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## Data in Brief

Aristolochic acids – Induced transcriptomic responses in rat renal proximal tubule cells *in vitro*Katarzyna M. Bloch <sup>\*</sup>, Andrew Evans, Edward A. Lock

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## ABSTRACT

Aristolochic acids (AAs) are the active components of herbal drugs derived from *Aristolochia* species that have been used for medicinal purposes since antiquity. However, AAs have recently been discovered to be highly nephrotoxic and induced urothelial cancer in humans and malignant tumors in the kidney and urinary tract of rodents. In this study, we exposed rat renal proximal tubule cells *in vitro* to a sub-cytotoxic level of AAs at three different time points (6 h, 24 h and 72 h). We then analyzed the gene expression profile after the compound exposure. Functional analysis with Ingenuity Pathways Analysis and DAVID tools revealed that at the late time point (72 h) there are many significantly altered genes involved in cancer-related pathways such as p53 signaling.

MIAMI-compliant microarray data are deposited in the NCBI GEO database under accession number GSE68687 and can be found at: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68687>.

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Specifications	
Organism/cell line/tissue	Rat/NRK-52E cells (ATCC, CRL-1571)
Sex	Male
Sequencer or array type	Affymetrix Rat Genome 230 2.0 Array
Data format	Raw and analyzed
Experimental factors	Normal rat renal proximal tubule cells exposed to 1.65 μM aristolochic acids for 6 h, 24 h and 72 h as well as time-matched controls exposed to DMSO alone
Experimental features	NRK-52E cells were exposed to aristolochic acids dissolved in DMSO (0.1%) at the IC <sub>10</sub> concentration at 72 h (1.65 μM) or DMSO only as control. After 6 h, 24 h and 72 h RNA was extracted from the cells. Three studies were conducted at each time point.
Consent	NA
Sample source location	NRK-52E cells (ATCC, CRL-1571), Liverpool, UK

## Direct link to deposited data

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68687>.

## Introduction

Aristolochic acids (AAs) are a mixture of structurally related nitrophenanthrene carboxylic acids, mainly 8-methoxy-6-

nitrophenanthro [3,4-*d*] 1,3-dioxolo-5-carboxylic acid (aristolochic acid I) and its 8-demethoxylated form (aristolochic acid II) (Fig. 1) that are secondary metabolites of *Aristolochia* and *Asarum* plant species.

Herbal drugs containing aristolochic acids have been used since antiquity, however in 1982, AAs were reported to be highly carcinogenic in rats causing renal and forestomach cancers [1,2]. Later similar findings were reported in mice [3]. In 1991, a unique and rapidly progressive renal fibrosis referred as Aristolochic Acid Nephropathy (AAN), was observed in around 5% of patients that took weight-reducing pills containing AAs [4]. Over 100 cases of AAN have been identified with around half needing renal transplantation [4,5]. AA consumption (as harvest contamination) is also hypothesized to be a causative agent in the development of a similar type of kidney fibrosis with malignant transformation of the urothelium – Balkan Endemic Nephropathy [6]. To date AAs have been shown to be among the 2% of the most potent known carcinogens [7]. IARC has classified herbal remedies containing species of the genus *Aristolochia* as carcinogenic to humans (Group 1) [8], whereas naturally occurring mixtures of AAs are classified into Group 2A (probably carcinogenic to humans). AA is a direct-acting mutagen in TA100 and TA1537 *Salmonella typhimurium* strains, but is not mutagenic in the nitroreductase-deficient strains (TA98NR and TA100NR) [9]. AAs were found to be positive in the L5178Y mouse lymphoma assay and MN assay [10]. Renal tubular epithelial cells are very sensitive to AAs and undergo apoptosis or necrosis in response to this compound, with AA1 being the more cytotoxic congener [11]. The carcinogenic effect of AAs is not fully elucidated but is associated with the formation of covalent

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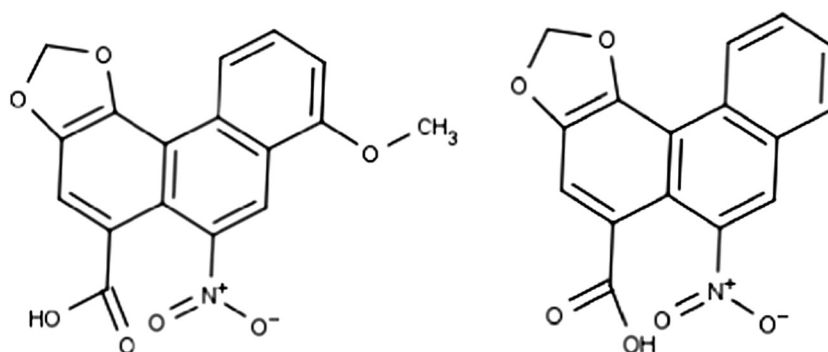


Fig. 1. Chemical structure of aristolochic acid I and II.

AA-DNA adducts. Rats treated with AAs develop mutations in p53 gene and the presence of AA-DNA adducts in renal cortex and p53 mutations in tumor tissue were reported in patients with endemic nephropathy [12].

## Materials and methods

### Materials

A mixture of AAI and AAI was purchased from Sigma-Aldrich, UK. TRIzol reagent and GlutaMAX were obtained from (Invitrogen, UK) and RNeasy Total RNA Mini Kit and RNA later from (Qiagen, UK).

### Cell culture

NRK-52E cells (ATCC, CRL-1571) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum, penicillin  $100 \text{ IU ml}^{-1}$ , and streptomycin solution  $100 \mu\text{g ml}^{-1}$  in a humidified 5%  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ .

### Cytotoxicity-determination of $\text{IC}_{10}$

A dose–response curve for AAs was determined using the MTT assay [13]. Cells were exposed for 72 h in 96-well plates to a wide range of concentrations of AAs dissolved in DMSO and diluted to give a final concentration of 0.1% v/v DMSO. Control cells were exposed to DMSO alone (0.1% v/v). At least three separate experiments were conducted. The dose that caused approximately 10% cytotoxicity ( $\text{IC}_{10}$ ) at 72 h was selected for the transcriptomics studies.

### Cell treatment

NRK-52E cells were cultured to confluence on 6-well plates. For transcriptomics studies, cells were exposed to AAs dissolved in DMSO (0.1% v/v) at the  $\text{IC}_{10}$  concentration at 72 h ( $1.65 \mu\text{M}$ ) or DMSO (0.1% v/v) alone. After 6 h, 24 h and 72 h the medium was removed and RNA was extracted from cells. For replication, three studies were conducted at each time point.

Table 1

KEGG pathways enriched after 72 h exposure to AAs.

KEGG pathway	No. of genes involved in pathway	Total no. of genes involved in the pathway (%)	<i>P</i> -value (Benjamini)
p53 signaling pathway	16	1.9	$8.5\text{E}-8$ ( $1.3\text{E}-5$ )
Pathways in cancer	30	3.6	$8.6\text{E}-5$ ( $6.4\text{E}-3$ )

### RNA isolation and microarray

From in vitro studies total RNA was isolated from DMSO (control) and AA-treated cells. TRIzol reagent was used for RNA isolation. Total RNA was purified using the RNeasy Total RNA Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was checked for purity and integrity using Agilent 2001 Bioanalyzer (Agilent Technologies GmbH, Germany) before processing. Transcriptomics data was generated using GeneChip Rat Genome 230 2.0 The rat array provides comprehensive coverage of the transcribed rat genome and comprised of more than 31,000 probe sets, analyzing over 30,000 transcripts and variants from over 28,000 well-substantiated rat genes.

### Microarray hybridization

#### Target preparation

cDNA was prepared using the Affymetrix IVT express kit (Affymetrix, Santa Clara). cDNA synthesis and labeling were performed according to the manufacturer's procedures. Subsequent labeling of the samples was conducted by synthesis of Biotin-labeled complementary RNA (cRNA) using the GeneChip IVT labeling kit (Affymetrix). Purified cRNA was quantified using a spectrophotometer, and unfragmented samples were checked on the Bioanalyzer. Subsequently, cRNA samples were fragmented for target preparation according to the Affymetrix manual and checked on the Bioanalyzer. Samples were stored at  $-20^\circ\text{C}$  until ready to perform hybridization.

#### Hybridization

cRNA targets were hybridized on high-density oligonucleotide gene chips (Affymetrix Human Genome U133 Plus 2.0 and GeneChip Rat Genome 230 2.0 Arrays) according to the Affymetrix Eukaryotic Target Hybridization manual. The gene chips were washed and stained using the Affymetrix Fluidics Station 450 and Genechip Operating Software and scanned by means of an Affymetrix GeneArray scanner.

### Microarray analysis

The intensity values of different genes (probe sets) generated by Affymetrix GeneChip Operating Software were imported into GeneSpring

Table 2

Ingenuity pathways enriched after 72 h exposure to AAs.

Ingenuity canonical pathway	No. of genes involved in the pathway	<i>P</i> -value
p53 signaling	17/98	$9.65\text{E}-08$
Glutathione biosynthesis	3/3	$4.96\text{E}-05$
ATM signaling	10/59	$5.20\text{E}-05$
Cell cycle: G2/M DNA damage checkpoint regulation	9/49	$6.44\text{E}-05$
Molecular mechanisms of cancer	8/99	$6.74\text{E}-05$

**Table 3**  
GO enriched after 72 h exposure to AAs.

GO	No. of genes involved in pathway	Total no. of genes involved in the pathway (%)	P-value (Benjamini)
Regulation of apoptosis	61	7.4	1.80E-08 (5.10E-05)
Release of cytochrome c from mitochondria	8	1	1.40E-05 (3.70E-03)
Regulation of cell proliferation	51	6.2	3.30E-05 (7.60E-03)
Apoptotic mitochondrial changes	8	1	1.50E-04 (2.40E-02)
Blood vessel morphogenesis	20	2.4	2.70E-04 (4.20E-02)
Tissue remodeling	11	1.3	2.80E-04 (4.10E-02)
Response to abiotic stimulus	33	4	2.90E-04 (4.00E-02)
Cellular response to stress	35	4.2	3.20E-04 (4.00E-02)
DNA damage response, signal transduction resulting in induction of apoptosis	7	0.8	3.60E-04 (4.30E-02)
Regulation of cell adhesion	15	1.8	4.50E-04 (4.60E-02)
Response to DNA damage stimulus	24	2.9	4.80E-04 (4.70E-02)

version 11 software (Agilent) for data analysis. The raw data files (CEL files) containing signal values for individual probes were pre-processed to generate one value per probe set. Pre-processing of arrays was done using GC-RMA (Robust Multiarray Analysis algorithm). To identify the differentially expressed (DE) probe sets, two sampled *t*-test (unpaired *t*-test) with a *P*-value <0.05 and Benjamini–Hochberg correction for multiple testing correction and a fold cut off of 2 was used. Comparisons were made between control DMSO-exposed and AA-exposed cells. Three studies were conducted at each time point.

#### Functional annotation

To understand the biological meaning behind the list of DE genes, Kyoto Encyclopaedia of Genes and Genomes database (KEGG) pathways analysis and Gene Ontology (GO) from DAVID website (The Database for Annotation, Visualization and Integrated Discovery) v 6.7 (<http://david.abcc.ncifcrf.gov/>) were used. Only GO and pathways with *P*-value <0.05 (Benjamin–Hochberg corrected) and with 5 or more genes were analyzed and discussed. In addition, Ingenuity Pathway Analysis (IPA) was used. Only pathways and functions with *P* < 0.05 (Fisher's exact test right-tailed) were analyzed and discussed.

#### Data deposition

MIAMI-compliant microarray data were deposited in the NCBI GEO database under accession number GSE68687; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68687>.

## Results

#### Gene expression profile induced after aristolochic acids exposure

No genes were differentially expressed after 6 h and 24 h exposure to AAs, however at 72 h, 1204 genes were found to be differentially expressed. Both IPA and KEGG pathways identify p53 signaling as a top statistically significantly enriched pathway (Tables 1 and 2). Additionally after 72 h exposure genes involved in apoptosis, stress

response, DNA damage and glutathione biosynthesis were identified (Tables 2 and 3).

## Conclusion

Our results suggest that microarray analysis is a useful tool for detecting AA exposure *in vitro* and the gene expression analysis can identify the responses to toxicity and carcinogenicity of AAs in rat renal proximal tubule cells. In addition, the significantly altered genes were identified to be associated with cancer pathways and specifically p53 signaling, DNA damage responses and apoptosis.

## Acknowledgements

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