Bioactivation Mechanisms of N-Hydroxyaristolactams: Nitroreduction Metabolites of aristolochic Acids

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Aristolochic acids (AAs) are human nephrotoxins and carcinogens found in concoctions of Aristolochia plants used in traditional medicinal practices worldwide. Genotoxicity of AAs is associated with the formation of active species catalyzed by metabolic enzymes, the full repertoire of which is unknown. Recently, we provided evidence that sulfonation is important for bioactivation of AAs. Here, we employ Salmonella thyphimurium umu tester strains expressing human N-acetyltransferases (NATs) and sulfotransferases (SULTs), to study the role of conjugation reactions in the genotoxicities of N-hydroxyaristolactams (AL-NOH and AL-II-NOH), metabolites of AA-I and AAII. Both N-hydroxyaristolactams show stronger genotoxic effects in umu strains expressing human NAT1 and NAT2, than in the parent strain. Additionally, AL-II-NOH displays increased genotoxicity in strains expressing human SULT1A1 and SULT1A2, whereas AL-I-NOH shows enhanced genotoxicity in SULT1A1/2 and SULT1A3 strains. 2,6-Dichloro-4-nitrophenol, SULTs inhibitor, reduced umuC gene expression induced by N-hydroxyaristolactams in SULT1A2 strain. N-hydroxyaristolactams are also mutagenic in parent strains, suggesting that an additional mechanism(s) may contribute to the genotoxicities. Accordingly, using putative SULT substrates and inhibitors, we found that cytosols obtained from human kidney HK-2 cells activate N-hydroxyaristolactams in aristolactam-DNA adducts with the limited involvement of SULTs. Removal of low-molecular-weight reactants in the 3.5–10 kDa range inhibits the formation of aristolactam-DNA by 500-fold, which could not be prevented by the addition of cofactors for SULTs and NATs. In conclusion, our results demonstrate that the genotoxicities of N-hydroxyaristolactams depend on the cell type and involve not only sulfonation but also N,O-acetyltransfer and an additional yet unknown mechanism(s). Environ. Mol. Mutagen. 60:792–806, 2019. © 2019 Wiley Periodicals, Inc.

Key words: umu test; N,O-acetyltransfer; sulfonation; aristolactam-DNA adducts; HK-2 cytosols

Abbreviations: AA-I, aristolochic acid I or 8-methoxy-6-nitrophenanthro-[3,4-d]-1,3-dioxole-5-carboxylic acid; AA-II, aristolochic acid II or 6-nitrophenanthro-[3,4-d]-1,3-dioxole-5-carboxylic acid; AAN, aristolochic acid nephropathy; AAs or AA, collective terms for various aristolochic acids; AL-DNA, aristolactam-DNA adduct; AL-I, aristolactam I; AL-II, aristolactam II; AL-II-NOH, N-hydroxyaristolactam II; AL-I-NOH, N-hydroxyaristolactam I; AL-I-NOH, AL-I-NOH, AL-II-NOH, AL-I-NSO₃H, AL-II-NSO₃H, aristolactam-I-N-sulfate or N-sulfonyloxyaristolactam I; BEN, Balkan endemic nephropathy; CYP1A2/1, cytochrome P450 1A1 and cytochrome P450 1A2; DMSO, dimethyl sulfoxide; DNCP, 2,6-dichloro-4-nitrophenol; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HK-2, human kidney cell line; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NAT, N-acetyltransferase; NQO1, NAD(P)H:quinone oxidoreductase 1; NR, nitroreduction; O-AT, bacterial O-acetyltransferase; PAGE, polyacrylamide gel electrophoresis; PAP, 3-phosphoadenosine-5-phosphate; PAPS, 3-phosphoadenosine-5-phosphate; PCP, pentachlorophenol; Salmonella thyphimurium, S. thyphimurium; ssDNA, salmon sperm DNA; SULT, sulftotransferase.

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INTRODUCTION

Aristolochia botanicals have been used since antiquity to treat a variety of human conditions (Dawson 1927; Grollman and Marcus 2016). These herbs contain a family of structurally related nitrophenanthrene carboxylic acids, of which aristolochic acid I (AA-I) and aristolochic acid II (AA-II) are the most abundant and toxic principles (Fig. 1) (Kumar et al. 2003; Michl et al. 2014). Whereas both AA-I and AA-II induce aristolactam(AL)-DNA adducts (Schmeiser et al. 1988; Pfau et al. 1990b; Shibutani et al. 2007), mutations (Schmeiser et al. 1990; Schmeiser et al. 1991; Xing et al. 2012) and carcinogenesis in rodents (Mengs et al. 1982; Mengs 1988), only AA-I shows strong nephrotoxic effects in mice (Sato et al. 2004; Shibutani et al. 2007). In the literature, the mixture of AA-I and AA-II is often designated as AA.

In humans, AA ingestion is associated with aristolochic acid nephropathy (AAN) (Vanherweghem et al. 1993; Gillerot et al. 2001) and its environmental form known as Balkan endemic nephropathy (BEN) (Grollman et al. 2007). One of the unique features of AAN is its strong association with malignancies of the upper urinary tract (Cosyns et al. 1999; Nortier et al. 2000; Chen et al. 2012; Hoang et al. 2013). However, not all individuals are susceptible to the adverse effects of AA and there is a long latent period between exposure and effects (Vanherweghem 1998; Grollman 2013). Therefore, toxicities of AA in humans have long been overlooked. Recently, owing to the advances in mass spectrometry and next generation sequencing techniques, AA ingestion has been implicated in the etiology of renal cell carcinoma (Scelo et al. 2014; Jelakovic et al. 2015; Hoang et al. 2016; Turesky et al. 2016), bladder cancer (Poon et al. 2015), intrahepatic cholangiocarcinoma (Zou et al. 2014), and hepatocellular carcinoma (Poon et al. 2013; Totoki et al. 2014; Ng et al. 2017). These findings indicate that exposure to AA contributes significantly to kidney disease and cancer worldwide.

Since Aristolochia species are common in Chinese Traditional Medicine, it is estimated that in China alone there are 10–100 million people at risk of developing AA-associated kidney disease and cancer (Hu et al. 2004; Grollman 2013). In Taiwan, where one-third of the population has been exposed to AA (Hsieh et al. 2008), the link between AA exposure, upper urinary tract cancer (Chen et al. 2012), and hepatocellular carcinoma (Ng et al. 2017; Chen et al. 2018) is well documented. Despite abundant warnings (Administration 2001; World Health Organization 2008; National Toxicology Program 2011), AA-containing remedies are still marketed online and are available for purchase in many countries around the world, including the United Kingdom (Gold and Slone 2003), the European Union (Maggini et al. 2018), the United States (Vaclavik et al. 2014), and Australia (Tay 2019).

Approximately 5–10% of exposed individuals are prone to AA-related diseases (Vanherweghem 1998; Bamias and Boletis 2008). Polymorphisms in genes encoding xenobiotic-metabolizing enzymes may contribute to interindividual variation in sensitivity to the effects of toxicants. However, no clear association between such a candidate gene and AA-induced disease has been demonstrated. Therefore, identifying the full set of enzymes responsible for the metabolism of AA will provide indispensable clues that will define individual sensitivity to the toxic effects of AA.

Nitroreduction (NR) of AA is necessary for the formation of reactive intermediates (Fig. 1) that bind covalently

![Fig. 1. Proposed pathways for the metabolic activation of the aristolochic acids. Four-electron NR of AA-I and AA-II produces their respective N-hydroxyaristolactams, AL-I-NOH and AL-II-NOH. N-hydroxyaristolactams decompose to form electrophilic cyclic nitrenium/carbonium ions with delocalized positive charge, or undergo conjugation reactions catalyzed by sulfotransferases (SULTs) and N-acetyltransferases (NATs). Resulting N-sulfonylaristolactams (AL-I-NOSO_3H and AL-II-NOSO_3H) and N-acetoxyaristolactams (AL-I-NOAc and AL-II-NOAc) readily undergo solvolysis, forming the same active species as N-hydroxyaristolactams. N-hydroxyaristolactams are more stable than their esters and lead to DNA adduction at much slower rates.](image-url)
to the exocyclic amino groups of dA- and dG-forming AL-DNA adducts (Pfau et al. 1990c). The dA-AL adduct is highly persistent (Fernando et al. 1993; Grollman et al. 2007) and mutagenic (Attaluri et al. 2010) accounting for the AA-mutational signature that is observed in human cancers related to AA-exposure (Grollman et al. 2007; Chen et al. 2012; Hoang et al. 2013; Hollstein et al. 2013). Several mammalian enzymes, including NAD(P)H: quinone oxidoreductase (NQO1), xanthine oxidase, cytochromes P450:1A1/2 (CYP1A1 and CYP1A2), CYP:450 oxidoreductase, and prostaglandin H-synthase activate AA to form DNA-binding species (Stiborova et al. 2009). AA activation requires the transfer of four electrons and occurs, presumably, through the formation of N-hydroxyaristolactams, AL-I-NOH, and AL-II-NOH (Pfau et al. 1990a; Pfau et al. 1990c).

We recently found that N-hydroxyaristolactams react only weakly with DNA (Sidorenko et al. 2014), findings consistent with the low efficiency of NR activation of AA in vitro. Commonly, esterification to O-acetylated and O-sulfonated derivatives stimulates covalent binding of the N-hydroxylated metabolites of nitroarenes and arylamines to DNA (Bartsch 1987; Glatt 2006; Purohit and Basu 2000). Nevertheless, the involvement of cytosolic sulfotransferases (SULTs) and N-acetyltransferases (NATs) in AA bioactivation remains a matter of debate. First, it was shown that expression of human SULT1A1 and SULT1B1 in bacterial and human cells increases the mutagenicity of AA-I (Meinl et al. 2006). Later, conflicting evidence was presented demonstrating that bioactivation of AA by human NQO1 is not enhanced by the presence of several recombinant SULTs and NATs (Stiborova et al. 2011). Aiming to resolve this apparent discrepancy, Sidorenko et al. reported that O-sulfonation of AL-I-NOH and AL-II-NOH leads to the formation of highly reactive N-sulfonfuroxyster anions of AL-I-NOSO2H and AL-II-NOSO2H, respectively (Sidorenko et al. 2014). Subsequently, human SULT1B1, SULT1A1, SULT1A2, and SULT1A3 were found to be involved in AA activation in vitro and in cultured mammalian cells (Hashimoto et al. 2016). Nevertheless, the controversy regarding the enzymes involved in the bioactivation of AA remains, because it was reported that following AA-I exposure, transgenic mice carrying the functional human SULT1A1-SULT1A2 gene cluster accumulate comparable levels of AL-DNA to that detected in the parental strain deficient in the murine sult1a1 and sult1a2 (Arlt et al. 2016). Therefore, it is important to assess fully the mechanisms and enzymes involved in the activation of N-hydroxyaristolactams, which latter we are capable of synthesizing on demand (Attaluri et al. 2014).

The majority of studies addressing AA genotoxicity were mainly focused on AA-I, or a mixture of AA-I and AA-II. Although AA-I and AA-II are known mutagens in Salmonella typhimurium (S. typhimurium) strains efficient in NR (Schmeiser et al. 1984), no evidence regarding the mutagenic effects of N-hydroxyaristolactams in bacteria has been presented thus far. Moreover, despite the preliminary evidence that N,O-acetyltransfer may be involved in AA activation, this mechanism has not been pursued in detail. Previously, one of us has established various S. typhimurium umu tester strains that overexpress bacterial O-acetyltransferase, human N-acetyltransferases (NATs) or human sulfotransferases (SULTs) (Oda et al. 1985, 1993, 1995, 1999, 2012). The utility of these strains in evaluating the toxicities of xenobiotics has been validated by studying various nitroarenes and aromatic amines. In the present work, we examine the role of bacterial and human enzymes in the genotoxicities of AL-I-NOH and AL-II-NOH using SOS-based assay in umu tester strains. Furthermore, 32P-postlabeling DNA adduct analysis (Reddy and Randerath 1986; Shibutani et al. 2006) is employed to study the bioactivation mechanisms of the N-hydroxyaristolactams by means of cytosolic extracts obtained from cultured human kidney HK-2 cell line (Ryan et al. 1994; Hashimoto et al. 2016).

**MATERIALS AND METHODS**

**Chemicals and Enzymes**

AL-I-NOH and AL-II-NOH were synthesized in our laboratory as described (Attaluri et al. 2014), and AA-I was purified from A. indica by high-performance liquid chromatography. AA-related compounds were dissolved in dimethyl sulfoxide (DMSO) at 30–40 mM and stored at –20°C. 2,6-Dichloro-4-nitrophenol (DCNP) was purchased from Tokyo Chemical Industry Co. LTD (Japan). Enzymes used for 32P-postlabeling analysis were obtained from Worthington (Newark, NJ), New England Biolabs (Ipswich, MA), MP Biomedicals (Solon, OH), and Sigma Aldrich (St. Louis, MO). DMSO, 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (70% purity), salmon sperm DNA (ssDNA), pentachlorophenol (PCP), quercetin, β-estradiol, 4-nitrophenol were from Sigma-Aldrich. PAPS (>90% purity) and 3′-phosphoadenosine-5′-phosphate (PAP) were purchased from R&D Systems (Minneapolis, MN). dG-AL-II and dA-AL-II containing oligonucleotides were synthesized as described (Attaluri et al. 2010). γ-32P-ATP (6000 μCi/ml) was obtained from PerkinElmer (Boston, MA). Recombinant human SULT1B1 was purchased from MyBioSource Inc. (San Diego, CA).

**Bacterial Strains**

The following S. typhimurium umu tester strains were used: NM2009 (bacterial O-acetyltransferase (O-AT)-overproducing strain), TA1535/pSK1002 (parental strain), and NM2000 (O-AT-deficient strain) (Oda et al. 1985, 1993); NM6000 (O-AT-deficient parental strain), NM6001 (human NAT1 expressing strain), NM6002 (human NAT2-expressing strain) (Oda et al. 1999); and NM7000 (O-AT-deficient parental strain), NM7001 (human SULT1A1-expressing strain), NM7002 (human SULT1A2-expressing strain), and NM7003 (human SULT1A3-expressing strain) (Oda et al. 2012).

**Genotoxicity (SOS/umu) and Cytotoxicity Assays Involving**

**TA1535/pSK1002, NM2009, NM2000, NM6000, NM6001 and NM6002 Strains**

Umu assay was carried out as described (Oda et al. 1995). Briefly, after overnight culture, each strain was diluted 100-fold with TGA medium containing 1% bacitryptone (w/v), 0.5% NaCl (w/v), 0.2% glucose (w/v), 20 μg/mL ampicillin, and incubated on a shaker for 1 h at 37°C. After cell density reached an absorbance of 0.25–0.3 at 600 nm, 10 μL of AL-I-
NOH, or AL-II-NOH dissolved in DMSO was added. The resulting mixtures were incubated for 2 h as above. Induction of umuC gene expression as a response to DNA damage was determined by measuring cellular β-galactosidase activity as reported previously (Oda et al. 1985). Values obtained for β-galactosidase activity for each dose of N-hydroxysteriolactams were divided by that recorded in the absence of the compounds and compared to the fold-changes observed using respective parental strains. The cytotoxic effects of the N-hydroxysteriolactams were determined by monitoring the changes in the optical density of the cells at 600 nm. Genotoxicity and cytotoxicity results are presented as mean values of three independent experiments.

**Um assay in Strains Expressing Human SULTs**

The umu assay was conducted as described previously (Oda et al. 2012). Briefly, bacterial cells were grown overnight at 37°C in LB broth containing ampicillin (25 μg/mL) and kanamycin (25 μg/mL). The cultures were diluted 50-fold with TGA medium, supplemented with 1 mM isopropyl-β-D-thiogalactoside and incubated at 37°C for 3 h until the cell density reached an absorbance of ~0.3 at 600 nm. Aliquots (1 mL) of the TGA culture and 10 μL of compounds dissolved in DMSO were mixed and further incubated with agitation for 3 h at 37°C. Genotoxicities and cytotoxicities of compounds were evaluated and presented as described above.

**HK-2 Cell Culture and Preparation of Cytosolic Fractions**

A human kidney HK-2 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in K-SFM media as recommended by the manufacturer under 5% CO2 at 37°C. ATCC validates cell origin by Short Tandem Repeat analysis and conducts testing of cells for mycoplasma contamination prior to release of the product to customers. Cells from confluent cultures grown on 75 cm2 plates were collected, and cytosolic lysates were prepared by homogenization and ultracentrifugation in Tris–HCl pH 7.5 supplemented with inhibitors of proteases (Roche, Branchburg, NJ), as described for renal and hepatic murine tissues (Sidorenko et al. 2014). Alternatively, cellular fractions were prepared by using the nuclear cytosolic fractionation kit following manufacturer’s instructions (Cell Biolabs, San Diego, CA). Half of each cytosolic preparation was divided in small aliquots and stored at −80°C until used in an activation assay. The rest of the sample was dialyzed against Tris–HCl (pH 7.5) on membranes with 10 kDa or 3.5 kDa molecular weight cut off (Thermo Scientific, Rockford, IL). Two dialysis schemes for each molecular weight cut off were conducted. Depending on the size of the membrane, sample volume was 100 μL, for Slide-A-Lyzer MINI Dialysis Unit, or 1 mL, when using Slide-A-Lyzer Dialysis Cassette. The dialysis buffer was used at 500 times the volume of the sample. A typical procedure was as follows: dialysis for 2 h at 4°C followed by change of the buffer and dialysis for additional 2 h. Following these two steps, the buffer was replaced with the fresh portion and samples were further dialyzed overnight at 4°C. Dialysis experiments were reproduced several times on different days, which did not affect the outcomes and conclusions of this study. Protein content before and after dialysis was measured by the bicinchoninic acid assay or/and by the Bradford assays. Results obtained by both assays were consistent. Dialyzed samples were handled and stored the same way as nondialyzed.

**Activation of N-Hydroxyaristolactams by HK-2 Cytosols**

Unless indicated otherwise, the following components were included in each 100 μL of incubation mixture: 80 μg of ssDNA in 50 mM Tris–HCl pH 7.5, 0.5 mM EDTA, 15 mM MgCl2, and 1–100 μM AL-I-NOH. To study the effects of cofactors, either of the following compounds was added to mixtures at the following concentrations: 0.2 mM PAPS, 1 mM acetyl-CoA, 1 mM NADPH, 1 mM NADH, 1 mM FAD, and 1 mM FMN (all from Sigma-Aldrich). When necessary, various compounds (pentachlorophenol, 4-nitrophenol, PAP, quercitin, β-estradiol) were included in the reaction mixtures in the dose range of 0.1–1000 μM. Reactions were initiated by the addition of cytosols and incubated at 37°C for 15 min–4 h. Generally, we conducted serial dilutions of cytosolic protein to obtain a dose range between 6 and 25,000 ng/100 μL. Details are provided in the corresponding figures. At the indicated time, 100 μL volume was withdrawn from each reaction mixture and immediately combined with the equal volume of phenol-isooamyl-alcohol followed by rigorous vortexing for 15 s. Proteins were extracted by three steps of repeated shaking with phenol-isooamyl-alcohol, followed by centrifugation. DNA from aqueous upper fractions was precipitated by a standard procedure using ice-cold ethanol and sodium acetate at acidic pH. DNA pellets were dissolved in water and the yields were quantified by measuring the optical density of the samples at 260 nm. Collected DNA samples were stored at −20°C until adduct analysis as below. Control incubations were run routinely and were as follows: (1) without ssDNA, (2) without AL-NOH, (3) without cofactors, or (4) without cytosols. Each reaction was conducted in triplicate and/or repeated on a different day with new aliquots of cytosols and new dilutions of AL-I-NOH.

**32P-Postlabeling Adduct Analysis**

DNA adduct levels were evaluated as described previously (Dong et al. 2006; Sidorenko et al. 2012; Hashimoto et al. 2016). Briefly, DNA samples (5 or 10 μg) were enzymatically digested to 3'-phosphate nucleosides. The resulting AL-adducted nucleosides were enriched by butanol extraction, and the mixtures were evaporated to dryness by centrifugation under vacuum. Samples were reconstituted with water and labeled by radioactive phosphorus, transferred from γ-32P-ATP to 5'-hydroxyl groups of nucleosides by polynucleotide kinase deficient for 3'-phosphatase activity. Following labeling, the samples were dried as above and reconstituted in the loading buffer containing formamide and bromophenol blue. The labeled products were resolved by gel electrophoresis using 30% nondenaturing polyacrylamide (PAGE). The following synthetically obtained oligonucleotides (Attaluri et al. 2010; Sidorenko et al. 2012) were digested, as a mixture of 15, 30, or 60 fmol of each, and processed in parallel with DNA samples obtained from activation assay:

5'-CTTC TCT TCT GTG CXC TCT TCT TCT-3' X = dA-AL-II
5'-CTTC TCT TCT GTX CAC TCT TCT TCT-3' X = dG-AL-II

Gels were exposed to a phosphor screen (GE Healthcare) for various times (5 min—several hours, depending on adduct levels). The results were visualized by the Typhoon system and densitometry was conducted using Image QuaNT v5.2 (Molecular Dynamics). Representative fragments of PAGE are presented. AL-DNA adduct levels were plotted in Sigma Plot v13.0 (SPSS Inc.) and shown as mean values and standard deviations obtained from triplicate experiments, or as values obtained from the most representative experiment.

**RESULTS**

**Cytotoxicity and Genotoxicity of AL-I-NOH and AL-II-NOH in TA1535/pSK1002, NM2009, and NM2000 S. typhimurium Strains**

To assess whether bacterial O-acetyltransferases (O-AT) are involved in the genotoxictics of the N-hydroxysteriolactams, we employed NM2009 (bacterial O-AT overexpressing strain),
NM2000 (O-AT deficient strain) and TA1535/pSK1002 (parental strain) umu tester strains. Background β-galactosidase activity in the absence of compounds was evaluated for each strain and indicated as “1” on the y-coordinate (Fig. 2A, B). For each tester strain, any multifold changes in the baseline activity of β-galactosidase in response to treatments with N-hydroxyaristolactams are presented as functions of compound concentrations. The SOS/umu test showed that both N-hydroxyaristolactams are genotoxic in all three strains, with AL-II-NOH being more genotoxic than AL-I-NOH (Fig. 2A, B). Importantly, changes in β-galactosidase activities in NM2009 and NM2000 cells were similar to those found in the parent strain. In addition, AL-II-NOH was slightly cytotoxic (~20%–30% reduction in cell growth) to TA1535/pSK1002 and NM2009 cells but did not affect NM2000 strain (Fig. 2D), whereas AL-I-NOH was mildly cytotoxic only to the parental strain (Fig. 2C). These results suggest that bacterial O-acetyltransferases are not important for the genotoxicities of the N-hydroxyaristolactams.

Effects of AL-I-N-OH and AL-II-NOH on Cytotoxicity and umuC Induction in the S. typhimurium Tester Strains: NM6001, NM6002, NM6003, NM6000, NM6001, NM6002, NM7000, NM7001, NM7002, and NM7003

NM6001 (human NAT1 expressing), NM6002 (human NAT2 expressing), and NM6000 (parental) umu strains were used to examine the involvement of human NATs in toxicities of the N-hydroxyaristolactams. Background β-galactosidase activity in all strains, established in the absence of N-hydroxyaristolactams, increased with increasing concentration of either compound, AL-I-NOH and AL-II-NOH (Fig. 3A, B). Although, the parental strain, lacking human enzymes, also responded to exposure, the effects were more pronounced in NAT-producing strains. Thus, in both NAT-expressing strains, the presence of 3 μM AL-I-NOH or AL-II-NOH increased induction of the umuC gene by 2.6- or 4.1-fold, respectively. In the NM6000 strain, the same concentration of compounds induced 1.5- (AL-I-NOH) and 1.8-fold (AL-II-NOH) changes relative to their corresponding background

Fig. 2. Induction of umuC gene expression (A and B) and cytotoxicity response (C and D) induced by AL-I-NOH and AL-II-NOH in S. typhimurium tester strains TA1535/pSK1002 (◆), NM2009 (■) and NM2000 (●). Experiments were carried out as described in the section Material and Methods. In (A) and (B), each point was derived as the ratio of β-galactosidase activity for each dose of the compound to the activity in the corresponding untreated cells. In all graphs, points represent mean values of triplicate determinations.
β-galactosidase activities (Fig. 3A, B). We also found that AL-II-NOH was more genotoxic than AL-I-NOH across all three strains. With respect to cytotoxicity, AL-I-NOH at the dose range up to 30 μM did not induce significant toxicity in these umu strains (Fig. 3C), whereas AL-II-NOH was slightly cytotoxic showing approximately a 10% reduction in cell number in NAT1- and NAT2-expressing strains (Fig. 3D). These results indicate that expression of human NAT1 and NAT2 renders bacterial cells sensitive to the genotoxic effects of AL-I-NOH and AL-II-NOH.

Subsequently, to study the role of human SULTs in the bioactivation of AL-I-NOH and AL-II-NOH, we evaluated umuC gene expression and cytotoxicity in response to these compounds in the following four tester strains: parental (NM7000) strain and strains expressing human SULT1A1 (NM7001), SULT1A2 (NM7002), and SULT1A3 (NM7003) isoforms. The background and N-hydroxyaristolactams-induced activities of β-galactosidase in all four strains were established and presented (Fig. 4A, B) as described above for other bacterial strains. In the presence of 10 μM AL-I-NOH, the background activities of umuC gene were increased, respectively, by 4.9- and 5.4-fold in SULT1A1 and SULT1A2-expressing strains (Fig. 4A). Under the same conditions, AL-I-NOH led to 2.5- and 2.8-fold elevated activities of β-galactosidase in the parental and SULT1A3-producing cells, respectively, indicating that SULT1A3 may not be involved in the genotoxicity of AL-I-NOH. Overall, AL-II-NOH was more genotoxic than AL-I-NOH across all four tester strains. Thus, this compound at 10 μM induced an approximate 8-fold increase in β-galactosidase activity in both SULT1A2- and SULT1A3-expressing strains. The amplitude of response in the parental and SULT1A1 strains showed about a 6-fold difference from corresponding background values in untreated cells. With respect to cytotoxicity, both compounds were slightly cytotoxic in SULT1A-expressing bacteria, showing maximally a 20% reduction in cell density compared to the value for untreated cells, but not that of the parental strain (Fig. 4C, D).

To provide further evidence for the importance of SULTs in the genotoxicities of N-hydroxyaristolactams in SULT-expressing bacteria, we evaluated the effects of 2,6-dichloro-4-nitrophenol (DNCP), an inhibitor of SULT1A (Seah and

![Fig. 3. Induction of umuC gene expression (A and B) and cytotoxicity response (C and D) induced by AL-I-NOH and AL-II-NOH in S. typhimurium tester strains NM6000 (◆), NM6001 (■), and NM6002 (●). In (A) and (B), results are presented as values corresponding to β-galactosidase activity normalized to untreated control. In all graphs, points are mean values of triplicate determinations.](https://example.com/fig3.png)
Wong 1994), on the umuC gene expression induced by AL-I-NOH and AL-II-NOH in the SULT1A2-expressing strain (Table I). The presence of 50 μM DNCP reduced genotoxicities of AL-I-NOH and AL-II-NOH by 2.3- and 2.1-fold, respectively. These results corroborate our previous observations that human SULT1A enzymes are involved in the bioactivation of N-hydroxyaristolactams. Intriguingly, in all of our parental strains, we detected the activity of β-galactosidase, which showed dose dependence in response to the treatment with N-hydroxyaristolactams, thus suggesting that they induce other mechanisms of mutagenesis in bacterial and, potentially, mammalian cells.

Bioactivation of N-Hydroxyaristolactams in AL-DNA by HK-2 Cytosols

In order to explore mechanisms of bioactivation of N-hydroxyaristolactams in human cells, we studied their activation in AL-DNA in the presence of ssDNA and cytosolic extracts obtained from cultured immortalized human kidney HK-2 cells. The basis for the bioactivation assay of N-hydroxyaristolactams was previously established by one of us (Sidorenko et al. 2014) for murine renal and hepatic cytosols. In this type of experiment, proteins and cofactors present in cell lysates are capable of transforming low reactive fig. 4. Induction of umuC gene expression (A and B) and cytotoxicity response (C and D) induced by AL-I-NOH and AL-II-NOH in S. typhirum tester strains NM7000 (◆), NM7001 (■), NM7002 (●), and NM7003 (▲). Experiments were conducted as described in the section Material and Methods. In (A) and (B), values for β-galactosidase activity, observed for each dose of compound, were divided by that measured in corresponding untreated strain. In all graphs, points represent mean values of triplicate determinations.

| Inhibitor | AL-I-NOH | AL-II-NOH |
|-----------|-----------|-----------|
| None      | 355 ± 13 (100) | 621 ± 14 (100) |
| DNCP*     | 156 ± 6 (44)   | 292 ± 32 (47)  |

*DNCP: 2,6-dinitro-4-nitrophenol.

Incubations of cells were conducted with or without 50 μM DCNP in the presence of AL-I-NOH (10 μM) or AL-II-NOH (20 μM). The induction of umuC gene expression was determined as described in the section Materials and Methods. Experiments were carried out in triplicate for each condition, and the results are presented as mean values ± standard deviation. Percent of β-galactosidase activity from control incubations without DCNP is shown in parenthesis.
N-hydroxyaristolactams into highly reactive species, which bind DNA covalently forming AL-DNA adducts according to the mechanism outlined in the Figure 1.

Consistent with our previous reports, AL-I-NOH by itself showed only weak reactivity toward DNA (Fig. 5A). HK-2 cytosols prepared by ultracentrifugation efficiently activated AL-I-NOH in dG-AL-I and dA-AL-I in the presence of ssDNA. This activity showed linear dependence on the protein amount (Fig. 5C) and the concentration of AL-I-NOH (Fig. 5B). In the presence of 6 ng of cytosolic proteins per 100 μL of reaction mixture, 10 AL-DNA adducts per 10⁶ nucleotides were formed in 2 h. This number was raised by 500 times in the presence of 25 μg of the protein, resulting in 15-20 AL-DNA per 10⁴ nucleotides, suggesting that at least 2% of AL-I-NOH was transformed in AL-DNA (Fig. 5C). Surprisingly, addition of PAPS, cofactor for SULTs, was active, its quality was evaluated in the activation assay of AL-I-NOH (Fig. 5D), showing that the presence of PAPS, cofactor for SULTs, did not stimulate this activity (Fig. 5B, C). Because similar results were found for AL-II-NOH (not shown), we used AL-I-NOH in all subsequent experiments.

To ensure that PAPS, cofactor for SULTs, was active, its activity only by half and addition of PAPS had no effect on the reaction rate (Fig. 8). To assess whether prolonged incubation at 4°C affects the AL-DNA formation, we incubated the cytosolic fractions on the dialysis membrane.

Subsequently, we dialyzed HK-2 cytosols using membranes with a 10 kDa molecular weight cutoff and found that following dialysis the activity of cytosols dropped drastically, showing a 500-fold reduction as compared to the HK-2 sample before dialysis. This activity could not be restored by the addition of PAPS (Fig. 6C). Moreover, neither of the other compounds, alone or introduced as a mixture, including AcCoA, NADH, NADPH, FAM, or FNM was capable of restoring AL-I-NOH activation (data not shown). In contrast, dialysis on 3.5 kDa units reduced this activity only by half and addition of PAPS had no effect on the reaction rate (Fig. 8). To assess whether prolonged incubation at 4°C affects the AL-DNA formation, we incubated the cytosolic fractions on the dialysis membrane

![Image](image-url)

**Fig. 5.** Activation of AL-I-NOH in AL-DNA by HK-2 cytosols, fortified, and not fortified by PAPS. Cytosols from HK-2 cells (6; 25; 100; 400; 1500; 6250 and 25,000 ng/100 μL) or recombinant SULT1B1 protein (1 ng/100 μL) were incubated with 100 μM AL-I-NOH in the presence of ssDNA with or without 0.2 mM PAPS for 2 h at 37°C. DNA was extracted and two micrograms from each sample was subjected to electrophoresis and exposure for 2 (A and B) or 10 min (D) on a phosphor screen. (A) and (B) are unaltered fragments of the same gel shown at the same contrast, and analysis in (D) was done in a separate experiment and shown at the similar contrast level but longer exposure time. Control 1 in (A) is DNA/cytosol; Control 2—DNA/AA-I; Controls 3–5—DNA/AL-I-NOH; Control 6—DNA/AL-I-NOH/PAPS. AL-DNA levels in control incubations with AL-I-NOH were at 0.2 ± 0.02 adducts/10⁶ nucleotides. (C) Quantitative representation of results shown in (B). Filled circles—reactions with PAPS; empty circles—without PAPS. (D) s1 (8 AL-DNA/10⁷ nucleotides) and s2 (6 AL-DNA/10⁷ nucleotides) represent adducted DNA from reactions conducted with PAPS from R&D and Sigma-Aldrich, respectively. St—standard mixture of oligonucleotides containing dG-AL-II and dA-AL-II adducts, 30 fmol each. AL-DNA is a combined term for dG-AL and dA-AL adducts.
overnight at 4°C and found no difference in activity between this fraction and the dialyzed sample, indicating that reduction in the bioactivation activity of cytosols following incubation on 3.5 kDa units results from defrosting and/or subsequent prolonged incubation of the protein sample in cold. These results suggest that there is an unidentified mechanism(s) capable of promoting AL-DNA formation mediated by AL-I-NOH and HK-2 cytosols.

DISCUSSION

Summary of Results

N-hydroxyaristolactams are formed by a four-electron reduction process of the nitro group of the AA followed by intramolecular modifications (Priestap et al. 2010; Priestap et al. 2012). In this article, we investigate mechanisms underlying the genotoxocities of the N-hydroxyaristolactams in bacterial and human kidney cells. First, we evaluated the genotoxocities of AL-I-NOH and AL-II-NOH using umu assay in S. typhimurium tester strains, which express bacterial O-AT, or various isoforms of human NATs and SULTs. Furthermore, we employed cytosolic fractions obtained from human HK-2 cells to separate the pathways of bioactivation of AL-I-NOH in mammalian cells. Based on our results, we conclude the following: (1) bacterial O-AT are not involved in genotoxicity of AL-I-NOH and AL-II-NOH in Salmonella cells, (2) human NAT1, NAT2, SULT1A1, and SULT1A2 increase genotoxocities of AL-I-NOH and AL-II-NOH in S. typhimurium, whereas (3) human SULT1A3 is important for activation of AL-II-NOH but does not play a major role with respect to AL-I-NOH
genotoxicity, and (4) additional mechanisms are implicated in the genotoxicities of these compounds in bacterial and mammalian cells.

NR and Genotoxicities of AA

The toxicities of the AA are closely related to the stepwise reduction of their nitro group. NR may follow different routes and result in various outcomes depending on the nature of NR enzymes and oxygenation of tissues responsible for biotransformation (Purohit and Basu 2000). Thus, four-electron reduction of AA eventually results in electrophilic cyclic nitrenium aristolactam ions (Pfau et al. 1990a; Pfau et al. 1990c) that bind DNA covalently yielding highly mutagenic and persistent dA-AL adducts (Fernando et al. 1993; Grollman et al. 2007; Attaluri et al. 2010; Schmeiser et al. 2014). In the classical Ames assay, AA-I is mutagenic in Salmonella strains that are proficient in NR (Schmeiser et al. 1984), whereas the aristolactam—six electron reduction products of AA—are genotoxic in these strains only in the presence of hepatic S9 microsomal fractions (Schmeiser et al. 1986). Therefore, based on these and other observations (Pfau et al. 1990c), it was suggested that reductive and oxidative metabolism of AA and aristolactams, respectively, result in N-hydroxyaristolactams, which spontaneously decompose to cyclic nitrenium ions, yielding covalent adducts with DNA, and resulting in mutagenesis in bacterial and mammalian cells. In contrast to this hypothesis, we recently demonstrated that N-hydroxyaristolactams in vitro react only weakly with DNA and require further activation by human SULTs (Sidorenko et al. 2014; Hashimoto et al. 2016). However, these compounds display dose-dependent genotoxic responses in our umu strains that lack bacterial O-acetyltransferases and other parental strains that do not express human conjugation enzymes. The single-electron reduction mechanism and the existence of additional activation mechanism(s) involving conjugation of the N-O-group, but not carried by SULTs and NATs, may account for this discrepancy. These possibilities are discussed below.

The Role of Single-Electron Reduction and Oxidative Stress in AA Effects

One-electron step-wise reduction of various nitroaromatic compounds produces a nitro anion radical, known to engage in the redox cycle with molecular oxygen with concomitant formation of hydrogen peroxide, resulting in depletion of cellular nucleophiles and oxidative DNA damage (Purohit and Basu 2000; Boelsterli et al. 2006). Exposure to N-hydroxyamines has also been implicated in oxidative stress and mutagenesis in bacterial and mammalian cells (Purohit and Basu 2000; Boelsterli et al. 2006). Although reduction of the AA by mammalian Type I two-electron reductases, such as NQO1, is not oxygen sensitive, bacterial nitroreductases, and several other mammalian enzymes, known to activate AA, operate by a single-electron mechanism (Stiborova et al. 2013; Hashimoto et al. 2016). However, the evidence for the involvement of oxidative stress in the toxicities of AA in mammalian cells is limited (Yu et al. 2011; Romanov et al. 2015; Wu et al. 2015), and the AA-mutational signature in human tumors is strongly associated with dA-AL adducts lacking the signatures known for oxidative DNA damage (Hoang et al. 2013; Alexandrov et al. 2015; Hoang et al. 2016; Petljak and Alexandrov 2016; Rosenquist and Grollman 2016). Accordingly, it appears that in mammalian cells formation of AL-DNA through the cyclic nitrenium ion is the key step for AA-genotoxicity and mutagenesis, whereas in facultative anaerobes, such as S. thyphimurium in the active growth stage, both oxidative DNA damage and AL-DNA may contribute to the genotoxicities of AA and N
hydroxylaristolactams. In contrast to most common Salmonella Ames strains, which are sensitive to either deletions, intercalations, or base substitutions at G46, our umu strains easily detect a wide range of genotoxic events that induce expression of the umu operon (Oda et al. 1985). Therefore, in our experiments, genotoxicities of N-hydroxylaristolactams in S. typhimurium result not only from the AL-DNA formation and also from oxidative stress. To fully assess the role of oxidative pathways in the genotoxicities of the N-hydroxylaristolactams in bacterial cells, it will be important to study their mutagenicity in anaerobically grown bacteria, and bacteria lacking either classical nitroreductases or an 8-oxoguanine DNA repair system.

Involvement of Conjugation Reactions in Genotoxicities of AA

Until recently, it was believed that N-hydroxylaristolactams, due to their instability were the major source of the cyclic nitrenium ions (Schmeiser et al. 1986; Pfau et al. 1990c;b). Indeed, some reductively activated nitroarenes react with cellular nucleophiles without further activation (Purohit and Basu 2000; Boelsterli et al. 2006). However, this reactivity can be amplified by N-O-conjugation. The most common esterification reactions of the hydroxylamines are carried by bacterial O-acetyltransferases, human NAT1 and NAT2, and various human SULT enzymes yielding N-O-acetyl and N-O-sulfonyl esters (Hein et al. 1993; Oda et al. 1993; Oda et al. 1999; Glatt 2000; Oda et al. 2012). These transformations are enzyme-, xenobiotic-, and species-specific and lead to the formation of easily excreted and, in many cases, highly unstable and reactive metabolites. Consistently, Meinl et al. (2006) reported that expression of human SULT1A1 in Salmonella and human cells moderately enhances the mutagenicity of AA-I. In contrast, follow-up studies using recombinant enzymes in conjunction with adduct analysis suggested that human NATs and SULT1A enzymes do not play a major role in AA-I bioactivation, if initiated by cytosolic renal or hepatic extracts or recombinant NQO1 (Stiborova et al. 2011). To resolve this controversy, our collaborators synthesized N-hydroxylaristolactams, their sulphonated and acetylated derivatives, and we provided evidence that sulfonation likely is more important for AA-I bioactivation than NAT, and we provided evidence that sulfonation likely is more important for AA-I bioactivation than NAT, but not NAT2, obtained from insect cells overexpressing human proteins, stimulated DNA adduct formation triggered by the presence of N-hydroxylaristolactams, DNA and AcCoA. The levels of AL-DNA in these reactions were two orders of magnitude lower than that observed for recombinant SULT1A1/2 and SULT1B1. Therefore, at that time, we concluded that sulfonation is the primary bioactivation route of AL-I-NOH and AL-II-NOH. However, the source of the enzymes in the in vitro studies was commercial and NAT enzymes were present as a complex mixture of proteins, and no positive control with other xenobiotics was conducted. Therefore, we may have previously underestimated the role of NATs in bioactivation of AA, and the current studies provide evidence that N,O-acetyltransfer may be as important as sulfonation for the bioactivation of N-hydroxylaristolactams.

With respect to sulfonation, AL-I-NOH and AL-II-NOH were mutagenic in umu strains expressing human SULT1A enzymes corroborating our earlier findings (Sidorenko et al. 2014). AL-I-NOH was genotoxic in SULT1A2- and SULT1A1-expressing bacteria, with only slight effects in the SULT1A3 strain. AL-II-NOH was genotoxic in all three S. typhimurium-expressing human SULT1A1, 1A2, or 1A3. Most importantly, the differential role of SULT1A isoforms in the current study recapitulates previously observed results in vitro and in cultured cells (Sidorenko et al. 2014; Hashimoto et al. 2016). Although DCNP reduced the genotoxicities of N-hydroxylaristolactams in SULT1A-expressing bacteria, supporting the direct role of SULTs in producing their genotoxic intermediates, the umuC gene expression was reduced only by half. This finding along with the observed mutagenicity of N-hydroxylaristolactams in the parent strains suggests that there are other mechanisms, which will be discussed somewhere below, involved in the generation of genotoxic species.

Activation of N-Hydroxylaristolactams by HK-2 Cytosols

In the umu test, the exact nature of genotoxic species that arises, because of the treatment with test compounds, remains obscure and the potential role of reactive oxygen species in the mutagenicity of the nitroarenes has been discussed above. To explore further whether there are additional bioactivation mechanisms of N-hydroxylaristolactams that yield cyclic nitrenium ions, we selected HK-2 cells as the source of bioactivation enzymes. These cells originate in the human proximal tubule (Ryan et al. 1994), which is one of the main targets of AA toxicity (Deipierreux et al. 1994). Importantly, AA-I and AL-I-NOH are cytotoxic and genotoxic to HK-2 cells in culture, and these effects are inhibited by pentachlorophenol, SULT1A inhibitor, and to some extent depend on SULT1A1 an SULT1A2 expression (Hashimoto et al. 2016). First, we obtained cytosolic extracts from HK-2 cells and found that when these fractions are combined with DNA and AL-I-NOH, substantial levels of AL-DNA are formed. Importantly, in this assay,
the genotoxicity of N-hydroxyaristolactolams is not mediated by oxidative DNA damage and directly depends on the generation of a cyclic nitrenium ion. Consistent with our previous observations, AL-I-NOH reacts only weakly with DNA without the presence of cytosols, and high levels of the compound and long incubations are necessary to be able to detect AL-DNA, even when using highly sensitive postlabeling method (~5 adducts per 10^9 nucleotides per 5 μg DNA, corresponding to 0.075 femtomoles of adducts in total).

Surprisingly, when mixtures of AL-I-NOH, cytosol, and DNA were fortified by PAPS, it did not promote further AL-DNA formation. To verify whether this observation was due to the presence of a sufficient amount of PAPS in the cytosolic preparation, we dialyzed HK-2 cytosols using membranes with the 10 kDa molecular weight cutoff to remove any cofactors present in the original preparation of the cytosols. Following dialysis, HK-2 fractions showed a drastic loss in their ability to activate AL-I-NOH, showing a 500-fold reduction in activation capacity. This activity could not be restored by the addition of either/or a combination of the following: PAPS, AcCoA, NADPH, NADH, and FAD, suggesting the presence of an unidentified constituent(s) capable of promoting adduct formation. Subsequently, dialysis on 3.5 kDa filter units reduced activation of AL-I-NOH only by two-fold and this was partly due to some loss of protein activity upon defrosting and overnight incubation at 4°C. Since general cofactors have a molecular weight below 1 kDa, these results suggest that there is yet an unknown mechanism(s) that activates N-hydroxyaristolactolams into DNA binding species.

Other Mechanisms Involved in Activation of Hydroxylated Compounds

It remains to be explored, whether a previously unidentified conjugation reaction(s) promotes N-O-esters formation and AL-DNA formation, or other changes in N-hydroxyaristolactolams facilitated by oxidation or reduction by renal cytosols lead to AL-DNA. For example, during reduction of AA, the formation of various short-lived metabolites has been proposed, some of which, for example, oxazinone, may, theoretically, serve as precursors of electrophilic species and AL-DNA adducts (Priestap et al. 2010; Priestap et al. 2012). Likewise, biotransformation of N-hydroxyaristolactolams in cells and extracts may result in various yet to be established metabolites.

With respect to conjugation, in addition to the common esterification reactions, there are other mechanisms that may create electrophilic species (Purohit and Basu 2000). For example, some N-O-glucuronides exhibit electrophilic properties (Bartsch 1981). O-methylation may potentially create reactive species, but we previously synthesized N-O-methylester of aristolactam I and it did not bind DNA covalently in contrast to its sulfated species (unpublished data).

Effects of phosphorylation, and enzymatic and spontaneous conjugation with glutathione on activities of the N-hydroxyaristolactolam remain yet to be explored. Additionally, NATs are not the only enzymes that may be responsible for O-acylation. Some t-RNA syntheses were proposed to be involved in activation of certain nitroaromatic compounds in eukaryotes (Tada and Tada 1975).

In conclusion, our data shed light on the bioactivation pathways of the aristolochic acids and their proximate carcinogenic species, emphasizing the unusual nature of these compounds, and suggests the existence of a variety of pathways involved in their genotoxities. Our current research results warrant further investigation of the mechanisms of bioactivation of these important human carcinogens and nephrotoxins.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR’S CONTRIBUTION

Y. Okuno carried out the umu assay.

Y. Oda and VS developed the conceptual aspects of the investigation and designed the studies, coordinated the project, analyzed data obtained in umu assay, and drafted the manuscript. VS also developed approaches to study the bioactivation of the N-hydroxyaristolactolams by cell lysates, conducted experiments involving HK-2 cytosols, analyzed the results, and prepared the corresponding figures.

RB, SA, and FJ developed the synthesis of and prepared purified AL-I-NOH, AL-II-NOH, AA-I, also dA-AL-, dG-AL-containing oligonucleotides.

FJ and APG aided in study design and in editing the manuscript.

All authors contributed to manuscript writing and editing.

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