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Bloch, KM, Evans, AR and Lock, EA (2015) Aristolochic acids - Induced transcriptomic responses in rat renal proximal tubule cells in vitro. Genomics Data, 5. pp. 254-256. ISSN 2213-5960
Data in Brief

Aristolochic acids — Induced transcriptomic responses in rat renal proximal tubule cells in vitro

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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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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 LS178Y 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 AAI being the more cytotoxic congener [11]. The carcinogenic effect of AAs is not fully elucidated but is associated with the formation of covalent
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 endemnic nephropathy [12].

**Materials and methods**

**Materials**

A mixture of AAI and AAII 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 IU ml⁻¹, and streptomycin solution 100 μg ml⁻¹ in a humidified 5% CO₂ incubator at 37 °C.

**Cytotoxicity-determination of IC₁₀**

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 (IC₁₀) 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 IC₁₀ concentration at 72 h (1.65 μM) 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 (%) | P-value (Benjamini) |
|----------------|---------------------------------|-----------------------------------------------|--------------------|
| p53 signaling pathway | 16 | 1.9 | 8.5E⁻⁸ (1.3E⁻⁵) |
| Pathways in cancer | 30 | 3.6 | 8.6E⁻⁵ (6.4E⁻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 °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 | P-value |
|-----------------------------|-------------------------------------|--------|
| p53 signaling               | 17/98                               | 9.65E⁻⁰⁸ |
| Glutathione biosynthesis    | 3/3                                 | 4.96E⁻⁰⁵ |
| ATM signaling               | 10/59                               | 5.20E⁻⁰⁵ |
| Cell cycle: G2/M DNA damage | 9/49                                | 6.44E⁻⁰⁵ |
| Molecular mechanisms of cancer | 8/99                                | 6.74E⁻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) |

Conclusions

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.

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