Mutation Research 743 (2012) 52–58

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Comparison of the mutagenicity of aristolochic acid I and aristolochic acid II in the gpt delta transgenic mouse kidney

Guozhen Xing[a,b], Xinming Qi[a], Min Chen[a,b], Yuanfeng Wu[a,b], Jun Yao[a,b], Likun Gong[a], Takehiko Nohmi[c,1], Yang Luan[a,*], Jin Ren[a,*]

[a] Center for Drug Safety and Evaluation Research, State Key Laboratory of New Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Haike Road 501, Shanghai 201203, China
[b] Graduate School of the Chinese Academy of Sciences, Shanghai 201203, China
[c] Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan

ARTICLE INFO

Article history:
Received 3 November 2011
Received in revised form
23 December 2011
Accepted 26 December 2011
Available online 5 January 2012

Keywords:
Aristolochic acid
DNA adducts
Mutagenesis

ABSTRACT

Aristolochic acid (AA) is known to be a potent mutagen and carcinogen. Aristolochic acid I (AAI) and aristolochic acid II (AAII), the two major components of AA, differ from each other by a single methoxy group. However, their individual mutagenic characteristics *in vivo* are unclear. In the present study, we compared their DNA adduct formation and mutagenicities in the gpt delta transgenic mouse kidney. The dA-AAI, dG-AAI, dA-AAII and dG-AAII were identified in the kidney two days after intragastric administration of AAI or AAII at 5 mg/kg. The concentration of DNA adducts formed by AAII was approximately 2.5-fold higher than that formed by AAI ($p < 0.05$). The mutant frequency induced by AAII was nearly two-fold higher than that induced by AAI ($p < 0.05$) following administration of 5 mg/kg AAI or AAII, five times per week for six weeks. Investigation of the mutation spectra showed no statistically significant difference between AAI- and AAII-treated mice ($p > 0.05$). A:T to T:A transversion was the predominant type of mutation in both treated groups, the GC-associated mutation rates, however, differed between the AAI and AAII treatments. The *in vivo* metabolic pathways of AAI and AAII are different, and this may affect their mutagenicity. In the present study, we measured the levels of AAI and AAII in the kidney and plasma of gpt delta transgenic mice at multiple time points after a single intragastric dose of 1 or 5 mg/kg of either component. Our results showed that the levels of AAII in both kidney and plasma were considerably higher than those of AAI ($p < 0.01$). The present study indicated that AAII showed more carcinogenic risk than AAI *in vivo*, and this may be, at least partly, the result of its increased levels in kidney and plasma.

1. Introduction

Aristolochic acid (AA), derived from *Aristolochia* plant species, is associated with aristolochic acid nephropathy [1,2] and Balkan endemic nephropathy [3]. Herbal remedies containing species of the genus *Aristolochia* were classified as carcinogenic to humans (Group 1) and the AA mixture was upgraded from Group 2A to Group 1 by the International Agency for Research on Cancer in 2009 [4]. Although all pharmaceutical preparations containing AA were withdrawn from the market in many countries, a wide variety of *Aristolochia* plants are still in use in many regions of the world [5,6].

AA is a mixture of structurally related nitrophenanthrene carboxylic acids mainly consisting of 8-methoxy-6-nitro-phenanthro-(3,4-D)-1,3-dioxolo-5-carboxylic acid (aristolochic acid I, AAI) and 6-nitro-phenanthro-(3,4-D)-1,3-dioxolo-5-carboxylic acid (aristolochic acid II, AAII). The molecular structure of AAI differs from AAII by only a single methoxy group which may have important implications for their metabolic and toxicity properties. Clarification of such structure–toxicity relationships will enable better understanding of the mechanisms that underlie their mutagenicity. Moreover, the content ratio of AAI to AAII in *Aristolochia* plants varies with *plant* species and geographical regions [7], and may also change during processing [8]. Determination of which of the two components is more toxic *in vivo* will be helpful with regards to quality control and toxicity monitoring of *Aristolochia* plants.

The metabolic pathways of AAI and AAII differ [9,10]. AAI can be O-demethylated and nitroreduced *in vivo* to form 8-OH-aristolochic acid I (AAIa) and aristolactam I (ALI); however, AAII is only nitroreduced to form aristolactam II (ALII). Both ALI and ALII can form 8-hydroxyaristolactam Ia, which

* Corresponding authors. Tel.: +86 021 20231000x1303; fax: +86 021 20231000x1303.
E-mail addresses: yluan@mail.simm.ac.cn (Y. Luan), jren@mail.shcnc.ac.cn, gzxing@cdser.simm.ac.cn (J. Ren).
[1] Tel.: +81 03 3700 9872.

1383-5718/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2011.12.021

undergoes phase II conjugation [11–13]; however, an intermediate with a cyclic nitrenium ion, generated by the nitroreduction of AAI and AAII, can covalently bind the exocyclic amino groups of DNA bases [14]. The predominant DNA adducts detected in rodents and humans include 7-(deoxyadenosin-N^6-yl) aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl) aristolactam I (dG-AAI), 7-(deoxyadenosin-N^6-yl) aristolactam II (dA-AAII) and 7-(deoxyguanosin-N^2-yl) aristolactam II (dG-AAII), and can result in mutagenesis and carcinogenesis [15–17].

AA-DNA adducts can be detected in many organs *in vivo* and higher levels of DNA adducts are found in the kidney, forestomach and urethral epithelium in rodents [15]. ^{32}P-postlabeling has been used classically to determine AA-DNA adducts; however, different results have been obtained using different ^{32}P-postlabeling methods. Schmeiser et al. [17] found the same levels of AAI- and AAII-DNA adducts in the kidney using ^{32}P-postlabeling-thin layer chromatography (TLC), whereas in the bladder only AAII gave rise to DNA adduct formation. However, Pfau et al. [18] and Dong et al. [19] found higher levels of AAII-DNA adducts than AAI-DNA adducts in the kidney and bladder of rats using ^{32}P-postlabeling-TLC-Ion-pairing-high performance liquid chromatography (HPLC) and ^{32}P-postlabeling Ion-pairing-polyacrylamide gel electrophoresis (PAGE), respectively. Shibutani et al. [20] used ^{32}P-postlabeling-PAGE to investigate DNA adducts formed by AAI and AAII in mice, and found equivalent amounts of DNA adducts formed by AAI and AAII.

Recently, liquid chromatography–mass spectrometry (LC–MS) has been used more extensively than ^{32}P-postlabeling to determine DNA adducts. LC–MS has advantages in that it not only provides information on the chemical identity of the detected DNA adducts, but also avoids the health risks of radioactivity [21]. Chan et al. [22] determined the level of DNA adducts formed by AAI and AAII using a hybrid quadrupole time-of-flight tandem mass spectrometer coupled with electrospray ionization source (ESI), in which the limit of detection was one adduct per 10^9 normal nucleosides. Chan et al. found that the level of DNA adducts formed by AAII was 1.6-fold higher than that formed by AAI in rat kidney. The deoxycytidine adduct, which in turn was formed *via* an intermediate with deoxycytosine, was also identified in the kidneys of rats injected with a high dose of AAII.

AA is mutagenic in bacteria, mammalian cells, mice and rats [15,23,24]. Schmeiser et al. [25] found that AAI and AAII were mutagenic in same manner in *Salmonella typhimurium* TA1537 and TA100, whereas Götzl and Schimmer [26] found that AAII was more mutagenic than AAI in *S. typhimurium* TA98, TA100 and TA1537. Maier et al. [27] found that AAI was more mutagenic than AAII in subcutaneous granulation tissue in rats; however, this study took only oxygen tension into account, without considering the effects of metabolism and the distributions of AAI and AAII *in vivo*.

The transgenic mouse mutation assay is a powerful tool to study chemical mutagenesis *in vivo*, data from transgenic mouse assays have more relevance in studies mechanism of initiation of carcinogenesis. Studies of the mutagenicity of the AA mixture, using Muta™ Mice [24] and Big Blue rats [23], however, did not provide the information for their respective mutagenicities. In the present study, we used LC-ESI-MS–MS and *gpt* delta transgenic mouse [28] to determine the DNA adducts and mutant frequency of *gpt* gene induced by AAI and AAII in the kidney as well as their individual mutation spectrum characteristic. We also determined the levels of AAI and AAII in plasma and kidney at different time-points.

2. Materials and methods

2.1. Chemicals

AA I (96%) and the mixture of AA (65% AAI and 27% AAII) were obtained from sigma (St. Louis, MO, USA). ALI was obtained from ChemPartner (Shanghai, China). 2-Deoxyadenosine (dA), 2-deoxyguanosine (dG), DNase I and phosphodiesterase I were obtained from Sangon Biotech Co. (Shanghai, China). Alkaline phosphatase was obtained from Sigma. HPLC-grade methanol and acetonitrile were obtained from Merk (Darmstadt, Germany). Other reagents were analytically pure.

2.2. Isolation of AAII

The AA mixture was separated by polyamide column chromatography, subsequently dissolved in chloroform-methanol solvents and mixed with polyamide [100 mg AA mixed with 0.5 g polyamide (100–200 mesh)], and then dried. The dry mixture was loaded on a polyamide column and eluted with water/methanol/formic acid (1:4:0.25). The AAI and AAII levels in different fractions were detected by polyamide thin layer chromatography. Fractions containing only AAI or AAII were combined and evaporated to dry. Fractions containing both AAI and AAII were combined and subsequently condensed to a small volume. This mixture of AA was then separated using Sephadex LH-20 column and eluted with methanol–water (30:70). The fractions which contained AAII were combined and dried. Using this method, 17 mg of AAII was obtained from a 100 mg mixture of AA. The purity of AAII (>95%) was confirmed by HPLC.

2.3. Synthesis of ALII

Ten mg AAII and 500 mg zinc powder were added into 50 ml 0.05 M K_3PO_4 buffer (pH 5.8) and stirred for 48 h at 37 °C in dark. The reaction mixture was extracted by 60 ml ethyl acetate for four times. The combined extracts were dried by reduction vaporization and resolved in 2 ml of tetrahydrofuran and then was isolated by Waters preparative HPLC with methanol–water (80:20) contained 0.1% formic acid. ALII was identified by Mass Spectrometry and the purity (>95%) was confirmed by HPLC.

2.4. Synthesis of AA derived DNA adducts

2.4.1. Synthesis of dA-AAI, dG-AAI, dA-AAII and dG-AAII by zinc reduction

Added 0.5 mg AAI or AAII, 1 mg dA or dG and 10 mg zinc powder into 500 μl K_3PO_4 buffer (50 mM, pH 5.8). The reaction system was incubated in a water-bath at 37 °C for over 16 h. Then the reaction mixture was extracted with 500 μl ethyl acetate for 3 times. The combined extracts were dried under nitrogen at room temperature. The residue was resolved with 200 μl acetonitrile in water (50:50) for LC-ESI-MS–MS analysis.

2.4.2. Preparation of oligodeoxynucleotides containing a single AAI-derived DNA adduct

A 10-mer oligodeoxynucleotide (330 μg; 5′-TTTTTATTTT-3′ was dissolved in 1 ml 50 mM K_3PO_4 buffer (pH 5.8) was incubated with 0.5 mg AA I and 20 mg zinc dust in 2 ml Eppendorf tube over 16 h at 37 °C in the dark, as described by Schmeiser et al. [29]. The reaction mixture was then centrifuged and the supernatant was removed. The supernatant was separated by HPLC as described in [19]. Based on the extinction coefficient at 260 nm, the concentrations of the 10-mer containing a single dA-AAI was 30.3 μg (9.24 nmol)/ml = 1.0 $OD_{260\,nm}$.

2.5. Animal experiment

The *gpt* delta transgenic mice were developed by Dr. Nohmi of the National Institutes of Health Sciences, Japan. All animal experiments were approved by the Shanghai Animal Care and Use Committee [Certificate No.SCXK (Shanghai) 2002–0010]. Animals were acclimatized in Specific Pathogen Free rooms with the temperature at 20–26 °C, the humidity at 30–70% and a 12-h light/dark cycle for at least 1 week. Regular laboratory chow and filtered tap water were allowed *ad libitum*.

2.5.1. To detect formation of AA-DNA adducts in kidney

Eight-weeks-old male *gpt* delta transgenic mice were randomly divided into 2 groups (n = 5 per group) and treated intragastically with AAI or AAII 5 mg/kg for two days. The mice were killed by cervical dislocation 24 h after last treatment and kidneys were removed and stored in a freezer frozen at −40 °C until being analyzed.

2.5.2. To detect mutant frequency in kidney induced by AAI and AAII

Eight-weeks-old male *gpt* delta transgenic mice were randomly divided into 5 groups, including vehicle control (n = 5), AAI 1 mg/kg group (n = 4), AAI 5 mg/kg (n = 4), AAII 1 mg/kg group (n = 4), and AAII 5 mg/kg (n = 4). The mice were treated intragastically with AAI or AAII dissolved in 1% $NaHCO_3$ five days a week for consecutive six weeks. The vehicle control group received 1% $NaHCO_3$ at same manner. One week after the last treatment, the mice were killed by cervical dislocation. Kidneys were removed and quickly frozen in liquid nitrogen, then stored in a deep freezer frozen at −80 °C until being analyzed.

2.6. Mutation assay

High-molecular-weight genomic DNA was extracted from kidneys using the RecoverEase DNA Isolation Kit (Stratagene; LaJolla, CA, USA), and stored at 4 °C for