Research Article

Alternariol acts as a topoisomerase poison, preferentially affecting the IIα isoform

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Alternariol (AOH), a mycotoxin formed by *Alternaria alternata*, has been reported to possess genotoxic properties. However, the underlying mechanism of action is unclear. Here, we tested the hypothesis that interactions with DNA–topoisomerases play a role in the DNA-damaging properties of AOH. First we compared DNA-damaging properties of AOH with other *Alternaria* mycotoxins such as AOH monomethyl ether (AME), altenuene and isoaltenuene. AOH and AME significantly increased the rate of DNA strand breaks in human carcinoma cells (HT29, A431) at micromolar concentrations, whereas altenuene and isoaltenuene did not affect DNA integrity up to 100 μM. Next, we selected AOH as the most DNA-damaging *Alternaria* metabolite for further studies of interactions with DNA topoisomerases. In cell-free assays, AOH potently inhibited DNA relaxation and stimulated DNA cleavage activities of topoisomerase I, IIα and IIβ. Stabilisation of covalent topoisomerase II–DNA intermediates by AOH was also detectable in cell culture, and here, the IIα isoform was preferentially targeted. AOH is thus characterised as a poison of topoisomerase I and II with a certain selectivity for the IIα isoform. Since topoisomerase poisoning and DNA strand breakage occurred within the same concentration range, poisoning of topoisomerase I and II might at least contribute to the genotoxic properties of AOH.

Keywords: Alternariol / DNA-topoisomerase / Mycotoxin

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1 Introduction

Mycotoxins are natural low molecular weight products formed as secondary metabolites by a wide range of different moulds. Well-known genera of moulds producing mycotoxins are *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*. Contamination of food and feed with toxin-producing fungi represent a major risk for diseases in humans and animals [1]. There are numerous studies on the toxicological effects of mycotoxins such as the aflatoxins, ochratoxin A and the fumonisins. However, little is known so far about the toxicological relevance of *Alternaria* toxins. *Alternaria* spp. are prevalent ubiquitously appearing on the surface of cereals [2], vegetables [3] or fruits [4] as well as in the ground, on wood or walls. Exposure to *Alternaria*, especially *Alternaria alternata* has been associated with adverse health effects. High levels of contamination with *A. alternata* in cereals have been discussed to be associated with enhanced incidence of oesophageal cancer in the province of Linxian, China [5, 6]. Extracts of *A. alternata* have been reported to possess genotoxic and mutagenic properties *in vitro* [7, 8]. So far, little is known about the impact of the individual mycotoxins produced by this genus. Alternariol (AOH, Fig. 1A), one of the main metabolites of *A. alternata*, has been reported to possess fetotoxic, teratogenic, genotoxic and mutagenic properties [9–11]. However, the underlying mechanisms of action have not been fully elucidated so far.

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Abbreviations: A431, human vulva carcinoma cell line; **AME,** alternariol monomethyl ether; **AOH,** alternariol; **fpg,** formamidopyrimidine–DNA–glycosylase; **HT29,** human colon adenocarcinoma cell line; **kDNA,** kinetoplast DNA; **LDH,** lactate dehydrogenase; **MCF-7,** human breast adenocarcinoma cell line

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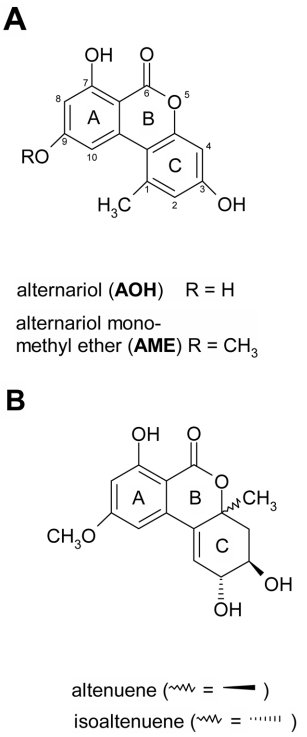

Figure 1. Structure of *Alternaria* metabolites. (A) AOH and AME; (B) altenuene and isoaltenuene.

AOH has been suggested to possess DNA-intercalating properties [12]. High affinity to dsDNA often results in the interference with the catalytic activity of topoisomerases [13, 14]. DNA topoisomerases regulate the topology of the DNA during transcription, replication, chromosome condensation and segregation [15]. These enzymes introduce transient breaks in the phosphodiester backbone of the DNA, enabling the release of torsion stress. In mammalian cells, two major classes of topoisomerases are expressed, topoisomerase I and II. Topoisomerase I introduces transient single strand breaks in the phosphate backbone of the DNA enabling the broken strand free to rotate around the intact second strand. Topoisomerase II is an ATP-dependent homodimeric enzyme that generates transient DNA double-strand breaks through which a second DNA double helix is passed [16]. During these processes a transient covalent enzyme–DNA intermediate is formed, the so-called cleavable complex [17]. Two isoforms of topoisomerase II have been identified in human cells, IIα and IIβ. The highest expression of topoisomerase IIα is observed during the late S/G2 phase of the cell cycle due to its function in chromosome condensation and segregation. In contrast, topoisomerase IIβ is expressed constitutively throughout the cell cycle [18].

Interference with the activity of topoisomerase I and II can occur at different steps of the catalytic cycle. Depending on the mode of interaction the maintenance of DNA integrity might be affected. Catalytic inhibitors bind to the enzyme prior to DNA-binding thus inhibiting the formation of the cleavable complex. In contrast, topoisomerase poisons target and stabilise the cleavable complex after its formation thus preventing its release and the religation of the DNA strand. As a consequence, DNA double strand breaks might result from the collision of the stabilised covalent DNA–topoisomerase intermediate with the approaching replication fork, a serious DNA-damaging incident.

In the present study, we addressed the question whether the *Alternaria* toxins AOH and AOH monomethyl ether (AME) interfere with the activity of human topoisomerases and whether topoisomerase inhibition might be of relevance for the DNA-damaging properties of these mycotoxins. For comparison, the *Alternaria* metabolites altenuene and isoaltenuene were included in the testing. The DNA strand-breaking potential of the selected *Alternaria* mycotoxins was determined by single cell gel electrophoresis (comet assay). The potential interference with human topoisomerases was investigated in cell-free systems as well as in intact cells with special emphasis on the mode of interaction with the target enzyme.

2 Materials and methods

2.1 Chemicals and recombinant enzymes

Alternaria toxins AOH, altenuene and isoaltenuene were synthesised as described previously [19, 20]. AME and the other substances used in these studies were purchased from Sigma–Aldrich (Germany) and Roth (Germany). Recombinant topoisomerase I, IIα and IIβ were purified as described [21].

2.2 Cell Culture

The human colon adenocarcinoma cell line (HT29), the human vulva carcinoma cell line (A431) and the human breast adenocarcinoma cell line (MCF-7) were derived from the German collection of microorganisms and cell cultures (DSMZ, Germany) and cultured as described previously [21].

2.3 Viability and cytotoxicity

2.3.1 Trypan blue exclusion

HT29 or A431 cells (3×10^5) were seeded into Petri dishes (∅ 5.5 cm) and allowed to grow for 48 h. Cells were incubated for 1 h with the substances and thereafter collected and mixed with a trypan blue solution. Cell viability was determined as percentage of living cells.

2.3.2 Lactate dehydrogenase (LDH) assay

Eight thousand HT29 cells or ten thousand A431 cells *per* well were seeded into 96-well plates and cultured for 48 h. Cells were treated for 1 h with the solvent control DMSO,

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AOH, AME or Triton X-100 as a positive control for maximal cell membrane damage. The assay (Cytotoxicity Detection Kit (LDH), Roche) was performed according to the manufacturer's protocol. Absorption was measured at 492 nm.

2.4 Single cell gel electrophoresis (comet assay)

Single cell gel electrophoresis was performed according to the method of Tice *et al*. [22]. HT29 or A431 cells (3×10^5) were seeded into Petri dishes (∅ 5.5 cm) and allowed to grow for 48 h. Subsequently cells were treated for 1 h with *Alternaria* toxins, the solvent control DMSO (1% v/v) or 10 μM menadione as a positive control in serum-free medium. Cells were collected and the viability was determined by trypan blue exclusion. Thereafter, aliquots corresponding to 70 000 cells were centrifuged at $425 \times g$ for 10 min. The pelleted cells were resuspended in 65 μL of low melting agarose and distributed onto a frosted glass slide, precoated with a layer of normal melting agarose. The slides were covered with a glass slide and kept on ice for 10 min to allow solidification of the agarose. After the cover glass was removed, slides were immersed overnight at 4°C in lysis solution (89 mL of lysis stock solution: 2.5 mM NaCl; 100 mM EDTA; 10 mM Tris-HCl; pH 10; 1% w/v *N*-laurylsarcosylsodium salt; 10 mL DMSO and 1 mL Triton-X-100). Additional treatment of the samples with the DNA repair enzyme formamidopyrimidine–DNA–glycosylase (fpg) allows the detection of potential oxidative DNA damage. The slides were washed three times in fpg-buffer (40 mM HEPES; 0.1 M KCl; 0.5 mM EDTA; pH 8; 0.2 mg/mL BSA). Fpg-treated samples were incubated with a 1:3000 diluted fpg solution for 30 min. Subsequently, DNA was allowed to unwind for 20 min at 4°C (300 mM NaOH; 1 mM EDTA; pH 13.5) followed by horizontal gel electrophoresis at 4°C for 20 min (25 V, 300 mA). Thereafter, the slides were washed three times with 0.4 M Tris-HCl; pH 7.5 and stained with ethidium bromide (40 μL *per* coverslip, 20 μg/mL). Fluorescence microscopy was performed with a Zeiss Axioskop ($\lambda_{ex} = 546 \pm 12$ nm; $\lambda_{em} \geq 590$ nm). Slides were subjected to computer-aided image analysis (Comet Assay III System, Perceptive Instruments, Suffolk, Great Britain), scoring 50 images *per* slide. For each concentration of drug, two slides were independently processed and analysed. The results were parameterised with respect to tail intensity (intensity of the DNA in the comet tail calculated as percentage of overall DNA intensity in the respective cell).

2.5 Fluorescence competition assays

The assays were performed according to the method of Morgan *et al*. [23] with slight modifications adapted to 96-well plate format [21]. The replacement of the intercalator ethidium bromide (1 μM) from double-stranded calf thymus DNA resulting in a decreased fluorescence intensity was measured at $\lambda_{ex} = 512$ nm and $\lambda_{em} = 590$ nm. The replacement of the minor groove-binding ligand Hoechst 33258 (1 μM) was determined at $\lambda_{ex} = 355$ nm and $\lambda_{em} = 460$ nm.

2.6 Preparation of nucleic extract

Nucleic extract was prepared from MCF-7 cells as described by Habermeyer *et al*. [21] and used in relaxation assays.

2.7 Effects on topoisomerase I

2.7.1 Relaxation assay

The interference with the catalytic activity of topoisomerase I was determined using a plasmid relaxation assay. Plasmid DNA (250 ng of pUC 18) was incubated in a final volume of 30 μL containing 0.3 μL of nucleic extract; 10 mM Tris-HCl; pH 7.9; 100 mM KCl; 10 mM $MgCl_2$; 0.5 mM DTT; 0.5 mM EDTA and 0.03 mg/mL BSA for 30 min at 37°C. The reaction was stopped by the addition of one-tenth volume of 5% w/v SDS. Afterwards the samples were treated with 1 mg/mL proteinase K at 37°C for 30 min. Gel electrophoresis was performed at 4.5 V/cm in 1% w/v agarose gels with Tris-acetate/EDTA (TAE) buffer (40 mM Tris-HCl; 1 mM EDTA; pH 8.5 and 20 mM acetic acid). Subsequently, the gel was stained in 10 μg/mL ethidium bromide solution for 20 min. The fluorescence of ethidium bromide was detected with the LAS-3000 system (Fujifilm, Raytest, Germany).

2.7.2 Cleavage assay

Effects on the stabilisation of the cleavable complex between topoisomerase I and DNA were studied using the cleavage assay. Its performance is similar to that of the relaxation assay with some differences. Human recombinant topoisomerase I (40 ng) is used instead of nucleic extract and gel electrophoresis is accomplished at 0.4 V/cm in 1% w/v agarose gels with 0.5 μg/mL ethidium bromide in TAE buffer overnight. The detection of nicked, supercoiled and relaxed DNA was carried out as described above.

2.8 Effects on topoisomerase II

2.8.1 Decatenation assay

The catalytic activity of either topoisomerase IIα or IIβ was detected using catenated kinetoplast DNA (kDNA) in a cell-free decatenation assay. The kDNA is an aggregate of interlocked DNA minicircles (mostly 2.5 kb), which can be released by topoisomerase II. kDNA (200 ng) (TopoGen, OH, USA) were incubated in a final volume of 30 μL containing 40 ng of topoisomerase IIα or IIβ; 50 mM Tris; pH 7.9; 120 mM KCl; 10 mM $MgCl_2$; 1 mM ATP; 0.5 mM DTT; 0.5 mM EDTA and 0.03 mg/mL BSA at 37°C for 1 h. The reaction was stopped by the addition of one-tenth vol-

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ume of 1 mg/mL proteinase K in 10% w/v SDS followed by an incubation at 37°C for 30 min. Gel electrophoresis was performed in the absence of ethidium bromide at 4.5 V/cm in 1% w/v agarose gels with TAE buffer. Fluorescence of DNA minicircles and aggregated kDNA was detected as described above.

2.8.2 Cleavage assay

Effects of *Alternaria* toxins on the stabilisation of the cleavable complex between topoisomerase IIα or IIβ and the pUC18 plasmid DNA were detected using the cleavage assay. Plasmid DNA (140 ng pUC18) was incubated in a final volume of 20 µL containing 1.36 µg topoisomerase; 2 mM Tris-HCl; pH 7.9; 10 mM $MgCl_2$; 10 mM NaCl; 0.02 mM EDTA; 0.8 mM ATP and 50 mM KCl at 37°C for 6 min. The reaction was stopped by the addition of one-tenth volume of 1 mg/mL proteinase K in 10% w/v SDS with 20 mM EDTA and incubation at 45°C for 30 min and further 5 min after addition of loading buffer. Gel electrophoresis was performed in the presence of ethidium bromide at 4.5 V/cm in 1% w/v agarose gels with TAE buffer. Detection of the fluorescence has been carried out as described above.

2.8.3 Immunoband depletion assay

A431 cells (2.5×10^6) were seeded and allowed to grow for 48 h. After incubation with the respective compounds cells were lysed in lysis buffer (250 mM Tris pH 6.8, 4% w/v SDS, 0.04% w/v bromophenol blue, 4% v/v glycerol, 20 mM DTT, 20 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, 2.5 M urea). The cell suspension was sonicated and heated to 95°C for 10 min. Samples (20–30 µL) were separated by SDS-PAGE (7% acrylamid gel) and proteins were transferred onto a nitrocellulose membrane. Blocking the membrane for nonspecific protein binding was carried out for 1 h in 5% w/v dry milk (Roth, Germany) in 20 mM Tris pH 7.6, 130 mM NaCl, 0.1% Tween-20. Human topoisomerase IIα or IIβ was developed with rabbit polyclonal antibodies (Santa Cruz, Heidelberg, Germany, diluted 1:500 v/v in blocking solution) at 4°C overnight. Thereafter, the membranes were washed and incubated with horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody (diluted 1:2000 v/v in blocking solution). The respective chemoluminescent signals (LumiGLO®, Cell Signaling Technology, USA) were analysed using the LAS-3000 system with the Image Analyzer software (AIDA 3.52) for quantification (Fuji, Raytest). Arbitrary light units were plotted as test over control (%) with the control being cells treated with 1% DMSO.

3 Results

3.1 Induction of DNA strand breaks

The impact of the *Alternaria* metabolites AOH, AME, altenuene and isoaltenuene (Fig. 1) on DNA integrity was determined as DNA strand breaks in HT29 cells and in A431 cells by single cell gel electrophoresis (comet assay). In addition to the conventional comet assay, treatment with the base excision DNA repair enzyme fpg was performed to indicate potential oxidative DNA damage. To exclude experimental artefacts by cytotoxicity, cell viability was determined by trypan blue exclusion in parallel. Under the applied incubation conditions cell viability (>85%) was maintained throughout the experiment (data not shown). Additionally, cytotoxicity was evaluated as LDH leakage. AOH and AME did not significantly affect the leakage of LDH in HT29 and A431 cells after 1 h of incubation up to 50 µM (data not shown).

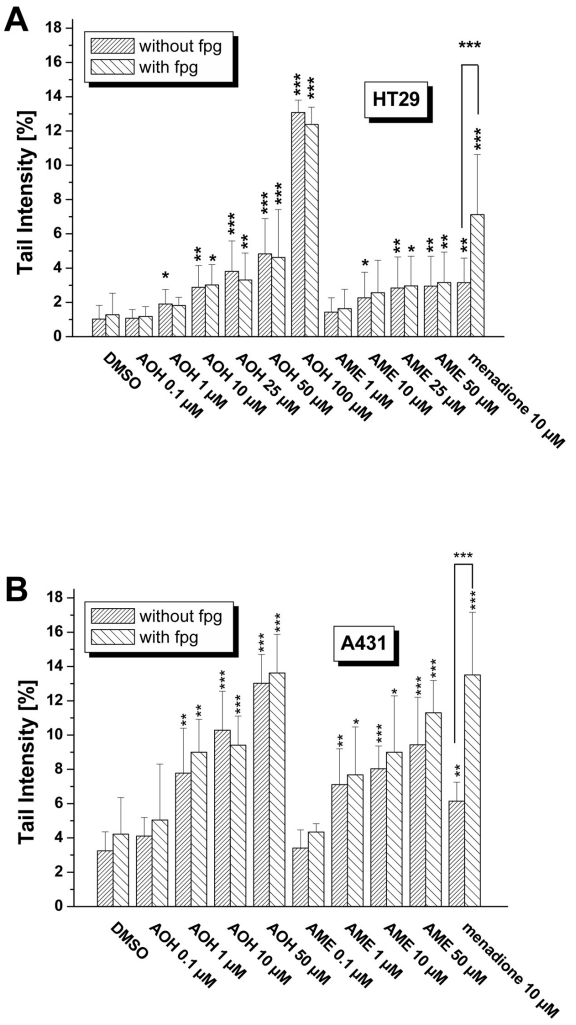

Figure 2. DNA strand breaks in (A) HT29 and (B) A431 cells, determined by single cell gel electrophoresis (comet assay). Cells were treated adherent in serum-free medium for 1 h. The data presented are the means ± SD of at least three independent experiments, each performed in duplicate. Significances indicated refer to the significance level as compared to the respective control (* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$).

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After 1 h of incubation AOH and AME were found to significantly increase the rate of DNA strand breaks in HT29 and A431 cells at concentrations ≥1 μM (Figs. 2A and B). AME exhibited slightly but not significantly lower DNA strand-breaking properties than AOH in both cell lines. However, although the onset of enhanced DNA damage occurred in both cell lines with AOH and AME at a similar concentration (1 μM), the extent of DNA damage, measured as tail intensity, was significantly higher in A431 cells. In contrast to AOH and AME, altenuene and isoaltenuene did not affect DNA integrity up to 100 μM (data not shown).

Menadione, a reference compound for the induction of oxidative stress, included as a positive control in the assay, significantly increased the rate of DNA strand breaks in HT29 as well as in A431 cells. By the addition of fpg, menadione-mediated DNA strand breaks were significantly enhanced, indicating as expected oxidative DNA damage (Figs. 2A and B). In contrast, the rate of DNA strand breaks induced by AOH and AME did not significantly differ from the fpg-treated samples suggesting that these compounds do not cause oxidative DNA damage (Figs. 2A and B).

3.2 Competition with ethidium bromide and Hoechst 33258

The affinity of the selected *Alternaria* metabolites to the major or minor groove of the DNA was investigated by competition experiments. The replacement of the intercalator ethidium bromide from double-stranded calf thymus DNA results in a loss of fluorescence. The selected *Alternaria* metabolites did not compete with ethidium bromide up to a concentration of 25 μM (Fig. 3A), thus indicating no substantial affinity to the major groove of the DNA. In contrast, actinomycin D, used as a reference compound, effectively displaced ethidium bromide within the DNA double helix (Fig. 3A).

The affinity of the mycotoxins to the minor groove of the DNA was tested by competition with the minor groove-binding ligand Hoechst 33258. AOH effectively competed with Hoechst 33258 for binding to the minor groove of the DNA with an EC_{50} value of 8.1 ± 1.2 μM (Fig. 3B). In comparison to AOH, the minor groove binder netropsin, included as a positive control in the assay, replaced Hoechst 33258 with an EC_{50} of 0.4 ± 0.04 μM (Fig. 3B). Due to the fluorescence of AME and the limited solubility under these assay conditions, AME was not included in the testing. Altenuene and isoaltenuene did not compete with Hoechst 33258 for binding to the minor groove of DNA up to 25 μM (Fig. 3B).

3.3 Effect on the catalytic activity of topoisomerase I

Topoisomerase I activity was determined as relaxation of supercoiled pUC18 plasmid DNA by nucleic extract from MCF-7 cells. The assay was performed in the absence of

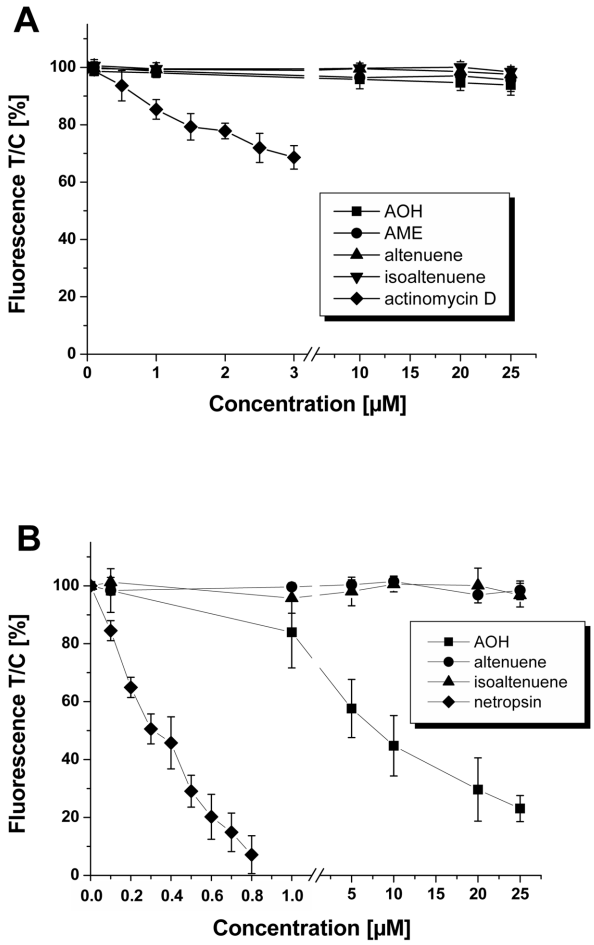

Figure 3. Fluorescence competition assays with calf thymus DNA. (A) Competition with the DNA intercalator ethidium bromide (1 μM). (B) Competition with the minor groove binder Hoechst 33258 (1 μM). The ordinate shows the fluorescence of ethidium bromide (A) or Hoechst 33258 (B) calculated as percent of control (T/C%). Competition with ethidium bromide or Hoechst 33258 is detected as a loss of fluorescence. The data presented are the means ± SD of at least three independent experiments, each performed in quintuplicate.

ATP, to exclude topoisomerase II activity. AOH effectively inhibited the catalytic activity of topoisomerase I at a concentration of 50 μM (Fig. 4A, lane 5). AME, as well as altenuene and isoaltenuene showed no effect on topoisomerase I up to 100 μM (data not shown).

3.4 Stabilisation of the cleavable complex of recombinant topoisomerase I

Based on the inhibitory effect of AOH on topoisomerase I activity, we addressed the question whether AOH acts as a catalytic inhibitor or as a topoisomerase I poison, stabilising the cleavable complex. Enzyme–DNA intermediates can be detected after digestion of the topoisomerase by proteinase K as open circular plasmid DNA, as illustrated

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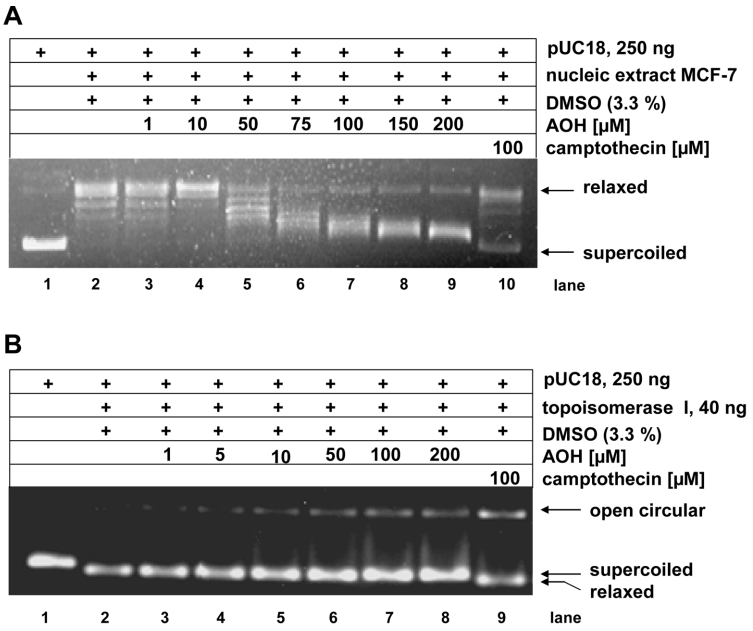

Figure 4. (A) Catalytic activity of topoisomerase I measured by DNA relaxation. Supercoiled pUC18 plasmid DNA (250 ng, lane 1) was incubated for 30 min at 37°C with nucleic extract from MCF-7 cells (lanes 2–10) in the absence (3.3% DMSO, lane 2) or presence of increasing AOH concentrations (lanes 3–9) or with camptothecin as a positive control (lane 10). The reaction was stopped with 1% w/v SDS, and after digestion with proteinase K, samples were separated in 1% agarose gels in the absence of ethidium bromide. The ethidium bromide-labelled DNA was documented under UV light by digital photography. Active topoisomerase I converts the supercoiled pUC18 plasmid DNA into the relaxed form (see arrows). Shown is a representative gel out of three independent experiments with similar result. (B) Topoisomerase I–DNA cleavage assay. Supercoiled pUC18 plasmid DNA (250 ng, lane 1) was incubated for 30 min at 37°C with 40 ng of recombinant human topoisomerase I (lanes 2–9) in the absence (3.3% DMSO, lane 2) or presence of increasing AOH concentrations (lanes 3–8) or with camptothecin (lane 9). The reaction was stopped with 1% w/v SDS. After digestion with proteinase K, samples were subjected to 1% agarose gel electrophoresis in the presence of ethidium bromide. UV-transluminated gels were documented by digital photography. Open circular DNA appears when the covalent enzyme–DNA intermediate is stabilised indicative for topoisomerase I poisons. Shown is a representative gel from three independent experiments with similar outcome.

by the positive control camptothecin (Fig. 4B, lane 9). AOH was found to enhance the proportion of open circular plasmid DNA in a concentration-dependent manner (Fig. 4B). The inhibition of topoisomerase I-catalysed relaxation (Fig. 4A) was observed in a concentration range where the stabilisation of the topoisomerase I–DNA complex was induced (Fig. 4B) thus identifying AOH as a topoisomerase I poison *in vitro*.

3.5 Impact on the catalytic activity of recombinant topoisomerase IIα and IIβ

The question whether the selected *Alternaria* metabolites target human topoisomerase IIα and IIβ was addressed by determining the catalytic activity of both topoisomerase II isoforms in a decatenation assay. In its catenated form, kDNA cannot enter the agarose gel, whereas minicircles, released by catalytically active topoisomerase II, migrate into the gel. AOH and AME (data not shown) inhibited the activity of topoisomerase IIα at concentrations ≥25 μM (Figs. 5A and B, lane 7). Doxorubicin, a topoisomerase II poison, served as a positive control (Fig. 5B, lane 10). The stereoisomeres altenuene and isoaltenuene did not affect topoisomerase IIα activity up to 200 μM (data not shown). The catalytic activity of topoisomerase IIβ was inhibited by AOH at a concentration ≥100 μM (Figs. 5C and D, lane 8). AME, altenuene and isoaltenuene showed no inhibitory effect on topoisomerase IIβ up to 200 μM (data not shown).

3.6 Stabilisation of the cleavable complex of recombinant topoisomerase IIα and IIβ

Considering the finding that AOH inhibited topoisomerase II activity, the question was raised whether this mycotoxin acts as catalytic inhibitor of human topoisomerase II or as a topoisomerase II poison, stabilising the cleavable complex. Moreover, it was of interest whether the preference of AOH for inhibition of the topoisomerase IIα isoform is also evident in the topoisomerase II–DNA cleavage assay. The stabilisation of the respective cleavable complex leads to linear pUC18 plasmid DNA and open circular plasmid DNA

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as a result of DNA strand breaks in supercoiled DNA induced by topoisomerase IIα or IIβ. AOH was found to stabilise the topoisomerase IIα–DNA intermediate at concentrations ≥10 μM (Fig. 6A). In contrast, the topoisomerase IIβ–DNA complex was stabilised by AOH at concentrations ≥50 μM (Fig. 6B), indicating a less potent effect of AOH on topoisomerase IIβ. Thus, AOH was identified as a topoisomerase II poison with about five-fold preference for topoisomerase IIα.

3.7 Impact on topoisomerase II within the cell

The question whether the stabilisation of the cleavable complex of topoisomerase II by AOH is of relevance within the

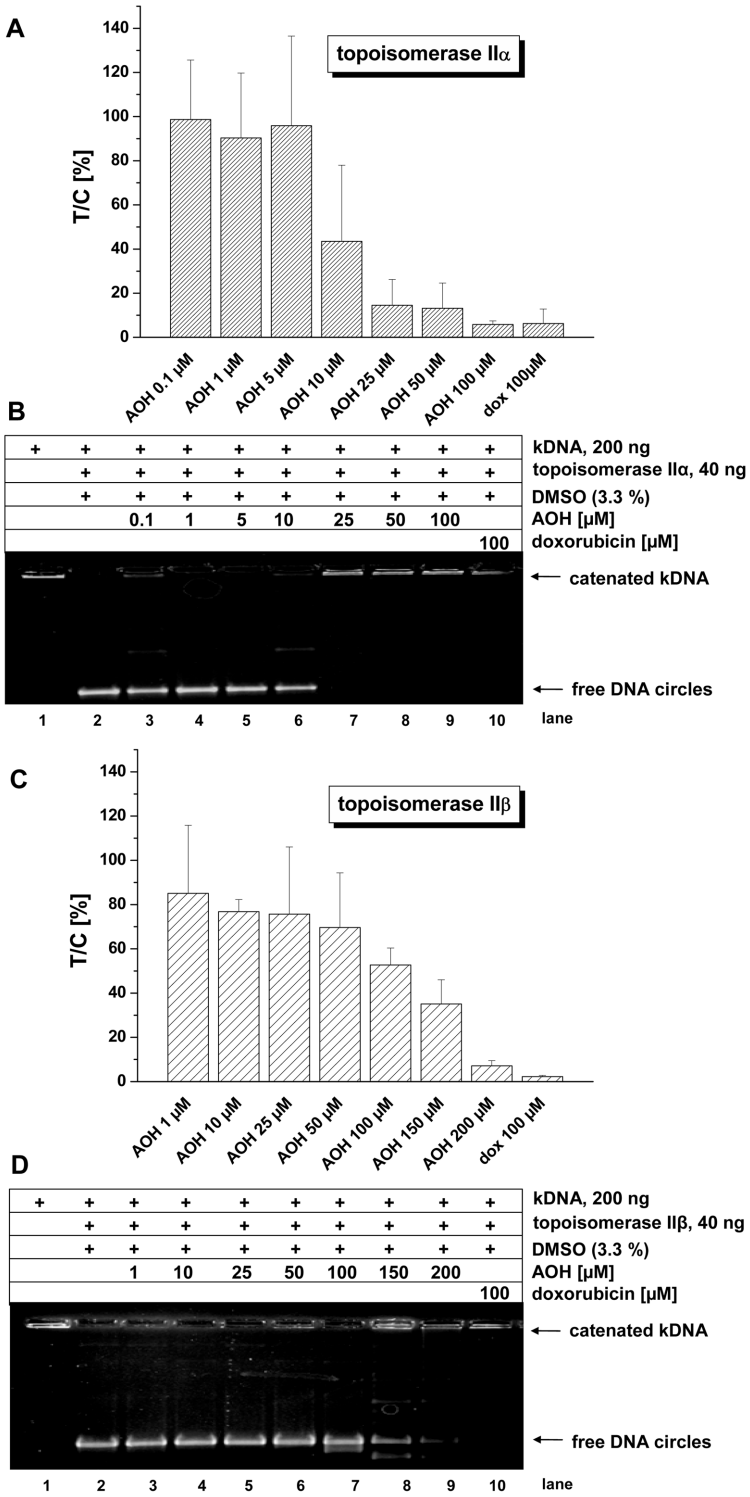

Figure 5. Catalytic activity of recombinant human topoisomerase II determined as the decatenation of kDNA. Topoisomerase II was incubated for 60 min at 37°C in the absence or presence of increasing concentrations of AOH or with doxorubicin as positive control. The reaction was stopped with 1% w/v SDS, and after digestion with proteinase K, samples were separated by 1% agarose gel electrophoresis. The ethidium bromide-labelled DNA was documented under UV light by digital photography. (A) Fluorescence signals of decatenated kDNA treated with topoisomerase IIα and AOH or doxorubicin were calculated as test over control (T/C%) in comparison to the solvent control DMSO. Data show the means ± SD of at least three independent experiments. (B) Representative gel of a decatenation assay with topoisomerase IIα. Lane 1 shows catenated kDNA not exposed to topoisomerase IIα enzyme. Active topoisomerase II releases single free DNA circles from the catenated DNA network treated with the solvent control DMSO (lane 2). Increasing concentrations of AOH show an inhibitory effect of the catalytic activity ≥25 μM (lanes 7–9) as well as the positive control doxorubicin (lane 10). (C) Fluorescence signals of decatenated kDNA treated with topoisomerase IIβ and AOH or doxorubicin were calculated as test over control (T/C%) in comparison to the solvent control DMSO. Data show the means ± SD of at least three independent experiments. (D) Representative gel of a decatenation assay with topoisomerase IIβ. Increase in concentrations of AOH show an inhibitory effect of the catalytic activity ≥150 μM (lanes 8–9) as well as the positive control doxorubicin (lane 10).

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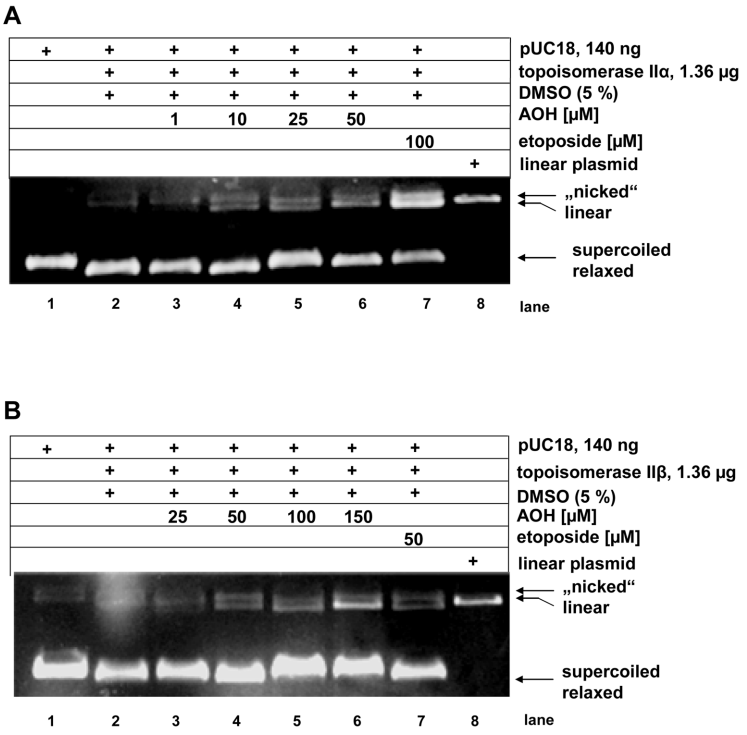

Figure 6. Topoisomerase II–DNA cleavage assay. 140 ng supercoiled pUC18 plasmid DNA (lane 1) was incubated for 6 min at 37°C with recombinant human (A) topoisomerase IIα (1.36 µg, lanes 2–7) or (B) topoisomerase IIβ (1.36 µg, lanes 2–7) in the absence (5% DMSO, lane 2) or presence of increasing AOH concentrations (lanes 3–6) or with etoposide as positive control (lane 7). The reaction was stopped by the addition of 1 mg/mL proteinase K in 10% w/v SDS with 20 mM EDTA and incubation at 45°C for 30 min and further 5 min after addition of loading buffer. Samples were subjected to 1% agarose gel electrophoresis in the presence of ethidium bromide. DNA was detected under UV light and documented by digital photography. Linearised plasmid DNA (lane 8) and nicked DNA is formed when the covalent enzyme–DNA intermediate is stabilised indicative for topoisomerase IIα or IIβ poisons. Shown is a representative gel from three independent experiments with similar results.

cell was addressed by an immunoband depletion assay in A431 cells. Interference of AOH with the homeostasis of free cellular topoisomerase and the amount of topoisomerase protein covalently linked to the DNA was investigated by Western blot analysis. Topoisomerase protein, trapped in a stabilised cleavable complex, is not able to migrate into a SDS-polyacrylamide gel, which leads to a decrease of the immunoband signal in the Western blot analysis. After incubation of A431 cells for 1 h with AOH, a significant reduction of the topoisomerase IIα signal, as compared to the solvent control, was observed (Fig. 7A). The topoisomerase II poison etoposide, used as a positive control, also reduced the intensity of the protein signal (Fig. 7B, lane 2). To a similar degree the topoisomerase IIβ signal was declined by etoposide (Figs. 7C and D, lane 2). AOH also clearly decreased the topoisomerase IIβ signal, but less pronounced compared to the IIα isoform. These results indicate that AOH acts as a topoisomerase poison within the cell with a preference to the IIα isoform.

4 Discussion

In the present study, we show that AOH and its monomethylether AME possess potent DNA-damaging properties in human colon carcinoma and vulva carcinoma cells, in contrast to the structurally related *Alternaria* metabolites altenuene and isoaltenuene (Figs. 2A and B). These data are in line with earlier reports on the genotoxic potential of AOH and AME in other mammalian cell models [10, 11]. The potent DNA strand-breaking effect of AOH *in vitro*, already detectable at 1 µM (in serum-free medium), indicates a substantial genotoxic potential. Pfeiffer *et al*. [24] recently reported DNA strand-breaking activity of AOH and AME at comparable concentrations under serum containing conditions using the method of alkaline unwinding. However, little is known so far about the underlying mechanism of action. Using additional fpg treatment in the comet assay for the detection of specific oxidised DNA bases, we investigated, whether oxidative DNA damage might also play a role in the genotoxicity of *Alternaria* toxins. Xenobiotic metabolism might generate reactive catechols or hydrochinones, metabolites probably arising from aromatic hydroxylation of AOH or AME [25]. These metabolites may form reactive semiquinones and quinones or undergo redox cycling inducing the generation of reactive oxygen species (ROS). In HT29 as well as in A431 cells no induction of fpg-sensitive sites was observed (Figs. 2A and B) indicating that oxidative stress does not play a predominant role in the induction of DNA damage by AOH and AME. However, further studies are needed to address the question whether ROS might be generated within the cell. In cells with enhanced metabolic capacity such as, *e.g*. hepatic cells the induction of oxidative DNA damage might indeed play a role.

In the present study, we focussed on the question whether the interference with the regulation of DNA topology might be responsible for the observed clastogenic effects of AOH

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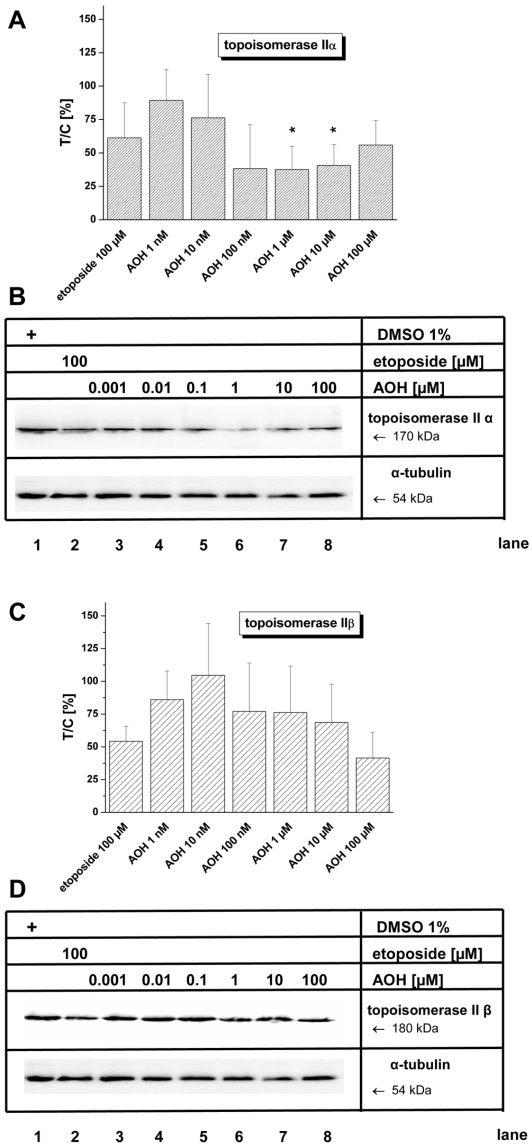

Figure 7. Immunoband depletion assay with A431 cells for topoisomerase IIα + β. A431 cells were incubated for 60 min at 37°C in 100 µL drug containing medium. Lysis buffer was added, the cell suspension was sonicated and heated to 95°C for 10 min. Cell lysates were applied to SDS-gel electrophoresis on 7% polyacrylamide gels, followed by Western blotting and immunostaining with rabbit peptide antibodies specific for topoisomerase IIα, IIβ or with mouse peptide antibodies specific for α-tubulin. Light units of detectable (A) topoisomerase IIα and (C) topoisomerase IIβ were plotted as test over control (T/C%) for the incubation with AOH or etoposide in comparison to the solvent control DMSO. Data show the means ± SD of at least four independent experiments. The significances indicated are calculated compared to the control using Student's *t*-test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0{,}001$). (B and D) Representative Western blots for immunoband depletion of (B) topoisomerase IIα and (D) topoisomerase IIβ by DMSO (lane 1), etoposide (lane 2) and AOH (lanes 3–8). The content of α-tubulin was determined as a loading control (bottom).

and AME. DiCosmo and Straus [12] proposed that AOH interacts with DNA by intercalation, detected as the formation of positive windings in the negative winded plasmid DNA thus reducing the mobility in the agarose gel. However, in the present study up to a concentration of 25 µM none of the selected *Alternaria* metabolites succeeded to replace the intercalator ethidium bromide in a respective fluorescence competition assay, thus lacking substantial affinity for intercalation into the DNA double helix (Fig. 3A). But AOH competed with the minor groove-binding ligand Hoechst 33258 indicating an affiliation to the heterogenic group of minor groove-binding ligands (Fig. 3B). AME, which structurally differs from AOH only at position 9, bearing a methoxy group instead of a hydroxyl residue, could not be included in the testing due to its limited solubility and its fluorescence prevalent under this assay conditions. The *Alternaria* metabolites altenuene and isoaltenuene showed no affinity for Hoechst 33258 replacement, presumably due to the lack of planarity resulting from the nonaromatic B- and C-rings together with the consequent axial/equatorial positions of the substituents at the C-ring. The substantial affinity of AOH to the minor groove of the DNA, resulting from its planar aromatic structure, might indeed contribute to the DNA-damaging and mutagenic properties of this mycotoxin [26]. A number of DNA minor groove-binding ligands have been reported to affect mammalian topoisomerase I and II [13, 14]. Therefore, we investigated the impact of the selected *Alternaria* toxins on the different classes of human topoisomerases. We identified AOH as an inhibitor of topoisomerase I and II, with preference for the IIα isoform (Figs. 4A and 5). In contrast, AME, bearing a methoxy group at position 9, did not affect the catalytic activity of topoisomerase I up to 100 µM. The importance of hydroxyl groups for the catalytic inhibition of topoisomerases I and II has been shown, *e.g.* for anthocyanidins [21]. The results show that the interference of AOH with topoisomerase I and II is not limited to the inhibition of the catalytic activity. At concentrations suppressing the catalytic activity of topoisomerase I, AOH was found to stabilise the covalent topoisomerase I–DNA intermediate (Fig. 4B), thus acting as a topoisomerase I poison *in vitro*. In addition, AOH potently inhibited the catalytic activity of topoisomerase II, stabilising the topoisomerase II–DNA intermediates (Figs. 5–7), identifying this mycotoxin as a topoisomerase II poison. Of note, AOH was found to target preferentially topoisomerase IIα thus discriminating between the two topoisomerase II isoforms. A similar tendency towards topoisomerase IIα was observed for AME, although AME was found to be less potent. Altenuene and isoaltenuene were completely inactive towards topoisomerase I and II. Therefore, the aromatic ring system appears to be crucial for topoisomerase inhibitory properties of these *Alternaria* metabolites. For effective topoisomerase I inhibition a free hydroxyl group at position 9 appears to be essential.

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Topoisomerase IIβ is expressed ubiquitously in tissues, whereas topoisomerase IIα is primarily expressed in proliferating tissue. So far, only a few compounds have been reported to discriminate between the topoisomerase II isoforms [27]. Teniposide, a semisynthetic derivative of podophyllotoxins shows preference for the α-isoform [28]. In contrast, the phenanthridine alkaloid lycobetaine and the synthetic quinoxaline phenoxypropionic acid derivative XK469 act as a selective topoisomerase IIβ poison [29, 30].

The majority of topoisomerase-targeting compounds are known to act as topoisomerase poisons, stabilising the covalent DNA topoisomerase intermediate [31]. The collision of the stabilised complex with an approaching replication fork leads to double-strand breaks, an effect which is desired for therapeutically used topoisomerase-targeting drugs [32]. Recent studies with topoisomerase IIβ deficient mice suggest that the therapeutic benefit of anticancer drugs acting through topoisomerase II poisoning is solely mediated by the IIα-isoform of the enzyme, whereas poisoning of the IIβ-isoform seems mostly responsible for undesired side effects of the therapy, such as secondary cancerogenesis [33]. From these findings the postulate was derived to develop new cancer drugs that act as selective poisons of topoisomerase IIα. Given the inherent proclivity of AOH towards topoisomerase IIα, the compound might serve as a lead structure for such a drug development. As that may be, in the case of food contamination with AOH, the poisoning of topoisomerases will inevitably result in undesired DNA damage and enhanced mutagenicity. In HT29 and A431 cells DNA strand breaks by AOH were observed in the concentration range where AOH was found to stabilise topoisomerase–DNA intermediates. These results support the assumption that the interference of AOH with topoisomerases at least contributes to the clastogenic effect of this mycotoxin.

In conclusion, our data show that AOH potently binds to the minor groove of the DNA and acts as a topoisomerase I and II poison, which is likely to cause or at least contribute to the impairment of DNA integrity in mammalian cells.

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