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Immunomodulatory, Genotoxic, and Antiestrogenic Properties of the *Alternaria* Mycotoxins Tricycloalternarene 2b and Tricycloalternarene 3a

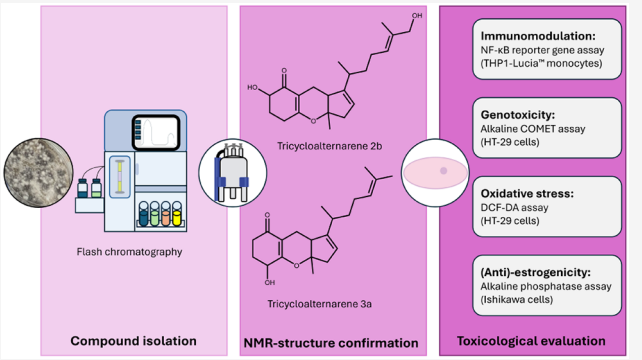
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ABSTRACT: *Alternaria* species produce a broad range of mycotoxins that frequently contaminate food and feed. While the toxicological properties of some *Alternaria* mycotoxins (e.g., alternariol, alternariol monomethyl ether, tenuazonic acid, and some perylene quinones) have been extensively investigated, toxicological information remains scarce for the majority of the *Alternaria* metabolites. In the present study, the two poorly characterized *Alternaria* mycotoxins tricycloalternarene 2b and tricycloalternarene 3a (TCA 2b and TCA 3a) were isolated from an *Alternaria* extract, structurally characterized by NMR spectroscopy, and evaluated with respect to their immunomodulatory, genotoxic, oxidative stress-inducing, and (anti)estrogenic properties. Both compounds were tested at concentrations ranging from 0.1 to 40 μM. Immunomodulatory effects were assessed using the nuclear factor κB (NF-κB) reporter gene assay in THP1-Lucia monocytes. Genotoxicity and intracellular oxidative stress were determined in HT-29 cells using the alkaline COMET assay in the absence and presence of formamidopyrimidine DNA glycosylase (FPG) and the DCF-DA assay, respectively. The ability of the compounds to interfere with the estrogenic pathway was investigated using the alkaline phosphatase (AlP) assay in Ishikawa cells. Both compounds suppressed lipopolysaccharide (LPS)-induced NF-κB pathway activation, with significant effects observed from 10 μM for TCA 2b and 20 μM for TCA 3a. Furthermore, both compounds induced concentration-dependent DNA damage, which was markedly enhanced by FPG treatment, resulting in significant DNA damage starting from 10 μM. In contrast, neither compound induced detectable intracellular oxidative stress under the applied experimental conditions. Of note, both compounds significantly reduced estradiol-induced AlP activity, indicating antiestrogenic effects at concentrations $\geq$20 μM for TCA 2b and $\geq$30 μM for TCA 3a. Overall, this study provides the first comprehensive toxicological characterization of TCA 2b and TCA 3a, and identifies both metabolites as biologically active *Alternaria* mycotoxins with immunosuppressive, genotoxic, and antiestrogenic properties *in vitro*.



1. INTRODUCTION

Fungal contamination of food and feed represents a major global challenge to food safety and security. Contamination can occur before harvest in the fields, during processing and even during storage of the products.[1] The major fungal genera contaminating crops include *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*.[2] These fungi produce a wide range of low-molecular weight toxic secondary metabolites known as mycotoxins.[3] It was long assumed that approximately 25% of food crops were contaminated by mycotoxins.[4] However, current estimates suggest that the prevalence of mycotoxin contamination is considerably higher, with 60−80% of food crops containing detectable levels of at least one mycotoxin.[5] Despite their widespread occurrence, only selected mycotoxins, including aflatoxins, deoxynivalenol (DON), zearalenone (ZEN), and fumonisins, are currently regulated in the European Union.[6] Among the mycotoxin-producing fungi, species of the genus *Alternaria* have attracted increasing attention owing to their widespread occurrence and their ability to produce a structurally diverse spectrum of mycotoxins.[3] *Alternaria* species are ubiquitous plant pathogens that infect a wide range of agricultural commodities, including cereals, oilseeds, fruits, and vegetables, resulting in frequent contamination of food and feed.[3,7] Mycotoxins produced by *Alternaria* species are still considered "emerging mycotoxins" due to limited knowledge of their occurrence and toxicological

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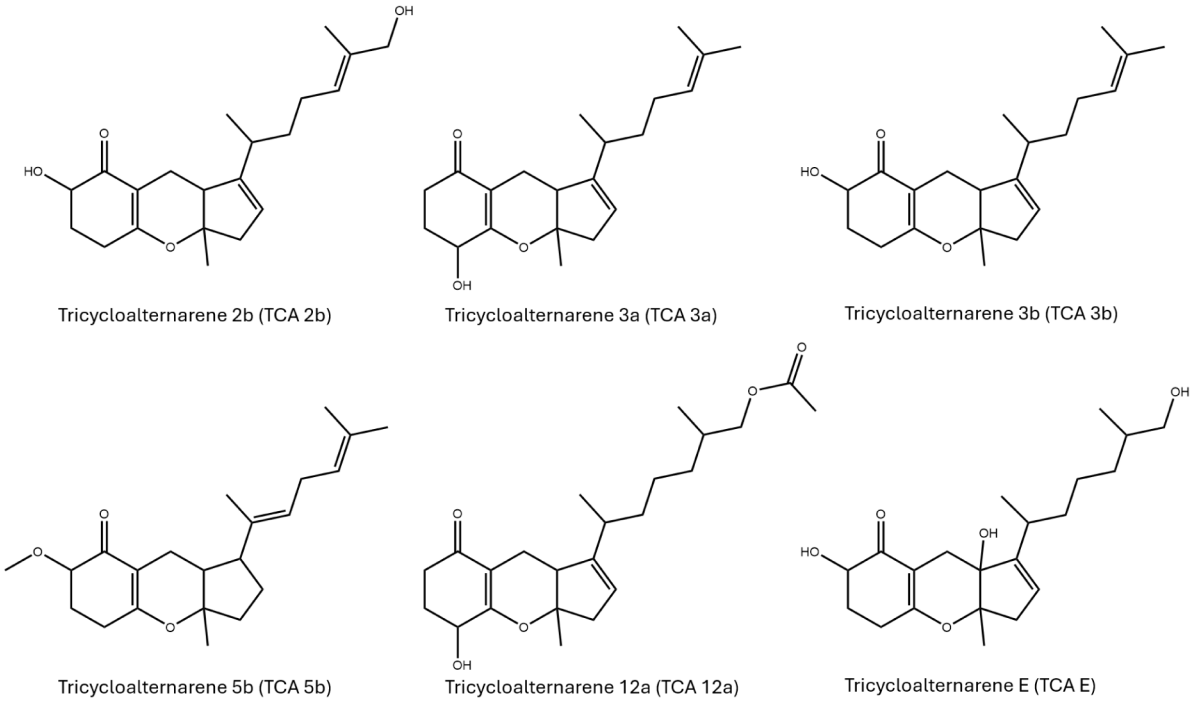


Figure 1. Chemical structures of selected tricycloalternarenes (created with ChemDraw).

properties, which hinders proper risk assessment. To date, more than 70 toxic secondary metabolites produced by *Alternaria* species have been identified.[8] However, toxicological information is available for only a small number of these metabolites. Of these metabolites, alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TeA) are among the best characterized *Alternaria* mycotoxins with respect to their occurrence and toxicological properties.[9] In recent years, altersetin (AST) and the perylene quinones (PQs) altertoxin I (ATX-I), altertoxin II (ATX-II), and alterperylenol (ALTP) have attracted increasing attention and have become the focus of several studies.[10−13] Collectively, *Alternaria* mycotoxins have been associated with a broad spectrum of adverse biological effects *in vitro*, such as genotoxicity, endocrine disruption, immunomodulation, and oxidative stress. However, the underlying mechanisms of actions differ substantially between individual mycotoxins.[9] Several *Alternaria* mycotoxins have been reported to interfere with DNA integrity. In this context, the dibenzo-α-pyrones AOH and AME have been shown to cause DNA strand breaks in various human cell lines, including HT-29, Caco-2, and HepG2 cells, through poisoning of human topoisomerases I and II.[14,15] Both mycotoxins have also been reported to induce oxidative stress, which may further contribute to their genotoxic effects.[16] Among the different classes of *Alternaria* mycotoxins, PQs are regarded as particularly potent genotoxic metabolites. In particular, the epoxide-bearing PQ ATX-II has been shown to induce substantially greater levels of DNA damage than AOH or AME.[17,18] ATX-II has additionally been reported to trigger oxidative stress in human intestinal cells, although this effect was observed only at concentrations above those that induced genotoxicity.[19] In contrast, TeA, one of the most abundant *Alternaria* mycotoxins in food, did not induce DNA damage in γH2AX assays performed with HepG2 and HT-29 cells.[9,20] Besides their genotoxic potential, several *Alternaria* mycotoxins have also been shown to interfere with endocrine signaling pathways. Among the best characterized examples, the dibenzo-α-pyrones AOH and AME have been shown to act as estrogen receptor (ER) agonists *in vitro*, exhibiting a higher affinity for ERβ than ERα.[21] In contrast, crude *Alternaria* extracts have shown potent antiestrogenic effects *in vitro*.[22] Subsequent studies identified AST, along with the PQs ALTP, ATX-I and ATX-II, as antiestrogenic *Alternaria* mycotoxins.[11,23] In addition to their interaction with ER signaling, several *Alternaria* mycotoxins have also been shown to exhibit immunomodulatory properties. AOH and AME significantly suppressed lipopolysaccharide (LPS)-induced immune responses in human bronchial epithelial (BEAS-2B) cells and mouse macrophages (RAW264.7),[24] and AOH was also shown to suppress IL-1β−induced immune responses in human Caco-2 cells.[25] PQs, including ATX-I, ATX-II, and ALTP have likewise been reported to exert pronounced immunosuppressive effects through inhibition of LPS−induced nuclear factor κB (NF-κB) signaling pathway activation.[11] Despite considerable progress in elucidating the toxicological properties of *Alternaria* mycotoxins such as AOH, AME, TeA, AST, ATX−I-III, and ALTP, toxicological data remain unavailable for the majority of the more than 70 identified *Alternaria* mycotoxins. For example, metabolites belonging to the subclass of tricycloalternarenes (TCAs) were previously described as nonspecific phytotoxins, reflecting that neither their specific biological function nor their mode of action has been elucidated yet.[26] TCAs are a structurally diverse subclass of meroterpenoid metabolites sharing a common tricyclic carbon skeleton. Individual TCAs differ mainly in the number, position, and nature of oxygen-containing substituents attached to this core scaffold, giving rise to numerous structurally related analogues (Figure 1).[27] Available toxicological information on TCAs is limited and largely restricted to reports of moderate cytotoxicity and weak antimicrobial activity.[28] Notably, a recent study demonstrated that selected members of this structural subclass exert immunosuppressive

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effects in several mammalian cell lines without significantly affecting cell viability.[29] Together, these observations suggest that TCAs may exhibit biological activities beyond those previously described, yet their broader toxicological relevance remains largely unexplored.

Building upon this knowledge, the aim of the present study was to isolate TCAs from a complex *Alternaria alternata* extract and to perform an initial toxicological characterization of the isolated compounds. In particular, based on toxicological effects previously reported for other *Alternaria* mycotoxins, a selected panel of *in vitro* assays was applied to investigate their potential to interfere with NF-κB-mediated inflammatory signaling, cause DNA damage, induce oxidative stress, and modulate estrogen receptor signaling.

2. MATERIALS AND METHODS

2.1. Materials

Dulbecco's Modified Eagle medium (DMEM; high glucose), fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin (P/S) were obtained from Thermo Fisher Scientific Inc. (Waltham, USA). Media and supplements needed for the cultivation of Ishikawa cells and THP1-Lucia NF-κB monocytes were obtained from Gibco Life Technologies (Karlsruhe, Germany). Quanti-Luc, normocin, and zeocin, were bought from Invivogen (Toulouse, France). Dexamethasone (Dexa) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (Steinheim, Germany), whereas 4-nitrophenyl phosphate and 17β-Estradiol (E2) were acquired from Sigma-Aldrich (Schnelldorf, Germany). Charcoal-dextran stripped-FBS (Catalog #A3382101) was obtained from Thermo Fisher Scientific Inc. (Waltham, USA). Dimethyl sulfoxide (DMSO) and Triton X-100 were supplied by Carl Roth GmbH & Co (Karlsruhe, Germany). 2′,7′-Dichlorfluorescein-Diacetat (DCF-DA) was purchased from Merck (Darmstadt, Germany), whereas Cell Titer-Blue was bought from Promega (Waldorf, Germany). Cell culture consumables were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and Sarstedt AG + Co. KG (Nümbrecht, Germany). Formamidopyrimidine DNA glycosylase (FPG) was obtained from New England Biolabs (Frankfurt, Germany). Deuterated methanol for the NMR measurements was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The complex *Alternaria* extract used for the isolation of the TCAs was obtained by cultivating the *Alternaria alternata* strain ATCC66981 on mCDB agar for 1 week, followed by mycotoxin extraction. The procedure was performed as described by Gao et al. (2022), without modifications.[30]

2.2. Fractionation and Isolation

To characterize the mycotoxins present in the complex *Alternaria* extract, ultrahigh performance liquid chromatography-ultraviolet-evaporative light scattering detector/mass spectrometry (UHPLC-UV-ELSD/MS) was performed. For this purpose, a Waters Acquity UPLC H-Class system equipped with a sample manager, quaternary solvent manager, column manager, photo diode array (PDA) detector, isocratic solvent manager, quadrupole dalton (QDa) detector and ELSD was used. A BEH C18 column (1.7 μm, 2.1 × 100 mm, Waters) at 30 °C was used as stationary phase. The flow rate was set to 0.3 mL/min using water +0.1% formic acid (A) and acetonitrile +0.1% formic acid (B) as mobile phases. The following gradient was applied: 65% B at 0.0 min, 65% B for 3.5 min, 65%−98% B in 0.1 min, and 98% B for 4.4 min. For MS detection, electrospray ionization (ESI) was applied, using 10 mM ammonium formate in water:-methanol (9:1, v/v) as makeup solvent with a flow rate of 0.4 mL/min. The samples were analyzed in positive (cone voltage: 30 V, capillary voltage: 0.8 kV) mode in a mass range of m/z 100−1200. The instrument was controlled by the software Empower 3.

For the isolation of the mycotoxins of interest, flash chromatography was conducted on an Interchim puriFlash 4250 system (Montluçon, France), endowed with ELSD, PDA and a fraction collector, controlled by Interchim Software. An Interchim PuriFlash C18-HQ column (15 μm, 5 g) was used, with the mobile phases consisting of water (A) and acetonitrile (B). The following gradient was applied: 5% B at 0 min, 5%−50% B in 30 min, 50% B for 10 min, 50%−98% B in 30 min, 98% B for 10 min. The flow rate was set to 2 mL/min, and fractions of 2 mL were collected. In total, 31.13 mg of extract were separated in two chromatographic runs. Thin layer chromatography (TLC) analysis of the obtained fractions was performed and fractions showing a similar TLC fingerprint were pooled. TLC was carried out on Merck silica gel 60 PF254 plates. The mobile phase consisted of ethyl acetate and was analyzed under UV254, and at visible light after derivatization with vanillin (1% in methanol)/sulfuric acid (5% in methanol). From the purification process, 3.47 mg of TCA 2b and 5.04 mg of TCA 3a with purities of ≥95% and ≥98% were obtained, respectively.

2.3. Structure Elucidation (NMR)

The structures of the isolated compounds were elucidated and confirmed by nuclear magnetic resonance (NMR) spectroscopy. NMR experiments were conducted on a Bruker Ascend 500 MHz spectrometer (Bruker, Billerica, MA, USA) equipped with a 5 mm triple-resonance Prodigy CryoProbe, an Avance NEO console and a SampleJet automated sample changer. Samples were measured at 298 K in deuterated methanol, with chemical shifts referenced to the residual nondeuterated solvent signals (δH 3.31 ppm; δC 49.0 ppm). The resonance frequencies were 500.19 and 125.77 MHz for ^{1}H NMR and ^{13}C NMR, respectively. Standard 1D (^{1}H, and ^{13}C) and gradient-enhanced (ge) 2D experiments, including COSY, HSQC, and HMBC, were used as supplied by the manufacturer. The ^{1}H and ^{13}C NMR chemical shifts and corresponding signal assignments obtained for the isolated compounds are summarized in Table S1.

2.4. Stock Solution Preparation and Selection of Test Concentrations

For cell-based experiments, the isolated mycotoxins TCA 2b and TCA 3a were dissolved in DMSO to prepare 20 mM primary stock solutions, which were stored at −80 °C. Assay-specific intermediate stock solutions were subsequently prepared by diluting the primary stocks in DMSO, taking into account the final concentrations of TCA 2b and TCA 3a required in the assay medium and the maximum final DMSO concentration permitted for each experimental setup. Stock solutions of compounds used as assay controls or to elicit specific biological responses were prepared in DMSO (i.e., Dexa and E2) or sterile distilled water (LPS, hydrogen peroxide, and Triton X-100). Within each experiment, the final solvent concentration was kept constant across all treatments and corresponding control conditions.

Across the different assays, TCA 2b and TCA 3a were tested over an overall concentration range of 0.1−40 μM, although the specific concentrations varied among assays. This range was selected to enable an initial toxicological characterization of the two compounds and to investigate their potential biological effects *in vitro*. The selected concentrations were not intended to represent realistic dietary exposure levels, as reliable data on the occurrence of these compounds in food and, consequently, on human dietary exposure are currently lacking.

Further details regarding the concentrations of TCA 2b and TCA 3a tested, the final solvent concentrations in the incubation media, and the incubation procedures are provided in the respective assay sections.

2.5. NF-κB Gene Reporter Assay

The interaction of the isolated compounds with the NF-κB signaling pathway was assessed in THP1-Lucia monocytes using the NF-κB reporter gene assay, as previously described.[11] The assay was performed in the absence and presence of bacterial lipopolysaccharide (LPS) to investigate immunostimulatory and immunosuppressive properties, respectively. Briefly, cells were seeded in 96-well suspension cell culture plates at a density of 3.4 × 10^{5} cells/cm^{2} in assay medium (RPMI 1640 supplemented with 10% FBS, 1% P/S (100 U/mL), 25 mM HEPES, and 100 μg/mL normocin) and treated with various concentrations of the test compounds (0.1−40 μM), 0.2% DMSO (solvent control), or 1 μM dexamethasone (inhibition

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control). To induce activation of the NF-κB pathway, 10 ng/mL LPS was added to all samples and controls (except the solvent control) 2 h after the start of incubation. Following an additional 18 h incubation period, NF-κB activity was quantified using a coelenterazine-based luminescence reagent (Quanti-Luc). Luminescence was measured with a microplate reader and used as a surrogate marker for NF-κB-dependent luciferase expression. Cells treated with 0.2% DMSO for 2 h and subsequently coincubated with LPS for 18 h served as the positive control. All test conditions were tested in at least three technical replicates and in three independent experiments, and results were normalized against the positive control (10 ng/mL LPS).

2.6. Alkaline Single Cell Gel Electrophoresis (Comet Assay)

The genotoxic potential of the TCAs was evaluated using a previously described protocol,[10] with minor modifications. Briefly, HT-29 human colon carcinoma cells were routinely cultured in Dulbecco's modified eagle medium (DMEM; high glucose, 4.5 $g \cdot L^{-1}$) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% P/S (100 U/mL), and 1% nonessential amino acids (NEAA). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and were subcultured twice a week at approximately 80% confluency. HT-29 cells were seeded at a density of 3×10^5 cells per Petri dish (Ø 3.5 cm) and cultured for 24 h. Subsequently, the cells were exposed to different concentrations of the test compounds (1−40 μM) for 1 h in assay medium (DMEM high glucose with 1% P/S). Cells treated with 1% DMSO served as the solvent control, whereas cells incubated with 1% DMSO followed by UV−B irradiation (31.2J for 1 min) served as the positive control. Following exposure to the respective test conditions, cells were washed two times with phosphate buffered saline (PBS), detached by trypsinization, and cell viability was assessed using trypan blue exclusion assay. Only samples with a viability greater than 80% were included in the analysis. Two microscope slides were prepared per test condition, each slide containing two agarose gel pads. A total of 30,000 cells per gel pad were dispersed in 0.8% low-melting agarose and transferred onto the slides precoated with a base of 0.8% normal-melting agarose. Agarose solutions were prepared in a phosphate buffer containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4. Cell lysis was performed by immersing the slides in lysis buffer (10% DMSO, 1% Triton X-100 and 0.1% lauroyl sarcosinate) and incubating them overnight at 4 °C. To enable the detection of FPG-sensitive DNA lesions, one slide per treatment condition was subsequently incubated with FPG enzyme for 30 min at 37 °C. Thereafter, slides were equilibrated in alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 20 min on ice, to allow DNA unwinding prior to electrophoresis. Electrophoresis was then conducted at 300 mA for 20 min. Subsequently, slides were neutralized using TRIS buffer (pH 7.5), and DNA was stained using an ethidium bromide solution (0.02 mg/mL). Imaging was performed using a Zeiss Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany) at excitation/emission wavelengths of 546/590 nm ($\lambda_{ex}/\lambda_{em}$). Comet images were analyzed using "Comet Assay IV" software (version 4.2.1; Perceptive Instruments, Suffolk, UK). For each condition, 50 cells per gel pad were evaluated, resulting in 100 measurements per condition across at least three independent biological replicates.

2.7. Dichlorfluorescein-Diacetate (DCF-DA) Assay

The DCF-DA assay was performed according to the method described by Keston and Brandt with slight modifications.[31] Briefly, HT-29 cells were seeded at a density of 6.9×10^5 cells/cm^2 in assay medium in black, clear-bottom 96-well plates. Cells were allowed to attach and grow for 48 h before being washed with PBS and incubated with 50 μM DCF-DA in phenol red-free DMEM for 15 min. Subsequently, the dye solution was removed, and cells were exposed to the test substances (1−40 μM) for 1 h in phenol red-free DMEM containing 1% DMSO (v/v). This short-exposure protocol was selected to match the 1 h treatment period used in the comet assay and to determine whether acute reactive oxygen species (ROS) formation could contribute to the FPG-sensitive DNA lesions observed under the same conditions. Media containing 1% DMSO and 1 mM H_2O_2 served as solvent and positive controls, respectively. At the end of the incubation period, DCF fluorescence was measured at λ_{ex} = 485 nm and λ_{em}= 535 nm to assess ROS formation. All experiments were performed in at least three independent biological replicates, each comprising three technical replicates per condition.

2.8. Alkaline Phosphatase (AlP) Assay

To assess the (anti)-estrogenic activity induced by the TCAs, the AlP assay was performed on Ishikawa cells, a human endometrial adenocarcinoma cell line. In brief, cells were seeded at a density of 5.2×10^4 cells/cm^2 in 96-well cell culture plates in assay medium (DMEM/F12 without phenol red, supplemented with 5% charcoal-dextran-stripped FBS and 1% P/S) and cultivated for 24 h. DMSO (0.25%) and 1 nM estradiol (E2) served as solvent and positive controls, respectively. Estrogenic activity was assessed by incubating cells with test substances in the absence of E2 for 48 h, whereas antiestrogenic activity was evaluated in the presence of 1 nM E2. Following the incubation period, cells were washed with PBS and frozen for 20 min at −80 °C. Subsequently, cells were thawed for 5 min at room temperature and 50 μL AlP buffer (0.24 mM $MgCl_2$, 1 M diethanolamine, 5 mM 4-nitrophenyl phosphate) was added to each well. Absorbance was measured at 2 min intervals over 1 h at 405 nm and 37 °C. All experiments were performed in at least three independent biological replicates, each comprising three technical replicates per condition.

2.9. CellTiter Blue (CTB) Assay

Cell viability was assessed in parallel with the NF-κB reporter gene, DCF-DA, and AlP assays using the CTB assay to exclude cytotoxicity as a confounding factor in the interpretation of immunosuppressive, ROS inducing or antiestrogenic effects. In all experimental setups, Triton X-100 (0.01%) was included as a positive control for cytotoxicity. For the NF-κB reporter gene assay, CTB reagent was added directly to each well following 20 h of exposure to the test substances at a final dilution of 1:10 (v/v). Regarding the DCF-DA assay, the incubation medium was removed after 1 h incubation and replaced by a CTB working solution (10% v/v in phenol-red-free DMEM). For the AlP assay, the incubation medium was removed after 48 h and replaced by a CTB working solution (10% v/v in DMEM/F12). The incubation time with the CTB reagent varied between the assays (2 h for the NF-κB reporter gene and DCF-DA assays and 50 min for the AlP assay) to account for differences in cell number and metabolic activity, affecting the rate of resazurin reduction. Subsequently, fluorescence was recorded using a microplate reader at an excitation wavelength of 530 nm and an emission wavelength of 560 nm. All experiments were performed in at least three independent biological replicates, each comprising three technical replicates per condition.

2.10. Statistical Analysis

Prior to the statistical analyses, normality was assessed using the Shapiro−Wilk test. Statistically significant differences between several concentrations (≥3) of the tested mycotoxins were determined using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Comparisons between two groups were assessed using Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed with OriginPro 2025 (Version 10.2.0.188; Academic, OriginLab Corporation, Northampton, MA, USA). All experiments were conducted using at least three independent biological replicates. Further details on the statistical tests applied are provided in the respective results section.

3. RESULTS

3.1. Immunomodulatory Effects

The NF-κB reporter gene assay in THP1-Lucia monocytes was applied, in the absence and presence of LPS costimulation, to evaluate the immunostimulatory and immunosuppressive properties of the isolated TCAs, respectively. To assess whether the observed effects were associated with changes in

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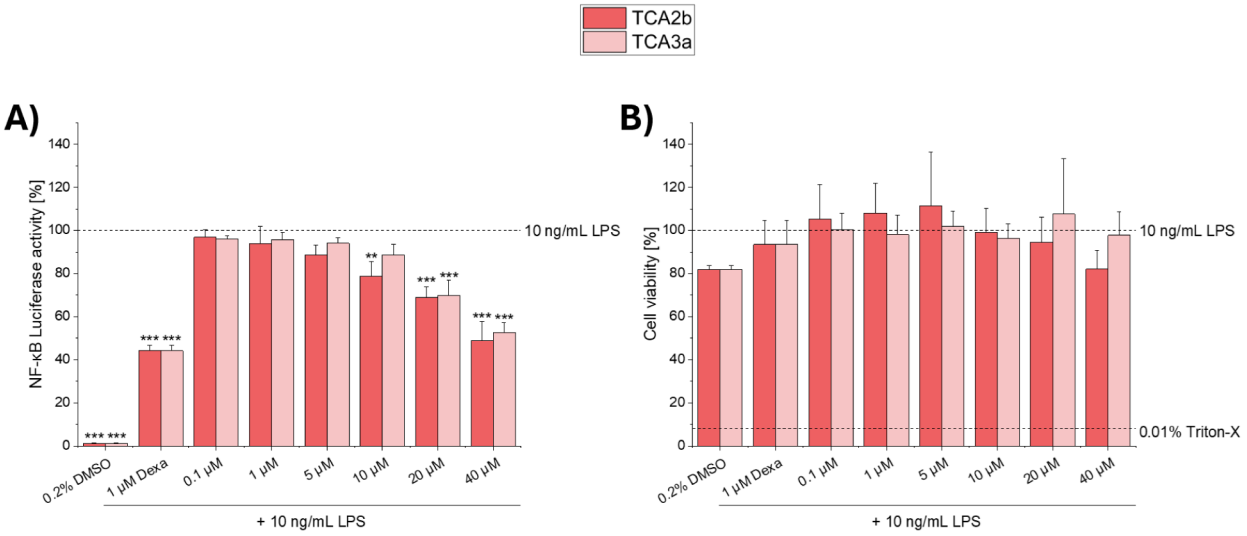


Figure 2. Immunosuppressive and cytotoxic effects of tricycloalternarene 2b (TCA 2b) and tricycloalternarene 3a (TCA 3a) in THP1-Lucia monocytes following 20 h of exposure. A) shows the results from the NF-κB reporter gene assay. Cells were treated with the indicated concentrations of the test compounds in the presence of 10 ng/mL lipopolysaccharide (LPS) to investigate immunosuppressive effects. DMSO (0.2%) served as solvent control, whereas dexamethasone (Dexa; 1 μM) was included as negative control. NF-κB activity is expressed relative to the positive control (0.2% DMSO + 10 ng/mL LPS), which was set to 100%. B) reports the impact of the test compounds on cell viability, determined using the CellTiter blue (CTB) assay. Data are presented as mean + standard deviation of three independent experiments. Cell viability is expressed relative to the positive control (0.2% DMSO + 10 ng/mL LPS), which was set to 100%. Triton X-100 (0.01%) served as positive control for cytotoxicity (indicated by the lower dotted line). Statistical significance was determined by one-way ANOVA followed by Bonferroni's post hoc test (*p < 0.05; **p < 0.01; ***p < 0.001).

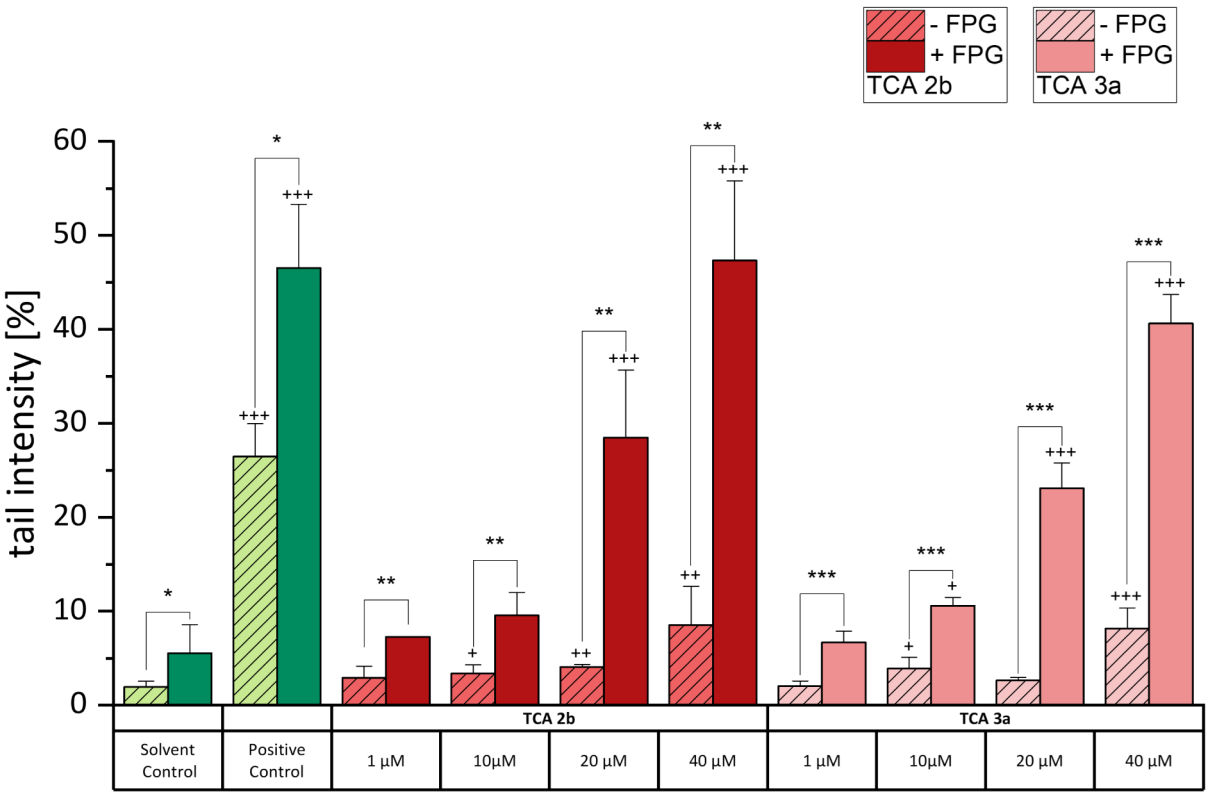


Figure 3. Genotoxic effects of tricycloalternarene 2b (TCA 2b) and tricycloalternarene 3a (TCA 3a) in HT-29 cells, assessed using the alkaline Comet assay. DNA damage was assessed after 1 h of exposure and is expressed as tail intensity (%). Measurements were performed in the absence and presence of formamidopyrimidine DNA glycosylase (FPG) enzyme. DMSO (1%, v/v) served as the solvent control, while cells exposed to UV−B radiation for 1 min were used as the positive control. Statistical significance was evaluated using Student's t test. Significant differences between the individual treatments and the respective solvent controls are indicated by ^{+}p < 0.05, ^{++}p < 0.01, and ^{+++}p < 0.001. Significant differences between the corresponding FPG-treated and non-FPG-treated conditions for each individual treatment are indicated by *p < 0.05, **p < 0.01, ***p < 0.001.

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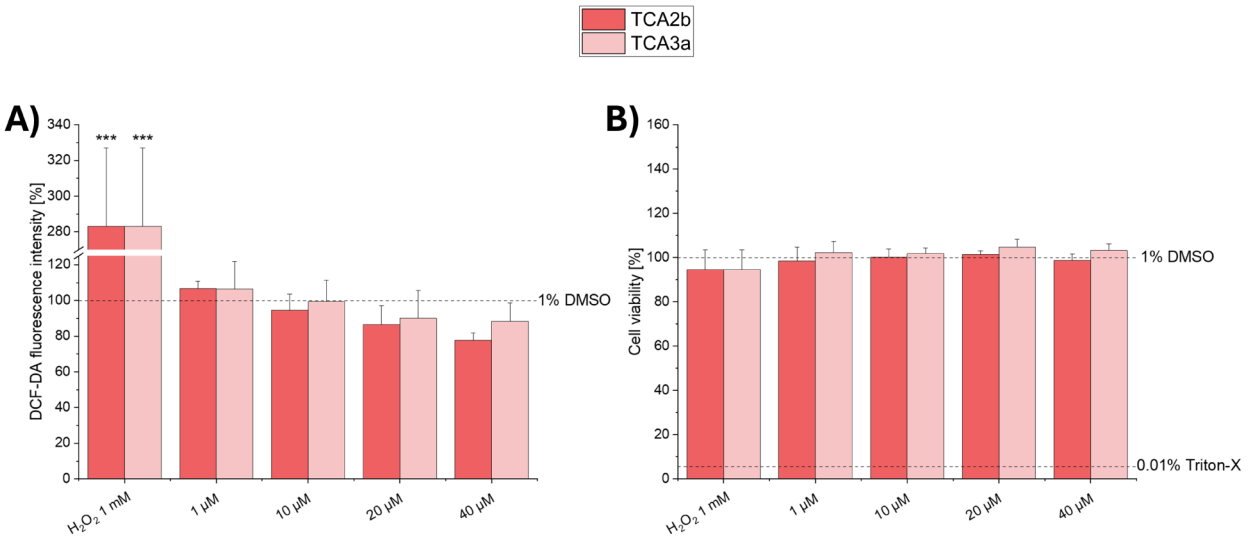


Figure 4. Oxidative stress induction and cytotoxic effects of tricycloalternarene 2b (TCA 2b) and tricycloalternarene 3a (TCA 3a) in HT-29 cells after 1 h of incubation. A) Induction of intracellular oxidative stress was assessed using the Dichlorfluorescein-diacetate (DCF-DA) assay. Cells were treated with the indicated concentrations of the test compounds, and DCF fluorescence was assessed after 1 h. DMSO (1%) served as solvent control, which was set to 100%, whereas H_2O_2 (1 mM) was included as positive control. B) reports the impact of the test compounds on cell viability, determined using the CellTiter blue (CTB) assay. Cell viability is expressed relative to the solvent control (1% DMSO), which was set to 100%. Triton X-100 (0.01%) served as positive control for cytotoxicity (indicated by the lower dotted line). All data are presented as mean + standard deviation of three independent experiments. Statistical significance was determined by one-way ANOVA followed by Bonferroni's post hoc test (*p < 0.05; **p < 0.01; ***p < 0.001).

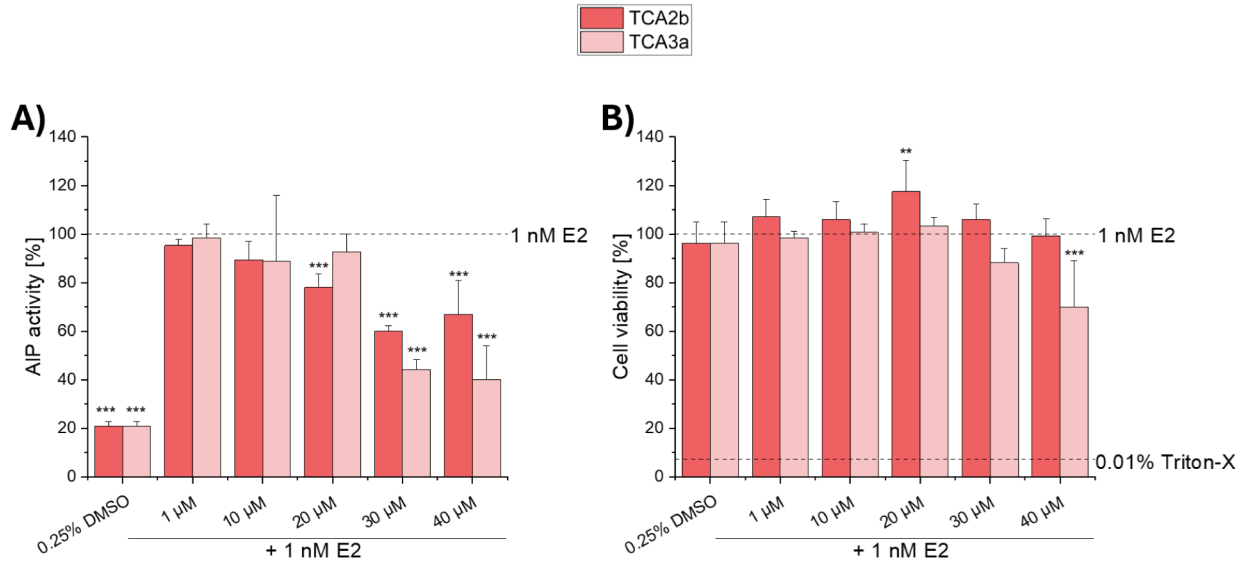


Figure 5. Antiestrogenic activity and effects on cell viability of tricycloalternarene 2b (TCA 2b) and tricycloalternarene 3a (TCA 3a) in Ishikawa cells after 48 h of exposure. (A) shows the results of the alkaline phosphatase (AlP) assay. Cells were treated with the indicated concentrations of the test compounds in the presence of 1 nM estradiol (E2) to evaluate antiestrogenic activity. DMSO (0.25%) served as the solvent control. AlP activity is expressed relative to the positive control (1 nM E2), which was set to 100%. (B) reports the impact of the test compounds on cell viability, determined using the CellTiter blue (CTB) assay. Cell viability is expressed relative to the positive control (1 nM E2), which was set to 100%. Triton X-100 (0.01%) served as positive control for cytotoxicity and is indicated by the lower dotted line. All data are presented as mean + standard deviation of three independent experiments. Significant differences compared to the solvent control were determined by one-way ANOVA followed by Bonferroni's post hoc test (*p < 0.05; **p < 0.01; ***p < 0.001).

cell viability, the CTB assay was conducted in parallel. As shown in Figure 2A, exposure of cells to the newly isolated mycotoxins TCA 2b and TCA 3a led to concentration-dependent suppressions of LPS-induced NF-κB pathway activation. TCA 2b significantly reduced the luciferase activity from 10 μM onward (78.9 ± 6.5% of the positive control), resulting in more than 50% suppression at the highest tested concentration (40 μM; 48.9 ± 8.8% of the positive control). TCA 3a also reduced NF-κB pathway activation, although the reduction at 10 μM did not reach statistical significance (88.7 ± 5.0% of the positive control; p = 0.051). At 20 and 40 μM, TCA 3a significantly suppressed pathway activation to 79.8 ± 7.0% and 52.6 ± 4.7% of the positive control, respectively. Neither compound induced NF-κB activation within the

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investigated concentration range (Supplementary Figure S1), and neither significantly affected cell viability at any tested concentration (Figure 2B).

3.2. Genotoxicity

To assess the genotoxic potential of TCA 2b and TCA 3a, the alkaline COMET assay was performed in HT-29 cells in the absence and presence of FPG enzyme to detect not only DNA single- and double-strand breaks but also FPG-sensitive DNA lesions, which are indicative of oxidative DNA damage and/or covalent guanine adduct formation.[32] As shown in Figures 3, 1 h exposure to both compounds resulted in increased DNA damage. In particular, treatment of cells with TCA 2b induced moderate but statistically significant and concentration-dependent DNA damage in the absence of FPG at concentrations ≥10 μM. TCA 3a also resulted in increased tail intensity in the absence of FPG enzyme; however, the response was less consistent. Incubation with 10 μM and 40 μM resulted in significantly increased tail intensities compared with the solvent control (3.9 ± 1.2%, 8.1 ± 2.2%, respectively), whereas no significant increase was observed at the intermediate concentration of 20 μM (2.6 ± 0.3%). The inclusion of FPG markedly enhanced the genotoxic effects of TCA 2b, resulting in significant increases in tail intensity at 20 μM and 40 μM (28.5 ± 7.2% and 47.3 ± 8.5%, respectively). Likewise, FPG treatment substantially increased the genotoxic response elicited by TCA 3a. Tail intensities increased in a concentration-dependent manner, reaching statistical significance from 10 μM onward. Specifically, tail intensity increased from 10.6 ± 0.9% at 10 μM to 23.1 ± 2.7% and 40.6 ± 3.1% at 20 and 40 μM, respectively. For both compounds, FPG treatment resulted in significantly higher tail intensities compared with the corresponding conditions in the absence of FPG.

3.3. Oxidative Stress

The ability of the TCAs to induce intracellular ROS formation was assessed in HT-29 cells using the DCF-DA assay following 1 h of exposure (Figure 4). Neither compound significantly altered intracellular ROS formation at any of the tested concentrations (1−40 μM; Figure 4A). Cell viability, determined in parallel using the CTB assay, was not significantly affected by either compound after 1 h of exposure (Figure 4B).

3.4. Antiestrogenic Effects

The ability of the TCAs to modulate the estrogenic pathway was evaluated using the AlP assay in Ishikawa cells. Incubations were performed in the absence and presence of 1 nM E2 to assess their estrogenic and antiestrogenic properties, respectively. To ensure that the observed effects were not confounded by cytotoxicity, cell viability was assessed in parallel using the CTB assay. As shown in Figure 5A, both TCAs exhibited significant antiestrogenic activity. Incubation with TCA 2b significantly reduced AlP activity at concentrations ≥20 μM, reaching 78.0 ± 5.7% of the positive control at 20 μM and further decreasing in a concentration-dependent manner to 66.9 ± 13.9% at the highest tested concentration (40 μM). These reductions occurred without changes in cell viability (Figure 5B). Incubation with TCA 3a significantly reduced AlP activity at 30 and 40 μM (44.3 ± 3.9% and 40.1 ± 14.0% of the positive control, respectively). However, a significant reduction in cell viability was observed at 40 μM (Figure 5B). To assess potential estrogenic activity, all compounds were additionally tested in the absence of E2. Under these experimental conditions, none of the test substances significantly induced AlP activity (Supplementary Figure S2).

4. DISCUSSION

Species belonging to the *Alternaria* genus can colonize a wide range of food crops and produce complex mixtures of mycotoxins, resulting in continuous dietary exposure for consumers through the ingestion of contaminated foods.[3,33] In this context, mycotoxins derived from *Alternaria* species have been associated with a broad range of adverse effects in both *in vivo* and *in vitro* studies, including, fetotoxicity, teratogenicity, genotoxicity, endocrine disrupting activity, and immunomodulatory effects.[3,9,10,34] Although several known *Alternaria* mycotoxins (e.g., AOH, AME, AST, ATX-I, ATX-II, ALTP) can, individually or in combination, account for some of the observed biological effects, in other cases these compounds do not sufficiently explain the responses induced by complex extracts.[10,14,15,17,35] This suggests that additional, not yet investigated or poorly studied metabolites may also contribute to the overall toxicological profile of the mycotoxin mixtures produced by *Alternaria* species. This highlights the need to investigate further metabolites that could account for biological effects observed in complex mixtures but remain unidentified or insufficiently characterized. Therefore, in the present study, the two isolated *Alternaria* toxins TCA 2b and TCA 3a were evaluated with respect to their immunomodulatory, genotoxic, oxidative stress-inducing, and antiestrogenic effects.

Immunomodulation represents an important toxicological end point of *Alternaria* mycotoxins, as several metabolites have been shown to interfere with inflammatory signaling pathways. In this context, the NF-κB pathway is a key mediator of innate immune response and inflammation, regulating the transcription of various genes involved in cytokine production and inflammatory processes. In the present study, TCA 2b and TCA 3a significantly suppressed LPS-induced NF-κB pathway activation in THP1-Lucia cells without affecting cell viability, supporting that the observed immunosuppressive effects reflect true pathway modulation (Figure 2). Of note, a previous study conducted on the structurally related ACTG-toxin H, isolated from a complex *A. alternata* extract, also reported concentration-dependent immunosuppressive effects in both human and murine macrophages. Yang et al. demonstrated that ACTG-toxin H significantly inhibited nitrite and IL-6 production from 25 μg/mL (72.6 μM) onward, whereas suppression of IL-1β production and NF-κB pathway activation required 50 μg/mL (145.2 μM).[29] Notably, in the present study the onset of immunosuppressive activity was observed at substantially lower concentrations for TCA 2b and TCA 3a than those previously reported for ACTG-toxin H, as significant inhibition of LPS-induced NF-κB pathway activation was already detected at concentrations ≥10 μM and 20 μM, respectively. In addition, Spina et al. reported immunosuppressive effects of a complex *A. alternata* extract containing TCA 2b and TCA 3a in THP1 macrophages.[36] However, because the extract also contained other immunosuppressive *Alternaria* mycotoxins, including ATX-I-III, the specific contribution of the TCAs could not be conclusively distinguished. By testing the isolated compounds, the present study provides direct evidence that both TCA 2b and TCA 3a themselves suppress LPS-induced NF-κB pathway activation. A

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comprehensive comparison of the immunomodulatory effects of some well-known *Alternaria* mycotoxins was recently reported by Partsch et al., who evaluated seven structurally diverse mycotoxins in THP1-Lucia monocytes and PMA-differentiated macrophages. Among the mycotoxins investigated, ALTP exhibited the strongest immunosuppressive activity, followed by ATX-I, AST, and tentoxin (TEN). AOH and AME exhibited only weak immunosuppressive effects, which were accompanied by cytotoxicity.[37] More recently, Partsch et al. reported immunosuppressive effects of TeA, one of the most abundant *Alternaria* mycotoxins, although significant inhibition of the NF-κB pathway was observed only from 25 μM onward.[38] Based on the currently available data, TCA 2b and TCA 3a appear to exhibit an intermediate immunosuppressive potency among the *Alternaria* mycotoxins investigated to date, with an earlier onset than TeA but a later onset than ALTP, ATX-I, AST, AOH, and TEN. While the present findings establish TCA 2b and TCA 3a as direct modulators of NF-κB signaling, the molecular mechanisms underlying these effects remain to be elucidated, and further studies are warranted to determine their cellular targets and potential relevance under realistic exposure conditions.

Besides their immunomodulatory properties, the genotoxic potential of TCA 2b and TCA 3a was also investigated, as DNA damage can become fixed during replication and thereby contribute to long-term adverse outcomes such as carcinogenesis.[39] As shown in Figure 3, both compounds induced significant DNA damage in HT-29 cells, as determined using the alkaline COMET assay in the presence and absence of FPG enzyme. Notably, in the absence of FPG, exposure to either compound caused only slight but significant increases in tail intensity (<10%), whereas FPG treatment markedly amplified the detected DNA damage. At the highest tested concentration (40 μM), tail intensities reached 47.3% and 40.6% for TCA 2b and TCA 3a, respectively, which were within the range observed for the positive control (46.5%). In previous studies, genotoxic effects have also been reported for the dibenzo-α-pyrones AOH and AME in several mammalian cell lines, including HT-29 cells, A431 cells, Caco-2 cells, and HepG2 cells, with evidence pointing to poisoning of human topoisomerases I and II as a key underlying mechanism.[14,15] In a study by Fehr et al., AOH and AME induced low but significant increases in tail intensity in the absence of FPG, detectable from 1 and 10 μM onward, respectively. The magnitude of this DNA damage was broadly comparable to that observed for TCA 2b and TCA 3a under the same conditions. In contrast to the present findings, FPG treatment did not further increase DNA damage induced by AOH and AME.[14] Interestingly, AOH and AME have been reported to induce intracellular oxidative stress, whereas neither TCA 2b nor TCA 3a increased DCF fluorescence under the applied experimental conditions (Figure 4).[16] This contrasting response pattern suggests that different mechanisms may contribute to the genotoxic effects of these structurally distinct classes of *Alternaria* mycotoxins. PQs, particularly epoxide-bearing metabolites such as ATX-II and stemphyltoxin III (STTX-III), are considered among the most potent genotoxic *Alternaria* mycotoxins. In particular, ATX-II has been shown to be at least 50-fold more potent than AOH and AME as a mutagen in mammalian cells and induces DNA strand breaks at substantially lower concentrations.[17,18] Aichinger et al., reported that exposure of HT-29 cells to 1 μM ATX-II induced significant DNA damage in the alkaline COMET assay. In the absence of FPG, tail intensities of approximately 7% were observed, whereas FPG treatment increased tail intensities to approximately 17%.[19] This FPG-dependent increase resembles the response pattern observed for the TCAs in the present study, although the effects of ATX-II occurred at lower concentrations. Similar to the present study, ATX-II did not induce detectable increase of intracellular ROS formation at concentrations that caused pronounced DNA damage in the COMET assay. It has therefore been suggested that its genotoxicity is mediated by the formation of DNA adducts, which may subsequently be converted into FPG-sensitive DNA lesions.[18,32] Similar to the findings reported for ATX-II, TCA 2b and TCA 3a showed pronounced FPG-sensitive DNA damage without an increase in DCF fluorescence under the applied experimental conditions (Figure 4). These findings suggest that the observed genotoxicity is unlikely to result from intracellular oxidative stress and that other mechanisms may have contributed to it. Nevertheless, given that the DCF-DA assay does not detect all ROS with equal sensitivity,[40] a limited contribution of ROS cannot be entirely excluded. A separate consideration is the potential of the tested TCAs to induce oxidative stress under other exposure conditions. The present results should not be interpreted as evidence that these compounds cannot induce ROS production, as the DCF-DA assay was performed only after 1 h of exposure to match the exposure duration used in the comet assay and thereby determine whether acute ROS formation could contribute to the genotoxic effects observed at the same time point. Consequently, these findings do not exclude the possibility that the tested TCAs may induce ROS production following longer exposure periods, and further studies are therefore needed to comprehensively characterize the ROS-inducing potential of these emerging mycotoxins.

In addition to their immunomodulatory and genotoxic properties, TCA 2b and TCA 3a were assessed for their ability to interfere with ER signaling (Figure 5). This end point is particularly relevant for *Alternaria* mycotoxins, as both estrogenic and antiestrogenic activities have been reported.[11,21] Among the *Alternaria* mycotoxins investigated so far, the dibenzo-α-pyrones AOH and AME have been shown to exhibit estrogenic activity, whereas ALTP, ATX-I, and AST were primarily identified as antiestrogenic compounds.[9−11] In the present study, both TCA 2b and TCA 3a significantly reduced E2-induced AlP activity in Ishikawa cells, indicating antiestrogenic activity and expanding the group of *Alternaria* mycotoxins with this property (Figure 5). TCA 2b significantly reduced AlP activity from 20 μM onward without affecting cell viability. The magnitude of this reduction remained largely unchanged between 30 and 40 μM, suggesting that the antiestrogenic response may have approached a plateau within the tested concentration range. For TCA 3a, significant reductions in AlP activity were observed at 30 and 40 μM. However, the reduction at 40 μM was accompanied by impaired cell viability, preventing a clear attribution of the effect at this concentration to a specific antiestrogenic mechanism (Figure 5). Compared with TCA 2b and TCA 3a, the previously characterized antiestrogenic *Alternaria* mycotoxins ALTP, ATX-I, and AST showed an earlier onset of antiestrogenic activity in Ishikawa cells, with significant effects observed from 0.4, 2, and 5 μM, respectively.[10,11] At 10 μM, ALTP and AST almost completely suppressed E2-induced AlP activity, although these pronounced effects were accompanied by reduced cell viability. In contrast, the

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antiestrogenic profile of ATX-I more closely resembled that of TCA 2b and TCA 3a. Despite the earlier onset of activity at 2 μM, the reduction in E2-induced AlP activity at 10 μM was comparable, reaching 13.6% for ATX-I and 10.6% and 11.3% for TCA 2b and TCA 3a, respectively. Overall, TCA 2b and TCA 3a exhibited comparatively weak antiestrogenic activity among the currently characterized *Alternaria* mycotoxins, with their effects more closely resembling those of ATX-I than those of ALTP and AST.

In conclusion, the present study expands the toxicological knowledge of the poorly investigated *Alternaria* mycotoxins TCA 2b and TCA 3a. To the best of our knowledge, this is the first study to demonstrate the genotoxic and antiestrogenic potential of these compounds, providing the first comprehensive toxicological characterization of members of the TCA subclass. Both compounds exhibited immunosuppressive, genotoxic, and antiestrogenic activities *in vitro*, demonstrating that TCAs represent biologically active *Alternaria* metabolites. Although these effects were observed only at relatively high concentrations, the toxicological relevance of TCA 2b and TCA 3a cannot be excluded, as occurrence and exposure data remain scarce. Moreover, these compounds occur together with numerous other biologically active *Alternaria* mycotoxins in naturally contaminated food and feed, raising the possibility that combinatory effects may contribute to the overall toxicity of complex *Alternaria* mycotoxin mixtures. Future studies are therefore warranted to elucidate the molecular mechanisms underlying the observed biological effects, to establish their toxicological relevance under realistic exposure conditions, and to determine the contribution of TCAs to the toxicity of naturally occurring *Alternaria* mycotoxin mixtures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.6c00497.

^{1}H and ^{13}C NMR (500/125 MHz) spectroscopic data of TCA 2b and TCA 3a, in MeOD (Table S1); immunostimulatory and cytotoxic properties of TCA 2b and TCA 3a in THP1-Lucia monocytes (Figure S1); estrogenic activity and effects on cell viability of TCA 2b and TCA 3a in Ishikawa cells (Figure S2) (PDF)

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Notes

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■ ABBREVIATIONS

AlP, alkaline phosphatase; ALTP, alterperylenol; AME, alternariol monomethyl ether; AOH, alternariol; AST, altersetin; ATX-I, altertoxin-I; ATX-II, altertoxin-II; CTB, CellTiter-Blue; DCF-DA, dichlorofluorescein diacetate; ELSD, evaporative light scattering detector; ER, estrogen receptor; FPG, formamidopyrimidine DNA glycosylase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; NMR, nuclear magnetic resonance; PDA, photodiode array; PQ, perylene quinone; Qda detector, quadrupole Dalton detector; ROS, reactive oxygen species; TCA, tricycloalternarene

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REFERENCES

(1) Streit, E.; Schatzmayr, G.; Tassis, P.; Tzika, E.; Marin, D.; Taranu, I.; Tabuc, C.; Nicolau, A.; Aprodu, I.; Oswald, I.P.; et al. Current situation of mycotoxin contamination and co-occurrence in animal feed−focus on Europe. *Toxins* **2012**, *4* (10), 788−809.

(2) Berthiller, F.; Crews, C.; Dall'asta, C.; Saeger, S. D.; Haesaert, G.; Karlovsky, P.; Oswald, I. P.; Seefelder, W.; Speijers, G.; Stroka, J. Masked mycotoxins: a review. *Mol. Nutr. Food Res.* **2013**, *57* (1), 165−186.

(3) Crudo, F.; Varga, E.; Aichinger, G.; Galaverna, G.; Marko, D.; Dall'asta, C.; Dellafiora, L. Co-Occurrence and Combinatory Effects of Alternaria Mycotoxins and other Xenobiotics of Food Origin: Current Scenario and Future Perspectives. *Toxins* **2019**, *11* (11), 640.

(4) Omotayo, O. P.; Omotayo, A. O.; Mwanza, M.; Babalola, O. O. Prevalence of Mycotoxins and Their Consequences on Human Health. *Toxicol. Res.* **2019**, *35* (1), 1−7.

(5) Eskola, M.; Kos, G.; Elliott, C. T.; Hajslova, J.; Mayar, S.; Krska, R. Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate' of 25. *Crit. Rev. Food Sci. Nutr.* **2020**, *60* (16), 2773−2789.

(6) European Commission Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. In 2023/915 Commission, E., 2023.

(7) Wenderoth, M.; Garganese, F.; Schmidt-Heydt, M.; Soukup, S. T.; Ippolito, A.; Sanzani, S. M.; Fischer, R. Alternariol as virulence and colonization factor of Alternaria alternata during plant infection. *Mol. Microbiol.* **2019**, *112* (1), 131−146.

(8) EFSA *E. F. S. A. Scientific Opinion on the risks for animal and public health related to the presence of Alternariatoxins in feed and food. In EFSA Journal, Contam), E. P. o. C. i. t. F. C.* Parma, Italy, 2011.

(9) Louro, H.; Vettorazzi, A.; Lopez de Cerain, A.; Spyropoulou, A.; Solhaug, A.; Straumfors, A.; Behr, A. C.; Mertens, B.; Zegura, B.; Faeste, C. K.; et al. Hazard characterization of Alternaria toxins to identify data gaps and improve risk assessment for human health. *Arch. Toxicol.* **2024**, *98* (2), 425−469.

(10) Partsch, V.; Crudo, F.; Piller, D.; Varga, E.; Del Favero, G.; Marko, D. Resolving complexity: Identification of altersetin and toxin mixtures responsible for the immunomodulatory, antiestrogenic and genotoxic potential of a complex Alternaria mycotoxin extract. *Food Chem. Toxicol.* **2025**, *198*, 115315.

(11) Crudo, F.; Partsch, V.; Braga, D.; Blazevic, R.; Rollinger, J. M.; Varga, E.; Marko, D. Discovery of the Alternaria mycotoxins alterperylenol and altertoxin I as novel immunosuppressive and antiestrogenic compounds in vitro. *Arch. Toxicol.* **2025**, *99*, 407.

(12) (a) Fleck, S. C.; Pfeiffer, E.; Metzler, M. Permeation and metabolism of Alternaria mycotoxins with perylene quinone structure in cultured Caco-2 cells. *Mycotoxin Res.* **2014**, *30* (1), 17−23. (b) Fleck, S. C.; Pfeiffer, E.; Podlech, J.; Metzler, M. Epoxide reduction to an alcohol: a novel metabolic pathway for perylene quinone-type alternaria mycotoxins in mammalian cells. *Chem. Res. Toxicol.* **2014**, *27* (2), 247−253.

(13) Gao, J.; Chen, L.; Steffen, K.; Schweder, H.; Schreckenberger, B.; Li, S.-M.; Auxier, B.; Rokas, A.; Fischer, R. Intermolecular carbon-carbon naphthalene coupling involves a radical mechanism driven by P450 cytochrome in Alternaria alternata. *Angew. Chem., Int. Ed.* **2026**, No. e2505284.

(14) Fehr, M.; Pahlke, G.; Fritz, J.; Christensen, M. O.; Boege, F.; Altemoller, M.; Podlech, J.; Marko, D. Alternariol acts as a topoisomerase poison, preferentially affecting the IIalpha isoform. *Mol. Nutr. Food Res.* **2009**, *53* (4), 441−451.

(15) Solhaug, A.; Eriksen, G. S.; Holme, J. A. Mechanisms of Action and Toxicity of the Mycotoxin Alternariol: A Review. *Basic Clin. Pharmacol. Toxicol.* **2016**, *119* (6), 533−539.

(16) Tiessen, C.; Fehr, M.; Schwarz, C.; Baechler, S.; Domnanich, K.; Bottler, U.; Pahlke, G.; Marko, D. Modulation of the cellular redox status by the Alternaria toxins alternariol and alternariol monomethyl ether. *Toxicol. Lett.* **2013**, *216* (1), 23−30.

(17) Fleck, S. C.; Burkhardt, B.; Pfeiffer, E.; Metzler, M. Alternaria toxins: Altertoxin II is a much stronger mutagen and DNA strand breaking mycotoxin than alternariol and its methyl ether in cultured mammalian cells. *Toxicol. Lett.* **2012**, *214* (1), 27−32.

(18) Fleck, S. C.; Sauter, F.; Pfeiffer, E.; Metzler, M.; Hartwig, A.; Koberle, B. DNA damage and repair kinetics of the Alternaria mycotoxins alternariol, altertoxin II and stemphyltoxin III in cultured cells. *Mutat. Res., Genet. Toxicol. Environ. Mutagen.* **2016**, *798*−*799*, 27−34.

(19) Aichinger, G.; Puntscher, H.; Beisl, J.; Kutt, M.-L.; Warth, B.; Marko, D. Delphinidin protects colon carcinoma cells against the genotoxic effects of the mycotoxin altertoxin II. *Toxicol. Lett.* **2018**, *284*, 136−142.

(20) Hessel-Pras, S.; Kieshauer, J.; Roenn, G.; Luckert, C.; Braeuning, A.; Lampen, A. In vitro characterization of hepatic toxicity of Alternaria toxins. *Mycotoxin Res.* **2019**, *35* (2), 157−168.

(21) Lehmann, L.; Wagner, J.; Metzler, M. Estrogenic and clastogenic potential of the mycotoxin alternariol in cultured mammalian cells. *Food Chem. Toxicol.* **2006**, *44* (3), 398−408.

(22) Aichinger, G.; Kruger, F.; Puntscher, H.; Preindl, K.; Warth, B.; Marko, D. Naturally occurring mixtures of Alternaria toxins: anti-estrogenic and genotoxic effects in vitro. *Arch. Toxicol.* **2019**, *93* (10), 3021−3031.

(23) Aichinger, G.; Grgic, D.; Beisl, J.; Crudo, F.; Warth, B.; Varga, E.; Marko, D. N-acetyl cysteine alters the genotoxic and estrogenic properties of Alternaria toxins in naturally occurring mixtures. *Emerging Contam.* **2022**, *8*, 30−38.

(24) Grover, S.; Lawrence, C. B. The Alternaria alternata Mycotoxin Alternariol Suppresses Lipopolysaccharide-Induced Inflammation. *Int. J. Mol. Sci.* **2017**, *18* (7), 1577.

(25) Schmutz, C.; Cenk, E.; Marko, D. The Alternaria Mycotoxin Alternariol Triggers the Immune Response of IL-1beta-stimulated, Differentiated Caco-2 Cells. *Mol. Nutr. Food Res.* **2019**, *63* (20), No. e1900341.

(26) Qiao, L.-R.; Yuan, L.; Gao, J.-M.; Zhao, P.-J.; Kang, Q.-J.; Shen, Y.-M. Tricycloalternarene derivatives produced by an endophyte alternaria alternata isolated from Maytenus hookeri. *J. Basic Microbiol.* **2007**, *47* (4), 340−343.

(27) Fraga, B. M.; Diaz, C. E. Proposal for structural revision of several monosubstituted tricycloalternarenes. *Phytochemistry* **2022**, *198*, 113141.

(28) Zhang, H.; Zhao, Z.; Chen, J.; Bai, X.; Wang, H. Tricycloalternarene Analogs from a Symbiotic Fungus Aspergillus sp. D and Their Antimicrobial and Cytotoxic Effects. *Molecules* **2018**, *23* (4), 855.

(29) Yang, X.; Zhang, G.; Tang, X.; Jiao, J.; Kim, S. Y.; Lee, J. Y.; Zhu, T.; Li, D.; Yun, Y. G.; Gu, Q.; et al. Toll-like receptor 4/nuclear factor-kappaB signaling pathway is involved in ACTG-toxin H-mediated anti-inflammatory effect. *Mol. Cell. Biochem.* **2013**, *374* (1−2), 29−36.

(30) Gao, J.; Wenderoth, M.; Doppler, M.; Schuhmacher, R.; Marko, D.; Fischer, R. Fungal Melanin Biosynthesis Pathway as Source for Fungal Toxins. *Microbiology* **2022**, *13* (3), No. e0021922.

(31) Brandt, R.; Keston, A. S. Synthesis of Diacetyldichlorofluorescin: A Stable Reagent for Fluorometric Analysis. *Anal. Biochem.* **1965**, *11*, 6−9.

(32) Soukup, S. T.; Fleck, S. C.; Pfeiffer, E.; Podlech, J.; Kulling, S. E.; Metzler, M. DNA reactivity of altertoxin II: Identification of two covalent guanine adducts formed under cell-free conditions. *Toxicol. Lett.* **2020**, *331*, 75−81.

(33) Lee, H. B.; Patriarca, A.; Magan, N. Alternaria in Food: Ecophysiology, Mycotoxin Production and Toxicology. *Mycobiology* **2015**, *43* (2), 93−106.

(34) (a) Call, F.; Crudo, F.; Marko, D. Impact of in vitro digestion on the bioaccessibility, genotoxicity and mutagenicity of mycotoxins in a complex Alternaria extract. *Food Chem.: X* **2026**, *33*, 103481. (b) Crudo, F.; Aichinger, G.; Mihajlovic, J.; Varga, E.; Dellafiora, L.; Warth, B.; Dall'asta, C.; Berry, D.; Marko, D. In vitro interactions of Alternaria mycotoxins, an emerging class of food contaminants, with

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the gut microbiota: a bidirectional relationship. *Arch. Toxicol.* **2021**, *95* (7), 2533−2549.
(35) Solhaug, A.; Karlsoen, L. M.; Holme, J. A.; Kristoffersen, A. B.; Eriksen, G. S. Immunomodulatory effects of individual and combined mycotoxins in the THP-1 cell line. *Toxicol. In Vitro* **2016**, *36*, 120−132.
(36) Spina, R.; Ropars, A.; Bouazzi, S.; Dadi, S.; Lemiere, P.; Dupire, F.; Khiralla, A.; Yagi, S.; Frippiat, J.-P.; Laurain-Mattar, D. Screening of Anti-Inflammatory Activity and Metabolomics Analysis of Endophytic Fungal Extracts; Identification and Characterization of Perylenequinones and Terpenoids from the Interesting Active Alternaria Endophyte. *Molecules* **2023**, *28* (18), 6531.
(37) Partsch, V.; Selimagic, A.; Crudo, F.; Marko, D. Immunomodulatory potential of Alternaria mycotoxins on immune and intestinal cells: a comparative systematic in vitro study. *Arch. Toxicol.* **2026**, *100*, 3519.
(38) Partsch, V.; Crudo, F.; Marko, D. Opposing immunomodulatory effects of the Alternaria mycotoxin tenuazonic acid in immune and intestinal epithelial cells. *Food chem. toxicol.* **2026**, *218*, 116428.
(39) Hartwig, A.; Arand, M.; Epe, B.; Guth, S.; Jahnke, G.; Lampen, A.; Martus, H.-J.; Monien, B.; Rietjens, I.; Schmitz-Spanke, S.; et al. Mode of action-based risk assessment of genotoxic carcinogens. *Arch. Toxicol.* **2020**, *94* (6), 1787−1877.
(40) Chen, X.; Zhong, Z.; Xu, Z.; Chen, L.; Wang, Y. 2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy. *Free Radical Res.* **2010**, *44* (6), 587−604.

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