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Destruxins: Fungal-derived Cyclohexadepsipeptides with Multifaceted Anticancer and Antiangiogenic Activities

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Abstract

Destruxins (Dtx) are secondary metabolites of the entomopathogenic fungus *Metarhizium anisopliae*. Recently, Dtx came into focus of interest as anticancer therapeutics. However, data on human and especially on cancer cells are fragmentary. In order to successfully establish novel anticancer therapeutics, a broad knowledge on the cellular and molecular mechanisms underlying their activity is essential. Consequently, this study aimed to investigate the impact of the most common Dtx derivatives A, B and E on human cancer cell growth and survival with a focus on colon cancer cell models. Summarizing, the experimental data showed that i) Dtx A and B exert potent antiproliferative activity in the micromolar and Dtx E in the nanomolar range in KB-3-1, A549, CaCo-2, and especially in HCT116 colon cancer cells, ii) all three Dtx derivatives cause imbalance of cell cycle distribution, iii) their cytostatic/cytotoxic effects are widely p53-independent but reduced by p21- and bax-deletion, respectively, iv) cytotoxicity is based on intrinsic apoptosis induction and associated with phosphoinositide-3-kinase (PI3K)/Akt pathway inhibition, v) anticancer activity of Dtx E but not Dtx A and B involves disturbance of the intracellular redox balance, vi) Dtx inhibit the migration and tube formation of human endothelial cells indicating antiangiogenic potential, and vii) all three Dtx derivatives possess ionophoric properties not differing in conductivity, ion selectivity and single channel kinetics. Thus, Dtx represent feasible, multifunctional anticancer drug candidates for preclinical development especially against colorectal cancer.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

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1 Introduction

The great majority of destruxins (Dtx) are secondary metabolites of the entomopathogenic fungus *Metarhizium anisopliae* which is extensively utilized as alternative to chemical insecticides in agricultural pest and disease-vector control programs. However, a few Dtx are also produced by plant pathogenic fungi [1, 2]. With regard to their chemical structure Dtx belong to a group of cyclic hexadepsipeptides composed of one α -hydroxy acid and five amino acid residues. Currently, about 35 derivatives of these cyclic hexadepsipeptides are known and it has been unequivocally proven that their presence is correlated with fungal virulence. The most common Dtx are A, B and E. Individual Dtx differ on their alpha hydroxy acid moiety, the N-methylation pattern, and the residues of the amino acids (Fig. 1) [1, 3]. Comparable to other cyclic hexadepsipeptides, Dtx are known for their insecticidal [1, 4, 5], phytotoxic [6], immunosuppressive [7] and cytotoxic effects [4, 8]. It is well documented that their insecticidal activities are based on initial tetanic paralysis attributed to muscle depolarisation by direct opening of Ca^{2+} channels in membranes finally leading to insect death [9]. Recently, Dtx were identified as vacuolar H^{+} -ATPase (V-ATPase) inhibitors. This is of special interest as V-ATPase was suggested as an attractive target for osteoporosis or cancer drug therapy [2]. Indeed, inhibitory effects of Dtx on the bone-resorbing activities of osteoclasts have been shown [10, 11]. Dtx B and E were found to inhibit pit formation without affecting osteoclast differentiation and survival. Dtx-induced morphological changes in osteoclasts were accompanied by disruption of the actin rings and the blockage of formation of prominent clear zones and ruffled borders [10]. Moreover, these bone resorbing activities were shown to act cell-specifically on the cytoskeleton of polarized osteoclasts, rather than generally on their cytoskeleton [11]. Since these properties resemble calcitonin, which has been approved for the treatment of osteoporosis, Dtx are currently in the focus of interest as new anti-osteoporosis therapeutics [2]. Additionally, recent studies suggested a potential quality of Dtx as anticancer drugs. For instance, subsequent Dtx E treatment was reported to diminish Bcl-xL-mediated resistance of diverse anticancer drugs with different modes of actions and suggested to be due to inhibition of V-ATPase activity [12]. Lately, promising *in vitro* and *in vivo* anticancer effects of Dtx B on human HT-29 colorectal cancer cells were found which were hypothesized to be based on inhibition of the Wnt/ β -catenin/Tcf signaling pathway [13, 14]. Altogether, hitherto existing data on the anticancer activities of Dtx are interesting but the underlying molecular mechanisms widely unexplored. In order to successfully establish novel anticancer therapeutics in the clinics a broad knowledge on the underlying cellular and molecular mechanisms of activity is essential. Consequently, this study aimed to clarify whether Dtx A, B and E have an impact on human cancer cell growth and survival with a focus on colon cancer cell models and to elucidate their modes of action.

2 Materials and Methods

2.1 Test compounds

Dtx A, B and E were isolated from a *M. anisopliae* culture broth by a three-step purification protocol including liquid–liquid extraction column chromatograph, apolar chromatography and high-speed counter-current chromatography for final purification [15]. Due to their lipophilic character stock solutions of all derivatives were frequently prepared in DMSO as solvent for cell culture studies and stored at 4°C. For fluorescence imaging studies stock solutions were prepared in Tween 80:MeOH = 1:2 to avoid reduced Ca²⁺ signalling induced by DMSO. All other compounds were supplied by Sigma-Aldrich GmbH (St.Louis, MO).

2.2 Cell culturing

The following human cancer cell lines were used for this study: the epidermal carcinoma-derived cell line KB-3-1 (generously donated by Dr. Shen, Bethesda, USA) [16], the KB-3-1 derivative KB-HU selected against hydroxyurea (generously donated by Dr. Cheng, Yale University, New Haven, CT) [17] the colon carcinoma cell line CaCo-2 (American Tissue Culture Collection), the colon carcinoma cell model HCT116 and respective sublines with deleted p53 (compare Fig. 6D), p21, or bax genes (generously donated by Dr. Vogelstein, John Hopkins University, Baltimore) [18–20], as well as the Bcl-2-negative non-small cell lung cancer cell line A549 and its bcl-2 pBabe/Puro-transfected subline [21]. Additionally, human umbilical vein endothelial cells (HUVEC) were used. HCT116 cell lines were grown in McCoy's and Caco-2 cells in MEME culture medium. For KB-3-1 as well as for A549 cells and the respective sublines RPMI 1640 medium was used. HUVECs were maintained in endothelial basal medium (EBM)-2 (Lonza, MD, USA) supplemented according to the instructions of the manufacturer. All other culture media were purchased from Sigma-Aldrich GmbH (St. Louis, MO) and supplemented with 10% fetal calf serum (PAA, Linz, Austria). Cultures were regularly controlled for *Mycoplasma* contamination.

2.3 Cell viability assays

Cells grown in a humidified 5% CO₂ incubator were collected, counted, and adjusted to a concentration of 2x10⁴ cells/ml for further transfer of 100 µl/well into 96-well plates. After 24 h recovery cells were treated with 100 µl of the three Dtx for 24, 48, and 72 h, respectively. To study the reversibility of the cytotoxic Dtx effect wash out experiments were performed. Thus, the test compounds were removed from the wells after an incubation time of 6, 24, and 48 h and substituted by fresh medium. Afterwards, cell viability of 6-, 24-, and 48 h- treated cells was measured after a total time of 72 h and compared to cells under continuous (72h) Dtx treatment. In all experimental settings the concentrations for Dtx A and B were ranging from 0.5 to 10 µM while for Dtx E lower concentrations (0.01–0.5 µM) were used. For modulator studies with N-acetyl cysteine (NAC) and buthionine sulfoximine (BSO), cells were pre-treated for 30 min at 37°C with 50 µl of a 1.25 and 2.5 mM NAC or for 24 h with a 50 and 100 µM BSO solution. Subsequently, Dtx were added in another 50 µl medium. In all experimental settings, the proportion of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)- based vitality assay according to the manual (EZ4U, Biomedica, Vienna, Austria). All experiments were

performed at least twice in triplicates and data evaluated using the GraphPadPrism 5 software.

2.4 Clonogenic assay

3×10^2 cells per well were seeded into 12-well plates. Following 24 h recovery, cells were treated with the test substances. After an incubation time of 24, 48 h and 72 h the test compounds were removed from the wells and substituted by fresh medium. Additionally, one 12-well plate was continuously treated with the test substances (192 h). At day 8 (192 h) after first drug contact, cells were washed twice with PBS, fixed with methanol at -20°C and stained with crystal violet. The number of colonies was determined by direct counting.

2.5 Apoptosis detection

Early apoptotic morphological changes of HCT116 cells treated 24 h or 48 h with the three Dtx derivatives were visualised by 4',6'-diamidino-2-phenylindole (DAPI) staining as described earlier [21]. Further, Hoechst 33258/propidium iodide (PI) co-stainings were performed with 48 h Dtx-treated HCT116 cells according to Kowol et al [22]. The Hoechst dye stains the nuclei of live and dead cells while PI uptake indicates the loss of membrane integrity characteristic of necrotic and late apoptotic cells. In addition, to investigate the impact of Dtx on the mitochondrial membrane potential, HCT116 cells (5×10^6 cells/well) were exposed to $10 \mu\text{M}$ Dtx A, B and $0.5 \mu\text{M}$ Dtx E for 48 h before staining with the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Mitochondrial Membrane Potential detection Kit; Stratagene, La Jolla, CA, USA) and FACS analysis was performed as described [21, 23].

2.6 DHE-assay for intracellular ROS/superoxide radical detection

Intracellular superoxide radical (O_2^-) production was determined by flow cytometry using the cell permeable dihydroxyethidium (DHE) [24]. DHE enters the cells and is oxidized to form ethidium which binds to DNA showing red fluorescence. KB-3-1 cells (5×10^5 per sample) were incubated 30 min with the indicated Dtx concentrations at 37°C in PBS. After 30 min drug treatment $10 \mu\text{M}$ DHE was added for another 30 min at 37°C . Antimycin A ($10 \mu\text{M}$) was used as positive control. Subsequently, the DHE fluorescence (535/617 nm) was measured using FACS Calibur (Becton Dickinson and Company, NY, USA). The median fluorescence intensity was quantitated by CellQuest software (Becton-Dickinson) analysis of the recorded histograms.

2.7 Cell cycle analyses

Adherent KB-3-1, CaCo-2, HCT116 p53(+/+), and HCT116 p53(-/-) as well as HCT116 p21(+/-) and HCT116 p21(-/-) (5×10^5 cells/well) were incubated in 6-well plates at 37°C for 24 or 48 h with rising Dtx concentrations. Cell cycle progression was examined by flow cytometry using FACS Calibur (Becton Dickinson, Palo Alto, CA) as described [25]. CellQuest Pro software (Becton Dickinson and Company, NY, USA) was used to analyse the resulting DNA histograms.

2.8 ³H-thymidine incorporation

4×10^4 per well HCT116 p21(+/+) and (-/-) cells were seeded into a 96-well plate. After 24 h of recovery, cells were exposed for another 24 h to 2.5–10 μ M Dtx A and B and 0.1–0.5 μ M Dtx E. After exposure, the culture medium was replaced for 4 h by a 2 nM ³H-thymidine solution (diluted in full culture medium; radioactivity, 25 Ci/mM) and cells were incubated at 37°C. Afterwards, cells were washed three times with PBS and cell lysates were prepared. Radioactivity was determined as described [26]. Experiments were carried out in triplicates, and results were expressed as counts per minute (cpm).

2.9 Western blot analyses

After 24 h drug exposure, cell proteins were isolated, resolved by sodium dodecyl sulfate (SDS)/PAGE and transferred onto a polyvinylidene difluoride membrane for Western blotting as described [21]. Antibodies used were as follows: p53 monoclonal mouse DO-1 (Neomarkers, CA, USA); p21^{Waf1} polyclonal rabbit C-19 (Santa Cruz Biotechnology, CA, USA); bcl-xL polyclonal rabbit (Santa Cruz Biotechnology); Apoptosis Sampler kit: PARP, caspase 7 and cleaved caspase 7 (Cell Signalling Technology, Beverly, MA) polyclonal rabbit; Pro-Apoptosis Bcl-2 Family Antibody Sampler kit: bax polyclonal rabbit and c-Oncogene Antibody Sampler kit: c-fos and c-myc (Cell Signalling Technology, Beverly, MA); β -actin monoclonal mouse AC-15 (Sigma); cyclin D1 polyclonal rabbit sc-246; NF- κ B p65 polyclonal rabbit (all from Santa Cruz Biotechnology). pERK, ERK, S6, p-S6, Akt, pAkt, p-c-jun, (all Cell Signaling Technologies) polyclonal rabbit. All primary antibodies were diluted 1:1000, except anti-cyclin D1 polyclonal rabbit were diluted 1:200. Secondary, horseradish-peroxidase-labelled antibodies from DakoCytomation, Denmark A/S were used at working dilutions 1:10000.

2.10 Capillary-like tube formation assay

Angiogenesis slides (Ibi, Martinsried, Germany) were coated with 10 μ l matrigel per well (Growth Factor Reduced, Becton Dickinson) and incubated for 30 min at 37°C to allow gelling. Endothelial cells were detached by trypsin treatment and centrifuged for 5 min at 300 g. The pellet was washed once with PBS and adjusted to 1×10^5 cells per ml in EBM-2 medium containing ascorbic acid and hydrocortisone according to the instructions of the manufacturer as well as 10 ng/ml VEGF. Subsequently, cells were incubated with rising Dtx concentrations for 20 h. Cells were stained with 1 μ M calcein (Sigma) and micrographs of fluorescent cells were taken at 4-fold magnification using a Nikon Eclipse Ti, the FITC filter set of the instrument and a Nikon Digital Sight DS-Fi1C camera. Tube formation was quantified as described by Guidolin et al. [27] using the Cell Profiler Software Package [28]. Briefly, images were converted into binary images by thresholding as described by Otsu [29]. Areas with an extension of more than 125 μ m in one direction were considered as tubes and selected for analysis, smaller areas were discarded. A single pixel topological skeleton representing the tubular network was constructed and network length was calculated by multiplying the pixel count with a scaling factor representing microns per pixel. All experiments were carried out in triplicates.

2.11 *In vitro* wound healing assay

To investigate the effect of Dtx on VEGF-induced endothelial cell migration, scratch assays were performed. Therefore, HUVEC cells were seeded in 24-well culture plates and grown to 90% confluence. Afterwards, in the centre of the monolayer a denuded zone (gap) of constant width was scratched with a micropipette tip. Subsequently, cellular debris was washed with medium, and HUVEC cells were exposed to 10ng/ml VEGF or 1 μ M Dtx A, B and 0.25 μ M Dtx E for 12 hours. Pictures of the artificial wounds were taken at 4-fold magnification at the beginning of the experiment and after 12h drug exposure using a Nikon Eclipse Ti and a Nikon Digital Sight DS-Fi1C camera. Changes of the open area during the experiment were measured using the image J programme and statistical significances were evaluated using the Student's t-test.

2.12 Electrophysiological measurements

Ionophoric properties were studied on CaCo-2 cells at room temperature as previously described [30, 31]. Briefly, currents were recorded with the patch clamp technique in the inside-out mode [32]. Electrodes pulled of borosilicate capillaries had a tip resistance of 5-10 M Ω . The pipette and bathing solutions used were of the same composition and contained 140 mM potassium aspartate, 2 mM CaCl₂, 2 mM MgCl₂, 2 mM Na₂ATP, 10 mM HEPES, titrated to pH 7.4 with KOH. For assessing permeability of the channel for anions, potassium aspartate was replaced by an equimolar concentration of KCl. For measurement of current carried by sodium cations, potassium aspartate was replaced by NaCl. Currents carried by divalent cations were recorded with solutions consisting of 110 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES for calcium ions as the charge carrier, titrated at pH 7.4 with Ca(OH)₂. After formation of a G Ω -seal and a control period of 5 min during which no electrical activity could be observed, Dtx were added to the bathing solution (1 and 10 μ M). The wash-in phase lasted about 1 to 2 min until single channel current could be detected. The same experimental protocol (holding potential E_h of -60, -40, -20, 0, +20, +40, +60 mV) was applied as during control recordings. Electrophysiological measurements were carried out with an Axopatch-1D patch clamp amplifier (Axon Instruments, CA) at a cut-off frequency (-3 dB) of 2 kHz. Currents were filtered at 5 kHz with a dual variable filter (VBF 8, Kemo Ltd, Beckenham, Kent, U.K.), digitized via an AD converter (TL-1 interface, Axon Instruments, CA) and sampled at 5-10 kHz. Data acquisition and storage were processed directly to a PC equipped with pCLAMP 6 software (Axon Instruments, CA). Single channel analysis was performed with the ASCD software (G. Droogmans, Leuven, Belgium).

2.13 Fluorescence imaging

Caco-2 cells were loaded with 1 μ M FURA 2AM, 0.5 μ M SBFI AM or 0.5 μ M PBFI AM for the measurement of changes in intracellular Ca²⁺, Na⁺, and K⁺ concentrations, respectively. An equivalent concentration of Pluronic 20% in methanol was added at room temperature for 30-45 min. Fluorescent dye and Pluronic were procured from Molecular Probes (Leiden, The Netherlands). After washout, cells were allowed to hydrolyze the dye for 30 min to 1 h. Then experiments were performed as previously described [33]. Ratiometric measurements were realized following background subtraction with the Axon

Imaging Workbench 2.2 software (Axon Instruments, CA) averaging 2 frames. Results are presented as the relative change ($-F/F_0$) of the F_{340}/F_{380} signal.

3 Results

3.1 Effects of Dtx on viability and proliferation of human tumor cell models

In a first approach, we investigated the impact of the three Dtx derivatives on human cancer cell proliferation and survival. Initially, the antiproliferative effects after 24, 48 h, or 72 h total exposure time to Dtx were evaluated against the four carcinoma cell lines KB-3-1, CaCo-2, HCT116, and A549 (Tables 1a-c). With regard to the tested cell lines of different tissue origin, the non-small cell lung cancer cell line A549 displayed distinct Dtx resistance compared to the three other cell lines. In contrast, the colon carcinoma cell lines HCT116 and CaCo-2 were most sensitive to Dtx exposure. Overall, for Dtx A and B at least 48 h were necessary to induce anticancer activity. For Dtx E minor cytotoxic effects were already detected after 24 h and at lower concentrations. Generally, in all tested cell lines Dtx E was more active compared to the other two Dtx derivatives. While Dtx A and B showed cytotoxic activity in the micromolar range, Dtx E was already active at low nanomolar concentrations. To further determine whether cells can recover from Dtx exposure, one representative cell line (HCT116) was exposed to varying Dtx concentrations for 6 h, 24 h, and 48 h, respectively. The substance was then washed out, and the cells were re-incubated in fresh media and viability was measured 72 h after starting treatment. A significant loss of viable cells could be observed after 24 h Dtx exposure followed by recovery to 72 h (Fig. 2A). The cell survival rate was reduced to about 60% and 75% at 10 μ M Dtx A and B, respectively, and 30% at 250 nM Dtx E. This antiproliferative effect was more prominent after 48 h Dtx exposure indicated by IC_{50} values of 6.14 μ M, 3.64 μ M, and 67 nM for Dtx A, B, and E, respectively. Interestingly, short-term Dtx exposure (6 h) slightly but significantly enhanced the number of viable cells measured 72 h following the start of drug exposure. Colony forming experiments using longer exposure (up to 192 h/8 days) and recovery times revealed similar results (Fig. 2B). Treatment of single tumor cells with Dtx A and B distinctly reduced colony formation in a dose- and time-dependent manner. Dtx E exposure resulted already after 24 h in almost full activity. Taken together, these results suggest that Dtx-induced cytotoxicity could not be reversed by a longer recovery time.

3.2 Activation of the intrinsic apoptosis pathway by Dtx

Since the tested Dtx reduced the number of viable cells, we further intended to investigate the underlying molecular mechanisms. As a first step, morphological changes of Dtx-treated HCT116 cells were examined. Using DAPI stainings, the nuclear morphology of control and Dtx-treated cells was compared. While 24 h Dtx treatment showed no effect (data not shown), 48 h Dtx exposure induced a significant increase of characteristic features of early apoptosis like chromatin condensation, or apoptotic body formation (about 11-fold at 10 μ M Dtx A, B and 17-fold at 0.5 μ M Dtx E higher as compared to the control) (Fig 3A, lower right panel). Additionally, a minor increase of PI-permeable cells indicative for late apoptotic and necrotic cells (about 2.7-fold at 10 μ M Dtx A and B, and 4.1-fold at Dtx E) was found. Moreover, 48 h Dtx treatment induced significant mitochondrial membrane depolarisation which is a critical step in the mitochondria-dependent apoptotic pathway (Fig.

3B). The number of cells with collapsed mitochondrial membrane potential increased from 8% in control samples to 16%, 19%, and 22% after treatment with 10 μ M Dtx A-, B- and 0.5 μ M Dtx E, respectively. In contrast, 24 h Dtx exposure did not impair the mitochondrial membrane potential. However, when using Western blot analyses slight modifications of specific apoptosis markers were already observable after 24 h treatment (Fig. 3C). Thus, a weak cleavage of poly (ADP-ribose) polymerase (PARP) was found for all three Dtx being accompanied by a minor loss of the respective uncut molecule. Remarkably, for Dtx E in addition also a concentration-dependent increase of cleaved caspase-7 could be shown. Interestingly, the expression levels of the pro-apoptotic bax protein slightly decreased while the anti-apoptotic bcl-xL protein remained widely unchanged. Together, these data suggest that especially Dtx E leads to unexpected activation of apoptotic caspases even before mitochondrial membrane depolarisation.

3.3 Impact of radical scavenging on Dtx E activity

Since Dtx E is the only derivative containing an epoxy group (compare Fig. 1) we hypothesised that the higher efficacy of Dtx E observed in the previous experiments might be based on radical stress induction. Consequently, to investigate whether reactive oxygen species (ROS) are involved in the cytotoxic activity of the diverse Dtx derivatives the established radical scavenger NAC was used. While 30 min pre-treatment with NAC had no significant influence on the cytotoxic activity of Dtx A and B, significant protective effects were detected for Dtx E in KB-3-1 as well as in HCT116 p53 wild-type cells and the respective subline with deleted p53 (Fig. 4A and B). Accordingly, depletion of the intracellular GSH pools by pre-treatment with the specific GSH synthesis inhibitor BSO enhanced the cytotoxic potential of Dtx E in CaCo-2 cells and the two HCT116 cell models (Fig. 4C). In contrast, BSO showed no effects on cytotoxicity of Dtx A and B (data not shown). The oxidative properties of Dtx E were further confirmed by the DHE assay. In this method, DHE can be oxidized in the presence of superoxide radicals to ethidium that intercalates into DNA and emits an intense red fluorescence [24]. Thus, in KB-3-1 cells treated with 250 nM Dtx E the ethidium-emitted fluorescence increased about 50% compared to control indicating superoxide formation by Dtx E (Fig. 4D). Remarkably, this effect was not enhanced at higher Dtx concentrations. Dtx A and B did not show superoxide radical induction. Summarizing, these results indicate that Dtx E but not the derivatives A and B exerts pro-oxidative properties. Accordingly, Western blot analyses of Dtx E-treated HCT116 cells revealed a moderate upregulation of p53 which is known to play a critical role in oxidative stress response (Fig 4E). Consistent to the protective effects of NAC obtained in cell viability assays, co-treatment with 2.5 mM NAC showed a profound down regulation of Dtx E-induced p53 as well as p21 expression. Consequently, these data suggest that the higher anticancer activity of Dtx E compared to the two other derivatives is based on ROS induction.

3.4 Impact of Dtx on cell cycle distribution

In order to investigate whether Dtx exposure leads to alteration in cell cycle distribution, flow cytometric analyses were performed in KB-3-1 and CaCo-2 cells. After 24 h drug exposure, a concentration-dependent reduction up to a complete loss of cells in G₂/M phase was observed in both cell lines (Fig. 5). Correspondingly, a concentration-dependent

accumulation of CaCo-2 cells in G₀/G₁ phase was detected (Fig. 5A). At the highest Dtx A, B and E concentrations the proportion of cells in G₀/G₁ cell cycle phase raised by 14%, 15% and 17%, respectively. Prolonged drug exposure (48 h) did not enhance this cell cycle arresting effect. Different results were obtained for KB-3-1 cells. At the highest test concentration all three Dtx led to a 9% increase of cells in G₀/G₁ phase while number of cells in S phase raised by about 9%, 31% and 32% after Dtx A, B and E treatment, respectively (Fig. 5B). Again, prolonged drug treatment (48 h) did not change these results substantially.

3.5 Impact of the cell cycle- and apoptosis-regulating proteins p53, p21, bax, and bcl-2 on Dtx activity

To gain deeper insights into the mechanisms of Dtx-induced cell death, HCT116 cells with disrupted p53, p21 or bax genes as well as A549 cells with stable bcl-2 overexpression as a consequence of gene transfection were incubated with the three Dtx derivatives for the indicated time points (Table 2a-c). While in cell viability assays p53 and bcl-2 showed only a minor impact on Dtx-induced cytotoxicity, the pro-apoptotic bax was at least in 72 h drug exposure experiments weakly but significantly contributing to the sensitivity against all Dtx. This was indicated by a 1.6-fold higher sensitivity of HCT116 bax(+/+) cells compared to HCT116 bax(-/-) cells. Moreover, HCT116 cells with deleted p21 responded significantly stronger (2.7-fold) to all Dtx derivatives compared to the respective parental cell clone already after 24 h drug exposure. Since the cyclin-dependent kinase inhibitor p21 is a negative cell proliferation regulator, ³H-thymidine incorporation assays were performed. As shown in Figure 6A, all Dtx derivatives led to a significant and concentration-dependent reduction of DNA synthesis in the HCT116 p21(+/+) cell line. These cytostatic effects were significantly weaker in HCT116 p21(-/-) cells. p21 inhibits DNA replication by preventing cells from exiting G₁ phase [19]. Thus, flow cytometric analyses of PI-stained cells were used to investigate the effect of p21 on the Dtx-induced cell cycle alterations. Interestingly, as compared to KB-3-1 and CaCo-2 cells (Fig. 5), Dtx exhibited completely different effects on the cell cycle distribution of HCT116 colon cancer cells as indicated by a S-phase arrest which was independent from the cellular p21 (Fig. 6B). A higher proportion of cells in subG₀/G₁ phase was found in Dtx-treated p21-deleted cells, correlating well their higher sensitivity in viability assays. Consistent with the results of cytotoxicity assays, no major impact of the p53 status on overall cell cycle distribution pattern were seen. Like HCT116 p21(+/+) cells the HCT116 p53(+/+) and (-/-) both tended to accumulate in S-phase after 24 h drug exposure (Fig. 6C) as well as after prolonged drug treatment of 48h and 72 h (data not shown). Accordingly, Western blot analyses revealed no significant induction of p53 or its downstream target p21 after 24 h Dtx A and B treatment (Fig. 6D). However, a pronounced increase of both investigated cell cycle proteins was detectable at 0.5 μM Dtx E. This is consistent with the results from the viability assay showing a higher sensitivity of the 24 h Dtx E-treated HCT116 p53 (-/-) compared to p53 (+/+) cells (Table 2c). As this effect was not obvious at prolonged Dtx E treatment (48 h and 72 h) an impact of p53 and p21 only at an early stage of Dtx E action (Table 2c) might be suggested. Interestingly, a p53-independent down-regulation of the G₁/S-specific cyclin-D1 and c-myc, the latter representing another protein which is suggested to be involved in the choice of cell fate between cytostasis and apoptosis [34], was observed.

3.6 Dtx interfere with the MAPK and/or PI3K/Akt signalling pathways

Very recently Yeh et al. [14] reported that Dtx B impairs the mitogen-activated protein kinase (MAPK) and phosphatidyl 3-kinase/Akt (PI3K/Akt) pathway, both well-known to be involved in cancer development [35]. With regard to the MAPK pathway, in our hands Western blot analyses of HCT116 cells revealed no changes of the expression as well as phosphorylation levels of ERK after 24 h Dtx A and B treatment (Fig 7A). In contrast, Dtx E led to a moderate down regulation of both parameters already at 0.5 μ M, while the proto-oncogene c-fos and the nuclear factor kappa B (NF- κ B) p65 were slightly down regulated by all three derivatives. Dtx interference with the PI3K/Akt signalling pathway was indicated by the reduced phosphorylation of Akt and S6 after Dtx A, B, and E exposure. In contrast, the respective unphosphorylated proteins remained widely unchanged after Dtx A and B treatment but were slightly down regulated by Dtx E (Fig 7B).

3.7 Dtx inhibit proliferation and migration of human endothelial cells

Endothelial cells are key players in angiogenic processes which are crucial for the progression of solid tumors and several hematologic malignancies [36]. Thus, we further investigated the effects of the three Dtx derivatives on human umbilical vein endothelial cells (HUVEC). Cell viability assays with a 48 h Dtx total exposure time revealed a moderate cell growth inhibition at Dtx A and B concentrations $> 2.5 \mu$ M and Dtx E concentrations $> 0.25 \mu$ M (Fig 8A). This was also not significantly influenced by presence of vascular endothelial growth factor (VEGF), one of the main angiogenic factors known to induce endothelial cell growth, migration and tube formation. To further test, the effect of Dtx on VEGF-induced migration activity of HUVEC cells, *in vitro* wound-healing assays were performed using sub-toxic Dtx concentrations (1 μ M Dtx A and B, and 0.25 μ M Dtx E). While the VEGF-supplemented cells were able to close the wound area within 12 h, 25%, 30% and 76% of the initial gap remained open under Dtx A, B and E treatment (Fig 8B). These results indicate that all tested Dtx are able to block VEGF-induced endothelial cell migration with Dtx E being most effective.

3.8 Dtx inhibit capillary-like tube formation of human endothelial cells

Finally, we examined the effect of the three Dtx derivatives on the *in vitro* tube formation capacity of HUVEC as reported [27]. HUVEC were treated with VEGF in presence of 1.25–10 μ M Dtx A, B, and 0.05–0.5 μ M E, respectively. Twelve hours later, the capillary-like tube structure formation on matrigel was checked under a microscope. Representative networks of tube structures are shown in Figure 9. All three Dtx inhibited tube formation in a concentration-dependent manner, further confirming their distinct antiangiogenic activity.

3.9 Pore-forming activity of Dtx and their effect on intracellular ion concentrations

Dtx are well-known for their ionophoric properties [37, 38]. Because of their lipophilic character ionophores easily incorporate into cell membranes enabling the transport of cations across phospholipid bilayer membranes. Thus, the cellular ionic homeostasis is affected which finally may lead to cell death [33]. To further investigate, whether the anticancer activity of Dtx is based on their ionophoric properties, the patch clamp technique in the inside-out configuration was used in Caco-2 cells. After a control period of 15 min,

during which no electrical activity was observed, the addition of Dtx A, B, or E to the bathing solution induced single channel current within 1-2 min. The Dtx-induced pores conducted mono- and divalent cations with no differences in ion selectivity between Na^+ , K^+ and Ca^{2+} . Also conductivities did not differ significantly from each other (Dtx A: 16.5 ± 0.5 pS, $n=3$; Dtx B: 17.4 ± 0.4 pS, $n=3$; Dtx E: 16.4 ± 1.2 pS, $n=7$), and single channel kinetics were similar for all tested Dtx. Time constants for the open (τ_O) and closed (τ_C) states were independent on holding potentials (Table 3). Open state probability was independent of voltage and conducting ion in the range between $P_O = 0.12$ and 0.36 , thereby for Ca^{2+} ions more frequently in the higher range. To prove whether the ionophoric activity of Dtx has an effect on intracellular ion concentrations CaCo-2 cells were loaded with fluorescent dyes and treated with 10, 30 and 100 μM Dtx A, B or E (Fig. 10). Consistent with the data obtained with the patch clamp technique revealing that Dtx-induced pores conduct mono- and divalent cations, the intracellular ion concentrations were changed in a dose-dependent manner. However, even at 100 μM Dtx A, B or E the increase in $[\text{Na}^+]_i$ (Fig. 10B) and the decrease in $[\text{K}^+]_i$ (Fig. 10C) was very small. Although the increase in $[\text{Ca}^{2+}]_i$ (compare Fig. 10A) was approximately 3-times higher than that in $[\text{Na}^+]_i$, the effect of Dtx on $[\text{Ca}^{2+}]_i$ was very weak as compared to other cyclohexadepsipeptidic ionophores.

4 Discussion

Dtx are bioactive agents synthesised by *Metarhizium anisopliae*. So far, they are well known for their insecticidal properties [1, 4, 5]. However, their activity against human and especially cancer cells is widely unexplored. A potent antiproliferative activity of Dtx A, B, and E was reported for L1210 and P388 murine leukaemia cells, for spleen lymphocytes [8, 39], and, very recently, for Dtx B on a panel of human colorectal cancer cells (HT29, SW480, and HCT116 cells) [14]. In accordance to these studies, also in our hands all three derivatives showed distinct antiproliferative potential against a panel of human cancer cell lines with being most effective in cancer cells of colorectal origin (IC_{50} of cell lines: $\text{HCT116} < \text{CaCo2} < \text{KB-3-1} < \text{A549}$) suggesting Dtx treatment as especially attractive for this tumor entity. In contrast, the non-small cell lung cancer cell line A549 was remarkably resistant to all tested Dtx derivatives. Notably, this cell model expresses considerable amounts of the ATP-binding cassette (ABC) transport proteins ABCC1, ABCC2, and ABCG2. Thus, we hypothesise that the relative insensitivity of A549 cells might be a consequence of ABC-transporter overexpression especially since for the cyclic hexadepsipeptides ENN and BEA also a protective effect of ABCG2 has been shown. The impact of ABC transporters on Dtx activity is matter of on-going studies [40]. Nevertheless, in all tested cell lines the effects of Dtx A and B occur at micromolar concentrations. Remarkably, Dtx E was already highly active at nanomolar levels. In addition, while for Dtx A and B 48 h treatment is necessary to exert profound cell death, at least in clonogenicity assays full cytotoxicity of Dtx E manifests already after 24 h exposure. Furthermore, our studies reveal that Dtx E is more effective in apoptosis induction. This is in accordance with data from literature, demonstrating Dtx E always as the most potent derivative [8, 39]. Notably, Dtx E contains a very reactive epoxy group where Dtx A has an alkene and Dtx B an isopropyl moiety. As epoxides are typically unstable in aqueous environments and are chemically highly reactive species, the difference in their activity might be based on the

generation of oxidative stress [4, 39]. Indeed, subsequent studies using the superoxide indicator DHE, the radical scavenger NAC, and the GSH synthesis inhibitor BSO indicated that Dtx E more severely interferes with the intracellular redox balance than the other Dtx. Additionally, the Dtx E-mediated p53 induction was reversible by the radical scavenger NAC. Together, our results suggest that the stronger activity of Dtx E is mediated through generation of reactive oxygen species. Accordingly, *Spodoptera litura* larvae treated with crude Dtx containing a mixture of Dtx A and E revealed clear indications of oxidative stress indicated by a decrease in thiol content and increased levels of oxidized glutathione (GSSG), dehydro-ascorbate, protein carbonyls as well as free radicals (hydrogen peroxide and hydroxyl radical) [41]. Considering our data, this oxidative stress observed in crude Dtx-treated *Spodoptera litura* is very likely induced predominantly by Dtx E in the used mixture.

Beside their potent cytotoxic effects in cancer cells, this study also shows that Dtx A, B and E exert promising antiangiogenic properties by inhibiting the migration and tube formation of HUVECs at non- or low-toxic concentration. Promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures play a key role in angiogenesis which is essential for tumors progression and metastasis. Thus, angiogenesis has become an attractive target for anticancer therapy [42]. Consequently, Dtx are interesting candidates for further investigation as antiangiogenic agents in anticancer therapy.

To gain more insights into their mode of action, in the here presented study it was shown that Dtx induce apoptotic cell death mainly via the intrinsic pathway in HCT116 cells. Previously, for Dtx E caspase-dependent apoptosis induction was reported in EGF-stimulated human epidermal carcinoma A431 cells while in unstimulated A431 cells Dtx E induced growth arrest [43]. Moreover, our data suggest Dtx-mediated inhibition of the phosphoinositide 3-kinase/Akt pathway indicated by decreased phosphorylation (activation) of Akt and its downstream targets ribosomal protein S6 accompanied by down-regulation of cyclin D1, the latter is associated with apoptosis and additionally cell cycle arrest [44]. This correlates well with data obtained by Yeh et al. showing profound induction of the internal apoptosis pathway and PI3K/Akt interference in another colorectal cancer cell line (HT29) by a 48 h Dtx B treatment [14]. In another study by Kobayashi et al. Dtx E was shown to induce instability of the cyclin D1 mRNA or to suppress the transcription of the cyclin D1 gene in anchorage-independently growing cells more strongly compared to anchorage-dependently growing ones. Thus, the authors suggested Dtx E as a candidate anticancer agent against cyclin D1-overexpressing tumor cells [45].

In addition, we showed that all three Dtx induce blockage of DNA synthesis and cell cycle arrest (dependent on the cell line either in G₁- or S-phase). These findings are in accordance with Odier et al., who also described cell cycle perturbations for Dtx A, B, and E in P388 cells. In their study Dtx A and B led to an increase of cells in S-phase while Dtx E induced a G₀/G₁ arrest. Interestingly, the authors suggested that cell cycle disturbance by Dtx might be based on interference with ribonucleotide reductase (RR) [39]. Correspondingly, preliminary studies in our lab using a hydroxyurea-resistant cell model show that RR-overexpressing cells respond less sensitive to Dtx exposure compared to wild-type cells (data not shown). Thus, direct or indirect RR targeting might contribute to the anticancer activity of Dtx and is matter of ongoing investigations.

However, the mechanisms underlying the Dtx-induced cell cycle arrest are widely unknown. Typically, the tumor suppressor p53 plays a pivotal role in cell cycle arrest regulation. Especially the G₁ cell cycle checkpoint is operated through the p53-dependent transcriptional response. After DNA damage, p53 induces the accumulation of the downstream target p21 which is capable of blocking the G₁-S promoting cyclinE/Cdk2 activity, thus leading to the sustained G₁ arrest. However, data obtained from vitality assays as well as from cell cycle analyses indicate that p53 is not a major regulatory factor of Dtx antiproliferative activity. In contrast, a protective effect was found in cell viability assays for the p53 downstream protein p21. Thus, cells with deleted p21 were significantly hypersensitive to Dtx. These results suggest that p21 may protect cells from Dtx-induced toxicity through activation of a transient DNA synthesis arrest allowing successful damage repair in a p53-independent manner. Comparable effects were shown for the structurally related cyclohexadepsipeptide ENN [21] or the isoprenoid antibiotic substance asochlorin [46]. In case of asochlorin the p53-independent p21 induction was related to the transcriptional repression of the oncogene c-myc which correlates well with the downregulation of c-myc expression levels after Dtx treatment obtained in our studies.

Ion segregation across biological membranes is critical for cell function. Since the anticancer activity of the structurally related cyclohexadepsipeptides ENN and BEA was in part suggested to be based on their ionophoric properties, similar effects might be suggested for Dtx. Our data reveal that all three Dtx incorporate into the cell membrane forming transmembrane spanning, cation complexing pores that conduct in the pS range with single channel kinetics in the order of milliseconds. No differences were observed with regard to ion selectivity or single channel kinetics for all three Dtx. However, although the ionophoric action of Dtx was found already at very low concentrations, the intracellular ion concentrations were only minimally affected even at high concentrations. This effect was almost negligible for Na⁺ and K⁺ and less pronounced than in experiments with beauvericin and enniatins [30, 47]. This is in accordance with the lower open probability of Na⁺- and K⁺-conducting Dtx-pores compared to BEA- and ENN-induced ones. Moreover, in comparison with BEA [33] and ENN [30], Dtx only caused a small increase in intracellular Ca²⁺ concentration, which reconciles with the smaller conductivity and faster kinetics of the Ca²⁺-conducting pores induced by Dtx. Consequently, we conclude that these minor changes in intracellular ion concentrations are -in contrast to ENN and BEA- unlikely to play a significant role in the anticancer activity of Dtx.

Taken together, our data demonstrate that Dtx A, B, and even more pronounced Dtx E exert promising antiangiogenic and potent anticancer activities especially in colorectal cancer cells. Notably, their antiproliferative activity was shown to be widely p53-independent but enhanced by p21 depletion. Since cancer cells often lack a functional p53-p21-pRb axis substances with p53-independent cytostatic/cytotoxic activities are of increasing interest. Thus, Dtx represent interesting candidates for further investigations as new anticancer drugs.

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Figure 1.
Chemical structures of Dtx A, B, and E

Figure 2.

Time dependency of the impact of Dtx on cell viability. (A) HCT116 cells were treated with the indicated Dtx concentrations. After the indicated treatment durations, Dtx solution was replaced by fresh culture medium. Vitality was measured by MTT-based survival assay 72 h after first Dtx contact and compared to cells under continuous (72h) Dtx treatment. (B) Clonogenic survival of HCT116 cells treated with the indicated Dtx concentrations was determined. After the respective treatment durations, Dtx solution was replaced by fresh culture medium. Cell colonies were visualized 8 days (192 h) after the first drug contact or

continuous treatment by crystal violet staining. Numbers of colonies are expressed as means $\pm$ SD of triplicates.

Figure 3.

Cytotoxic activity of Dtx in HCT116 cells. (A) Morphological changes of nuclei were determined using DAPI (left and upper right panel) and PI stainings (lower right panel) after 48 h treatment. Photomicrographs of DAPI stainings were taken using a 40x objective. Means $\pm$ SD were calculated from at least 500 nuclei from three independent experiments. *, **, significant ($p < 0.05$, < 0.01 , respectively) difference between Dtx-treated and -untreated cells was calculated using the Students t-test (right panel). (B) Loss of mitochondrial membrane potential was measured after 48 h Dtx treatment by JC-1 staining. Percentages of

apoptotic (green fluorescent, FL-1) cells, located in the right lower quarter, are indicated in the graph. (C) Caspase induced cleavage of PARP and caspase- 7 as well as bax and bcl-xL expression was determined via Western blotting in control cells (Co) and in cells treated 24 h with the indicated Dtx concentrations (left panel). β -actin served as loading control. Cisplatin (Cis) was used as control for apoptotic protein activation. Band intensities were quantified using the Image J program and normalized to the respective β -actin loading controls (right panel). Statistical analyses were performed by Students t-test (*, $p < 0,05$; **, $p < 0,01$, ***, $p < 0,001$).

Figure 4.

The role of ROS in Dtx-induced cytotoxicity. (A, B) Effects of 30 min pre-treatment with the radical scavenger NAC on the cytotoxic activity of Dtx in (A) KB-3-1 and (B) HCT116 p53(+/+) and HCT116 p53(-/-) cells. (C) Effects of 24 h pre-treatment with the GSH synthesis inhibitor BSO on CaCo-2, HCT116 p53(+/+) and HCT116 p53(-/-) cells were analysed after 72 h Dtx exposure by MTT-based survival assays. (D) Production of intracellular superoxide by incubation with the indicated Dtx A, B, and E concentrations was determined in KB-3-1 cells using the superoxide indicator DHE. Antimycin A (AMA) was

used as positive control. Fluorescence was measured by flow cytometry. *, **, *** significantly ($p < 0.05$ and $< 0.01 < 0.001$) different from medium control (Student's t-test). (E) The impact of Dtx exposure as single drug or in combination with NAC on the expression pattern of p53 and p21 in HCT116 p53(+/+) cells after 24 h Dtx treatment at the indicated concentrations was determined by Western blot analyses. Results are additionally quantified with the Image J program and shown as histograms with error bars. ^(a) p53 and p21 expression levels are significantly different from control cells; ^(b) p53 and p21 expression levels of Dtx and NAC cotreated cells are significantly different to the respective single Dtx concentrations (Student's t-test: *, ** $p < 0.05$ and < 0.01).

Figure 5.

Impact of Dtx on cell cycle distribution. PI staining and FACS analyses were performed on (A) CaCo-2 and (B) KB-3-1 cells after 24 h and 48 h Dtx exposure at the indicated drug concentrations. Percentages of cells in G_0/G_1 , S, and G_2/M phase of the cell cycle are indicated.

Figure 6.

Impact of apoptosis- and cell cycle-regulating proteins on Dtx activity. (A) DNA synthesis of HCT116 p21(+/+) and HCT116 p21(-/-) cells after 24 h Dtx exposure at the indicated concentrations was determined by ³H-thymidine incorporation. Data are expressed as means ± SD of triplicates. (B) Changes of cell cycle distribution in HCT116 p21(+/+), HCT116 p21(-/-), (C) in HCT116 p53(+/+) and HCT116 p53(-/-) cells after 24 h Dtx treatment at the indicated concentrations were analysed by PI staining and flow cytometry. (D) Impact of Dtx exposure on the expression pattern of apoptosis- and cell cycle-regulating proteins p53,

p21, c-myc and cyclin D1 in HCT116 p53(+/+) and HCT116 p53(-/-) cells after 24 h Dtx treatment at the indicated concentrations was determined by Western blot analyses. Cisplatin (Cis) was used as positive control and β -Actin served as loading control. Band intensities were quantified using the Image J program and normalized to the respective β -actin loading controls. Statistical analyses were performed by Students t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$: expression patterns of Dtx-treated cells are significantly different to control cells.

Figure 7.

Interference of Dtx with the MAPK and PI3K/Akt pathways. Changes of expression and/or phosphorylation levels of the indicated proteins involved in the (A) MAPK and (B) PI3K/Akt pathway after 24h Dtx exposure were analysed by Western blotting. Cisplatin (Cis) was used as positive control and β-actin served as loading control. Band intensities were quantified using the Image J program and normalized to the respective β-actin loading controls or ratios of pERK, pAkt and pS6 to the corresponding total proteins were calculated. Values are normalized to the respective untreated controls. Statistical analyses

were performed by Students t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$: expression patterns of Dtx-treated cells are significantly different to control cells.

Figure 8.

Dtx inhibit VEGF-induced HUVEC proliferation and migration. (A) Impact of Dtx on VEGF-induced HUVEC viability was determined after 48 h treatment using the MTT-based survival assay EZ4U. (B) Scratch assays were used to investigate the effect of a 12 h Dtx exposure at the indicated concentrations on VEGF-induced endothelial cell migration. Open wound area was measured using the image J programme and statistical significances were evaluated using the Student's t-test. * significantly ($p < 0.05$) different from medium control.

Figure 9.

Dtx inhibit VEGF-induced tubular structures formation in HUVEC. HUVECs were treated with VEGF alone (control) or in presence of Dtx A, B, and E for 20 h at the indicated concentrations. Tube formation assay was performed as described in Materials and Methods. Photomicrographs shown were taken at 4-fold magnification using a Nikon Digital Sight DS-Fi1C camera and presented as relative values to that of control cells from at least three independent experiments.

Figure 10.

Impact of Dtx on intracellular ion concentrations. The increase in intracellular Ca²⁺ concentration (expressed as ratio F340 nm/F380 nm) caused by 10, 30 and 100 μ M Dtx A, B, and E is shown for three representative cells in comparison to the positive control ionomycin (100 μ M). Representative for all three Dtx derivatives the effect of 100 μ M Dtx E on (B) intracellular Na²⁺ concentration and (C) on the intracellular K⁺ concentration is

demonstrated and compared to the respective positive controls gramicidin and valinomycin, respectively.

Table 1

a) Cytotoxic activity of Dtx A against different carcinoma cell models			
Cell line	24 h IC₅₀ (μM)	48 h IC₅₀ (μM)	72 h IC₅₀ (μM)
	Mean* ± SD	Mean* ± SD	Mean* ± SD
KB-3-1	>10	>10	7.63 ± 0.60
CaCo-2	>10	2.17 ± 0.05	2.18 ± 0.11
HCT116	>10	3.6 ± 0.05	2.06 ± 0.11
A549	>10	>10	>10

b) Cytotoxic activity of Dtx B against different carcinoma cell models			
Cell line	24 h IC₅₀ (μM)	48 h IC₅₀ (μM)	72 h IC₅₀ (μM)
	Mean* ± SD	Mean* ± SD	Mean* ± SD
KB-3-1	>10	>10	2.89 ± 0.39
CaCo-2	>10	2.05 ± 0.01	1.34 ± 0.04
HCT116	>10	4.72 ± 0.18	3.22 ± 0.04
A549	>10	>10	9.85 ± 0.01

c) Cytotoxic activity of Dtx E against different carcinoma cell models			
Cell line	24 h IC₅₀ (μM)	48 h IC₅₀ (μM)	72 h IC₅₀ (μM)
	Mean* ± SD	Mean* ± SD	Mean* ± SD
KB-3-1	> 0.5	0.43 ± 0.03	0.05 ± 0.00
CaCo-2	0.43 ± 0.05	0.08 ± 0.00	0.05 ± 0.00
HCT116	> 0.5	0.05 ± 0.00	0.04 ± 0.00
A549	> 0.5	> 0.5	0.22 ± 0.01

* IC₅₀ values were calculated from whole dose response curves. Values given are means ± SD from at least two independent experiments performed in triplicates.

Table 2

a) Impact of apoptosis- and cell cycle-regulating proteins p53, p21, bax, and bcl-2 on Dtx A-induced cytotoxicity			
Cell line	24 h IC ₅₀ (μM)	48 h IC ₅₀ (μM)	72 h IC ₅₀ (μM)
	Mean* ± SD	Mean* ± SD	Mean* ± SD
HCT116 p21(+/+)	>10	4.88 ± 0.08	4.38 ± 0.04
HCT116 p21(-/-)	5.99 ± 0.05 (***)	2.03 ± 0.09 (**)	1.85 ± 0.04 (***)
HCT116 p53(+/+)	>10	3.6 ± 0.05	2.06 ± 0.11
HCT116 p53(-/-)	9.46 ± 0.06	3.19 ± 0.14	2.09 ± 0.06
HCT116 bax(+/+)	>10	4.08 ± 0.1	1.49 ± 0.08
HCT116 bax (-/-)	>10	4.78 ± 0.02 (**)	2.28 ± 0.05 (***)
A549/vc	>10	>10	>10
A549/bcl-2	>10	>10	>10
b) Impact of apoptosis- and cell cycle-regulating proteins p53, p21, bax, and bcl-2 on Dtx B- induced cytotoxicity			
Cell line	24 h IC ₅₀ (μM)	48 h IC ₅₀ (μM)	72 h IC ₅₀ (μM)
	Mean* ± SD	Mean* ± SD	Mean* ± SD
HCT116 p21(+/+)	>10	4.00 ± 0.12	3.52 ± 0.13
HCT116 p21(-/-)	5.34 ± 0.00	1.43 ± 0.01 (***)	0.98 ± 0.07 (***)
HCT116 p53(+/+)	>10	4.72 ± 0.58	3.22 ± 0.04
HCT116 p53(-/-)	>10	3.01 ± 0.70	2.98 ± 0.21
HCT116 bax(+/+)	>10	4.31 ± 0.05	2.35 ± 0.03
HCT116 bax(-/-)	>10	5.71 ± 0.11 (***)	3.75 ± 0.05 (***)
A549/vc	>10	>10	9.85 ± 0.01
A549/bcl-2	>10	>10	9.03 ± 0.15
c) Impact of apoptosis- and cell cycle-regulating proteins p53, p21, bax and bcl-2 on Dtx E- induced cytotoxicity			
Cell line	24 h IC ₅₀ (μM)	48 h IC ₅₀ (μM)	72 h IC ₅₀ (μM)
	Mean* ± SD	Mean* ± SD	Mean* ± SD
HCT116 p21(+/+)	0.27 ± 0.04	0.1 ± 0.02	0.08 ± 0.00
HCT116 p21(-/-)	0.12 ± 0.01 (**)	0.06 ± 0.01 (**)	0.04 ± 0.00 (***)
HCT116 p53(+/+)	> 0.5	0.05 ± 0.00	0.04 ± 0.00
HCT116 p53(-/-)	0.11 ± 0.00	0.06 ± 0.00	0.04 ± 0.00
HCT116 bax(+/+)	> 0.5	0.06 ± 0.00	0.04 ± 0.00
HCT116 bax(-/-)	> 0.5	0.07 ± 0.00	0.07 ± 0.00 (**)
A549/vc	> 0.5	> 0.5	0.22 ± 0.01
A549/bcl-2	0.49 ± 0.01	> 0.5	0.27 ± 0.02

* IC₅₀ values were calculated from whole dose response curves. Values given are means ± SD from at least two independent experiments performed in triplicates.

(**, ***) significantly different from parental cells ($p < 0.01$, 0.001; Student's t-test)

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Table 3
Single channel kinetics of Dtx-induced pores

Time constants	τ_o		τ_c	
E_h	-60 mV	+60 mV	-60 mV	+60 mV
Na⁺				
Dtx A	3.6 ± 0.3	3.8 ± 0.5	7.7 ± 3.1	4.6 ± 0.7
Dtx B	3.4 ± 0.1	3.0 ± 0.3	5.5 ± 0.6	5.7 ± 0.5
Dtx E	2.8 ± 0.3	2.9 ± 0.3	4.8 ± 0.8	5.5 ± 0.8
K⁺				
Dtx A	4.2 ± 0.4	3.2 ± 0.5	9.0 ± 2.5	9.6 ± 2.9
Dtx B	2.9 ± 0.7	2.8 ± 0.6	4.4 ± 0.1	6.1 ± 1.9
Dtx E	4.0 ± 0.6	2.9 ± 0.2	7.5 ± 1.2	6.5 ± 0.5
Ca²⁺				
Dtx A	3.1 ± 0.4	4.1 ± 0.4	7.1 ± 1.9	6.4 ± 1.7
Dtx B	4.4 ± 0.7	3.9 ± 0.1	4.9 ± 0.3	9.3 ± 2.7
Dtx E	3.5 ± 0.5	3.7 ± 0.3	6.7 ± 0.2	5.7 ± 0.3