remedies containing plant species of the genus *Aristolochia* as a Group 1 carcinogen to humans,[18] and the National Toxicology Report on carcinogens classified AA as known to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in humans and supporting data on mechanisms of carcinogenesis.[19] In 2001, the FDA issued strong warnings to the public and banned the importation of herbal products containing AA. The United Kingdom, Germany, Canada, and Australia also banned *Aristolochia* herbs. Nevertheless, a recent survey of herbal products marketed in the United States *via* the Internet reported that some botanical products still contain AA.[20] The exposure to AA through the usage of herbal products is a world-wide public health issue.[10,13,14] Thus, there is a critical need to establish noninvasive AA biomarkers in humans to assess exposure and health risk.

AA are components of all *Aristolochia* sp. and are responsible for the clinical syndromes formerly known as Chinese herbs nephropathy[7] and Balkan endemic nephropathy (BEN).[17,21] Both disorders are associated with a high incidence of urothelial carcinomas of the upper urinary tract (UTUC),[10,16] constituting a disease entity now termed aristolochic acid nephropathy (AAN).[10] AAN is endemic in rural farming villages located near tributaries of the Danube River in the Balkan countries where exposure to AA occurred by ingestion of bread contaminated with seeds of *Aristolochia clematitis*, a theory originally proposed in 1969 by Ivić.[13,22] The actual levels of exposure to AA in the Balkan countries are uncertain.[23] However, end-stage renal failure of patients with BEN progresses more slowly than the disease in patients in Belgium who ingested herbal mixtures as slimming agents where the maximal daily dose of AA was 0.025 mg kg^{-1} bodyweight over 13 months.[7] The slower progression of renal disease points to lower levels of AA exposure in the Balkans.[24] The elevated incidence of AAN in Taiwan occurs by exposure to AA through usage of traditional Chinese herbal medicines.[25] Epidemiologic studies in Taiwan reveal that about one-third of the Taiwanese population had been prescribed remedies containing *Aristolochia* herbs where, in some patients, the total dose of AA ingested often exceeded 100 mg.[26,27] It is noteworthy that the risk of kidney failure and incidence of UTUC in Taiwan are the highest in the world.[9,26,27]

Metabolic activation, DNA adduct formation, and *TP53* mutations induced by AAs

8-Methoxy-6-nitrophenanthro-[3,4-*d*]-1,3-dioxole-5-carboxylic acid (AA-I) and 6-nitrophenanthro-[3,4-*d*]-1,3-dioxole-5-carboxylic acid (AA-II), are the most widely studied AA. These compounds can undergo metabolic activation *via* reduction of the nitro moieties of the phenanthrene ring, by NAD(P)H:quinone oxidoreductase 1 (NQO1), xanthine oxidase, prostaglandin H synthase, NADPH:CYP reductase or CYP1A1/2, to form AL-DNA.[28–30] Initially, *N*-hydroxyaristolactam (AL-NOH), a product of partial nitroreduction of AA, was proposed as an immediate precursor of reactive species in the pathway to AL-DNA adduct formation.[31] Recently, it became clear that *N*-hydroxyaristolactam has relatively low reactivity with DNA, and further enzymatic bioactivation, which is catalyzed by sulfotransferases, is required to form AL-DNA adducts at high levels.[32] Thus, AA bioactivation *via* nitroreduction alone is not as efficient as when coupled with the sulfonation of AL-NOH. Among the human sulfotransferases (SULTs) studied *in vitro* so far, SULT1B1 is the most active in producing *N*-sulfonyloxyaristolactam; the SULT1A family catalyzes this reaction to a lesser extent.[32] The sulfonated metabolite undergoes heterolytic cleavage to form the proposed reactive nitrenium intermediates, which covalently bind to the exocyclic amino groups of the purine nucleobases (Fig. 1).[31,33] The major AL-DNA adducts have been identified as 7-(deoxyadenosin-N^6-yl) aristolactam I (dA-AL-I), 7-(deoxyguanosin-N^2-yl) aristolactam I (dG-AL-I), 7-(deoxyadenosin-N^6-yl) aristolactam II (dA-AL-II), and 7-(deoxyguanosin-N^2-yl) aristolactam II (dG-AL-II) in rodent. (Fig. 1).[33–35] dA-AL-I is by far the most abundant adduct of AA found in the human kidney.[34,36–39]

There is compelling evidence that dA-AL adducts are carcinogenic lesions in humans. The mutational spectrum of the *TP53* gene in upper urinary tract urothelial carcinomas of patients of the Balkans and Taiwan with documented exposure to AA is dominated by A:T-to-T:A mutations,[13,21,25,38,40] located primarily on the non-transcribed strand of DNA (Fig. 2). These A-to-T transversions in the *TP53* gene are otherwise rare mutational events in urothelial cancer. Among the 27 000 mutations

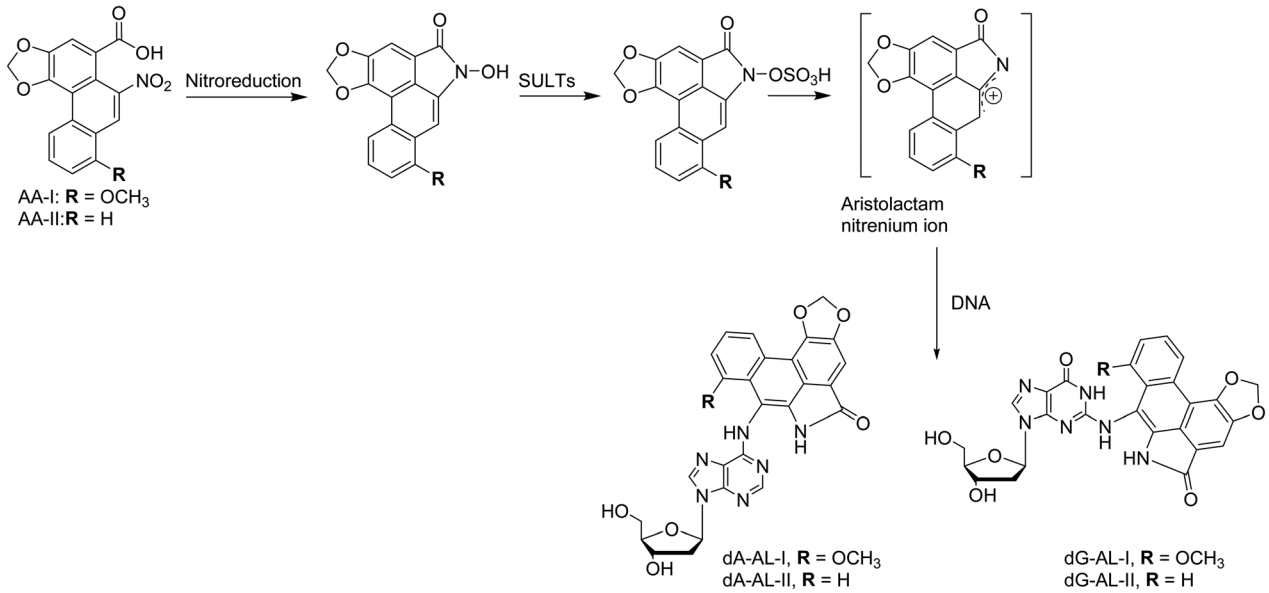

Fig. 1 Bioactivation of AA and DNA adduct formation. The nitroreduction of AA to form AL-NOH can lead to the formation of AL-DNA adducts. The reactivity of AL-NOH with DNA is significantly increased upon the sulfonation of AL-NOH.

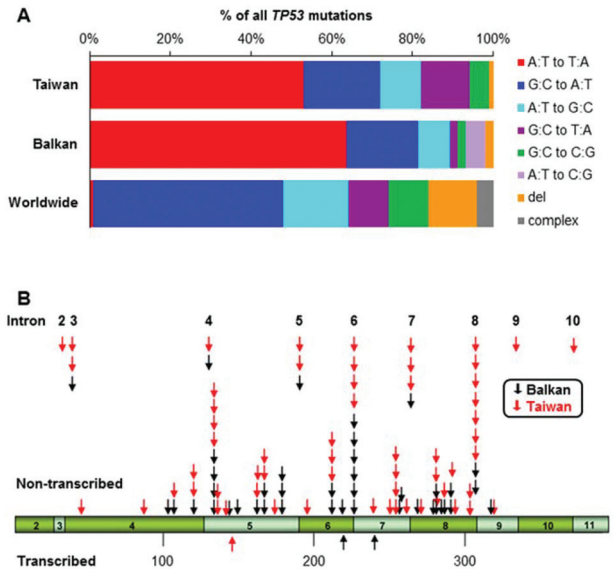

Fig. 2 dA-AL adducts induce the otherwise rare A-to-T transversion mutation in the *TP53* gene of patients with AAN in the Taiwan and the Balkans.[13] This figure was reproduced with permission from ref. 13.

in the IARC *TP53* database, A-to-T *TP53* mutations are found in 5.3% of all human cancers and only 1.4% of UTUCs overall.[13,41] AL-DNA adducts are strongly resistant to global genomic nucleotide excision repair,[42] which accounts for the strand bias in *TP53* mutations. The documented exposure to AA through detection of AL-DNA adducts combined with the high number of signature A-to-T mutations on the non-transcribed DNA strand implicate AA as the causative agent in UTUC in the Balkans and Taiwan.[21,25,38] Mechanistic studies support this conclusion. Site-specific mutagenesis studies in mammalian cells reveal that DNA polymerases frequently incorporate dAMP opposite AL-dA adducts, leading to A-to-T transversions.[43,44] Very recent studies, involving genome-wide sequencing of DNA in tumors of UTUCs from individuals with known exposure to AA, revealed an unusually large number of somatic mutations harboring this uncommon A-to-T transversion attributable to AA.[45,46] Another recent study also reported a high frequency of A-to-T mutations, but also G-to-T transversions as a complementary signature mutation in *TP53* of a group of non-smoking women from Belgium who ingested large amounts AA over a short period of time.[47] The authors proposed that the G-to-T transversions may be attributed to the dG-N^2 adduct of AA. AL-DNA adducts display remarkable persistence in humans and can be detected many years after exposure to *Aristolochia* has ceased.[34,36,38,48]

Biomonitoring of AL-DNA adducts in humans: ^{32}P-postlabeling *versus* UPLC-ESI/MS^n

Until our recent reports on the detection of dA-AL adducts by MS-based methods,[21,25,38,49–51] the biomonitoring of AL-DNA adducts utilized ^{32}P-postlabeling techniques.[16,34,36,37,40,48] The ^{32}P-postlabeling assay was established more than 30 years ago[52] and remains the most widely employed method to screen for DNA adducts in humans because of its high level of sensitivity.[53] Several different versions of the ^{32}P-postlabeling assay have been used to screen for an array of putative DNA adducts,[54–56] including AL-DNA, in rodents and humans.[21,28,33–36,38,40,57] ^{32}P-Postlabeling has been an impor-

tant technical advancement in the biomonitoring of DNA adducts of AL and other genotoxicants because it is one of the first methods that could be employed in human studies; however, ^{32}P-postlabeling has important drawbacks. The technique is labor-intensive and requires significant amounts of radioactive phosphorus, a strong β-emitter and potential health hazard; there are often a lack of suitable standards to account for adduct recovery and labeling efficiency, which can vary by more than 100-fold; and most importantly, there is no structural information about the lesion, which leaves the identity of the adduct ambiguous.[58]

During the course of our studies, we found that the linear quadrupole ion trap, the LTQ MS (Thermo Fisher), a highly sensitive instrument routinely used for peptide sequencing[59,60] could be employed for quantification of DNA adducts.[61] The powerful multi-stage consecutive reaction monitoring scan mode (MS^n) of the ion trap allows for quantitative measurement of DNA adducts. The MS^3 scan stage mode is effective in eliminating isobaric interferences often observed at the MS^2 scan stage and results in an improved signal-to-noise (S/N) ratio. The MS^3 scan stage also provides characteristic product ions of the aglycone (BH_2^+) to corroborate the identity of the DNA lesions.[61,62] We have employed UPLC-ESI/MS^n to identify and quantify DNA adducts of heterocyclic aromatic amines, aromatic amines, polycyclic aromatic hydrocarbons, and lipid peroxidation products in experimental laboratory animals or humans.[61–65] The linear quadrupole ion trap MS is an important technological advancement for biomonitoring of DNA adducts in humans. Triple quadrupole tandem mass spectrometry (TQ/MS) and quadrupole time-of-flight mass spectrometry also have been employed to measure AL-DNA adducts in rodents,[66,67] and there is one report of TQ/MS usage in a human study.[48]

We began our analyses by examining AL-DNA lesions in the kidney of an American woman who developed end-stage renal failure after treatment with an herbal remedy containing *Aristolochia*.[38] AL-DNA adducts were tentatively identified by ^{32}P-postlabeling/PAGE analysis (Fig. 3) in the renal cortex, medulla, and pelvis of this patient (1.1–3.4 adducts per 10^7 DNA bases for dA-AL and ≤1 adduct per 10^7 bases for dG-AL) years after ingestion of *Aristolochia* had ended.[38,68] The lower limit of quantification (LOQ) was about 1 adduct per 10^8 bases per 20 μg DNA. The identities of the dA-AL-I and dA-AL-II lesions were confirmed by UPLC-ESI/MS^3 scanning.[38] The dA-AL-I adduct was present at about 70-fold higher amounts than dA-AL-II.[38] Subsequently, stable isotopically labelled [$^{15}N_5$]-dA-AL-I and [$^{15}N_3$]-dA-AL-II were synthesized and employed for quantitative mass spectrometric measurements of these adducts.[51] Representative mass chromatograms and product ion spectra of dA-AL-I measured in the renal cortex of three patients with upper urothelial cancer from Taiwan, by UPLC-ESI/MS^3, are shown in Fig. 4. The level of adducts in the positive samples were 0.4 adducts and 5.9 adducts per 10^8 bases. The adduct level in the negative sample was below the LOQ, which was 3 adducts per 10^9 bases with 2 μg DNA assayed on column. The high quality product ion spectra

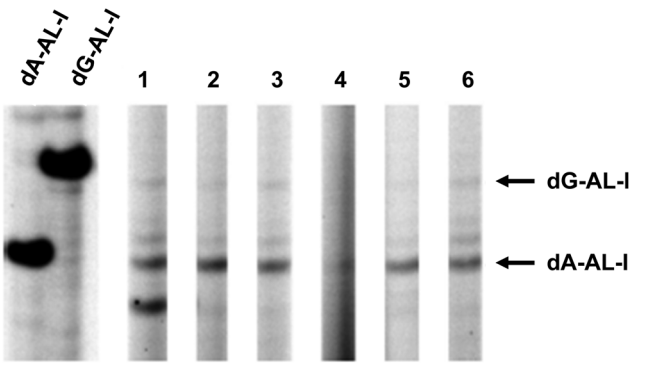

Fig. 3 Polyacrylamide gel electrophoresis (PAGE) of ^{32}P-Postlabeled DNA adducts from an American woman who developed end-stage renal failure after treatment with an herbal remedy containing *Aristolochia*. Samples in lanes 1–3 and 4–6 were excised from the right and left kidneys, respectively. Lanes 1 and 4 are from the renal cortex; lanes 2 and 5, from the renal medulla; and lanes 3 and 6, from the renal pelvis. Oligonucleotides containing dA-AL-I and dG-AL-I (1 adduct per 10^6 dNs), digested in parallel, were used as standards.[38] The figure was reproduced with permission from ref. 38 and "Copyright (2007) National Academy of Sciences, U.S.A."

acquired on the aglycone [BH_2^+] at the MS^3 scan stage corroborated the identity of the DNA lesion. The analytical method is robust: the within-day and between-day % coefficient of variation is less than 15% at the LOQ value.[51] Overall, the estimates of AL-DNA adducts by ^{32}P-postlabeling and UPLC-ESI/MS^3 showed good concordance.[50,51] However, UPLC-ESI/MS^3 is 10-fold more sensitive than ^{32}P-postlabeling for monitoring dA-AL-I and mass spectral data provide confirmation of identity of the lesion. We conclude that UPLC-ESI/MS^3 is the preferred method to biomonitor dA-AL-I in humans.

UPLC-ESI/MS^3 measurement of dA-AL-I

Paired renal cortex and upper urothelial tumor samples were obtained in Taiwan and the Balkans from patients with urothelial (transitional cell) carcinomas of the renal pelvis or ureter, and from patients with renal cell carcinomas. The demographics and other details about the subjects have been reported.[21,25,38] A summary of the levels of dA-AL-I adducts in renal cortex from the Taiwan cohort are shown in Fig. 5. dA-AL-I also was found in urothelial cancer tissue; however, adduct levels in that tissue were 15 to 100-fold lower than adduct levels in the renal cortex.[51] The elevated levels of dA-AL-I adducts in renal cortex likely are due to the active transport of AA-I into the cortex by organic ion transporters, which also may be involved in the site-selective toxicity and renal elimination of AA-I.[69] Of the 148 Taiwanese subjects assayed by MS, 132 subjects harbored dA-AL-I at a level above 0.3 adducts per 10^8 DNA bases (the LOQ value) in the renal cortex (unpublished data).[25,45,51] The mean level of dA-AL-I was 16.0 adducts per 10^8 DNA bases with a 95% confidence

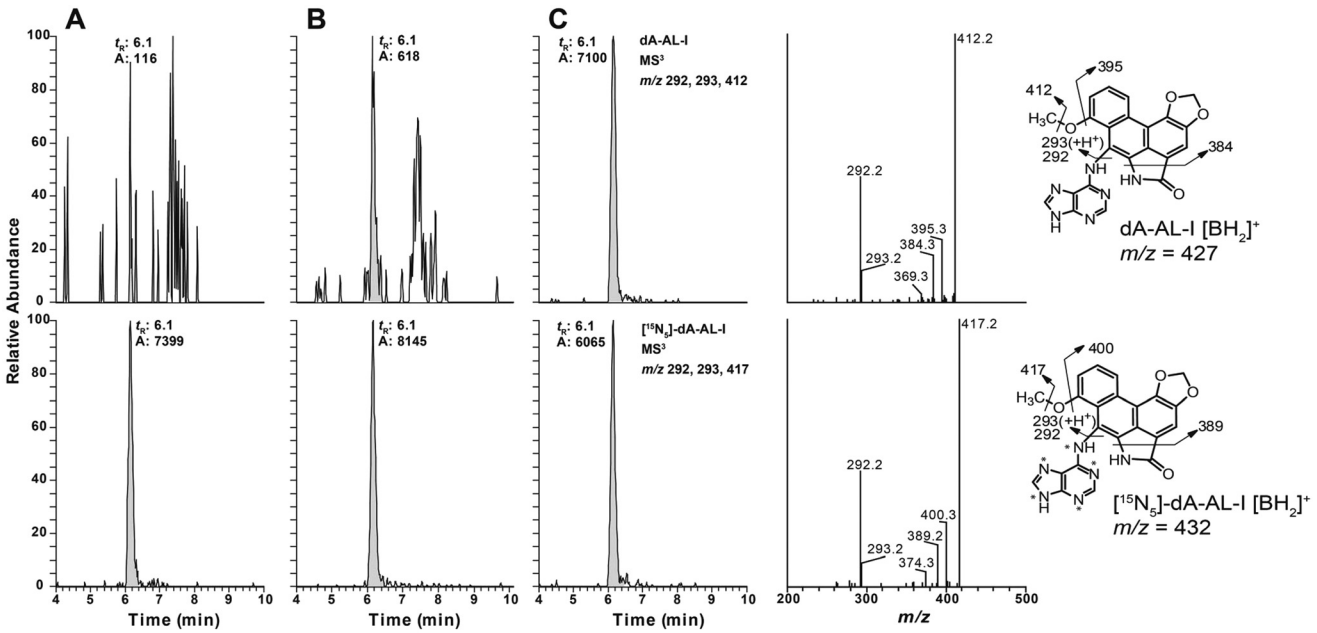

Fig. 4 UPLC-ESI/MS^3 reconstructed ion chromatograms of dA-AL-I from human kidney cortex of patients with upper urothelial cancer from Taiwan at levels (A) below the LOQ, and positive samples at (B) 0.4 adducts, and (C) 5.9 adducts per 10^8 bases. The product ion spectra of dA-AL-I from subject C is depicted along with the internal standard [$^{15}N_5$]-dA-AL-I (^{15}N labels are depicted with asterisks), which was added to DNA at a level of 5 adducts per 10^8 bases.

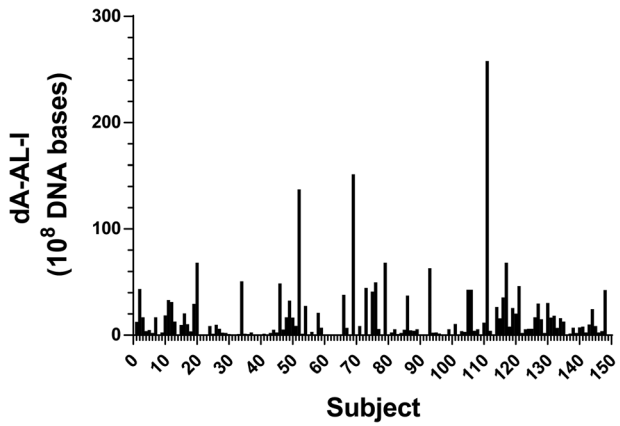

Fig. 5 Frequency and levels of dA-AL-I adduct identified in renal cortex of subjects from the Taiwan cohort. 132 out of the 148 subjects assayed harbored dA-AL-I at a level above 0.3 adducts per 10^8 DNA bases (the LOQ value).

interval range from 10.1 to 21.0 adducts per 10^8 DNA bases (a value of 0.15 adducts per 10^8 DNA bases, one-half the LOQ value for non-positive samples, was used for the calculation of the 95% CI).

The mean and range in the levels of dA-AL-I adducts in renal cortex tissue of subjects from Taiwan and the Balkans are depicted in Fig. 6. The numbers of subjects assayed from the Balkans are far fewer than the Taiwan cohort. Twelve of the 15 subjects of the Balkans harbored dA-AL-I adducts at levels above the LOQ, with a mean level of 2.0 adducts per 10^8 DNA bases.[21,49,50] Similar levels of dA-AL-I were detected with a larger set of subjects from the same cohort (67 subjects) by ^{32}P-postlabeling/PAGE.[21] Even though the amounts of AA-II present in *Aristolochia* herbs are usually within two- to five-fold of the amounts of AA-I,[19,70,71] the dA-AL-II adduct was detected at a frequency of only several percent and occurred at levels ~100-fold lower than the levels of dA-AL-I.[21,38,51] This DNA adduct biomarker data implicate dA-AL-I as the major genotoxic lesion in humans.

Formalin-fixed paraffin embedded (FFPE) tissues: an untapped biospecimen to assess AA exposure

The paucity of fresh tissue samples often precludes the measurement of DNA adducts in molecular epidemiology studies. In contrast, FFPE tissues are frequently available for biomarker discovery. Formalin fixation, followed by paraffin embedding (FFPE), has been used as the standard preservation technique for more than a century in laboratories worldwide.[72–74] Formaldehyde reacts with macromolecules and introduces intramolecular and intermolecular crosslinks between proteins and DNA. Chemical fixation preserves cell morphology and permits characterization of cells by light and electron microscopy or by immunohistochemistry (IHC).[73] The ability to examine, retrospectively, FFPE tissue for which there is clinical diagnosis of disease opens a previously untapped source of biospecimens for measurement of DNA adducts, by

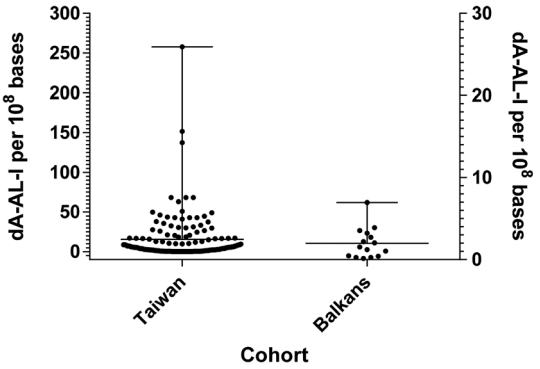

Fig. 6 The mean and maximum range of dA-AL-I adduct levels in cortex tissue of subjects from Taiwan (left scale axis) and the Balkans (right scale axis).

MS methods, to assess the exposure to environmental carcinogens. Until now, the screening of carcinogen DNA adducts in human FFPE tissues has been done largely by IHC methods.[75,76] The applicability of IHC to screen FFPE tissue-sections makes IHC an attractive method for the analysis of DNA adducts. However, an important drawback of IHC is that the specificity of many antibodies, even monoclonal antibodies, for DNA adducts is uncertain as they may cross-react with other DNA lesions, leading to errors in identification and quantification. Moreover, the production of antibodies is limited to select classes of carcinogen DNA adducts, and the generation of monoclonal antibodies is expensive. Thus, a robust, versatile and specific mass spectrometry method to measure DNA adducts in FFPE tissues is not subject to these limitations.

Interpretation of DNA adduct biomarker data from FFPE tissue must be done with caution. The identification of DNA adducts in clinical samples is frequently due to recent exposure, whereas the measurement of DNA adducts often is more relevant when assayed much earlier in time, when the multistage process of malignant tumor formation and progression has begun, rather than years later when the cancer has been diagnosed[2]. However, AA is unusual among many environmental carcinogens in its biopersistence: the dA-AL-I adduct is resistant to global genomic repair[42] and the adduct can be detected many years after ingestion of *Aristolochia* herbs has ceased.[34,38,48] Thus, the measurement of dA-AL-I adducts in the renal cortex likely represents an accumulation of adducts.

The quantitation of carcinogen-DNA adducts in FFPE tissues has been difficult because many such adducts are unstable at the elevated temperature or alkaline pH commonly used to reverse DNA cross-links in formalin-fixed tissues.[74,77,78] The recovery of fully digestible DNA from FFPE tissue requires the complete reversal of formalin cross-links of DNA under mild conditions where the DNA adducts are stable.[50] The incomplete reversal of DNA cross-links can impede the efficacy of nucleases to fully digest DNA and results in the poor recovery of DNA adducts. Some common conditions of DNA retrieval, including chloroform/phenol extraction or silica-based chromatography, did not provide fully digestible DNA, and the recovery of dA-AL-I was low, probably because dA-AL was present in the form of incompletely digested oligonucleotide adducts.[49,50] A major technical breakthrough was the development of commercial kits that employ mild retrieval conditions to recover DNA free of cross-links, which can serve as high fidelity templates for amplification with polymerase chain reaction (PCR). We examined the suitability of the DNA retrieval technique for application in the analysis of AL-DNA adducts in FFPE tissues.[49,50] With some modifications of the DNA isolation scheme, the commercial kits can be employed to measure dA-AL-I from FFPE tissues at the same level as those measured in matching fresh frozen tissue.[50] The FFPE DNA retrieval method was validated in male C57BL/6J mice exposed to AA-I (0.001–1 mg kg^{-1} body weight).[50] A linear-dose response in dA-AL-I adduct formation was observed in kidney, whereas a sublinear dose–response was seen for dA-AL-I adduct formation in liver (Fig. 7). The overall mean difference in adduct levels between freshly frozen and FFPE kidney and liver tissues, fixed in formalin for 24 hours across all doses was 21 ± 14% (mean ± SD).

Thereafter, we examined additional features of formalin fixation that could impact the quantity and quality of DNA and the recovery of AL-DNA adducts employing tissues of mice exposed to AA.[49] For example, the conditions employed for formalin fixation varies in different clinical laboratories across the world, and a prolonged time period of formalin fixation can result in increased amounts of DNA irreversibly cross-linked with protein and adversely affect the recovery of high quality, fully digestible DNA; thereby impacting the reliability of measurement of AL-DNA adducts. We examined the effect of the time of formalin fixation on the recovery of AL-DNA adducts in mice, following exposure to AA-I.[49] Although, the quantity of DNA recovered from tissues preserved in formalin for 1 week was ~30% of the amount recovered from frozen

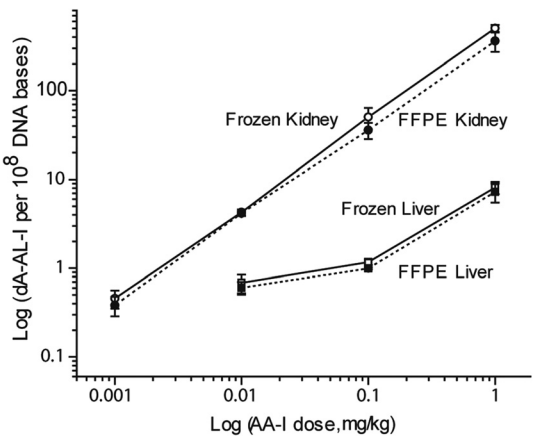

Fig. 7 dA-AL-I adduct formation in C57BL/6J mice exposed to AA-I (0.001–1 mg kg^{-1} body weight). The overall mean difference in adduct levels between freshly frozen and FFPE kidney and liver tissues, fixed in formalin for 24 hours across all doses was 21 ± 14% (mean ± SD, N = 4).[49] This figure was reproduced with permission from ref. 50.

tissue, indicative of irreversible cross-linking between DNA and protein,[72,74,79] the levels of AL-DNA adducts measured were similar to values determined in fresh frozen tissue. Therefore, the DNA retrieval technique is robust. Moreover, the DNA retrieved from FFPE tissue, following variable times of formalin fixation up to 7 days, could still serve as a template for PCR amplification, yielding sequence data of comparable quality to DNA obtained from fresh frozen tissue.[49] Thus, DNA isolated from FFPE tissues may be used to biomonitor DNA adducts and to amplify genes used for mutational analysis, providing clues regarding the origin of human cancers for which an environmental cause is suspected.

This method of DNA retrieval was then applied to measure dA-AL-I in tissue-sections of FFPE kidney from patients with upper urinary tract cancer from the Balkans.[50] The amounts of DNA recovered from two 10 μm thick section cuts of a surface area of 1.0 to 1.8 cm^2 ranged between 2.5 and 8 μg DNA, a quantity sufficient for DNA adduct measurements.[50] The levels of dA-AL-I adducts measured in freshly frozen tissue and matching FFPE tissue blocks of human kidney stored for up to 9 years at room temperature showed good concordance (Fig. 8). These findings show that archived human FFPE cortex biospecimens can be used to assess AA exposure by measurement of dA-AL-I adducts in cohorts where fresh frozen tissue is not available.

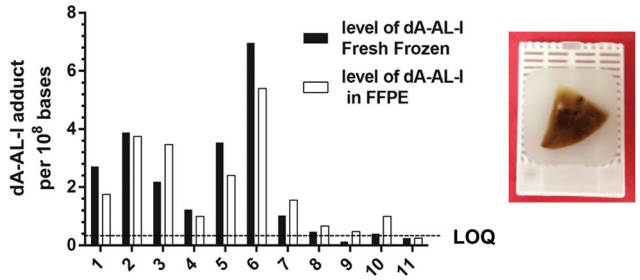

Fig. 8 dA-AL-I adducts in matching fresh frozen and FFPE kidney samples, containing both renal cortex and medulla, obtained from 11 individuals residing in endemic regions of Croatia and Serbia who underwent nephroureterectomy for UTUC, and a representative FFPE paraffin-embedded renal tissue block.

Biomonitoring of DNA adducts of AA and other genotoxicants in exfoliated urinary cells

Exfoliated urinary cells are comprised of urothelial cells shed from the upper urinary tract (renal pelvis and ureter) and from the lower tract (bladder and urethra); squamous epithelial cells from urethra, vagina; tubular epithelial cells from the kidney; and inflammatory cells (with infection). More than twenty years ago, pilot studies, using ^{32}P-postlabeling techniques, revealed the presence of putative DNA adducts in the urothelium and in exfoliated urinary cells of tobacco smokers.[80,81] One of the lesions was tentatively identified as the C-8 deoxyguanosine (dG) adduct of 4-aminobiphenyl, an aromatic amine implicated in the pathogenesis of bladder cancer in smokers.[80] ^{32}P-Postlabeling of exfoliated urinary cells of occupationally exposed factory workers also revealed putative DNA lesions.[82,83] Thus, urinary cells appear to be a promising biospecimen to non-invasively screen for DNA damage induced by chemicals at different portions of the kidney and urothelium. However, ^{32}P-postlabeling methods do not provide spectral data and the chemical identities of the lesions responsible for urothelial damage generally are unknown.

There are two reports on the analysis of AL DNA adducts in exfoliated cells of rodents subchronically treated with high doses of AA (10 mg kg^{-1}), employing ^{32}P-postlabeling[84] or TQ/MS.[85] The levels of dA-AL adducts were reported to range from 2–23 adducts per 10^9 DNA bases. Because of the exquisite sensitivity of our ion trap-based UPLC-ESI/MS^3 method, we sought to determine whether DNA from exfoliated urinary cells of human subjects with known history of ingestion of traditional Chinese medicines (TCMs) could be employed to biomonitor AL-DNA adducts. Exfoliated urinary cells obtained from five Chinese patients with compromised renal function treated by our collaborators, Tao Su, Li Yang, and Xiaomei Li, at the Peking University First Hospital, Beijing, were assayed for dA-AL-I (Table 1). Urine was collected over 24 h and exfoliated urinary cells were obtained by centrifugation. The DNA was isolated by a Qiagen™ kit and used for detection of adducts by MS.[86] The range in the level of DNA from urinary cells collected over 24 hours was 11–33 μg, a level sufficient for

Table 1 AL-DNA adduct levels in exfoliated urinary cells of patients from China[a]

Subject #	Gender	Age	AA-I intake (gram)	Drug form	Duration of drug treatment (year)	Gram/year	Time since discontinued	dA-AL-I/10^8 DNA bases
24	M	67	4.50	AA pill (GMT)	6	0.750	3 d	581
17	F	72	2.52	AA pill (QMX)	10	0.252	<3 mo	3.0
19	F	38	2.00	AA pill (QMX)	8	0.250	<3 mo	13.1
20	F	55	1.26	AA pill (GMT)	2	0.630	<3 mo	3.0
13	F	71	0	Aspirin	10	0	<3 mo	<0.3

[a] These preliminary data on dA-AL-I in exfoliated urinary cells have been reported.[86] There are several reports on the estimated composition of AA in Chinese drugs derived from *Aristolochia* sp.[19,70,71] QMX = Qingmuxiang-root of *Aristolochia debilis*. GMT = Guan mutong (GMT)-root of *Aristolochia manshuriensis*.

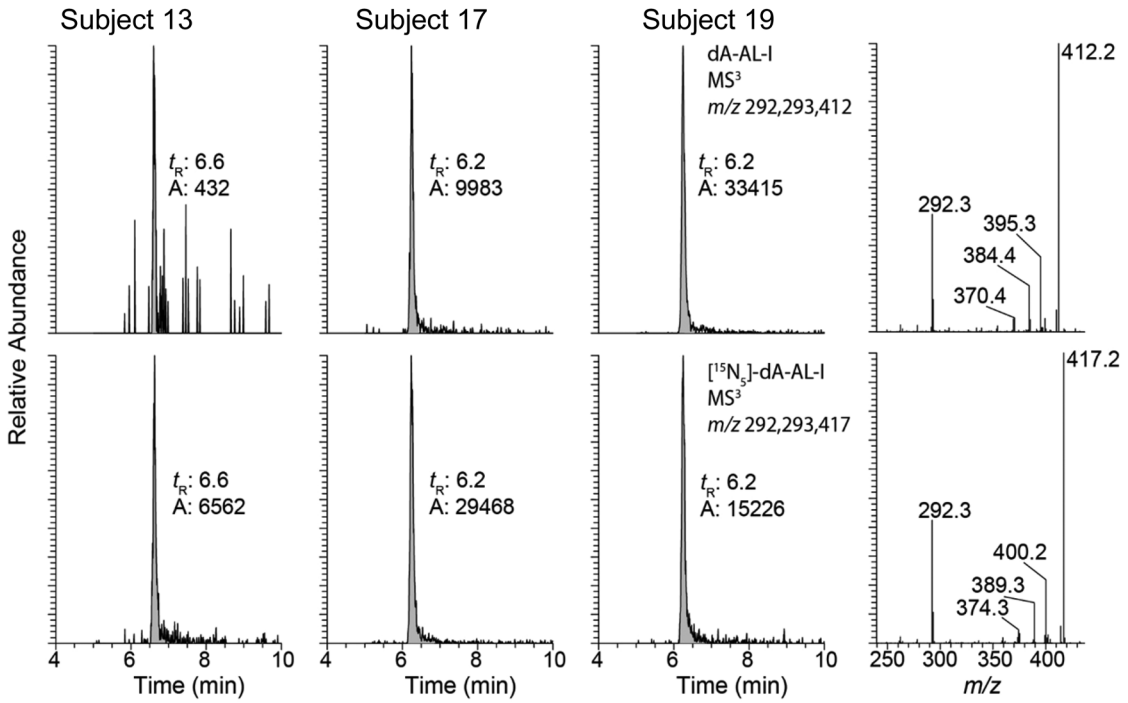

Fig. 9 UPLC-ESI/MS^3 chromatograms of dA-AL-I present in human exfoliated urinary cells. Analysis was done with 3 to 6.5 μg DNA. The internal standard, [$^{15}N_5$]-dA-AL-I, was added at a level of 3.9 adducts per 10^8 bases for subject 13; 8.3 adducts per 10^8 bases for subject 17; and 5.6 adducts per 10^8 bases for subject 19. The product ion spectrum of dA-AL-I and [$^{15}N_5$]-dA-AL-I are shown for subject 17.

measurement of AL-DNA adducts. Four of the patients had ingested herbs (cumulative dose 1.3–4.5 grams) containing variable amounts of *Aristolochia* (Table 1). dA-AL-I adducts were detected at levels above the LOQ value in all four subjects. One patient had exceptionally high levels of dA-AL-I (>500 adducts per 10^8 DNA bases) in exfoliated urinary cells collected three days after discontinuation of the drug treatment. dA-AL-I was not detected in exfoliated urinary cells of a subject, who did not have a recorded history of herbal usage. The reconstructed ion chromatograms at the MS^3 scan stage for dA-AL-I in DNA of urinary cells are shown in Fig. 9. Our pilot study demonstrates the feasibility of employing human exfoliated urinary cells as a non-invasive biospecimen to assess AL-DNA damage induced by TCMs.

Discussion

Aristolochic acid represents a highly relevant environmental toxicant model for translational studies of chemical carcinogenesis. The identification of AL-DNA adducts by state-of-the-art analytical mass spectrometry, combined with cancer genomics and mutational spectra databases to correlate mutations induced by AA and disease outcome have provided incontrovertible evidence for a causative linkage between AA exposure and the devastating environmental disease, now termed AAN. The usage of TCMs, some of which still contain AA, remains a world-wide public health issue. During the past several decades, over one-third of the population in Taiwan has been prescribed traditional herbal remedies containing *Aristolochia*.[26,27] The levels of dA-AL-I in some of the UUC patients of Taiwan are among the highest adduct levels reported for any known environmental genotoxic carcinogen,[55,56] and the recorded incidence of UTUC in Taiwan is the highest in the world.[15,27] The preliminary DNA adduct biomarker data reveal that there is also significant exposure to AA in mainland China.[86] The usage of TCM containing AA has been estimated to place tens of millions of people at risk in mainland China.[25] Thus, AAN and UTUC are likely to be prevalent in China and other Asian countries where *Aristolochia* herbs are used in traditional medicines.[25] The identification of AL-DNA adducts in renal tissue of populations in China, the United Kingdom, and the USA,[38,40] confirm that exposure to AA has occurred, most likely through usage of TCM containing *Aristolochia* sp.[20,70,71] Recent genome-wide sequence studies of tumors reveal the mutational signature associated with AA to be present in renal cell carcinomas from Romania[87] and Croatia[88] as well as hepatocellular carcinomas and intrahepatic cholangiocarcinomas from China.[46,89] These findings increase the scope of the potential public health risk associated with exposure to AA; however, these recent studies did not include confirmatory exposure to AA by measurement of AL-DNA adducts and the extent of AA exposure is unknown in these cohorts. A survey conducted in 2014 by the United States Food and Drug Administration on 30 herbal products marketed in the United States *via* the Internet reported the identification of AA-I and II in 20%

and 7%, respectively, of tested samples, despite a ban on the importation of herbs containing *Aristolochia*.[20] Thus, the exposure to AA and its attendant renal toxicity is a world-wide public health issue.

The employment of sensitive MS instrumentation has permitted the identification of AA exposure through its persistent DNA adducts in fresh frozen biospecimens. However, fresh tissue banks are often unavailable in many countries of Eastern Europe or Asia. The ability to retrieve AL-DNA adducts quantitatively from FFPE tissue blocks represents an important technical breakthrough that allows identification of AL-DNA adducts at the time of early exposure,[49,50] which is often 20–30 years prior to the development of the resulting urothelial cancer. The screening of FFPE tissues for dA-AL adducts permit the retrospective assessment of exposure to AA in populations where fresh tissues are unavailable and determine if AA is a causal agent of renal disease in these populations.

Our initial data show that exfoliated urinary cells are a promising noninvasive biospecimen to determine exposure to AA by measurement of dA-AL-I. Future studies that examine relationship between dA-AL-I adduct levels in exfoliated urinary cells and time of TCM exposure, the cumulative dose and time following withdrawal; renal functional status, and gender are required to validate exfoliated urinary cells as a reliable biospecimen to measure AA exposure. Biomonitoring AL-DNA adducts in exfoliated cells may avoid an invasive renal biopsy and offer a potentially powerful approach to screen people thought to be exposed to AA and at elevated risk for UTUC. Once validated, the screening of exfoliated urinary cells may have a far reaching impact on the detection, diagnosis, and prevention of AA-induced urothelial cancer. The technological approaches employed for screening AL-DNA adducts in FFPE tissue and exfoliated urinary cells may be adapted and clear the way for use of these largely underutilized biospecimens in molecular epidemiology studies that seek to assess the causal role of other hazardous chemicals in the risk of urothelial and other types of cancers.

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