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ARTICLE

# Absorption and metabolism of modified mycotoxins of alternariol, alternariol monomethyl ether, and zearalenone in Caco-2 cells

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## Abstract

**Background and objectives:** Various cereals, fruits, and vegetables are commonly contaminated with mycotoxins such as zearalenone (ZEN), alternariol (AOH), and alternariol monomethyl ether (AME). More recently, their glucosidic metabolites formed in the plant have gained increasing attention. Experimental data on the contribution of modified mycotoxins to total toxicity are either controversy or lacking. Thus, the aim of this study was to investigate the absorption and metabolism of ZEN-, AOH-, and AME-glucosides using the Caco-2 cell system.

**Findings:** No quantifiable amounts of ZEN-14-glucoside, ZEN-16-glucoside, free ZEN, and ZEN metabolites were found in Caco-2 cells and in the basolateral compartment. In contrast, glucosides of AOH and AME were absorbed and released their parental toxins, which were further metabolized to form glucuronides and sulfates to a variable extent. Metabolites were found on the basolateral site, too. There is also evidence that AOH-9-diglucoside is hydrolyzed to AOH-9-glucoside.

**Conclusion:** Our results demonstrate that modified ZEN is less absorbed whereas modified *Alternaria* toxins are taken up to a higher extent by Caco-2 cells, followed by deglucosylation, metabolization, and transport to the basolateral site, suggesting a potential contribution to overall toxicity of these modified mycotoxins.

**Significance and novelty:** For the first time, absorption studies using modified *Alternaria* toxins in the Caco-2 cell system were carried out.

**KEYWORDS**

alternariol monomethyl ether-glucosides, alternariol-glucosides, Caco-2 cells, In vitro absorption, modified mycotoxins, zearalenone-glucosides

**Abbreviations:** AME, Alternariol-9-O-monomethyl ether; AOH, Alternariol; DAD, Diode array detector; DMEM, Dulbecco's modified Eagle medium; DMSO, Dimethylsulfoxide; ESI, Electrospray ionization; Glc, Glucoside; Glc*A*, Glucuronide; HBSS, Hank's buffered salt solution; HPLC, High-performance liquid chromatography; LC, Liquid chromatography; MS, Mass spectrometry; $P_{app}$, Apparent permeability coefficient; PBS, Phosphate-buffered saline; S, Sulfate; UV, Ultraviolet; ZEL, Zearalenol; ZEN, Zearalenone.

# 1 | INTRODUCTION

Fungi are a natural part of our living environment and can infect almost any plant during either growth and/or post-harvest. A large number of these fungi are also able to form mycotoxins, mostly low-molecular metabolites that greatly differ in their structures. However, all mycotoxins have in common that they are toxic to humans and/or animals even in small quantities. Based on in vitro studies with mammalian cells, the *Alternaria* toxins alternariol (AOH) and alternariol monomethyl ether (AME) are assumed to be genotoxic (Pfeiffer et al., 2007), whereas the *Fusarium* toxin zearalenone (ZEN) causes reproductive problems in livestock, especially in pigs, due to its estrogenic activity (Metzler et al., 2010; Zinedine et al., 2007).

These mycotoxins are commonly detected in cereals, fruits, and vegetables (Broggi et al., 2013; Darsanaki et al., 2015; Scott et al., 2012), compromising food and feed safety. Modified mycotoxins can be formed by plants in their metabolism of xenobiotics, which proceeds in three phases, with phase II conjugation reactions being most important. Thus, several modified forms of AOH, AME, and ZEN were identified in the last decades including glucosides, malonylglucosides, and diglucosides (Engelhardt et al., 1988; Hildebrand et al., 2015; Kamimura, 1986). These phase II metabolites are believed to be cleaved during digestion (due to the acidic environment in the stomach and small intestine or gut microbiota hydrolytic enzymes) releasing the toxic parental form, thereby contributing to overall toxicity. To carry out a risk assessment, the European Food Safety Authority (EFSA) requested further data on toxicological effects of modified mycotoxins (EFSA, 2014, 2016, 2017).

Detailed knowledge regarding the fate of modified mycotoxins in the human intestine can be obtained by using cultured Caco-2 cells, representing a widely accepted in vitro system to evaluate human intestinal absorption and metabolism of various substances (Artursson & Karlsson, 1991; Press & Di Grandi, 2008; Yee, 1997). Although they derived from a human colon carcinoma, cultured Caco-2 cells have many characteristics of intestinal epithelial cells when grown on a permeable membrane under suitable culture conditions for three weeks. Cultured Caco-2 cells form a polarized monolayer with tight junctions between neighboring cells. The apical site, being the equivalent of the intestinal lumen side, develops microvilli whereas the basolateral site represents the blood side of the intestinal epithelium. Also, transporter proteins of the ABC superfamily are expressed, and various enzymes of phase I and phase II metabolism are activated (Press & Di Grandi, 2008).

Recently, the intestinal uptake of ZEN-glucosides and glucosidic conjugates of ZEN derivatives (α-/β-ZEL) in Caco-2 cells (Cirlini et al., 2016; Gratz et al., 2017) has been studied. Cirlini et al. (2016) observed that 16-*O*-β-D-glucopyranosyl-ZEN (ZEN16Glc) and 14-*O*-β-D-glucopyranosyl-ZEN (ZEN14Glc) pass the Caco-2 cell monolayer suggesting a transport from the intestine to the portal blood. In contrast, Gratz et al. (2017) showed that neither ZEN14Glc, nor α- and β-ZEL-14-glucoside (α- and β-ZEL14Glc) were absorbed in vitro. These two studies used different cell clones of Caco-2 cells as well as different experimental and culturing conditions, which may explain the contradictory results. Both studies have in common that only free ZEN was chosen as an indicator for deglucosylation and possible phase I and phase II metabolites of the modified mycotoxins, formed by the Caco-2 cells, have not been analyzed.

We are not aware of any comparable studies using modified forms of *Alternaria* toxins. Because glucosides of AOH and AME have been found in various food samples, it is important to assess their toxicological potential (Puntscher et al., 2018).

Because experimental data on the contribution of modified mycotoxins to total toxicity are controversy or completely lacking, in vitro absorption and metabolism studies were performed with human Caco-2 cells. Of particular interest was whether the modified forms are absorbed by the cells, hydrolyzed resulting in their parental mycotoxins, and further metabolized. Mass spectrometric analyses were used to examine both the incubation media and the cells for the free mycotoxins as well for their metabolites formed by the Caco-2 cells. In addition, experiments with the Millicell® system were performed to study transport processes (uptake, release) in order to predict a potential in vivo absorption.

# 2 | MATERIALS AND METHODS

## 2.1 | Chemicals and reagents

AOH and AME were isolated from a culture of the *Alternaria alternata* strain BFE 1346 grown on rice flour containing media for 24 days at 25°C as described earlier for the isolation of other *Alternaria* toxins (Fleck et al., 2012). The crude extracts of several approaches were analyzed by LC-DAD-MS, the extracts were combined, and the fractions containing AOH and AME were isolated by preparative HPLC. Evaporated and freeze-dried AOH and AME fractions were used for LC-DAD-MS and NMR analyses. Their identity was confirmed by NMR, and the purity was >98% according to HPLC analysis with UV detection at 254 nm. ZEN was purchased from Fermentek (Jerusalem, Israel) with a purity >98% according to the manufacturers´ certificates. HPLC-MS grade acetonitrile (ACN) and methanol (MeOH) were purchased from VWR International (Bruchsal, Germany). Accutase® solution, Lucifer Yellow, and Dulbecco's modified Eagle medium (DMEM/F12) was purchased from Sigma-Aldrich (St. Louis, USA).

9-*O*-β-D-glucopyranosyl-AOH (AOH9Glc), 3-*O*-β-D-glucopyranosyl-AOH (AOH3Glc), 9-*O*-[β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl]AOH (AOH9DiGlc), 7-*O*-β-D-glucopyranosyl-AME (AME7Glc), 3-*O*-β-D-glucopyranosyl-AME (AME3Glc), ZEN16Glc, and ZEN14Glc were isolated from extracts of cells of tobacco BY-2 suspension cell cultures that were incubated in the presence of AOH, AME, and ZEN as described earlier for the isolation of modified *Alternaria* toxins (Hildebrand et al., 2015). Mycotoxin conjugates were extracted into MeOH/dichloromethane ($CH_2Cl_2$) (2/1, v/v), followed by MeOH/water/acetic acid (79/20/1, v/v/v), and MeOH/$CH_2Cl_2$ (2/1, v/v) extraction steps. Extracts were freeze-dried and dissolved in MeOH to isolate modified mycotoxins by preparative HPLC. Isolation was performed on a reversed phase column (Phenomenex Luna C18(2), 250 × 15 mm, 5 µm) at a flow rate of 8 ml/min using 0.1% formic acid in acetonitrile (A) and 0.1% formic acid (B) in water as eluents. Conjugates of AOH and AME were separated with initially 80% B, held for 5 min, decreased to 60% B within 7 min, held for 6 min, decreased to 0%B, held for 2 min, and increased to 80% B followed by an equilibration step. For separation of ZEN conjugates, the following gradient was used: initially 90% B, decreased to 70% B within 35 min, decreased to 45% B within 10 min, decreased to 0% B within 5 min, held for 5 min, and increased to 90% B followed by an equilibration step. Detection was performed at 254 nm (AOH and AME conjugates) and 280 nm (ZEN conjugates). Stock solutions of each conjugate were prepared, and their concentrations were determined by quantitative $^1$H-NMR using coaxial tubes. Before analyzing sample concentrations, relative volumes of the coaxial tubes, and T1 times for each compound were determined. Compounds were dissolved in DMSO-$d_6$ and added to the outer part of coaxial tubes, coaxial inserts contained 150 µl of acetanilide (0.3 mg in DMSO-$d_6$) as internal standard. A standard Bruker $^1$H-NMR experiment (zg0) was performed using the following parameters: acquisition time 1.6 s, D1 30 s. Structure elucidation was performed by applying various 2D NMR experiments (Hildebrand et al., 2015). Data were interpreted individually and, where possible, compared to literature data (Borzekowski et al., 2018).

AOH-3-*O*-sulfate (AOH3S), AOH-9-*O*-sulfate (AOH9S), and AME-3-*O*-sulfate (AME3S) were available as reference compounds from earlier studies with cultured *Alternaria alternata* strains BFE1346 and BFE1348 (Soukup et al., 2016).

## 2.2 | Caco-2 cell culture and subculture

Caco-2 cells were from Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany; DSMZ No. ACC169). Cells were cultured at 37°C and 5% $CO_2$ in plates with 15 cm diameter containing 20 ml of medium and initially 1–5 x $10^6$ cells. The culture medium consisted of DMEM/F12 with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin and was replaced every 2–3 days. After 7 days, the medium was removed and attached cells were washed with 10 ml of phosphate-buffered saline (PBS), followed by treatment with 2.5 ml of Accutase® solution for 30 s. After removal of the Accutase® solution, cells were incubated at 37°C for 3 min and subsequently suspended in 20 ml of medium. Cell number was determined with a Casy® TTC Cell Counter and Analyser System (Roche).

## 2.3 | Stability assay

Stock solutions of AOH, AME, ZEN, AOH3Glc, AOH9Glc, AOH9DiGlc, AME3Glc, AME7Glc, ZEN14Glc, and ZEN16Glc were prepared in DMSO (approximately 2 mM each, depending on the amounts available). These solutions were further diluted in Hank´s buffered salt solution (HBSS, adjusted to pH 7.5) resulting in mycotoxin concentrations of 9–21 µM containing 1% DMSO (Figure 1). For the stability

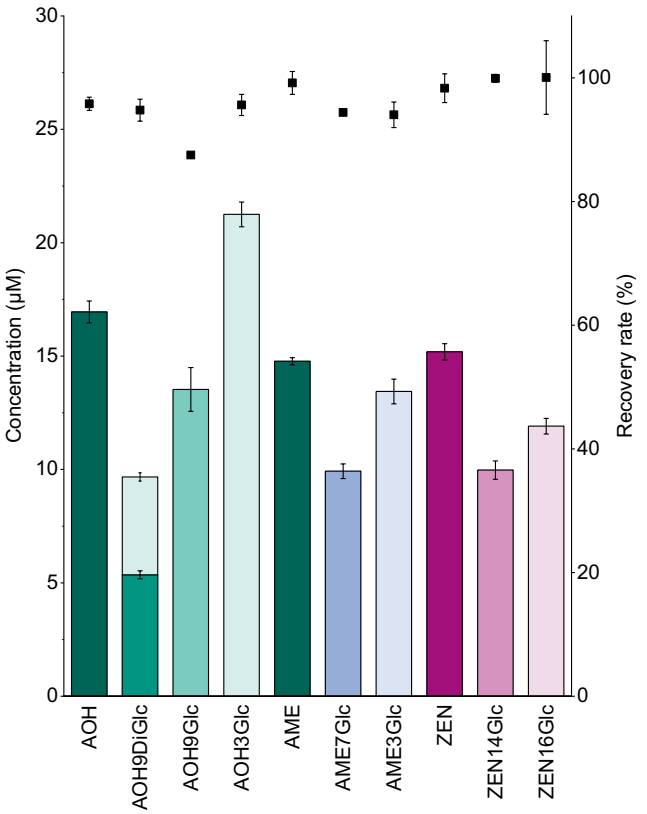


**FIGURE 1** Concentrations of the stock solutions of the free and modified mycotoxins measured by LC-DAD (bar chart). AOH9DiGlc is composed of 55.4% of AOH9DiGlc and 44.6% of AOH3Glc. The dot plot shows the recovery of the respective substances after 3 hr of incubation at 37°C. DiGlc, diglucoside; Glc, glucoside

assay, the solutions were incubated at 37°C for 3 hr in 24-well plates and subsequently frozen. As a control, solutions were frozen immediately after their production. Samples were freeze-dried and redissolved in 100 µl of MeOH for LC-DAD-MS analyses.

## 2.4 | Metabolism of the modified mycotoxins in Caco-2 cells

Metabolism studies of the modified mycotoxins in differentiated Caco-2 cells were performed in 24-well plates (Corning® Costar®, 1.9 $cm^2$ surface area). Cells were seeded ($4 \times 10^5$ per well) and grown for 21 days with renewal of the medium every 2–3 days. On day 21, medium was removed and 600 µl of mycotoxin solution (20 µM in HBSS, 1% DMSO) was added. After 3 hr of incubation, the incubation solution of each well was removed, frozen at −20°C, freeze-dried, and redissolved in 100 µl of MeOH for LC-DAD-MS analysis.

Attached cells of each well were washed twice with 600 µl of PBS, scraped off with a plastic spatula, suspended in 500 µl of purified water, and transferred into a 1.5-ml Eppendorf tube. The cells were lysed at −80°C for 24 hr and subsequently freeze-dried. For the first extraction of cells, 500 µl of MeOH/$CH_2Cl_2$ (2:1, v/v) was used, whereas 500 µl of MeOH/$H_2O$/acetic acid (79/20/1, v/v/v) was used for the second extraction, followed by a third extraction with 500 µl of MeOH/$CH_2Cl_2$ (2:1, v/v). During extraction, samples were sonicated in an ultrasonic bath for 5 min each. After centrifugation (3 min at 3,000 *g*), supernatants were combined and evaporated to dryness under a stream of nitrogen. Residues were redissolved in 100 µl of MeOH and analyzed by LC-DAD-MS. The metabolism studies described here were performed in duplicate; variability has been indicated by using range/2.

## 2.5 | Caco-2 Millicell® system: in vitro absorption of modified mycotoxins

For absorption experiments (performed in triplicate; precision indicated by using the standard deviation), 100 µl of medium containing $6 \times 10^5$ Caco-2 cells was filled in the apical compartment of inserts with semipermeable filters (Corning®, 0.33 $cm^2$ surface area, 0.4 µm pore size), whereas each well contained 600 µl of medium without cells in the basolateral compartment. Cells were grown into a differentiated monolayer for 21 days, with renewal of the medium in both compartments every 2–3 days. Then, the medium was removed in both compartments, and 100 µl of HBSS containing 20 µM of modified mycotoxin (with 1% DMSO) was filled into the apical compartment, whereas 600 µl of HBSS was added to the basolateral compartment. After 1 and 3 hr of incubation at 37°C, 90 µl of the apical compartment and 500 µl of the basolateral compartment were transferred to 1.5 and 2 ml Eppendorf tube, respectively. The solutions were freeze-dried and redissolved in 100 µl of MeOH for LC-DAD-MS analyses.

## 2.6 | Integrity of cell monolayers

In order to verify the integrity of the cell monolayer, both compartments were washed twice with HBSS directly after the absorption experiment, and 60 µl of Lucifer Yellow solution (100 µg/ml in HBSS) was added to the apical compartment, whereas the basolateral compartment contained dye-free HBSS. After an incubation period of 1 hr at 37°C, 200 µl of the basolateral solution was removed twice for duplicate fluorimetrical Lucifer Yellow analysis (excitation at 485 nm and emission at 535 nm). Only cell layers with less than 2% transfer of the dye from the apical to the basolateral side were considered intact (Debebe et al., 2012).

## 2.7 | LC-DAD-MS analysis

An LXQ Linear Ion Trap $MS^n$ system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Finnigan Surveyor HPLC-DAD system was used for LC-DAD-MS analysis. Separation was carried out on a reversed phase column (Phenomenex Luna, C18(2), 5 µm, 100 Å, 250 × 4.6 mm i.d.). Solvent A was $H_2O$ containing 5 mM ammonium acetate, and solvent B was ACN with 0.1% formic acid, and the flow rate was 0.5 ml/min. To analyze AOH, AME, and their derivatives, the linear gradient started with 20% B, was held for 5 min, was ramped to 50% B in 10 min, was ramped to 70% B in 9 min, reached 100% B within 5 min, was held for 2 min, and was decreased to 20% B in 1 min, and the system was equilibrated for 3 min before the next sample was injected. To analyze ZEN and its derivatives, the linear gradient started with 10% B, was ramped to 31% in 23 min, was ramped to 55% B in 10 min, was ramped to 100% B in 4 min, was held for 8 min, and was decreased to 10% B in 2 min, and the system was equilibrated for 5 min before the next sample was injected. The detection wavelength was 254 nm for AOH, AME, and their conjugates/metabolites, and 280 nm for ZEN and its derivatives/metabolites. UV absorbance at these wavelengths was used for quantification using external calibration curves of the parental mycotoxins (lowest calibration point was set as limit of quantification: AOH/AME = 1 µM, ZEN = 3 µM), assuming the same molar coefficient for the free mycotoxins and their conjugated metabolites. Because the absorption properties of the free mycotoxins, the modified mycotoxins (plant based), and the mycotoxin metabolites

(Caco-2 cell based) may more or less strongly deviate, this approach is semiquantitative only, depending on the application. This needs to be considered when, for example, recoveries are assessed for experiments, in which (modified) mycotoxins are further metabolized.

The mass spectrometer was operated in the negative electrospray ionization (ESI) mode for all mycotoxins. For the analyses of ZEN and its derivatives/metabolites, nitrogen was used as sheath gas, auxiliary gas, and sweep gas with flow rates of 26.0, 15.0, and 0.02 L/min, respectively. Spray voltage was 4.00 kV, spray current 3.15 μA, capillary voltage 45.10 V, capillary temperature 350°C, and the tube lens voltage was 125.59 V. To analyze AOH, AME, and their conjugates/metabolites, nitrogen was used as sheath gas, auxiliary gas, and sweep gas with flow rates of 9.0, 29.0, and 0.02 L/min, respectively. Spray voltage was 4.00 kV, spray current 3.15 μA, capillary voltage 45.10 V, capillary temperature 350°C, and the tube lens voltage was 125.49 V. Full scan mass spectra for all mycotoxins were recorded from *m/z* 100 to 800 in order to confirm the identity of the compounds by determining their $[M - H]^-$ ions. A signal to noise ratio (S/N) of 3 was used to decide whether a compound was detectable ($S/N \geq 3$) or not ($S/N < 3$).

### 2.8 | Calculation of $P_{app}$

$P_{app}$ values were calculated according to Artursson and Karlsson (1991) using the formula.

$$P_{app}(\text{cm s}^{-1}) = \frac{Q}{t\,A\,c_{api}}$$

where $Q$ is the amount of substance (μmol) in the basolateral compartment (either parental compound or the sum of the parental compound and metabolites), $t$ the incubation time (s), A the surface area of the monolayer (0.33 $cm^2$), and $c_{api}$ the initial concentration (mmol/L) of the mycotoxin in the apical compartment.

## 3 | RESULTS

### 3.1 | Stability assay of the modified mycotoxins

Because reference substances of glucosides of AOH, AME, and ZEN were not commercially available, small amounts of these mycotoxins were isolated of plant cell extracts (Hildebrand et al., 2015). Modified mycotoxins and their aglycones AOH, AME, and ZEN were first completely solubilized in DMSO, further diluted in HBSS solution (mycotoxin concentrations 9–21 μM, 1% DMSO), and incubated at conditions used during metabolism and adsorption studies. Free mycotoxins and their glucosides showed recovery rates of 88%–100% after 3 hr of incubation at 37°C (Figure 1). Thus, they are sufficiently stable for metabolism and absorption experiments. Minor losses may be due to reduced solubility of some compounds in HBSS, resulting in incompletely dissolved compounds that are available for further experiments. However, it should be considered that undissolved mycotoxins or modified mycotoxins can be redissolved during the Caco-2 cell experiments if some of the dissolved compound has been metabolized. This may result in slight over- or underestimations.

Furthermore, it was found that the solution of AOH9DiGlc was contaminated with AOH3Glc. The solution was composed of 55.4% AOH9DiGlc and 44.6% AOH3Glc, which was considered in the following experiments.

### 3.2 | Uptake and metabolism in Caco-2 cells

To investigate the metabolism of modified forms of AOH, AME, and ZEN, Caco-2 cell monolayers were grown on 24 well plates and treated with modified mycotoxins in solution (in HBSS, 1% DMSO) at 37°C for 3 hr. As positive control, cells were incubated with free mycotoxins, whereas incubations with HBSS containing 1% DMSO served as negative control. Supernatants and cell lysates were analyzed for free and modified mycotoxins as well as for metabolites formed by Caco-2 cells.

#### 3.2.1 | Free mycotoxins AOH, AME, and ZEN

The HPLC-DAD chromatograms of supernatants and cell lysates resulting from incubations with unconjugated mycotoxins are shown in Figure 2. AOH, AME, and ZEN were taken up into the cells. Several metabolites of the free mycotoxins were identified by LC-MS analyses mainly in the supernatant, which is in accordance with earlier findings (Burkhardt et al., 2009; Pfeiffer et al., 2011). Metabolites were characterized as monosulfates and glucuronides of AOH, AME, and ZEN, and as reductive metabolites of ZEN by their $[M–H]^-$ quasi molecular ions in the negative mode ESI mass spectra and by the characteristic release of the parental mycotoxin in $MS^2$ spectra (Table 1). The distribution of (semi-)quantifiable metabolites in both the supernatant and in the cells is shown in Figures 3–5.

Assignment of the linkage position of the metabolites and of the stereoisomers of the reductive derivatives of ZEN were either made tentatively based on their chromatographic properties and by comparison with literature data (Burkhardt et al., 2009; Pfeiffer et al., 2011) or in case of AOH and AME sulfates by comparison with reference compounds. However, NMR data are required for unambiguous determination of

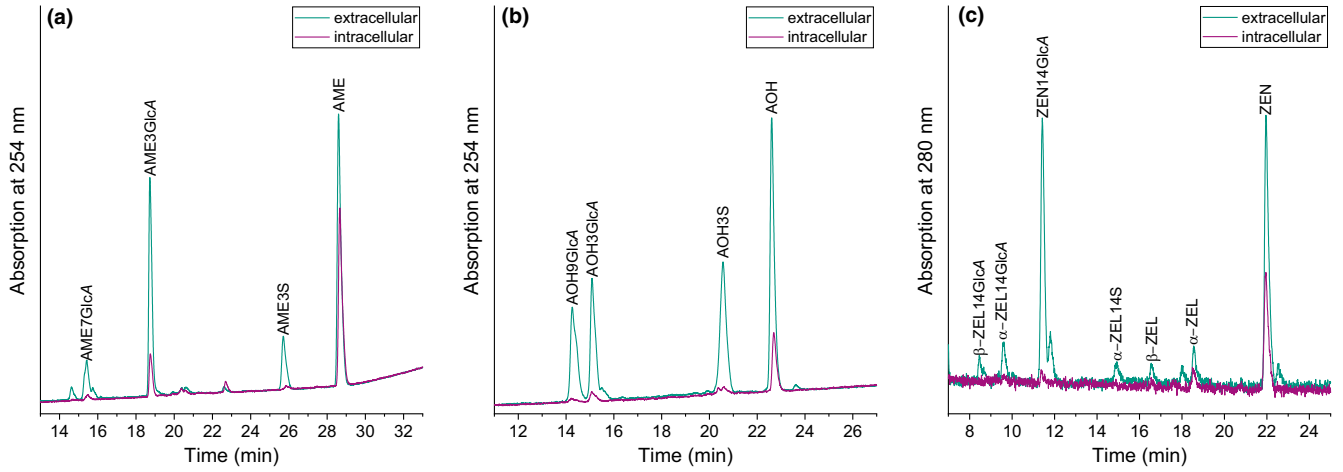


**FIGURE 2** HPLC-profiles of the extracts of the culture media and the cell extracts of Caco-2 cells incubated with AOH (a), AME (b), and ZEN (c) for 3 hr. Identification of metabolites was done by LC-MS analyses. Structure characterization has been tentative based on literature data (Burkhardt et al., 2009; Pfeiffer et al., 2011). To identify AOH and AME sulfates, reference compounds were available

**TABLE 1** Characterization of monoglucuronides (GlcA) and monosulfates (S) of AOH, AME, ZEN and, reductive metabolites of ZEN by LC-MS (RT = retention time in LC-MS)

| | $MS^2$ of $[M–H]^-$ | | | | $MS^2$ of $[M–H]^-$ | | | | $MS^2$ of $[M–H]^-$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mycotoxin** | **RT** | ***m/z* $[M–H]^-$** | **Glucuronide** | **RT (min)** | ***m/z* $[M–H]^-$** | ***m/z* $[M–H–GlcA]^-$** | **Sulfate** | **RT (min)** | ***m/z* $[M–H]^-$** | ***m/z* $[M–H–S]^-$** |
| AOH | 22.6 | 257 | 3-*O*-Glc*A* | 15.1 | 433 | 257 | 3-*O*-S | 20.6 | 337 | 257 |
| | | | 9-*O*-Glc*A* | 14.3 | 433 | 257 | | | | |
| AME | 28.6 | 271 | 3-*O*-Glc*A* | 18.7 | 447 | 271 | 3-*O*-S | 25.7 | 351 | 271 |
| | | | 7-*O*-Glc*A* | 15.4 | 447 | 271 | | | | |
| ZEN | 22.0 | 317 | 14-*O*-Glc*A* | 11.4 | 493 | 317 | | | | |
| α-ZEL | 18.6 | 319 | 14-*O*-Glc*A* | 9.6 | 495 | 319 | 14-*O*-S | 14.9 | 399 | 319 |
| β-ZEL | 16.5 | 319 | 14-*O*-Glc*A* | 8.5 | 495 | 319 | | | | |

the linkage position of the glucuronides. Because most of the metabolites were found in the supernatant (Figures 3–5), it can be suggested that the free mycotoxins were absorbed, metabolized, and subsequently transported to the extracellular space. This is consistent with results published by Burkhardt et al. (2009). The authors also found larger amounts of metabolites in the supernatant, whereas mainly the nonmetabolized toxins were present in the cells.

To assess the data from a quantitative point of view, it has to be noted that the sum of the recovered AOH, AME, ZEN, and their metabolites were 39.1 ± 1.1%, 50.5 ± 5.9%, and 38.6 ± 0.9%, respectively. These comparably low recovery rates may be due to an incomplete cell extraction, the poor solubility of the mycotoxins or analytical limitations based on quantitation at 254 nm (AOH, AME, and their metabolites) and 280 nm (ZEN and its metabolites) assuming a similar extinction coefficient of the metabolites and their parental mycotoxin. Additionally, formation of unknown metabolites is possible, but they are not expected to largely affect recovery rates. However, from a qualitative point of view, the metabolism of the free mycotoxins was comparable to previous publications.

### 3.2.2 | Modified forms of AOH, AME, and ZEN

*AOH conjugates*

Incubation of Caco-2 cells with AOH9Glc resulted in a similar metabolite distribution compared to the metabolite pattern of unmodified AOH (Figure 3). After 3 hr of incubation, AOH9Glc from the supernatant still represented 45% of the recovered compounds. In general, most of the metabolites were found in the supernatant, too, including AOH-3-glucuronide (AOH3Glc*A*) (15% of the recovered compounds), AOH-9-glucuronide (AOH9Glc*A*) (11% of the recovered compounds), AOH3S (14% of the recovered compounds), and unconjugated AOH (8% of the recovered compounds). In

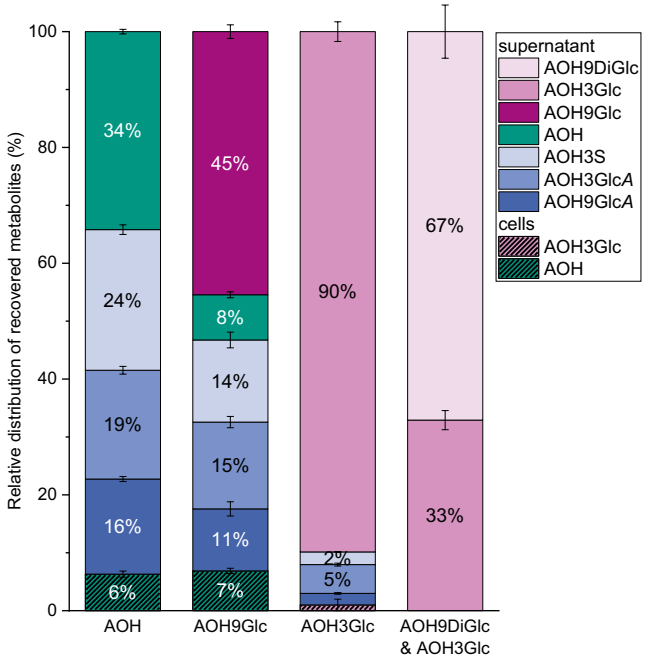


**FIGURE 3** Relative distribution of quantifiable compounds in the supernatant (filled bars) and in the extracted Caco-2 cells (shaded bars) after incubation of the cells with AOH, AOH9Glc, AOH3Glc, and AOH9DiGlc in 24-well plates for 3 hr ($n = 2$; range/2). It has to be mentioned that recoveries of AOH, AOH9Glc, AOH3Glc, and AOH9DiGlc including their identified metabolites were 39.1 ± 1.1%, 65.3 ± 6.3%, 71.4 ± 1.9%, and 85.9 ± 5.4%

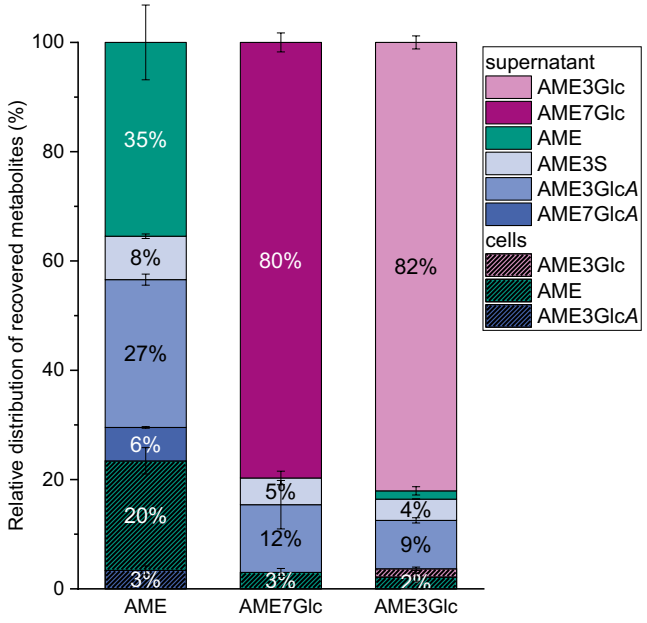


**FIGURE 4** Relative distribution of quantifiable compounds in the supernatant (filled bars) and in the extracted Caco-2 cells (shaded bars) after incubation of the cells in the presence of AME, AME3Glc, and AME7Glc in 24-well plates for 3 hr ($n = 2$; range/2). It has to be mentioned that the recoveries of AME, AME3Glc, and AME7Glc including their identified metabolites were 50.5 ± 5.9%, 92.6 ± 7.5%, and 109.7 ± 3.9%

the cells, AOH was present in quantifiable amounts besides traces of the two AOH-glucuronides and AOH9Glc. Thus, AOH9Glc was absorbed from the Caco-2 cells, deglucosylated to AOH, and further (partially) metabolized followed by an excretion of metabolites and AOH. This observation may support the hypothesis that modified mycotoxins contribute to overall toxicity of mycotoxins. However, it should be noted again that the sum of the recovered compounds was only 65.3 ± 6.3% of the initial amount of AOH9Glc.

Incubation of Caco-2 cells with AOH3Glc resulted in a different metabolite distribution compared to incubation with AOH9Glc. Again, most of the metabolites were identified in the supernatant. The plant metabolite AOH3Glc (in the supernatant) made up 90% of the total sum of the recovered metabolites. In addition, AOH3Glc*A* (5% of the recovered compounds), AOH9Glc*A* (2% of the recovered compounds), and AOH3S (2% of the recovered compounds) were (semi-) quantitated in the supernatant. Free AOH was detected in traces both in the supernatant and in the cells. In addition, small amounts of AOH3Glc were quantified in the cells together with traces of AOH3Glc*A* and AOH9Glc*A*. The sum of all quantifiable metabolites was 71.4 ± 1.9% of the initial amount of AOH3Glc. These data suggest that AOH3Glc is also absorbed, deglucosylated, and further metabolized, but to a considerably lesser extent than AOH9Glc. It can thus be assumed that the position of the glucosylation has an impact on the absorption and metabolism of modified mycotoxins in Caco-2 cells.

Metabolism studies with the mixture of AOH9DiGlc and AOH3Glc pointed out that no significant absorption, deglucosylation and/or metabolization occurred in Caco-2 cells. Only traces of AOH9DiGlc (and AOH3Glc) were detected in the cells, indicating a minor absorption of AOH9DiGlc (and AOH3Glc). Therefore, it can be suggested that mycotoxins carrying a disaccharide group are absorbed less efficiently than mycotoxins carrying a monosaccharide group. However, traces of AOH9Glc were found in the supernatant indicating a partial cleavage of AOH9DiGlc. Because AOH3Glc remained mainly intact in the supernatant in the individual experiment, this is consistent with the results of the combined experiment with AOH9DiGlc. The total recovery was 85.9 ± 5.4% of the initial applied compounds.

### *AME conjugates*

Figure 4 provides an overview of the metabolism of AME7Glc and AME3Glc in Caco-2 cells compared to the metabolism of free AME. After incubation of the Caco-2 cells with AME7Glc for 3 hr, mostly AME7Glc was quantitated in the supernatant (80% of all recovered compounds), followed by AME-3-glucuronide (AME3Glc*A*) (12% of the recovered compounds) and AME3S (5% of the recovered compounds), whereas the

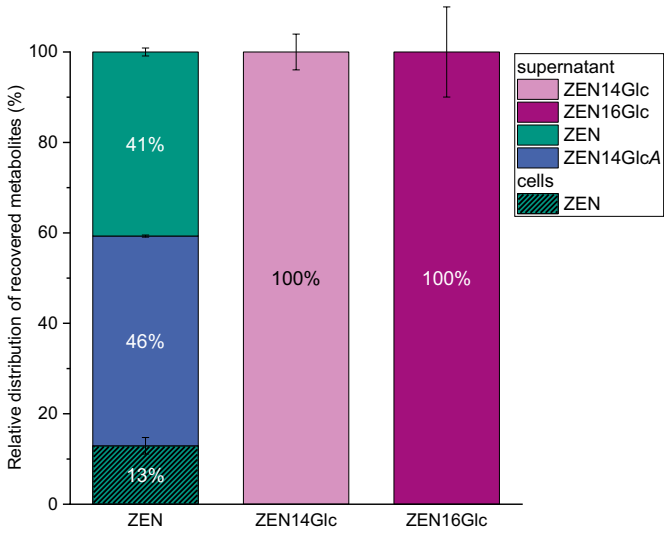


**FIGURE 5** Relative distribution of quantifiable compounds in the supernatant (filled bars) and in the extracted Caco-2 cells (shaded bars) after incubation of the cells in the presence of ZEN, ZEN14Glc, and ZEN16Glc in 24-well plates for 3 hr ($n = 2$; range/2). It has to be mentioned that the recoveries of ZEN, ZEN14Glc, and ZEN16Glc including their identified metabolites were 57.1 ± 1.7%, 117.2 ± 4.6%, and 33.3 ± 3.3%

aglycone AME was only detected in traces. Differently, however, AME was present in quantifiable amounts in the cells. In addition, AME7Glc, AME3Glc*A*, and AME3S were detected. The sum of all quantifiable metabolites was 92.6 ± 7.5% of the initially applied amount of AME7Glc.

A comparable distribution of metabolites was obtained after incubation of Caco-2 cells with AME3Glc. The majority (82%) of the recovered compounds was in form of the initial modified mycotoxin in the medium. Additionally, AME3Glc*A* (9% of the recovered compounds), AME3S (4% of the recovered compounds), and AME (2% of the recovered compounds) were quantified in the supernatant. AME3Glc, its aglycone AME, and traces of AME3Glc*A* were identified intracellularly, indicating the absorption, deglucosylation, and metabolization of AME3Glc in Caco-2 cells. The recovery of all compounds in the supernatant and in the cells was 109.7 ± 3.9% of the initially used amount of AME3Glc.

*ZEN conjugates*

In contrast to AOH- and AME-glucosides, the parental mycotoxin ZEN or ZEN metabolites were not detected in quantifiable amounts after 3 hr of incubation of ZEN-glucosides, neither in the cells nor in the medium (Figure 5). Nevertheless, traces of ZEN-14-glucuronide (ZEN14Glc*A*), ZEN-14-sulfate (ZEN14S), and α-ZEL-14-sulfate (α-ZEL14S) were detected after incubation of Caco-2 cells with ZEN14Glc, whereas only one sample of duplicate experiments with ZEN16Glc showed traces of ZEN14Glc*A*. However, it has to be mentioned that the overall recovery in the experiment with ZEN14Glc was 117.2 ± 4.6%, whereas recovery after incubation of ZEN16Glc was only 33.3 ± 3.3% of the initially applied ZEN glucoside. Therefore, the results of the experiment using ZEN16Glc have to be interpreted with caution. However, it appears reasonable to assume that the two glucosides of ZEN were absorbed, cleaved, and metabolized by Caco-2 cells to a lesser extent compared to AOH- and AME-glucosides.

## 3.3 | Absorption and metabolism in the Caco-2 Millicell® system

The Caco-2 Millicell® system is a well-established in vitro model to study the transition of compounds from the apical site to the basolateral site. Caco-2 cells were cultured in transwell inserts on semipermeable membranes, and the apical region was incubated for 1 or 3 hr at 37°C with the free mycotoxins AOH, AME, and ZEN (as control experiments) and with their glucosylated forms. After incubation, both the apical and the basolateral medium were removed and analyzed by LC-DAD-MS. The concentrations of AOH, AME, ZEN, and their Caco-2 metabolites (glucuronides and sulfates) were semiquantitatively determined using external calibrations calculated with the free mycotoxins. The integrity of the cell monolayer was tested as described above and evaluated by using previously described criteria (Debebe et al., 2012).

### 3.3.1 | Free mycotoxins AOH, AME, and ZEN

For comparison and to better evaluate the results of the modified mycotoxins, the absorption of the free mycotoxins AOH, AME, and ZEN was analyzed, too. The results of 1 and 3 hr incubations with AOH, AME, and ZEN (Table 2) are qualitatively comparable to previously published data (Burkhardt et al., 2009; Pfeiffer et al., 2011).

*AOH*

After 1 hr of incubation with AOH, the Caco-2 metabolite AOH3S and unconjugated AOH were detected in the apical compartment, whereas no metabolites or free AOH were identified in the basolateral compartment. However, total recovery was only 22%, which may be due to the fact that intracellular concentration was not measured in this assay. After incubation for 3 hr, AOH9Glc*A*, AOH3Glc*A*, and AOH3S were determined in the apical compartment. Traces of the two AOH-glucuronides, AOH3S, and parental AOH were detected in the basolateral compartment. The overall recovery was 35%, which is approximately equivalent to the recovery rate in the supernatant in the metabolism experiment. The results indicate a time-dependent absorption, metabolization,

**TABLE 2** Results of the absorption studies (mean ± standard deviation; $n = 3$) with the Millicell® system after incubation of the Caco-2 cells in the presence of AOH, AME, and ZEN

| Substance | Applied amount (nmol) | *t* | Compartment | Amount (nmol) | | | | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| | | | | **AOH** | **AOH9Glc*A*** | **AOH3Glc*A*** | **AOH3S** | |
| AOH | 1.69 | 1 hr | Apical | 0.23 ± 0.04 | nd | nd | 0.15 ± 0.04 | 22 |
| | | | Basolateral | nd | nd | nd | nd | |
| | | 3 hr | Apical | nd | 0.20 ± 0.06 | 0.22 ± 0.06 | 0.17 ± 0.05 | 35 |
| | | | Basolateral | nq | nq | nq | nq | |
| | | | | **AME** | **AME7Glc*A*** | **AME3Glc*A*** | **AME3S** | |
| AME | 1.48 | 1 hr | Apical | 0.22 ± 0.04 | nd | nq | nq | 15 |
| | | | Basolateral | nd | nd | nq | nd | |
| | | 3 hr | Apical | nq | nq | 0.39 ± 0.09 | 0.20 ± 0.03 | 40 |
| | | | Basolateral | nd | nq | nq | nd | |
| | | | | **ZEN** | **ZEN14Glc*A*** | **ZEN14S** | **α-ZEL14S** | |
| ZEN | 1.52 | 1 hr | Apical | nq | nq | nq | nq | 0[a] |
| | | | Basolateral | nq | nq | nq | nd | |
| | | 3 hr | Apical | nq | nq | nq | nq | 0 [a] |
| | | | Basolateral | nq | nq | nq | nq | |

Abbreviations: *t*, incubation time; DiGlc, diglucoside; Glc, glucoside; Glc*A*, glucuronide; S, sulfate; nd, not detectable; nq, detected, but not quantifiable

[a]Calculation of recovery rates was not possible due to nonquantifiable amounts of identified compounds.

and excretion (to the apical and basolateral compartment) of AOH/AOH metabolites by Caco-2 cells.

*AME*

Incubation of Caco-2 cells for 1 hr with AME resulted in a very low total recovery of 15% (including compounds in the apical and basolateral compartment), and only AME was quantified in the apical compartment. In addition, traces of AME3Glc*A* and AME3S were detected in the apical compartment. Only traces of AME3Glc*A* were detected basolaterally. With increasing incubation time, the concentration of AME in the apical compartment decreased to nonquantifiable amounts, accompanied by an increase of AME3Glc*A* and AME3S concentrations, and the detection of AME7Glc*A*. Additionally, it was possible to detect AME7Glc*A* (next to AME3*GlcA*) in the basolateral compartment, suggesting a time-dependent resorption of AME in Caco-2 cells, too. Overall recovery (including compounds in the apical and basolateral compartment) in the 3 hr experiment was 40% of the initially applied AME amount.

*ZEN*

The absorption studies with ZEN (both after 1 and 3 hr of incubation) resulted in detectable, but not in quantifiable amounts of ZEN and its main metabolites ZEN14Glc*A*, ZEN14S, and α-ZEL14S. All of them were present in apical and basolateral compartments, with the exception of α-ZEL14S, which was not detected in the basolateral compartment after 1 hr of incubation. The amount of initially used ZEN was "distributed" over several newly formed metabolites and quantitation was not possible (and therefore the determination of a recovery rate; artificially set to 0% in Table 2). Nevertheless, it can be assumed that small amounts of ZEN are absorbed by Caco-2 cells, metabolized, and transported to the basolateral compartment.

### 3.3.2 | Modified forms of AOH, AME, and ZEN

Results of the in vitro absorption studies of the modified mycotoxins in the Caco-2 Millicell® system are summarized in Table 3. The overall recoveries in the various experiments were between 30% and 77%. Low recoveries may result from intracellular localization of metabolites and/or from analytical limitations (see Section 2.7). However, as results of absorption studies with unconjugated mycotoxins are qualitatively comparable to literature data, the test system was considered appropriate.

*AOH glucosides and AOH diglucoside*

Absorption studies in Caco-2 cells revealed that AOH9Glc was absorbed and deglucosylated by Caco-2 cells resulting in the detection of AOH in the basolateral compartment after

both 1 and 3 hr of incubation. Unconjugated AOH was present in a quantifiable amount after 1 hr of incubation in the apical compartment, too. The only metabolite detected in the apical compartment after 1 hr of incubation was AOH3S. Longer incubation time resulted in further metabolization of AOH to AOH9Glc*A* and AOH3Glc*A*, which were identified in both compartments. In contrast, incubations with AOH3Glc for 1 and 3 hr resulted in the formation of AOH and AOH metabolites present only in the apical compartment, with the exception of AOH3Glc*A* that was detected in the basolateral compartment after 3 hr of incubation, too.

The results show that monoglucosylated AOH is absorbed time-dependently by Caco-2 cells, deglucosylated to AOH, and further metabolized to glucuronides and sulfates, which are also excreted to the basolateral site, indicating a potential intestinal absorption of monoglucosylated AOH with AOH conjugates reaching the portal blood.

Incubation of Caco-2 cells for 1 hr with a mixture of AOH9DiGlc and AOH3Glc did not result in a transition of compounds into the basolateral compartment, but small amounts of AOH and AOH3S were detected in the apical compartment. Longer incubation time induced the additional formation of AOH-glucuronides, which were both detected in the apical and basolateral compartment. Because the incubation solution was composed of AOH9DiGlc and AOH3Glc, it cannot be concluded that AOH9DiGlc contributed to these findings. However, metabolism studies using AOH9DiGlc showed the formation of AOH9Glc that indicates a hydrolysis of the diglucoside suggesting at least a (minor) contribution of AOH9DiGlc to the presence of AOH metabolites in the basolateral compartment.

*AME-glucosides*

Besides modified forms of AOH, glucosides of AME were studied for their potential epithelial transport. After 1 hr of incubation with AME7Glc, 51% of the initial compound was determined in the apical compartment, next to traces of AME, AME3Glc*A*, and AME3S. However, AME and AME metabolites were not detected in the basolateral compartment. Incubation for 3 hr resulted in the identification of AME3Glc*A* and AME3S in the basolateral compartment, too. Similarly, the plant metabolite AME3Glc did not cross the Caco-2 monolayer after 1 hr of incubation, whereas AME3Glc*A* was detected in the basolateral compartment after 3 hr of incubation. The results suggest a time-dependent absorption, deglucosylation, and metabolization of the two AME-glucosides, and excretion of metabolites formed by the Caco-2 cells to the basolateral site.

*ZEN-glucosides*

Absorption studies using glucosylated ZEN showed that ZEN14Glc was absorbed, cleaved, and metabolized to ZEN14S and α-ZEL14S after 1 hr of incubation, which were both detected in the apical compartment. Additionally, traces of ZEN and ZEN14Glc*A* were found in the apical compartment after 3 hr of incubation, but neither unconjugated ZEN nor ZEN metabolites were detected on the basolateral site. The initial compounds were recovered to 62 and 70% in the apical compartment. In comparison, ZEN16Glc was recovered to 75 and 71% of the initial amount in the apical compartment after 1 and 3 hr of incubation, respectively, but no metabolites were detected either in the apical or in the basolateral compartment. In accordance with the metabolism experiments, no significant release of ZEN or formation of ZEN metabolites occurred after incubation of Caco-2 cells for 3 hr with ZEN14Glc and ZEN16Glc. Both conjugates seem to be absorbed by Caco-2 cells to a minor extent only.

### 3.4 | Apparent permeability coefficient $P_{app}$ of modified AOH, AME, and ZEN

$P_{app}$ values express the transition of a compound from the apical to the basolateral compartment in the Caco-2 Millicell® system. The correlation between $P_{app}$ values from Caco-2 studies and the absorption rate after oral uptake in humans has been investigated on numerous substances with different structural properties (Artursson & Karlsson, 1991; Press & Di Grandi, 2008; Yee, 1997). $P_{app}$ values below $1 \times 10^{-6}$ cm/s implicate a poor absorption (0%–20%), whereas $P_{app}$ values between $1–10 \times 10^{-6}$ cm/s indicate a moderate absorption (20%–70%), and $P_{app}$ values over $10 \times 10^{-6}$ cm/s a well absorption (70%–100%) of the corresponding compound (Yee, 1997). In this study, it was only possible to calculate $P_{app}$ values for AOH3Glc and AME7Glc. $P_{app}$ values of other modified mycotoxins could not be calculated because quantitation of metabolites in the basolateral compartment was not possible. The calculated $P_{app}$ values in this study were $2.1 \times 10^{-6}$ cm/s for AOH3Glc and $1.7 \times 10^{-6}$ cm/s for AME7Glc. Therefore, the absorption of the two glucosylated *Alternaria* toxins is assumed to be moderately, and an intake of these modified mycotoxins should not be underestimated. However, it has to be taken into account that calculation of $P_{app}$ values does not consider compounds that were detected in nonquantifiable amounts.

## 4 | DISCUSSION

Data gathered here provide insights into the bioavailability of modified mycotoxins because predictions about the epithelial transition can be made. Metabolism studies with glucosidic

**TABLE 3** Results of the absorption studies (mean ± standard deviation; $n = 3$) with the Millicell® system after incubation of the Caco-2 cells in the presence of modified forms of AOH, AME, and ZEN

| | Applied amount (nmol) | *t* | Compartment | Amount (nmol) | | | | | Recovery (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Initial compound | AOH | AOH9Glc*A* | AOH3Glc*A* | AOH3S | |
| AOH9Glc | 1.35 | 1 hr | Apical | 0.24 ± 0.06 | 0.16 ± 0.04 | nd | nd | nq | 30 |
| | | | Basolateral | nd | nq | nd | nd | nd | |
| | | 3 hr | Apical | nd | nq | 0.17 ± 0.04 | 0.21 ± 0.04 | 0.16 ± 0.06 | 40 |
| | | | Basolateral | nq | nq | nq | nq | nd | |
| AOH3Glc | 2.12 | 1 hr | Apical | 1.17 ± 0.14 | nq | nd | nd | 0.09 ± 0.03 | 59 |
| | | | Basolateral | nd | nd | nd | nd | nd | |
| | | 3 hr | Apical | 0.52 ± 0.17 | 0.05 ± 0.03 | 0.14 ± 0.05 | 0.16 ± 0.06 | 0.20 ± 0.07 | 57 |
| | | | Basolateral | nd | nd | nd | 0.16 ± 0.03[a] | nd | |
| AOH9DiGlc & AOH3Glc | 0.97 | 1 hr | Apical | 0.67 ± 0.13 | nq | nd | nd | nq | 70 |
| | | | Basolateral | nd | nd | nd | nd | nd | |
| | | 3 hr | Apical | 0.59 ± 0.07 | nq | nq | nq | nq | 61 |
| | | | Basolateral | nd | nd | nq | nq | nd | |
| | | | | **Initial compound** | **AME** | **AME7Glc*A*** | **AME3Glc*A*** | **AME3S** | |
| AME7Glc | 0.99 | 1 hr | Apical | 0.50 ± 0.04 | nq | nd | nq | nq | 51 |
| | | | Basolateral | nd | nd | nd | nd | nd | |
| | | 3 hr | Apical | 0.13 ± 0.04 | nd | nd | 0.26 ± 0.03 | 0.23 ± 0.02 | 70 |
| | | | Basolateral | nd | nd | nd | 0.08 ± 0.04 | nq[a] | |
| AME3Glc | 1.34 | 1 hr | Apical | 1.04 ± 0.05 | nq | nd | nq | nq | 77 |
| | | | Basolateral | nd | nd | nd | nd | nd | |
| | | 3 hr | Apical | 0.42 ± 0.09 | nq | nd | 0.23 ± 0.06 | 0.20 ± 0.03 | 63 |
| | | | Basolateral | nd | nd | nd | nq | nd | |
| | | | | **Initial compound** | **ZEN** | **ZEN14Glc*A*** | **ZEN14S** | **α-ZEL14S** | |
| ZEN14Glc | 1.00 | 1 hr | Apical | 0.62 ± 0.05 | nd | nd | nq | nq | 62 |
| | | | Basolateral | nd | nd | nd | nd | nd | |
| | | 3 hr | Apical | 0.70 ± 0.10 | nq | nq | nq | nq | 70 |
| | | | Basolateral | nd | nd | nd | nd | nd | |
| ZEN16Glc | 1.19 | 1 hr | Apical | 0.90 ± 0.10 | nd | nd | nd | nd | 75 |
| | | | Basolateral | nd | nd | nd | nd | nd | |
| | | 3 hr | Apical | 0.85 ± 0.05 | nd | nd | nd | nd | 71 |
| | | | Basolateral | nd | nd | nd | nd | nd | |

Abbreviations: *t*, incubation time; DiGlc, diglucoside; Glc, glucoside; Glc*A*, glucuronide; S, sulfate; nd, not detectable; nq, detected, but not quantifiable.

[a]Value from two experiments.

metabolites of AOH and AME showed that the monoglucosides were taken up by Caco-2 cells, deglucosylated, and further metabolized to glucuronides and sulfates with AOH9Glc being metabolized to a higher extent than glucosides of AOH or AME linked at positions 3 or 7. Although no metabolites were quantified after incubation of Caco-2 cells with AOH9DiGlc, the detection of AOH9Glc indicates a slight hydrolysis of the diglucoside.

Further absorption studies indicate a possible intestinal transition of glucosylated AOH and AME in form of their aglycones as well as glucuronides and sulfates to the portal blood. In particular, AOH, AME, and their Caco-2 metabolites were identified in the basolateral compartment after 1 and/or 3 hr of incubation. Metabolites identified in the basolateral compartment were equivalent to the metabolites identified after incubation of the Caco-2 Millicell® system with unconjugated mycotoxins.

For the free mycotoxins AOH and AME, $P_{app}$ values of $34.9 \times 10^{-6}$ cm/s and $10.3 \times 10^{-6}$ cm/s were described in literature (Burkhardt et al., 2009), but could not be confirmed in this study because free mycotoxins and their metabolites were present in the basolateral compartment in concentrations below the limit of quantitation. However, $P_{app}$ values of AOH3Glc ($2.1 \times 10^{-6}$ cm/s) and AME7Glc ($1.7 \times 10^{-6}$ cm/s) were calculated, which are lower compared to the $P_{app}$ values of their aglycones described in literature. Therefore, it can be suggested that absorption of both modified mycotoxins is lower as compared to unconjugated AOH and AME. However, our calculation of $P_{app}$ values has some limitations, for example low recovery rates and nonquantifiable amounts of compounds in the basolateral compartment. In addition, comparison of our values with literature data is limited because different cell culture conditions may result in (minor) deviations. Nevertheless, the two glucosylated *Alternaria* toxins appear to be absorbed and should therefore be taken into account in the risk assessment of food and feed (Yee, 1997).

Metabolism studies of ZEN14Glc and ZEN16Glc in Caco-2 cells showed that both glucosides were absorbed and metabolized only to a small extent. In contrast to the experiments with modified *Alternaria* toxins, neither detectable unconjugated ZEN nor detectable ZEN metabolites were transported to the basolateral site within the experimental time frame. Nevertheless, our results indicate that the bioavailability of ZEN-glucosides is considerably lower than the bioavailability of unconjugated ZEN. In literature, studies with plant-based phase II metabolites of ZEN are contradictory concerning the intestinal absorption. In accordance with our data, Gratz et al. (2017) demonstrated that ZEN14Glc, α-ZEL14Glc, and β-ZEL14Glc were almost fully recovered (91%–93%) from the apical compartment after 24 hr of incubation, and only traces of ZEN14Glc and α-ZEL14Glc were detected on the basolateral compartment after 6 and 24 hr. Therefore, they concluded that limited bioavailability of glucose bound zearalenone compounds can be assumed. Contrary, however, Cirlini et al. (2016) observed that ZEN14Glc and ZEN16Glc were absorbed by Caco-2 cells and deglucosylated to ZEN, which was further transported to the basolateral compartment. Therefore, they suggested a possible intestinal absorption of the two glucosylated forms of ZEN, although ZEN16Glc seemed to be absorbed and deglucosylated to a lesser degree than ZEN14Glc. Such variabilities in cell culture studies can be attributed to various factors such as different concentration levels, Caco-2 cell clones, cell culture conditions (passage number, medium, pH, usage of fetal bovine serum, integrity of the cells, DMSO concentration), and analytical methods (Press & Di Grandi, 2008). However, none of the two studies analyzed possible Caco-2 metabolites (glucuronides and sulfates). In the current study, sulfates and glucuronides of ZEN were identified, however, not in the basolateral compartment.

Besides the in vitro studies with modified ZEN, in vivo studies with pigs have been carried out, too, indicating that modified forms of ZEN were hydrolyzed during digestion, thereby contributing to the overall toxicity of ZEN (Binder et al., 2017; Catteuw et al., 2019; Gareis et al., 1990). Also, the behavior of modified ZEN in the presence of fecal batch cultures was studied, demonstrating a rapid hydrolysis of ZEN- and α/β-ZEL-glucosides by the human colonic microbiota (Dall'Erta et al., 2013; Gratz et al., 2017; Kovalsky Paris et al., 2014). These studies indicate that other deglucosylation routes need to be considered for the risk assessment of modified mycotoxins, too, especially for ZEN-glucosides, which were deglucosylated by Caco-2 cells only to a minor extent.

## 5 | CONCLUSION

It was shown for the first time that plant-based phase II metabolites of AOH and AME are absorbed and further metabolized by Caco-2 cells, which implicates a possible in vivo absorption in the intestine of humans or animals, resulting in a potential contribution of modified *Alternaria* toxins to overall toxicity. Therefore, they should be considered in further studies for risk assessment of modified mycotoxins. In contrast, our results show that ZEN-glucosides are absorbed and metabolized to a lesser extent by Caco-2 cells, suggesting a lower bioavailability compared to modified *Alternaria* toxins and unconjugated ZEN itself.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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