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Identification and Apoptotic Potential of T-2 Toxin Metabolites in Human Cells

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ABSTRACT: The mycotoxin T-2 toxin, produced by various *Fusarium* species, is a widespread contaminant of grain and grain products. Knowledge about its toxicity and metabolism in the human body is crucial for any risk assessment as T-2 toxin can be detected in processed and unprocessed food samples. Cell culture studies using cells of human origin represent a potent model system to study the metabolic fate of T-2 toxin as well as the cytotoxicity in vitro. In this study the metabolism of T-2 toxin was analyzed in a cell line derived from human colon carcinoma cells (HT-29) and primary human renal proximal tubule epithelial cells (RPTEC) using high-performance liquid chromatography coupled with Fourier transformation mass spectrometry (HPLC-FTMS). Both cell types metabolized T-2 toxin to a variety of compounds. Furthermore, cell cycle analysis in RPTEC proved the apoptotic effect of T-2 toxin and its metabolites HT-2 toxin and neosolaniol in micromolar concentrations.

KEYWORDS: *T-2 toxin, HT-2 toxin, mycotoxin, metabolism, FTMS, apoptosis, primary cells, renal proximal tubule epithelial cells (RPTEC), HT-29, cell cycle*

INTRODUCTION

Trichothecene-producing fungi of the genus *Fusarium* are known for causing both toxic effects to animals and humans as well as serious economic losses due to contaminated cereal grains (estimated economic loss of about U.S. $3 billion in the 1990s in the United States).[1] Trichothecenes are mycotoxins commonly found in cereal grains and their products mainly derived from contaminated maize, wheat, rye, barley, oats, and rice.[2−4] T-2 toxin belongs to the type A subgroup of trichothecenes and is produced by different *Fusarium* species (e.g. *Fusarium sporotrichioides*, *Fusarium poae*, and *Fusarium acuminatum*).[5] In general, trichothecenes are chemically characterized by their sesquiterpenoid ring and the 12,13-epoxytrichothecene skeleton. The epoxide group at position C-12,13 is essential for the toxicological activity,[4,6] and T-2 toxin is known to be the most potent toxin of the group of trichothecenes in animal studies.[4]

Human exposure to T-2 toxin via the ingestion pathway and the contamination of human food and animal feed is a continuing worldwide problem.[2] Recently, many studies of cereal grain samples from different countries in which T-2 toxin was detected have been published.[7−11] Another source of intake might be the possible presence of toxic residues in edible animal products because T-2 toxin has been detected in meat, liver, heart, and milk after application of T-2 toxin to a lactating cow.[12] The Scientific Committee on Food of the European Commission has established a temporary tolerable daily intake (t-TDI) for the sum of T-2 and HT-2 toxin of 0.06 μg/kg bw in 2002.[13] On the basis of the current evaluation the panel on contaminants in the food chain (CONTAM) of the European Food Safety Authority has published a full TDI for the sum of T-2 and HT-2 toxin of 100 ng/kg bw in 2011.[14] However, apart from some studies with human liver homogenates and cell culture experiments, not much information is known about the metabolism of T-2 toxin in humans. In studies with human skin samples HT-2 toxin and T-2 tetraol were detected as the major metabolites,[15] whereas incubations of human liver homogenates with T-2 toxin and cell culture experiments with human fibroblasts identified HT-2 toxin as the sole metabolite.[5,16] In human blood cells HT-2 toxin and neosolaniol were detected, and carboxylesterase activity was shown to be responsible for the hydrolysis of T-2 toxin to these two metabolites.[17] A study of T-2 toxin applied to human renal proximal tubule epithelial cells (RPTEC) and normal human lung fibroblasts (NHLF) revealed HT-2 toxin as the main metabolite besides small amounts of neosolaniol in RPTEC.[18] However, data on the metabolism of T-2 toxin in humans remain rare. With regard to the metabolism of T-2 toxin in animals, various in vivo as well as in vitro studies are described in the literature showing a thorough metabolism of T-2 toxin strongly depending on the analyzed species. After absorption, trichothecenes are not accumulated in high doses in specific organs but distributed in different tissues and organs due to their relatively high water solubility.[12,19] In vivo studies in animals showed that T-2 toxin and formed metabolites were rapidly excreted via urine and feces.[12,20−22] During metabolism, T-2 toxin is mainly subjected to phase I as well as phase II reactions, resulting in more polar substances. Deacetylation, hydrolysis, and hydroxylation are among the common metabolic reactions leading to a variety of metabolites (Figure 1).[3,12,19,21,22] Nevertheless, no information on the toxicokinetics of T-2 toxin in the human body is

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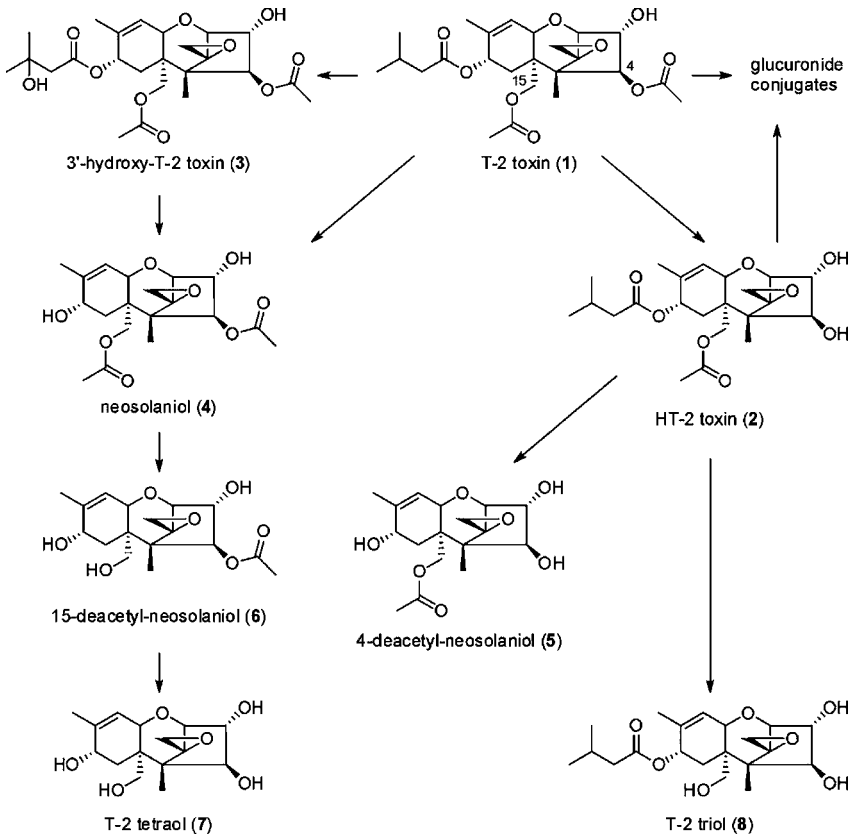

Figure 1. T-2 toxin and its metabolic pathways in vivo and in vitro.

available,[14] and studies on the metabolic fate and the cytotoxic effects of T-2 toxin and its metabolites in human cells are therefore important for further risk assessment.

In this study the metabolism of T-2 toxin in human colon carcinoma cells (HT-29) as well as in human primary cells (renal proximal tubule epithelial cells, RPTEC) was investigated, and the cytotoxic potential of the formed metabolites in vitro was compared to the toxicity of T-2 toxin using cell cycle analysis in RPTEC. Both cell types (RPTEC and HT-29) have been shown to be effective for studying the metabolism of different mycotoxins.[18,23] Because T-2 toxin metabolites were detected in the urine of various animals[12,21] and large amounts of toxin and its metabolites were eliminated into the intestinal tract through the biliary excretion system or transformed in the intestine,[20,22,24] RPTEC and HT-29 cells appear to be suitable model cell types to study the metabolism of T-2 toxin. The identification of formed metabolites in HT-29 and RPTEC cells was carried out using high-performance liquid chromatography in combination with Fourier transformation mass spectrometry (HPLC-FTMS). Because only trace amounts of certain metabolites were formed, HPLC-FTMS can be employed as a technique to identify metabolites according to their exact mass and fragmentation pattern as well as comparison to literature data.

For the toxicity of T-2 toxin, the inhibition of eukaryotic protein synthesis[22,25,26] and the induction of apoptosis in various cell lines in vitro[25−27] as well as in vivo[14,28] were described as the main effects. In human primary tubule epithelial cells, T-2 toxin induced apoptosis, whereas its metabolites (HT-2 toxin, T-2 triol, T-2 tetraol) showed lower cytotoxic effects but still induced apoptosis at higher concentrations.[18] Other studies with human cell types described a lower toxicity in vitro for the T-2 toxin metabolites T-2 triol and T-2 tetraol.[29] Consequently, cell cycle analysis in human primary cells was carried out to compare the apoptotic potential of the main T-2 toxin metabolites HT-2 toxin and neosolaniol in this study.

MATERIALS AND METHODS

Chemicals. T-2 toxin and HT-2 toxin were biosynthetically prepared and isolated in our laboratory (purity > 90%, ^{1}H NMR).[30] Neosolaniol for cell culture studies was purchased from Sigma-Aldrich (Steinheim, Germany). Isotopically labeled T-2 toxin (d_3-T-2) was synthesized in our laboratory.[30] T-2 and HT-2 toxin glucuronide were prepared in our laboratory according to a previous paper,[31] with slight modifications.

Media for cell culture studies (DMEM/Ham's F-12 and DMEM High Glucose) were obtained from PAA Laboratories (Pasching, Austria). Fetal calf serum (FCS) was purchased from Biochrom AG (Berlin, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma-Aldrich (Deisenhofen, Germany). Purified water was generated by a Milli-Q gradient A10 system (Millipore, Schwalbach, Germany).

Cell Culture. The human colon carcinoma cell line HT-29 was obtained from DSMZ (Braunschweig, Germany). Human renal proximal tubule epithelial cells (RPTEC) were purchased from Lonza Group AG (Basel, Switzerland). RPTEC maintain their tubule characteristics for at least 10 passages.[18]

HT-29 cells were cultivated using DMEM containing 1% penicillin/streptomycin and 10% FCS. After reaching 90% confluence, the cells were subcultivated twice a week to a ratio of 1:10 as described previously.[32] RPTEC were cultivated in DMEM/Ham's F-12 enriched with supplements.[32] During cultivation, the medium was changed twice a week and the cells were subcultivated every 14−17 days at a ratio of 1:3.

For determination of the cytotoxicity using the CCK-8 assay, cells were seeded in 96-well plates with 1×10^4 cells and at least 100 μL of

medium. For metabolism studies 5×10^4–1×10^5 cells were seeded in 12-well plates with at least 1.5 mL of medium. Experiments for cell cycle analysis were performed in 100 mm cell culture dishes with 5×10^5 cells and at least 10 mL of medium.

After reaching microscopic confluence (approximately 90%) at about 2 days after seeding, the culture medium was changed to serum-free medium 24 h prior to toxin incubation. T-2 toxin and its metabolites (HT-2 toxin and neosolaniol) were diluted in serum-free medium from a stock solution (toxins dissolved in acetonitrile/water (50:50; v/v); 10 mM) in concentrations ranging from 1 nM to 200 μM for 24 and 48 h. Negative control cells were incubated with serum-free medium containing an equal amount of solvent without toxin (control). All studies with primary cells were carried out using passages 3–10. HT-29 cells were kept at 37 °C under an atmosphere of 8.5% CO_2 and high humidity during cultivation and incubation periods. Primary cells were cultivated and incubated at 37 °C under 5.0% CO_2 atmosphere with high humidity.

Determination of the Cytotoxicity (CCK-8 Assay). The cytotoxic potential of T-2 toxin was analyzed with the Cell Counting Kit-8 (CCK-8) from Dojindo Laboratories (Tokyo, Japan). The CCK-8 assay was performed as described previously.[18] Cells were seeded in 96-well plates and incubated with T-2 toxin for 48 h in concentration ranges from 1 nM to 200 μM after 24 h of preincubation in serum-free medium.

Metabolism Studies of T-2 Toxin. To identify formed metabolites after T-2 toxin incubation, the supernatant cell culture medium was analyzed by HPLC-FTMS. Retention time and exact mass measurements as well as fragmentation patterns in comparison to literature data or standard substances, respectively, were used to identify formed metabolites. After 24 and 48 h of T-2 toxin incubation (100 μM), the supernatant cell medium was centrifuged (10000*g*, 10 min, room temperature) and directly used for HPLC-FTMS analysis. The remaining cells on the culture plate were washed twice with phosphate-buffered saline (PBS) before cell lysis with 1% Triton X-100 solution at 4 °C for 15 min. The cell lysates were collected and centrifuged in the same way as the cell medium and then used for HPLC-FTMS analysis. The stability of T-2 toxin in aqueous cell culture medium at 37 °C under 5% CO_2 conditions for at least 48 h has been proven previously in our group (data not shown).[18]

Quantitative Determination of T-2 Toxin and Its Metabolites. For the quantitative determination of T-2 toxin and its metabolites HT-2 toxin and neosolaniol, a matrix-matched calibration curve with standard solutions in different concentration ranges of the analytes (T-2 toxin, 5–225 ng/mL; HT-2, toxin 500–8000 ng/mL; neosolaniol, 2000–8000 ng/mL) was used. The mixture of standard substances was evaporated under N_2 at 40 °C and refilled to a defined volume with matrix cell medium. To generate matrix medium, cells were cultivated in the same way as described before for metabolism studies but without toxin incubation. The medium was collected 48 h after the medium had been changed to serum-free medium and centrifuged according to the sample preparation for metabolism studies. For the quantitative evaluation, the concentrations of T-2 toxin, HT-2 toxin, and neosolaniol were plotted against the sum of the peak areas of the $[M + NH_4]^+$ and $[M + Na]^+$ adducts of the analytes.

HPLC-FTMS. For analyzing T-2 toxin and its metabolites, an HPLC system was coupled to a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization (HESI) interface operated in the positive ionization mode. Data analysis was carried out using Xcalibur 2.0.7 SP1 (Thermo Fisher Scientific). Chromatographic separation was performed by an Accela LC 60057-60010 system (Thermo Fisher Scientific) on a 150 mm × 2 mm i.d., 4 μm, Synergi Fusion-RP (C18) column with a 4 mm × 2 mm i.d. guard cartridge of the same material (Phenomenex, Aschaffenburg, Germany) using a binary gradient with acetonitrile supplemented with 5 mM ammonium acetate (solvent A) and water supplemented with 5 mM ammonium acetate (solvent B). The gradient started at 20% A, increasing to 90% A after 9 min, maintaining the conditions for 1 min, before reaching 20% A at 11 min and equilibrating for 7 min, resulting in a total run time of 18 min. Flow rate was set to 250 μL/min, and injection volume was 20 μL. For removal of salt and other polar substances from cell culture media the first minute of each run was discarded using a divert valve. Further MS conditions were as follows: capillary temperature, 225 °C; vaporizer temperature, 350 °C; sheath gas flow, 50 arbitrary units; auxiliary gas flow, 10 arbitrary units; sweep gas flow, 5 arbitrary units; source voltage, 3.5 kV; capillary voltage, 33 V; tube lens, 100 V. FTMS experiments were based on a total ion scan of mass ranges from m/z 100 to 800 with a resolution of 30000. Product ion spectra of T-2 toxin metabolites as sodium and ammonium adducts were recorded with data-dependent fragmentation experiments for the detection of the specific m/z of $[M + Na]^+$ or $[M + NH_4]^+$, respectively, from a preset parent ion list, with collision-induced dissociation (CID), 25%; isolation width, 2.0; resolution, 15000; and activation, $Q = 0.250$.

Identification of T-2 Toxin Metabolites. T-2 toxin metabolites 4-deacetylneosolaniol (**5**), T-2 triol (**8**), 3′-hydroxy-T-2 toxin (**3**) (for structures see Figure 1), HT-2 toxin glucuronide, and T-2 toxin glucuronide were identified via exact mass measurements and fragmentation patterns. The observed MS data are as follows.

4-Deacetylneosolaniol (**5**): exact mass (FTMS), m/z 358.1855 for the NH_4^+ adduct (calculated for $[C_{17}H_{28}O_7N]^+$ as 358.1860); MS/MS CID 25% m/z (%) 197.0957 (100), 215.1063 (89), 227.1063 (53), 245.1169 (77), 263.1273 (85).

T-2 triol (**8**): exact mass (FTMS), m/z 400.2326 for the NH_4^+ adduct (calculated for $[C_{20}H_{34}O_7N]^+$ as 400.2330); MS/MS CID 25% m/z (%) 197.0957 (32), 215.1063 (100), 227.1063 (9), 245.1169 (18), 263.1274 (50), 365.1955 (34).

3′-Hydroxy-T-2 toxin (**3**): exact mass (FTMS), m/z 505.2034 for the Na^+ adduct (calculated for $[C_{24}H_{34}O_{10}Na]^+$ as 505.2044); MS/MS CID 25% m/z (%), 245.1169 (6), 267.0988 (6), 327.1199 (43), 387.1407 (100).

Glucuronide conjugates of T-2 and HT-2 toxin were identified by their characteristic loss of the glucuronic acid moiety: T-2 toxin glucuronide as NH_4^+ adduct, neutral loss of 176.0304 u (calculated for $[C_6H_8O_6]$ as 176.0332 u); HT-2 toxin glucuronide as NH_4^+ adduct, neutral loss of 176.0316 u (calculated for $[C_6H_8O_6]$ as 176.0332 u).

Cell Cycle Analysis. To compare the apoptotic potential of T-2 toxin to its metabolites, DNA analysis for cell cycle studies was performed with 5×10^5 primary cells seeded on 100 mm cell culture dishes. Before toxin incubation, medium was changed to serum-free medium and the cells were incubated for 24 and 48 h with T-2 toxin, HT-2 toxin, or neosolaniol (100 nM, 1 μM, 10 μM), respectively. Further cell cycle analysis was performed as previously reported.[32] The formation of a sub G1 peak was compared to the sub G1 area of the solvent treated control (control set to 100%).

Statistical Analysis. All measurements are presented as the mean value ± SD. Experiments were performed in at least three different cell passages using a minimum of six wells per group for determination of cytotoxicity (CCK-8) and a minimum of three individual experiments for metabolite quantitation and cell cycle analysis. Consequently, at least 9–18 samples were analyzed for each parameter. To determine significant differences, the unpaired Student's t test with $p \leq 0.05$ as statistically significant was used. The medium effective concentrations (IC_{50} values) were calculated on the basis of cytotoxicity data after 48 h of incubation using SigmaPlot version 12.0 according to the literature.[33]

RESULTS AND DISCUSSION

Metabolism of T-2 Toxin. To study the metabolism of T-2 toxin close to the human situation in vivo, primary RPTEC were used in comparison with an established human colon carcinoma cell line (HT-29). T-2 toxin metabolites were analyzed in the cell culture media by HPLC-FTMS. The results show that the initial T-2 toxin concentration decreased over 24 and 48 h in both cell types. HT-2 toxin (**2**), neosolaniol (**4**), T-2 triol (**8**), 3′-hydroxy-T-2 toxin (**3**), 4-deacetylneosolaniol (**5**) (for structures see Figure 1), T-2 toxin glucuronide, and HT-2 toxin glucuronide were identified as metabolites in HT-29 cells after 48 h of incubation with 100 μM T-2 toxin. Figure

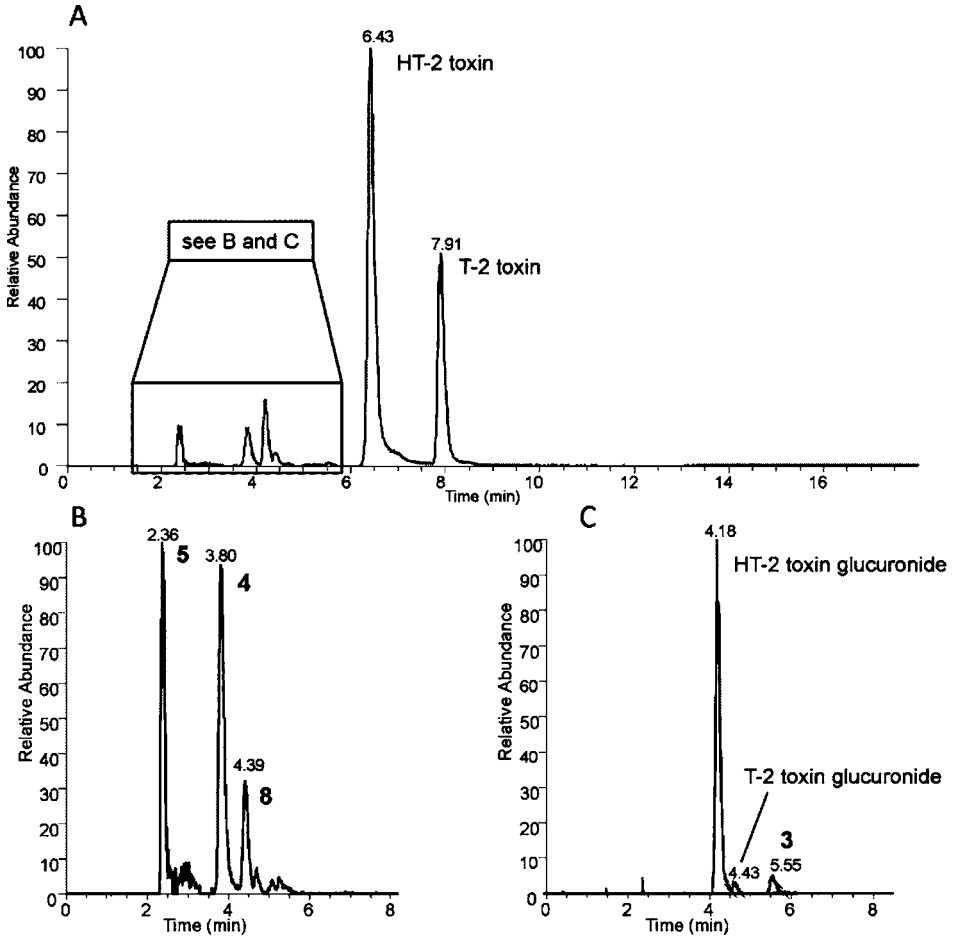

Figure 2. HPLC-FTMS extracted ion chromatogram of supernatant cell medium of HT-29 cells after incubation with 100 μM T-2 toxin for 48 h. T-2 toxin and formed metabolites are indicated via their exact masses as $[M + NH_4]^+$ with T-2 toxin, m/z 484.2541, and HT-2 toxin, m/z 442.2435 (A). Further formed metabolites are displayed in panels B and C with **5** (4-deacetylneosolaniol, m/z 358.1860); **4** (neosolaniol, m/z 400.1966); **8** (T-2 triol, m/z 400.2330); HT-2 toxin glucuronide (m/z 618.2756); T-2 toxin glucuronide (m/z 660.2862); and **3** (3′-hydroxy-T-2 toxin, m/z 500.2490) (for structures see Figure 1).

2 shows the obtained HPLC-FTMS chromatogram for HT-29 cells. In RPTEC a similar metabolite pattern was observed, and only T-2 triol was not detectable.

Besides T-2 toxin, the main metabolites HT-2 toxin and neosolaniol were quantified in cell media after 24 and 48 h of incubation (Figure 3). In both cell types T-2 toxin is rapidly metabolized and less than 15% (RPTEC) or 5% (HT-29 cells) of the initial T-2 toxin concentration remains after 48 h of incubation. Concurrently, an increase of HT-2 toxin is detectable in the cell media for both cell types but to a different extent for HT-29 and RPTEC cells. In HT-29 cells (Figure 3B) HT-2 toxin represents the main metabolite and is already formed after 24 h in concentrations up to 77%, increasing only slightly after 48 h. Neosolaniol is detectable after 24 and 48 h in amounts of about 12% in HT-29 cells. In RPTEC (Figure 3A), HT-2 toxin and neosolaniol are formed in approximately the same concentration range of about 30% each after 24 h, reaching 45% each after 48 h of incubation time. T-2 toxin again represents a minor component with amounts <15% after 24 and 48 h. The sum of the formed main metabolites HT-2 toxin and neosolaniol combined with the amounts of the remaining T-2 toxin corresponds well to the initially applied concentration of T-2 toxin (Figure 3). Further metabolites such as T-2 triol, 3′-hydroxy-T-2 toxin, T-2 toxin glucuronide, HT-2 toxin glucuronide, and 4-deacetylneosolaniol could still be identified by exact mass measurements, even though a quantitation was not possible. These formed metabolites account for less than approximately 1−5% of the total amount of T-2 toxin metabolites in vitro according to their peak area (Figure 2). The cell lysate of both cell types was also analyzed, but neither metabolites nor T-2 toxin was detectable in the samples, and for further analysis only the supernatant cell medium was considered.

The metabolites found in vitro in our study correspond well to known metabolites described in the literature. The two cell types used in this study show the same spectrum of formed metabolites with slight differences. T-2 triol (**8**) was detectable only in HT-29 cells, whereas T-2 tetraol (**7**) could not be found in any of the cell types used. The metabolic reactions forming the main metabolites HT-2 toxin (**2**) and neosolaniol (**4**) are the hydrolysis of the acetyl group at the C-4 position (HT-2 toxin) and additionally the hydrolysis and release of the isovaleryl moiety at the C-8 position (neosolaniol). The capability to perform this metabolic pathway was already seen for RPTEC[18] as well as for human and rat blood cells.[17] In inhibition experiments the specific enzymes catalyzing these reactions were suggested to be specific carboxylesterases,[17] which seem to be present in RPTEC as well as in HT-29 cells.

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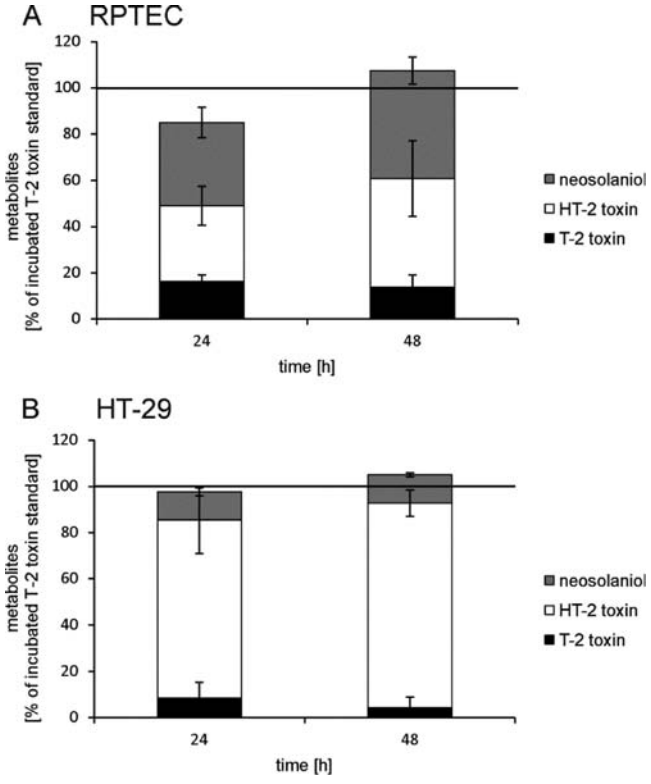

Figure 3. Quantitative determination of T-2 toxin metabolites (HT-2 toxin; neosolaniol) in RPTEC (A) and HT-29 (B) supernatant cell media after 24 and 48 h of T-2 toxin incubation. HT-2 toxin and neosolaniol are formed in corresponding amounts in RPTEC. HT-2 toxin is the main metabolite in HT-29 cells. Amounts are presented as percent of originally incubated T-2 toxin concentration. Mean ± SD; number of analyzed samples $n = 6$ over three individual passages.

In general, HT-2 toxin and neosolaniol are the most abundant metabolites formed in HT-29 and RPTEC cells in vitro in our study.

Identification of 3′-Hydroxy-T-2 Toxin. The T-2 toxin metabolite 3′-hydroxy-T-2 toxin (**3**) was formed in both cell types. The structure was validated via fragmentation experiments in comparison to a T-2 toxin standard because no reference compound was available. At a CID of 25% the first fragment to be cleaved from the pseudomolecular ion of T-2 toxin $[M + Na]^+$ is the isovaleryl side chain ($C_5H_{10}O_2$), which is characterized by a neutral loss of 102.0687 u (calculated for $C_5H_{10}O_2$ as 102.0675 u). For 3′-hydroxy-T-2 toxin, the pseudomolecular ion of the sodium adduct $[M + Na]^+$ also undergoes the loss of the isovaleryl moiety as the first fragment at CID 25% ($C_5H_{10}O_3$ neutral loss of 118.0637 u). The calculated sum formula of the fragment, based on the exact mass measurements, proved an additional hydroxy group to be present at the isovaleryl side chain of the formed metabolite, which is cleaved off with the isovaleryl moiety. The further fragmentation pattern of the formed metabolite is identical to that of T-2 toxin, and therefore the structure of the formed metabolite was identified as 3′-hydroxy-T-2 toxin. The exact position (3′) of the hydroxy group cannot be elucidated completely, but it is most likely to be the 3′ position because this metabolite is described in most literature.[5,19] With regard to its toxicity, 3′-hydroxy-T-2 toxin is characterized as being as potent as T-2 toxin in terms of the induction of apoptosis in mice.[28] In studies with liver homogenates of monkeys and rats the acute toxicity of 3′-hydroxy-T-2 toxin is considered to be slightly higher than that of the parent toxin.[24,28,34] Therefore, the metabolic reaction would result rather in an activation than a detoxification, but because there was no reference compound available, further studies with this compound could not be performed.

Glucuronide Conjugates. In this study a phase II metabolism of T-2 toxin in human primary cells as well as in an established human cell line resulting in the formation of T-2 toxin and HT-2 toxin glucuronides was shown. These polar conjugates have until now mainly been detected in in vivo studies.[21,22] In rat hepatocytes, however, glucuronide conjugates of HT-2 toxin and 3′-hydroxy-HT-2 toxin were indirectly identified after application of [^{3}H]-T-2 toxin. After β-glucuronidase treatment of the fraction containing unknown polar metabolites, [^{3}H]-HT-2 toxin and [^{3}H]-3′-hydroxy-T-2 toxin were detectable via thin layer chromatography combined with radioisotopic scanning.[35] Glucuronide conjugates of HT-2 toxin were additionally detected in perfused rat liver, and it was shown that they are rapidly eliminated via bile.[36]

The identification of T-2 and HT-2 toxin glucuronides in our study was performed on the basis of FTMS measurements and reference compounds and not by β-glucuronidase treatment as described in the literature.[37] Small amounts of T-2 toxin and HT-2 toxin glucuronides were synthesized in our laboratory according to a previous paper[31] with slight modifications and could be used for the identification in the cell media via exact mass measurements, fragmentation pattern experiments, and comparison of retention times (data not shown). On the basis of these data the metabolites formed in vitro in RPTEC and HT-29 cells were identified as the glucuronide conjugates of T-2 and HT-2 toxin with m/z 660.2859 for the NH_4^+ adduct of T-2 toxin glucuronide (calculated for $[C_{30}H_{46}NO_{15}]^+$ as m/z 660.2862) and m/z 618.2745 for the NH_4^+ adduct of HT-2 toxin glucuronide (calculated for $[C_{28}H_{44}NO_{14}]^+$ as m/z 618.2756). HT-2 toxin and T-2 toxin glucuronides were detectable in incubation experiments with RPTEC and HT-29 cells with T-2 toxin glucuronide being present in lower concentrations compared to HT-2 toxin glucuronide (Figure 2C). This indicates that T-2 toxin is rapidly metabolized to HT-2 toxin or neosolaniol, respectively (Figure 2), and is only limited accessible for glucuronidation reactions.

Identification of 4-Deacetylneosolaniol. A polar metabolite characterized as 4-deacetylneosolaniol (**5**) has been described before in the literature after incubation of T-2 toxin with rat liver S9 fraction and in in vivo as well as in vitro experiments.[12,17,22] In our study both cell types formed a metabolite showing an exact mass consistent with a deacetylated form of neosolaniol in the HPLC-FTMS spectrum (m/z of NH_4^+ adduct, 358.1860). In the literature two possible metabolites are described with the same mass, namely, 4-deacetylneosolaniol (**5**) and 15-deacetylneosolaniol (**6**) (Figure 1).[19] Because a differentiation between these two compounds was not possible by mass spectrometric fragmentation experiments, a different approach was used. RPTEC cells were incubated with three different substrates (10 μM): T-2 toxin (**1**), which can theoretically be transformed into both forms, 4-deacetyl- and 15-deacetylneosolaniol (**5** and **6**); HT-2 toxin (**2**), which is already hydrolyzed at the C-4 position and can consequently only be metabolized to 4-deacetylneosolaniol (**5**); and d_3-T-2 toxin (**9**), which can be metabolized to 4-deacetylneosolaniol (**5**) and 15-deacetylneosolaniol deuterated at the acetyl moiety attached to the hydroxy group at position

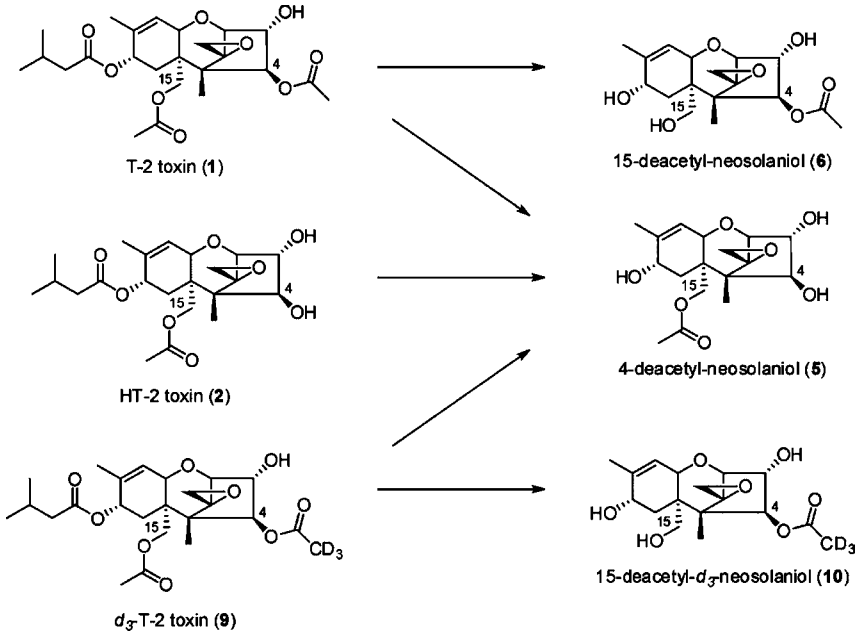

Figure 4. Possible formation of 4-deacetylneosolaniol and 15-deacetylneosolaniol from T-2 toxin, HT-2 toxin, and d_3-T-2 toxin.

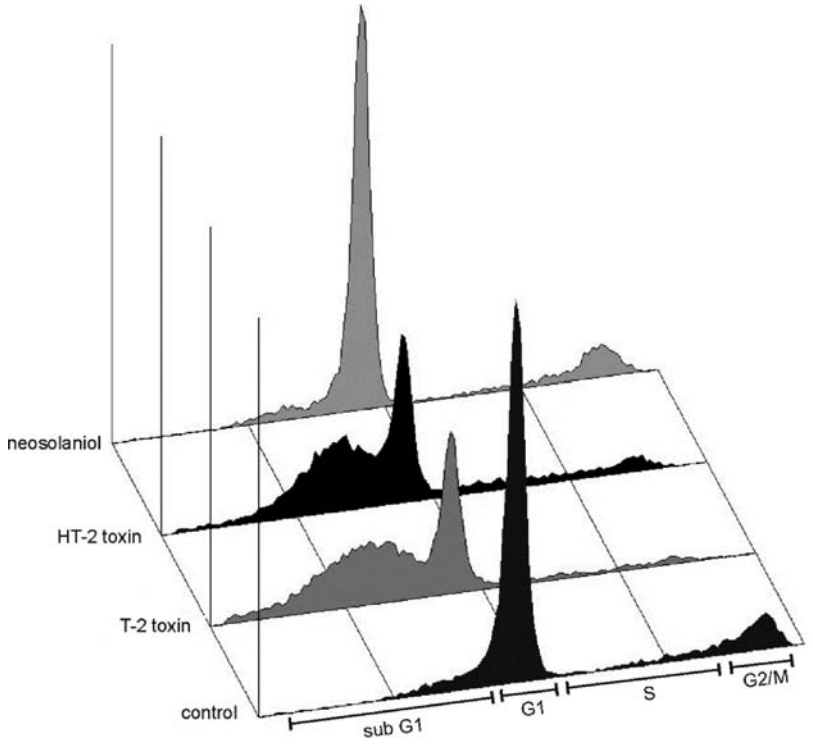

Figure 5. Histogram plot of cell cycle analysis in RPTEC after 48 h of incubation with T-2 toxin, HT-2 toxin, neosolaniol (1 μM), and solvent-treated control cells. Note the similar sub G1 peak formation of T-2 and HT-2 toxin (second and third histograms). Neosolaniol displays no sub G1 peak formation (similar to control; first and fourth histograms).

C-4 (**10**) (Figure 4). After 48 h of incubation of RPTEC with the three test compounds, T-2 toxin had been metabolized to HT-2 toxin and the NH_4^+ adduct of the unknown metabolite with m/z 358.1860 besides small amounts of remaining T-2 toxin. The HT-2 toxin incubation resulted in a metabolite with the exact mass of 4-deacetylneosolaniol besides nonmetabolized HT-2 toxin. The same metabolite was detectable by FTMS after incubation with d_3-T-2 toxin, but no deuterated 15-deacetylneosolaniol was formed after incubation with d_3-T-2 toxin (data not shown). Because the unknown metabolite was formed after incubation with HT-2 toxin and d_3-T-2 toxin and shows the same retention time in all samples, we can conclude that the metabolite formed in our study after T-2 toxin incubation is 4-deacetylneosolaniol (**5**) (Figure 4). This is further confirmed by the fact that incubating RPTEC with d_3-T-2 toxin, isotopically labeled at the acetyl group attached to the hydroxy group at position C-4, did not result in the

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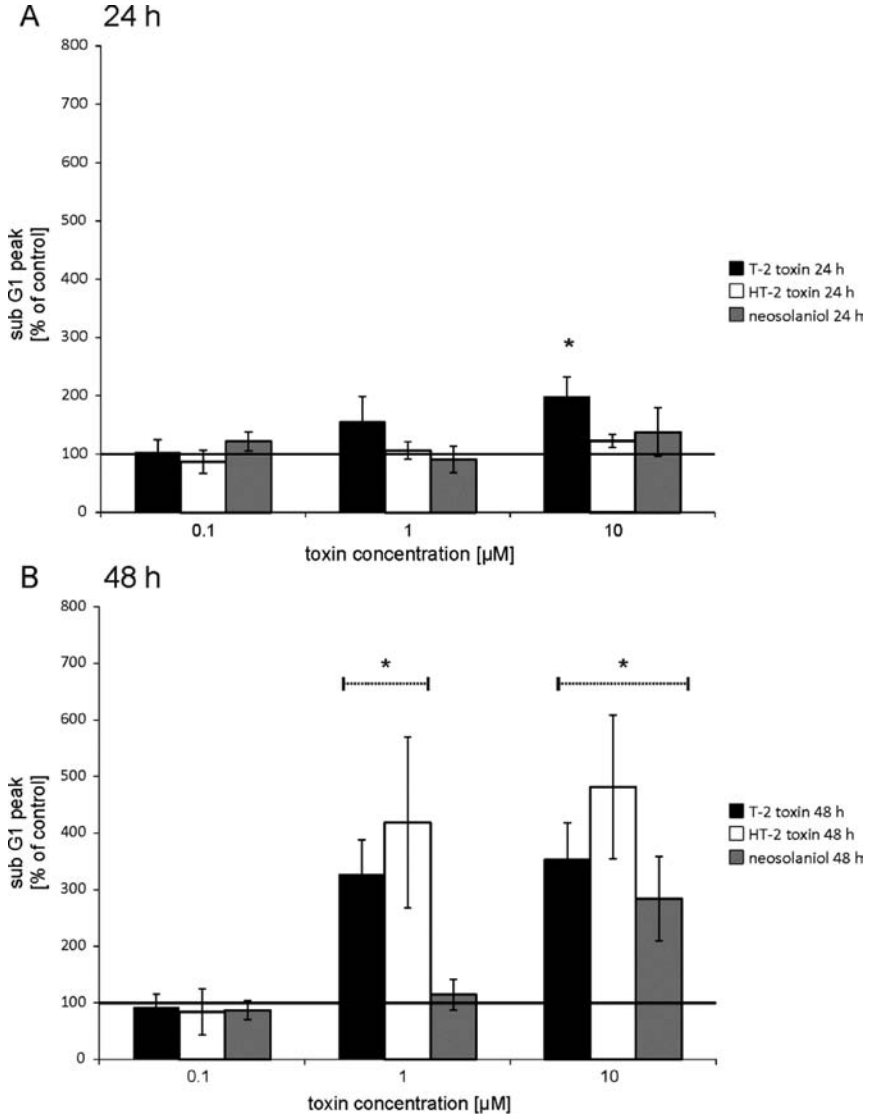

Figure 6. Time, concentration, and substrate dependent sub G1 peak formation in RPTEC after 24 h (A) and 48 h (B) of incubation with T-2 toxin, HT-2 toxin, and neosolaniol. Quantitation is shown as percent in comparison to solvent-treated control cells. Mean ± SD; number of analyzed samples $n = 6$ over three individual passages; ∗ indicates significant differences from the control ($p \leq 0.05$).

formation of deuterated 15-deacetylneosolaniol (**10**) (Figure 4).

Apoptotic Potential of T-2 Toxin, HT-2 Toxin, and Neosolaniol. Primary human RPTEC were used earlier in our group for cytotoxicity studies of T-2 toxin.[18] To compare the results of this study, which were performed with RPTEC from a different donor, with the previously reported results, we evaluated the general cytotoxicity of T-2 toxin on RPTEC using the CCK-8 assay in the first step. The IC_{50} value for RPTEC was calculated with 230 nM (±18 nM) and is therefore similar to the IC_{50} value of 200 nM (±60 nM) as described for RPTEC from a different donor used earlier in our group.[18]

To study the apoptotic potential of the main T-2 toxin metabolites HT-2 toxin and neosolaniol, sub G1 peak analysis as a marker for apoptosis was analyzed in human primary kidney cells. T-2 toxin is known for its high apoptotic potential and has been used as a positive control for cell cycle analysis.[32] RPTEC were incubated for 24 and 48 h with 100 nM, 1 μM, and 10 μM of the test compounds (T-2 toxin, HT-2 toxin, neosolaniol), and the quantitation of the sub G1 peak formation was normalized to solvent-treated control cells (set to 100%). The typical distribution of the cell cycle with an intensive G1 peak is visible in the control cells, whereas T-2 toxin and HT-2 toxin show a sub G1 peak formation at concentrations of 1 μM after 48 h of incubation with neosolaniol having no significant effect (Figure 5). Under these conditions (48 h of incubation; 1 μM), the apoptotic effect of HT-2 toxin resulting in a sub G1 peak formation of 418 ± 151% compared to control cells is similar to the effects caused by T-2 toxin (T-2 toxin, 326 ± 60%), whereas neosolaniol induced no apoptotic effect and caused no sub G1 peak formation in comparison to control cells (114 ± 27%) (Figure 6B). At concentrations of 10 μM and after 48 h of incubation, all three compounds showed clear formation of a sub G1 peak with similar intensities (T-2 toxin, 350 ± 65%; HT-2 toxin, 480 ± 126%; neosolaniol, 280% ± 75%) (Figure 6B). Weaker effects were observed after 24 h of incubation, and only T-2 toxin showed a significant effect at 10 μM forming a sub G1 peak of about 200% compared to control cells (Figure 6A). No apoptotic effect was detected after incubation of

0.1 μM T-2 toxin and its main metabolites for 24 and 48 h, respectively.

These results are consistent with literature data describing T-2 and HT-2 toxin as having a comparable apoptotic effect on RPTEC with neosolaniol being less toxic at concentrations of 1 μM and having a similar effect at concentrations of 10 μM.[18] In human Jurkat cells T-2 toxin caused apoptosis at concentrations of 10 μM (similar effects for HT-2 toxin, T-2 triol, T-2 tetraol),[26] whereas in human HL-60 cells HT-2 toxin showed a slightly lower apoptotic potential than T-2 toxin.[27] In vivo apoptosis was also detected after T-2 toxin application in the thymus of mice, with HT-2 toxin having a lower apoptotic effect and neosolaniol not being capable of inducing apoptosis.[28]

In summary, our results show that T-2 toxin is metabolized in human cells in primary culture (RPTEC) as well as in an established human cell line (HT-29) in a time-dependent matter to a variety of metabolites. Besides the main metabolite HT-2 toxin, neosolaniol was detected in similar amounts in primary cells. Further identified metabolites were 3′-hydroxy-T-2 toxin, T-2 triol (only in HT-29 cells), and 4-deacetylneosolaniol. Phase II metabolism resulting in glucuronide conjugates of T-2 as well as HT-2 toxin was additionally detected in vitro with HT-2 toxin glucuronide and T-2 toxin glucuronide being formed in both human cell types in low concentrations. The metabolic pattern found in human primary cells used in this study resembles the situation in vivo with a rapid decrease of the initially incubated amount of T-2 toxin and the concurrent formation of HT-2 toxin and neosolaniol as the main metabolites and more polar compounds as minor metabolites.[5] Because the chosen human primary cells (RPTEC) simulate the situation in vivo closely, the detected metabolite pattern of T-2 toxin in this study is likely to occur to a similar extent in vivo in humans. With regard to the apoptotic potential of the main metabolites formed in RPTEC (HT-2 toxin and neosolaniol), cell cycle analysis revealed an equal potential of HT-2 toxin compared to T-2 toxin (sub G1 peak formation to a similar extent after 48 h incubation at 1 μM), whereas neosolaniol showed no sub G1 peak formation under these conditions. The extent of the apoptotic potential at concentrations of 10 μM and 48 h of incubation was similar for all three tested compounds in our study. These findings are important for further risk assessment, because HT-2 toxin and neosolaniol are among the most frequently described metabolites after T-2 toxin application in vivo and could thus be present in food samples of animal origin as possible contaminants. Furthermore, neosolaniol and T-2 triol were detected in oat samples in low concentrations as coconta-minants in the presence of high concentrations of the primary contaminants T-2 and HT-2 toxin.[38] Therefore, it has to be highlighted that the metabolic fate of T-2 toxin in human primary cells does not necessarily result in a detoxification and the main metabolites should also be considered in risk assessment.

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Notes

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