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Alternaria toxins: Altertoxin II is a much stronger mutagen and DNA strand breaking mycotoxin than alternariol and its methyl ether in cultured mammalian cells

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HIGHLIGHTS

- Altertoxin II induces gene mutations and DNA strand breaks in V79 cells.
- This is the first report on mammalian mutagenicity of a perylene quinone-type toxin.
- Altertoxin II has a more than 50-fold higher mutagenic potency than alternariol.
- Unlike alternariol, altertoxin II does not interfere with cell cycle.

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ABSTRACT

Altertoxin II (ATX II) is one of the several mycotoxins produced by *Alternaria* fungi. It has a perylene quinone structure and is highly mutagenic in Ames *Salmonella typhimurium*, but its mutagenicity in mammalian cells has not been studied before. Here we report that ATX II is a potent mutagen in cultured Chinese hamster V79 cells, inducing a concentration-dependent increase of mutations at the hypoxanthine guanine phosphoribosyltransferase gene locus at concentrations similar to that of the established mutagen 4-quinoline-N-oxide. Thus, ATX II is at least 50-times more potent as a mutagen than the common *Alternaria* toxins alternariol (AOH) and alternariol methyl ether (AME). In contrast to AOH and AME, ATX II does not affect the cell cycle of V79 cells. ATX II also causes DNA strand breaks in V79 cells, with a potency again exceeding that of AOH and AME. The high mutagenic and DNA strand breaking activity of ATX II raises the question of whether this *Alternaria* toxin poses a risk for public health, and warrants studies on the occurrence of ATX II and other perylene quinone-type mycotoxins in food and feed.

1. Introduction

Fungi of the genus *Alternaria* are widely distributed plant pathogens and decay organisms of fruits and vegetables (EFSA, 2011, Ostry, 2008). Because the mold grows at low temperatures, *Alternaria* spoilage may also occur in fruits and vegetables kept under refrigeration. *Alternaria* species produce a wide variety of secondary metabolites, which belong to different chemical classes, e.g. dibenzo-α-pyrones, perylene quinones, peptides, and the fumonisin-like AAL-toxins (EFSA, 2011). The chemical structures of the most common dibenzo-α-pyrones alternariol (AOH) and alternariol monomethyl ether (AME) are depicted in Fig. 1, together with the structures of the perylene quinones altertoxins I–III (ATX I–ATX III) and stemphyltoxin III.

Exposure to *Alternaria* toxins has been linked to esophageal cancer in Linxian Province, China, as well as areas of South Africa where high levels of *A. alternata* contamination have been found in grain (Liu et al., 1991; Liu et al., 1992; Panigrahi, 1997). The putative carcinogenicity of *Alternaria* toxins has evoked interest in their genotoxic potential, and several laboratories have studied the mutagenic and DNA strand breaking effects of crude *Alternaria* extracts as well as some purified toxins.

Abbreviations: AME, alternariol-9-*O*-methyl ether; AOH, alternariol; ATX, altertoxin; DAD, diode array detector; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; ESI, electrospray ionization; FCS, fetal calf serum; HPRT, hypoxanthine guanine phosphoribosyl transferase; MS, mass spectrometry; NMR, nuclear magnetic resonance; NQO, 4-nitroquinoline-N-oxide; PBS, phosphate-buffered saline; PE, plating efficiency; 6-TG, 6-thioguanine; TK, thymidine kinase; UV, ultraviolet.

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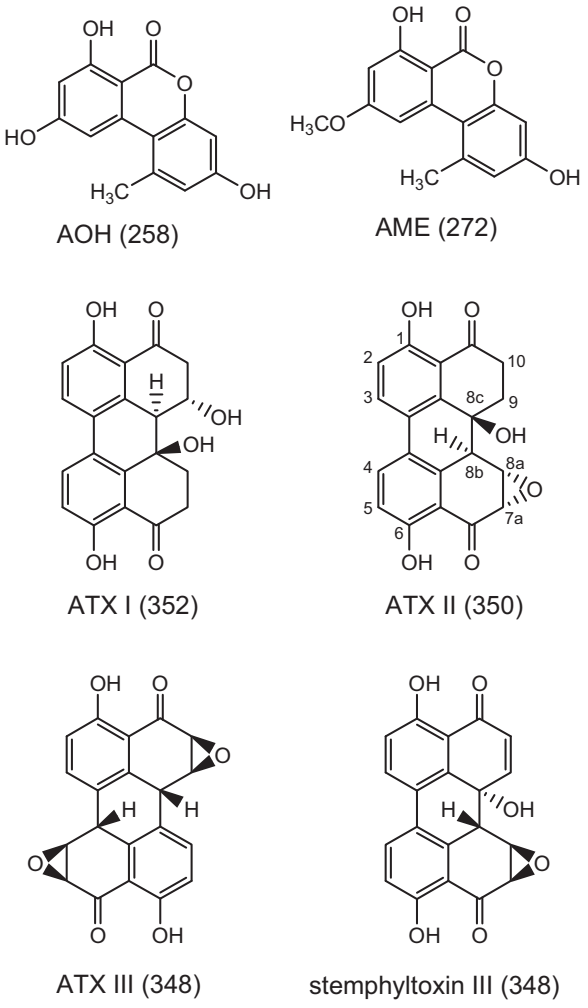

Fig. 1. Chemical structures and molecular weights of the dibenzo-α-pyrones AOH and AME, and the perylene quinones ATX I–ATX III and stemphyltoxin III.

Inconsistent results were obtained for AOH and AME in bacterial mutagenicity assays (summarized in Schrader et al., 2006): Both toxins were strongly mutagenic in both the *Bacillus subtilis rec* assay and *Escherichia coli* ND160 reverse mutation assay, but non-mutagenic or marginally mutagenic in the Ames *S. typhimurium* test using strains TA98, TA100 and TA1537 both in the absence and presence of fortified rat liver supernatant for metabolic activation (Davis and Stack, 1994). In contrast, ATX I–ATX III proved clearly mutagenic in TA98, TA100, and TA1537 both without and with metabolic activation, with a ranking order of ATX I < ATX II < ATX III (Stack and Prival, 1986). Stemphyltoxin III was also mutagenic in TA98 and TA1537 and gave a marginal response in TA100, independent of metabolic activation (Davis and Stack, 1991).

In mammalian cells, only very few studies exist on the genotoxicity and mutagenicity of AOH and AME, and only one on perylene quinone-type toxins. Lehmann et al. (2006) have reported that AOH acts as a strong clastogen in human endometrial carcinoma cells and in Chinese hamster V79 lung fibroblasts. A concentration-dependent induction of micronuclei without kinetochores was observed in both cell lines after treatment with AOH. The same laboratory reported on the induction of mutations at the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene locus in V79 cells and at the thymidine kinase (TK) locus in mouse lymphoma L5178Y $tk^{+/-}$ cells by AOH in a concentration-dependent manner (Brugger et al., 2006). DNA strand breaks were induced by AOH and AME in V79 cells, human hepatoma HepG2 cells, and human colon carcinoma HT29 cells (Pfeiffer et al., 2007). Fehr et al. (2009) showed that AOH and AME induced DNA strand breaks in HT29 and A431 human lung carcinoma cells and identified AOH as a topoisomerase poison, in particularly for the II-alpha isoform. Schrader et al. (2006) reported that ATX I was not mutagenic in V79 cells or rat hepatoma H4IIE cells.

In view of the strong mutagenicity of ATX II and ATX III in the Ames test (as shown above), it is important to know the genotoxicity of these altertoxins in mammalian cells. In this communication, we report that ATX II is a very powerful mutagen and DNA strand breaking agent in V79 cells, exceeding the mutagenicity of AOH and AME at least 50-fold.

2. Materials and methods

2.1. Chemicals

AOH was chemically synthesized in the laboratory of Podlech, Karlsruhe Institute of Technology (KIT), as previously reported (Koch et al., 2005) and contained 1.1% AME as determined by HPLC. AME was purchased from Sigma/Aldrich/Fluka (Taufkirchen, Germany) and had a purity of >96% according to HPLC analysis, containing 2.2% AOH. 4-Hydroxy-AME was at hand from our previous study on the microsomal metabolism of AME (Pfeiffer et al., 2007). Altenusin was purchased from Axxora (Lörrach, Germany), ATX I was a gift of M. Solfrizzo, Istituto di Scienze delle Produzioni Alimentari (Bari, Italy). 4-Nitroquinoline-N-oxide (NQO) and other chemicals and reagents were obtained from Sigma/Aldrich/Fluka.

ATX II was isolated from 10 L of a culture of *Alternaria alternata* strain TA7 grown for 24 days at 25 °C on a medium containing rice flour. The mycelium was mixed with 500 mL of 0.15 M sodium acetate buffer pH 5.0, homogenized in a blender, and extracted with ethyl acetate. The organic phase was dried over magnesium sulfate, concentrated to dryness under reduced pressure, and the residue dissolved in 60 mL methanol. The crude extract was analyzed by LC–DAD–MS, using a LXQ Linear Ion Trap MSn system (ThermoFisher Scientific, Waltham, MA, USA) and electrospray ionization (ESI) in the negative mode as described earlier (Pfeiffer et al., 2010). A 250 mm × 4.6 mm (inside diameter), 5 μm, reversed-phase Luna C8 column (Phenomenex, Torrance, CA) was used with a linear gradient. Solvent A was deionized water adjusted to pH 3.0 with formic acid, and solvent B was acetonitrile, which changed from 30 to 100% over 25 min. The flow rate was 0.5 mL/min. Aliquots of 1.5 mL of the crude extract were then fractionated by preparative HPLC on a 250 mm × 15 mm (inside diameter), 5 μm, reversed-phase Luna C18 column (Phenomenex), using deionized water as solvent A and methanol as solvent B. The gradient changed from 60 to 100% B over 20 min and the flow rate was 8 mL/min. The UV detector was set to 254 nm. The methanol was removed from the HPLC eluate using a rotary evaporator, and the compounds were extracted from the aqueous phases with ethyl acetate. The fraction containing ATX II of high purity (>98% according to LC–MS analysis) was characterized by mass spectrometry, UV and ^{1}H NMR spectroscopy. ^{1}H NMR signals are reported in parts per million referenced to internal tetramethylsilane (0.0 ppm).

ESI-MS (negative mode): $[M-H]^-$ *m/z* 349 (100%); MS^2 of *m/z* 349: *m/z* 331 (100%).

UV absorbance (methanol): 215, 259 (maximum), 287 (sh), 298 (sh), 360 nm. Minima at 232 and 315 nm. The UV spectrum was identical with that reported for ATX II by Stack et al. (1986).

^{1}H NMR (600 MHz, $CDCl_3$, numbering according to Fig. 1): δ 2.39 (dt, *J* = 4.0, 13.3 Hz, 1 H, H-9 ax); 2.84–2.86 (*m*, 1 H, H-10 eq); 2.78–2.91 (ddd, *J* = 2.5, 4.8, 3.2 Hz, 1 H, H-9 eq); 3.23–3.26 (*m*, 1 H, H-10 ax); 3.54 (*d*, *J* = 2.5 Hz; 1 H, H-8b); 3.70 (dd, *J* = 1.2, 3.6 Hz, 1 H, H-7a); 4.22 (*d*, *J* = 3.6 Hz, 1 H, H-8a); 7.05 (*d*, *J* = 8.8 Hz, 1 H, H-5); 7.10 (*d*, *J* = 8.8 Hz, 1 H, H-2); 7.84 (*d*, *J* = 8.8 Hz, 1 H, H-4); 7.91 (*d*, *J* = 8.8 Hz, 1 H, H-3); 12.12 (br, 1 H, 6-OH); 12.72 (br, 1 H, 1-OH). The ^{1}H NMR spectrum was identical with that reported for ATX II by Stack et al. (1986).

2.2. Cell culture

V79 cells were obtained from the German collection of microorganisms and cell cultures (Braunschweig, Germany) and cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS, Invitrogen, Karlsruhe, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a water-saturated atmosphere containing 5% carbon dioxide. Stock solutions of AOH, AME and NQO were made in dimethylsulfoxide (DMSO). ATX II was dissolved in ethanol, because solutions in DMSO proved to be highly unstable. The concentrations of the stock solutions were double-checked by measuring the UV absorbance after appropriate dilution with ethanol at the following wavelengths/extinction coefficients: AOH, 256 nm (4.06×10^4); AME, 256 nm (4.76×10^4); ATX-II, 258 nm (3.17×10^4) and 358 nm (5.3×10^3). The molar extinction coefficients of AOH and AME were from Asam et al. (2009) and that of ATX II from