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cyclohexadepsipeptides“

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Table of contents

1	Introduction	1
1.1	Overview	1
1.2	Current state of research.....	2
1.2.1	Enniatin B and beauvericin	2
1.2.2	Destruxin A and E	3
1.3	Calcium homeostasis	5
1.4	Calcium-mediated apoptosis	6
1.5	Calcium level-affecting substances	9
1.5.1	TMB-8.....	9
1.5.2	Thapsigargin.....	10
1.5.3	Ryanodine	11
2	Aim of this thesis	12
3	Materials and methods	13
3.1	Test substances	13
3.2	Cell line and culture medium	15
3.3	Cultivating of the cells	15
3.3.1	Thawing frozen cells	15
3.3.2	Subculturing adherent cells.....	15
3.3.3	Determination of the cell count.....	16
3.4	EZ4U cytotoxicity assay	18
3.4.1	Principle of the EZ4U assay.....	18
3.4.2	Procedure	18
3.4.3	Test evaluation	19
3.5	DAPI staining	21
3.5.1	Principle of DAPI staining	21
3.5.2	Procedure	21
3.5.3	Imaging cells.....	22
3.5.4	Morphological alterations in cell nuclei during mitosis and apoptosis	22
4	Results	24
4.1	Results of EZ4U assay	24
4.1.1	Impact of TMB-8	25
4.1.2	Impact of thapsigargin.....	29
4.1.3	Impact of ryanodine	33
4.2	Results of DAPI staining.....	37
4.2.1	Cytotoxicity of mycotoxins.....	37

4.2.2	Impact of TMB-8	39
4.2.3	Impact of thapsigargin.....	41
4.2.4	Impact of ryanodine	43
5	Discussion.....	45
6	Conclusion and future prospects	48
7	Abstract.....	50
8	References.....	52
9	List of figures.....	64
10	List of abbreviations	66

1 Introduction

1.1 Overview

Enniatin B (Enn), beauvericin (Bea), destruxin A (Dtx A) and destruxin E (Dtx E) are secondary metabolites which are related in terms of structure, occurrence and effects. They are mycotoxins which belong to the group of cyclic hexadepsipeptides (Desjardins, 2006; Pašs et al., 1981; Suzuki et al., 1966), which consist of alternating amide and ester bonds. Enn and Bea are predominantly produced by *Fusarium* species (Audhya and Russell, 1974; Logrieco et al., 1998), Dtx A and E by *Metarhizium anisopliae* (Kodaira, 1961; Pašs et al., 1981). Enn and Bea are also known to frequently occur in contaminated food and grain (Covarelli et al., 2015; Fotso et al., 2002; Jestoi et al., 2004; Logrieco et al., 2002; Munkvold et al., 1998; Uhlig et al., 2006), so that their effects on the human body should not be disregarded.

Many studies investigated the biological activities of these mycotoxins: Their insecticidal, antibacterial, phytotoxic and ionophoric character has widely been studied (for review, see Escrivá et al., 2015 and Pedras et al., 2002). In recent years, their cytotoxicity was brought into focus. The comparison of their effect in both cancer cells and non-malignant cells has shown that the former are more sensitive to these secondary metabolites (Dornetshuber et al., 2007), making them potential candidates for cancer therapy.

Calcium (Ca^{2+}) is a second messenger which is of utmost importance for cell homeostasis. The results of many studies indicate that the properties of these mycotoxins can be traced back at least partly to a Ca^{2+} dependency (Dumas et al., 1996a; Jow et al., 2004; Kamyar et al., 2004; Ojcius et al., 1991; Samuels et al., 1988; Tonshin et al., 2010). Hence, this thesis aims to establish a connection between the cytotoxicity of the cyclohexadepsipeptides and the intracellular Ca^{2+} concentration.

1.2 Current state of research

1.2.1 Enniatin B and beauvericin

Enn was first isolated from *Fusarium orthoceras* var. *enniatinum* (later renamed *Fusarium oxysporum*) by Gäumann et al. (1947), Bea from *Beauveria bassania* by Hamill et al. (1969). Enns and Bea belong to a family of fungal N-methylated cyclic hexadepsipeptides. Both contain three residues of hydroxyisovaleric acid; with Bea, they alternate with three residues of N-methylated phenylalanine, whereas Enns consist of three N-methylated branched chains of L-amino acids as valine, leucine or isoleucine instead (Desjardins, 2006).

Several investigations have shown the substantial cytotoxicity of these fusariotoxins in various cell types (Calò et al., 2003; Calò et al., 2004; Fornelli et al., 2004; Ivanova et al., 2006; Meca et al., 2010; Meca et al., 2011; Uhlig et al., 2005). Cell death evoked by Enn and Bea is the consequence of apoptotic events, as morphological changes typical of programmed cell death were observed, e.g. DNA fragmentation and apoptotic body formation (Dornetshuber et al., 2007; Jow et al., 2004).

Their cytotoxicity is considered to result primarily from their disruption of ionic homeostasis of the cell. Cation-selective channel forming properties have been reported for both Enn (Kamyar et al., 2004) and Bea (Kouri et al., 2003). However, many intracellular pathways are relevant as well: Bea-induced cell death relies on the mitochondrial pathway of apoptosis on the basis of release of cytochrome c and activation of caspase 3 (Jow et al., 2004). In addition, the involvement of the Bcl-2 family was shown (Lin et al., 2005). Both Enn and Bea were found to activate the p38 MAPK pathway (Tedjiotsop Feudjio et al., 2010). MAPK signaling and angiogenesis inhibition are also the basis for the synergism of Enn and the tyrosine kinase inhibitor sorafenib against human cervical carcinoma (Dornetshuber-Fleiss et al., 2015). The phosphorylation of ERK, a kinase which is permanently active in several tumors, was found to be decreased; a fact that also leads to the induction of apoptotic cell death (Wätjen et al., 2009). Bea was also shown to inhibit migration and to exert antiangiogenic activity in human cell lines (Zhan et al., 2007). Furthermore, Enn was found to induce cell cycle arrest in the G0/G1 phase (Gammelsrud et al., 2012). However, oxidative stress and DNA interactions do not participate in Enn- and Bea-mediated apoptosis (Dornetshuber et al., 2009), and possible genotoxic or mutagenic properties of Enn were disproved (Behm et al., 2009).

Dornetshuber et al. (2007) observed that micromolar Enn concentrations and treatments of at least 24 hours result in apoptotic effects, whereas short-term exposure to nanomolar

concentrations promotes cell proliferation, indicating possible tumor promoting properties of fusariotoxins which enter the food chain only in traces.

Involvement of Ca^{2+} :

Although also Enn affects the intracellular Ca^{2+} -level (Kamyar et al., 2004), especially Bea-mediated cell death is associated with Ca^{2+} . It was demonstrated that intracellular Ca^{2+} plays a significant role, as the administration of a Ca^{2+} chelator prior to Bea treatment attenuates apoptotic events (Jow et al., 2004). In addition, an increased Ca^{2+} influx provoked by Bea was also reported for malignant cells (Chen et al., 2006).

1.2.2 Destruxin A and E

Dtx A was first isolated by Kodaira (1961) from *Oospora destructor*, after which this group of secondary metabolites was named. This entomopathogenic fungus was later renamed *Metarhizium anisopliae* and was also the first source of Dtx E (Païs et al., 1981). They are cyclic hexadepsipeptides consisting of one α -hydroxy acid and five amino acids. Dtx A and Dtx E exhibit the same amino acid backbone and differ in the hydroxy acid residue (Liu and Tzeng, 2012).

Metarhizium species formerly served as alternatives to synthetical insecticides and as phytotoxic agents in agriculture. In recent years, the potential of their metabolites as therapeutic agents has been recognized, especially in the field of cancer research. In 1983, Morel et al. were the first to report their cytotoxic activity, with Dtx E being the most potent compound. This property was confirmed by other studies (Dumas et al., 1994; Odier et al., 1992).

Detailed investigations of Dtxs by Dornetshuber-Fleiss et al. (2013) have revealed their promising effects: Their cytotoxic and antiproliferative properties are a consequence of intrinsic apoptosis induction and partially rely on p21, the pro-apoptotic protein Bax and inhibition of the phosphoinositide-3-kinase pathway; however, they act independently from tumor suppressor p53. Besides, they interfere with DNA synthesis and cell cycle distribution. An additional antiangiogenic activity was observed, thus Dtxs could also prevent tumors from metastasizing. It was suggested that the epoxy group of Dtx E is responsible for the generation of reactive oxygen species and subsequent disruption of intracellular redox balance, making this compound the most effective one in the induction of cell death.

Dtxs are also known to inhibit vacuolar-type H⁺-ATPase (V-ATPase) (Muroi et al., 1994). Closely correlated to this characteristic, Dtx E was shown to induce EGF-dependent apoptosis in cells that overexpress EGFR, which is a Fas/FasL-mediated process (Yoshimoto and Imoto, 2002). Moreover, V-ATPase inhibitors decrease the anti-apoptotic activities of Bcl-2 and Bcl-xL and hence are potential candidates in the treatment of malignancies that overexpress these oncoproteins (Sasazawa et al., 2009), which are frequently responsible for chemoresistance.

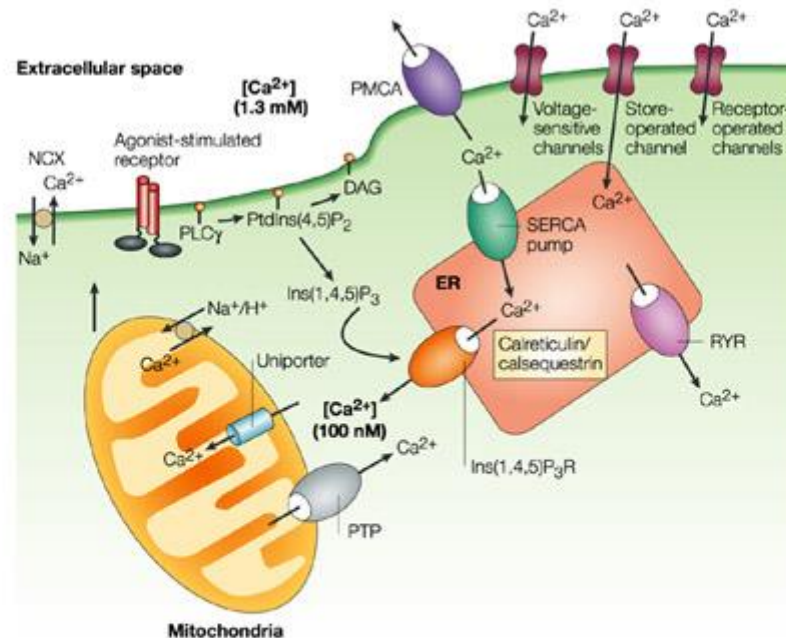
Involvement of Ca²⁺:

Dtxs induce depolarization of the muscle cell membrane and subsequent paralysis, which is abolished after treatment with nifedipine or cadmium chloride (CdCl₂), both inhibitors of Ca²⁺ channels, or prevented by using Ca²⁺-free medium (Samuels et al., 1988). Morphological changes and alterations of the cell surface can also be suppressed by administration of CdCl₂, in contrast to the influence on the nucleus (Dumas et al., 1996b). In addition, Dtx A was found to facilitate Ca²⁺ transport across the membrane by forming a sandwich complex that binds Ca²⁺ ions and translocates them to the center of the cell after incorporating into the membrane (Hinaje et al., 2002).

These findings suggest that the effects of Dtxs are strongly connected to an impact on the cell's Ca²⁺ balance. However, Dumas et al. (1996a) characterized this Ca²⁺ influx as a delayed event which possibly occurs secondary to protein phosphorylation. Other data also provide evidence that Dtxs act in a Ca²⁺-independent manner as well. For instance, inhibition of desert locust Malpighian tubule fluid secretion evoked by Dtx A could be prevented neither by Ca²⁺-free conditions, nor by Ca²⁺ channel blockers (James et al., 1993). These results support the existence of a mode of action that is based on many complex levels.

1.3 Calcium homeostasis

Ca^{2+} is a fundamental intracellular messenger which occurs ubiquitously. A cell's Ca^{2+} balance is controlled by various mechanisms.



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Figure 1: Regulation of cytosolic Ca^{2+} concentration (Orrenius et al., 2003).

Ca^{2+} ions reach the cytosol by passing voltage-sensitive, store-operated and receptor-operated channels in the membrane. In order to keep the cytosolic concentration at a low level of 100 nM, the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pumps Ca^{2+} into the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) which act as Ca^{2+} stores. There, it can be bound to calreticulin and calsequestrin. Ca^{2+} release from these stores is triggered by the stimulation of a G protein-coupled receptor and subsequent activation of phospholipase C- γ (PLC γ), an enzyme that hydrolyzes phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) into inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG). When Ins(1,4,5)P₃ binds to its receptors in the ER/SR-membrane, Ca^{2+} is released, increasing its cytosolic concentration abruptly up to 5 μM . In addition, Ca^{2+} can be released through ryanodine receptors (RYR).

The mitochondria take up Ca^{2+} through a uniporter and release it through exchange with Na⁺/H⁺, or by opening of the permeability transition pore (PTP).

The plasma membrane Ca^{2+} -ATPase (PMCA) is primarily responsible for the efflux of Ca^{2+} into the extracellular space. Another way is a Na⁺-dependent exchange.

1.4 Calcium-mediated apoptosis

As inevitable Ca^{2+} is for maintaining physiological cell function, as fatal it can be when cellular Ca^{2+} overload or disruption of intracellular Ca^{2+} compartmentalization occurs. Fleckenstein et al. (1974) were the first to discover a connection between Ca^{2+} and cell death when they observed excess Ca^{2+} entry into cardiomyocytes prior to myocardial fiber necrosis caused by ischaemia. Today, we know that Ca^{2+} can also play a crucial role in many pathways inducing apoptosis. Figure 2 gives an overview of targets that are relevant in cell death.

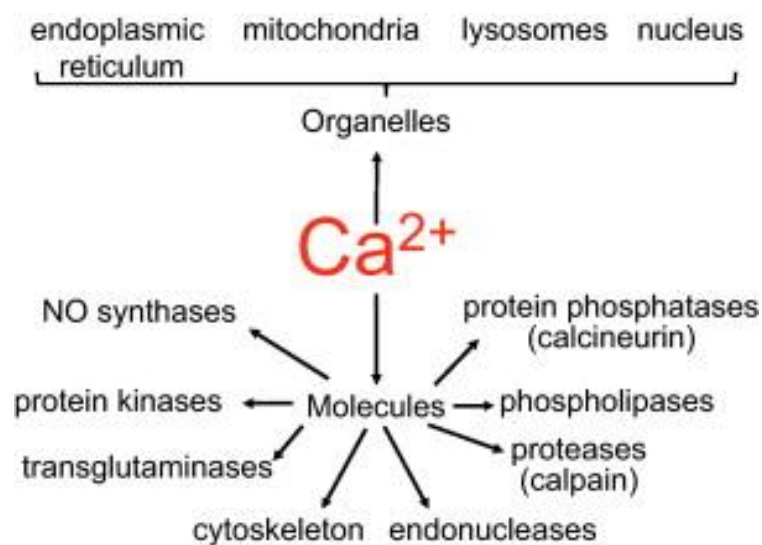


Figure 2: Intracellular targets in Ca^{2+} -mediated cytotoxicity (Zhivotovsky and Orrenius, 2011).

Some isoenzymes of *nitric oxide synthase* are stimulated by Ca^{2+} . Glutamate-mediated Ca^{2+} influx through NMDA-receptors is a consequence of increased production of nitric oxide (NO) and provokes excitotoxicity in neuronal cells (Dawson et al., 1991).

Transglutaminases catalyze reactions in a Ca^{2+} -dependent manner, involving cellular processes such as cell adhesion, regulation of the *cytoskeleton*, and finally cell death (Fesus and Piacentini, 2002).

Ca^{2+} overload also causes *endonuclease* activation and subsequent DNA fragmentation (Wyllie, 1980).

Proteases play a major role in apoptosis, especially *caspases* and the *calpain* family. The latter is a Ca^{2+} -activated cysteine protease which cleaves a multitude of cellular proteins during cell death. Its involvement in apoptosis varies dependent on the cell type (Vanags et al., 1996).

Ca^{2+} is able to activate *phospholipase A₂*, which generates arachidonic acid. Accumulation of intracellular arachidonic acid induces PTP opening and cytochrome c release and hence forces apoptosis through the mitochondrial pathway (Gugliucci et al., 2002).

Calcineurin, a Ca^{2+} -activated *protein phosphatase*, dephosphorylates Bad, a pro-apoptotic member of the Bcl-2 family, which leads to heterodimerization with Bcl-xL and thereby induces apoptosis (Wang et al., 1999).

Figure 3 gives an example of the way Ca^{2+} induces apoptosis via cytochrome c and caspases.

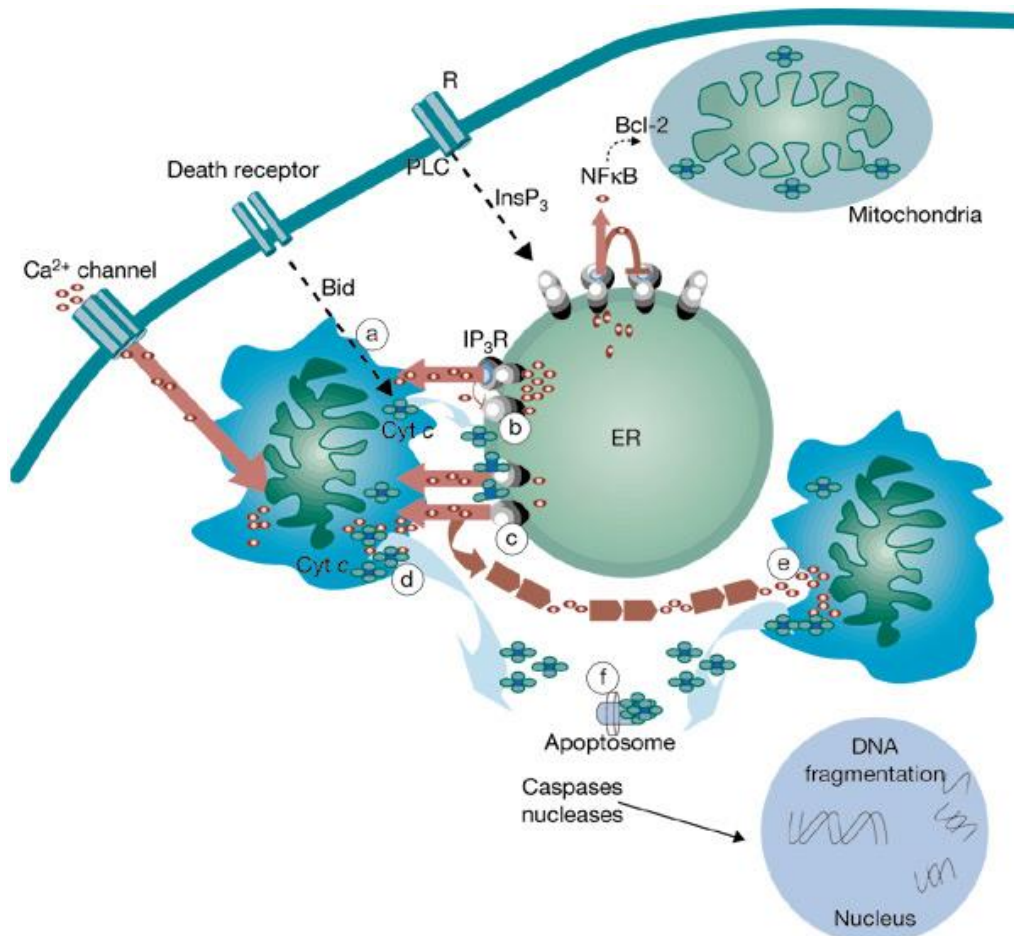


Figure 3: The role of Ca^{2+} as a messenger in apoptosis (Mattson and Chan, 2003).

Figure 3 shows the intracellular pathway of programmed cell death mediated by Ca^{2+} as established by Boehning et al. (2003). Ca^{2+} influx into a mitochondrion or activation of a death receptor located on the cell surface induces cytochrome c (Cyt c) release from that mitochondrion (a). Cyt c binds to the inositol-1,4,5-trisphosphate receptor (IP_3R) on the ER membrane (b) and thus mediates Ca^{2+} release from this intracellular store (c). The drastic increase of cytosolic Ca^{2+} concentration (d) leads to the uptake of Ca^{2+} by mitochondria and thereby to an enhanced release of Cyt c (e). This provokes formation of the apoptosome (f), a protein complex, and subsequent activation of caspases and nucleases, which accomplish

the process of apoptosis. Figure 3 also shows the involvement of Ca^{2+} in pathways of proteins responsible for cell survival, such as the transcription factor NF κ B and the anti-apoptotic protein Bcl-2.

Hence, the cell's sensitivity to apoptotic stress depends on the ER Ca^{2+} content, which is affected by many factors. For instance, a decrease in ER Ca^{2+} load and thereby protection of the cells from apoptosis is obtained by genetic ablation of calreticulin (Nakamura et al., 2000) or overexpression of PMCA (Brini et al., 2000) or Bcl-2 (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000). Conversely, cell death is facilitated by increasing ER Ca^{2+} load by overexpression of SERCA (Pinton et al., 2001), calreticulin (Arnaudeau et al., 2002) or pro-apoptotic proteins Bax/Bak (Nutt et al., 2002).

In addition, a sustained increase in intracellular Ca^{2+} level participates in the processing and release of apoptosis-inducing factor (AIF), a flavoprotein that provokes chromatin condensation and formation of large-scale DNA fragments (Norberg et al., 2008).

Elevated Ca^{2+} level in the mitochondrial matrix might induce PTP opening, which implicates mitochondrial swelling and membrane rupture.

Finally, Ca^{2+} contributes to the final step of apoptosis, namely phagocytosis. In order to be recognized by macrophages, apoptotic bodies expose phosphatidylserine (PS) on their surface. By inactivation of the PMCA, Ca^{2+} accumulates in the apoptotic bodies, whereas their content of ATP drops. The consequence of this disrupted Ca^{2+} /ATP balance is the activation of scramblase, which generates the translocation of PS (Yoshida et al., 2005).

However, one has to consider that not only a Ca^{2+} overload, but also depletion of the ER Ca^{2+} pool can harm a cell, since Ca^{2+} is essential for an enormous number of cellular processes including mitochondrial energy metabolism.

1.5 Calcium level-affecting substances

1.5.1 TMB-8

The benzoic acid derivative 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) is an established inhibitor of Ca^{2+} release from ER and SR. Based on their investigations on TMB-8 being a potent inhibitor of the contractility of smooth (Malagodi and Chiou, 1974a) and skeletal (Malagodi and Chiou, 1974b) muscles, Chiou and Malagodi (1975) were the first to describe the mechanism of action. Their observations indicate that although the Ca^{2+} uptake into SR is not altered, the quantity of Ca^{2+} released from the SR is significantly decreased, and the influx of Ca^{2+} into the cell is inhibited as well, resulting in a decreased cytosolic Ca^{2+} concentration. This makes TMB-8 a useful agent for the investigation of Ca^{2+} -dependent cellular processes.

Besides the effects on intracellular Ca^{2+} mobilization, TMB-8 shows a wide range of other properties, which have to be considered when it is used in various experiments. It also acts as an antagonist at both nicotinic (Bencherif et al., 1995) and muscarinic (Gordon and Chiang, 1989) acetylcholine receptor subtypes, inhibits the ATP-sensitive K^+ channel (Szewczyk et al., 1992) and other membrane currents (Himmel and Ravens, 1990), modifies phospholipid metabolism by inhibition of choline uptake (Palmer et al., 1992), induces tyrosine phosphorylation and dephosphorylation (Oda et al., 1995), stimulates pancreatic hormone release (Yasuda et al., 1989) and inhibits mitochondrial ATP production (Brand and Felber, 1984).

However, TMB-8 produces paradoxical effects: For instance, an elevation of cytosolic free Ca^{2+} level was also observed (Douthel and Paschen, 1999; Smallridge et al., 1991). In addition, this compound was shown to protect neuronal cells from the Ca^{2+} -dependent component of glutamate-induced toxicity, although TMB-8 itself exhibits intrinsic toxicity as well (Malcolm et al., 1996). The mechanism underlying the cytotoxicity of this agent is largely unclear. A possible explanation could be the suppression of global protein synthesis observed by Douthel and Paschen (1999), or any of the other various effects mentioned above. It has already been suggested that the behaviour of TMB-8 should be characterized before using it, as it can differ depending on the cell line (Smallridge et al., 1991). Thus, the effects concerning cell viability have to be put into question.

1.5.2 Thapsigargin

Thapsigargin (Thaps) is a sesquiterpene lactone which was first isolated from *Thapsia garganica* by Rasmussen et al. (1978). Its far-reaching properties have been investigated ever since: In addition to the histamine release in rat mast cells that was found by the original describers, it was identified as a tumor promoter in mouse skin carcinogenesis (Hakii et al., 1986). A possible participating cause could be the fact that Thaps inhibits mRNA translation, protein processing and gene expression rapidly (Wong et al., 1993). This ER stress inducer was also reported to act as a neurotoxin in perinatal rodent brain (Silverstein and Nelson, 1992) and as a blocker of the induction of long-term potentiation in rat hippocampal slices (Harvey and Collingridge, 1992).

The various activities of Thaps might result from its influence on the cell's Ca^{2+} level. It was first shown in 1985 by Ali et al. that it increased the cytoplasmic free Ca^{2+} level in platelets dose-dependently. The mechanism was studied intensively, and it was discovered that this effect is not caused by the hydrolysis of phosphoinositides and protein kinase C activation (Jackson et al., 1988), but by a selective inhibition of SERCA (Lytton et al., 1991). This pump binds two Ca^{2+} ions from cytosol and translocates them to the luminal side of SR and ER by active transport. Even at subnanomolar Thaps concentrations, the enzyme binds to both Ca^{2+} and Thaps equivalently at the absence of ATP. When Ca^{2+} dissociates due to ATP utilization, the dead-end Thaps-ATPase complex is formed and the catalytic activity of the pump is inhibited subsequently (Sagara and Inesi, 1991). By blocking SERCA, the cell loses its ability to pump Ca^{2+} into SR and ER, which serve as intracellular Ca^{2+} stores, hence Ca^{2+} is able to accumulate in the cytosol.

Since the elucidation of its mode of action, Thaps has been extensively used as a valuable agent for investigations of the ATPase mechanism and particularly for manipulations of the Ca^{2+} concentration in both cytosol and organelles, and thus for the evaluation of cellular events related to Ca^{2+} , as also in the present thesis.

The elevated Ca^{2+} level induces apoptosis, which could be taken advantage of in cancer therapy. As the non-selective effect of cytostatic drugs is a common problem, prodrugs of Thaps were designed. It was conjugated with peptides that are only substrates for prostate specific antigen (PSA) for the treatment of androgen-independent prostatic cancer (Jakobsen et al., 2001) or later with ones for prostate specific membrane antigen (PSMA). PSMA is – contrary to its name – not specific for the prostate, but is also expressed by cells of other tumors, but not by the corresponding benign tissue examples (Chang et al., 1999). One of these compounds targeted to PSMA is mipsagargin, which has been in phase II clinical trials

for patients suffering from advanced stages of hepatocellular carcinoma recently (ClinicalTrials.gov, 2016) and is therefore now expected to be launched soon.

1.5.3 Ryanodine

Rogers et al. (1948) first isolated this alkaloid from *Ryania speciosa* Vahl (belonging to the former family of Flacourtiaceae, today's Salicaceae), discovered its highly insecticidal activity, described its chemical properties and designated it ryanodine (Rya). After these observations, Rya was widely used as an insecticide, until it was replaced by synthetic compounds.

Rya targets the ryanodine receptor (RyR), which is a membrane protein occurring in many different cell types. In mammalian tissue, RyRs are classified into three isoforms: RyR1, which is predominantly expressed in skeletal muscles (Takeshima et al., 1989); RyR2, which is especially found in the cardiac muscle (Nakai et al., 1990; Otsu et al., 1990); and RyR3, also termed the “brain type” after its original tissue of identification (Hakamata et al., 1992), although it is also expressed in other cell types. These receptors play a major role in intracellular Ca^{2+} release. They serve as ion channels which are located in the membrane of SR and ER and are, together with the inositol trisphosphate receptor, responsible for the release of Ca^{2+} from these intracellular stores in their open state. Thus, RyRs affect many Ca^{2+} -related events, most notably the activation of muscle contraction by releasing Ca^{2+} from the SR.

The complexity of the interactions of Rya with RyRs has widely been studied and still leaves many questions unanswered. However, it is repeatedly confirmed that Rya binds to RyRs with high affinity and specificity and modifies the action of this channel in a concentration-dependent manner. At nanomolar concentrations, the channel is locked in its open state, whereas high concentrations of over 100 μM cause inhibition of the channel (Meissner, 1986).

As Ca^{2+} is a second messenger with far-reaching effects, mutations in the RyR genes evoke severe diseases, with malignant hyperthermia being the one which is best known (MacLennan et al., 1990).

2 Aim of this thesis

In spite of the Ca^{2+} -mediated cytotoxicity described above, unpublished data suggest that cyclohexadepsipeptides are more effective when the Ca^{2+} level is kept down. In order to confirm or disprove these paradoxical observations, this thesis deals with the question in which way substances that influence the intracellular Ca^{2+} level modify the cytotoxicity exerted by Enn, Bea, Dtx A and Dtx E.

The following objectives were included in the investigations:

- Determination of concentration- and time-dependent in vitro cytotoxicity of every compound as single agent
- Determination of concentration- and time-dependent in vitro cytotoxicity of mycotoxins in combination with TMB-8, Thaps or Rya in order to detect possible synergistic effects
- Visualization of drug-exposed nuclei in order to observe morphological alterations

3 Materials and methods

3.1 Test substances

The mycotoxins used in these experiments are:

- **Enniatin B (Enn)**, dissolved in methanol + Tween 80 (2:1)
- **Beauvericin (Bea)**, dissolved in methanol + Tween 80 (2:1)
- **Destruxin A (Dtx A)**, dissolved in dimethyl sulfoxide (DMSO)
- **Destruxin E (Dtx E)**, dissolved in DMSO

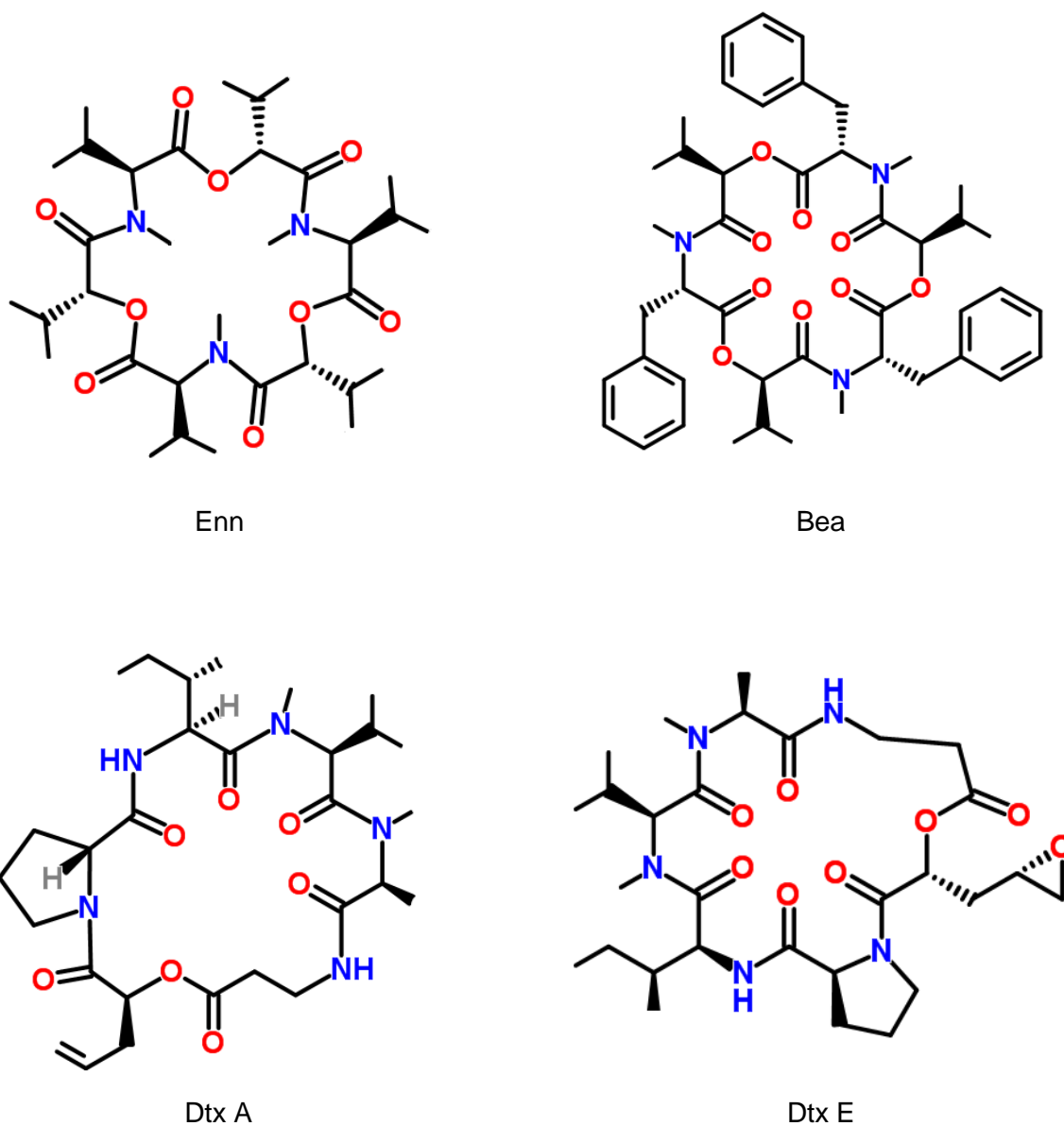


Figure 4: Structures of Enn, Bea, Dtx A and Dtx E (based on ChemSpider, 2015).

They were combined with the following Ca^{2+} level-affecting compounds:

- **8-(Diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8)**, dissolved in methanol + Tween 80 (2:1)
- **Thapsigargin (Thaps)**, dissolved in DMSO
- **Ryanodine (Rya)**, dissolved in methanol + Tween 80 (2:1)

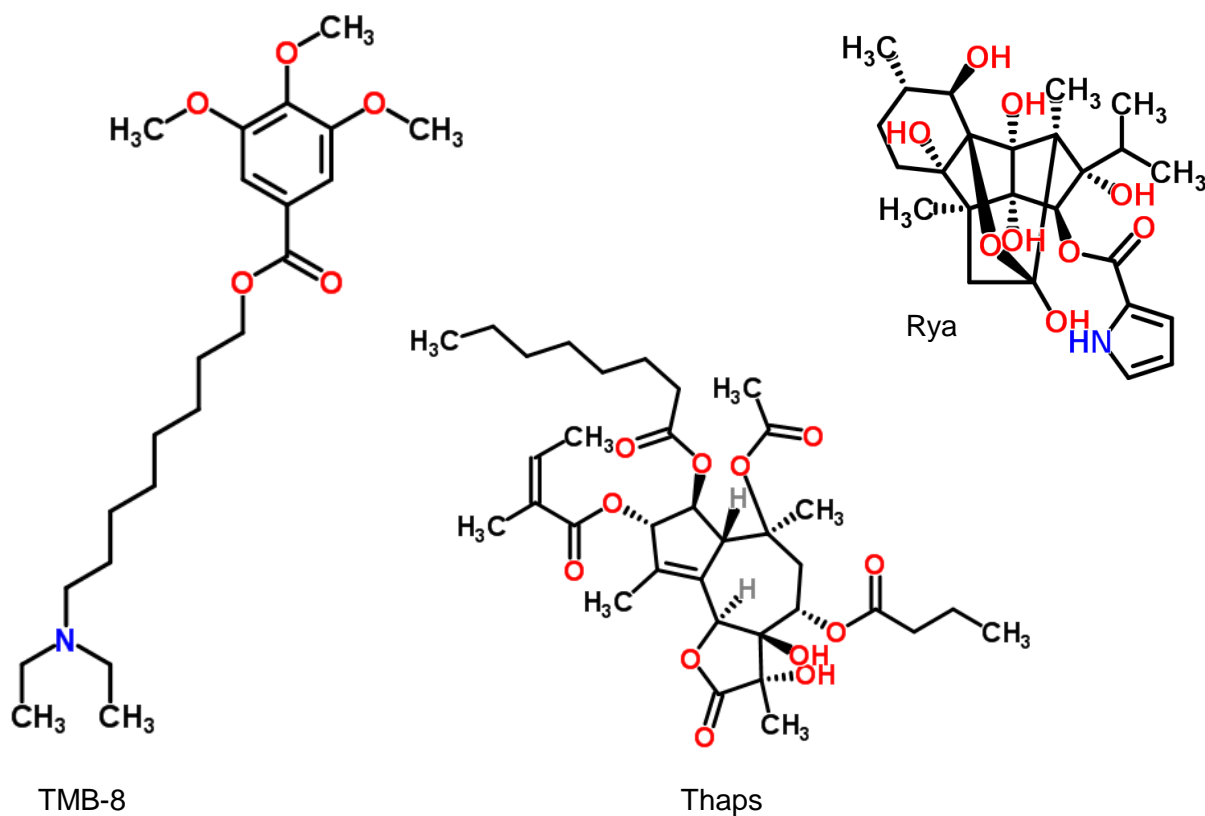


Figure 5: Structures of TMB-8, Thaps and Rya (ChemSpider, 2015).

Enn, Bea, TMB-8, Thaps and Rya were purchased from Sigma-Aldrich, Austria.

Dtx A and Dtx E were generously donated by Dr. Seger (Leopold Franzens University Innsbruck).

The solutions were stored at -20 °C.

Concentrations:

Enn, Bea and Dtx A: 0,5 µM, 1 µM, 2,5 µM, 5 µM, 10 µM

Dtx E: 0,01 µM, 0,05 µM, 0,1 µM, 0,25 µM

TMB-8: 12,5 µM, 25 µM, 50 µM

Thaps: 0,5 µM, 1 µM, 2 µM

Rya: 1 µM, 2 µM

3.2 Cell line and culture medium

The substances were tested in cells of the KB-3-1 cell line (generously donated by Dr. Shen, Bethesda, USA). Their tissue of origin was primarily an epidermoid carcinoma of the oral cavity of a male Caucasian. However, it has been proved that they contain marker chromosomes typical of HeLa cells and express type A glucose-6-phosphate dehydrogenase (G6PD), which is also characteristic of HeLa. Furthermore, the absence of the Y chromosome has been shown. Therefore, the KB-3-1 cell line is considered as a HeLa derivative and hence as a model for human cervical carcinoma cells (Lavappa et al., 1976).

The cells were cultivated in RPMI-1640 medium, which had been mixed with 10% fetal bovine serum (FBS) before (both purchased from Sigma-Aldrich, Austria). FBS is necessary in order to provide the cells with growth and adhesion factors, hormones, lipids and minerals. The medium was stored at 2 to 8 °C and was pre-warmed in a 37 °C water bath before use.

3.3 Cultivating of the cells

3.3.1 Thawing frozen cells

The cells were long-term stored in a DMSO-containing medium in liquid nitrogen or in a usual freezer at -80 °C. DMSO acts as a cryoprotective agent here. This way, the cells are preserved for a long time.

Frozen cells were thawed in a 37 °C water bath and transferred to a culture flask of 25 cm² with 5 ml of pre-warmed medium subsequently. To imitate conditions similar to the human body, the cells were maintained in an incubator which was set at 37 °C and an atmosphere of 5% CO₂. Since DMSO is harmful to the cells, the medium was changed the next day.

3.3.2 Subculturing adherent cells

To maintain cell proliferation, subculturing, also referred to as “passaging”, is necessary. KB-3-1 cells are adherent to the surface of the flask, and should always be subcultured before reaching confluency. They were detached by enzymatic means: After removing the medium and washing the cells with trypsin-EDTA (purchased from Sigma-Aldrich, Austria), 500-600 µl of trypsin-EDTA were added and the cells were incubated. After 2 minutes, detachment of the cells was checked under the microscope. If at least 90% of the cells were dissociated by now, 2-3 ml of pre-warmed medium were added to block the effect of trypsin. After dispersing

thoroughly, part of the cell suspension was transferred to a new culture flask and filled up to 5 ml with medium again. The other part was discarded.

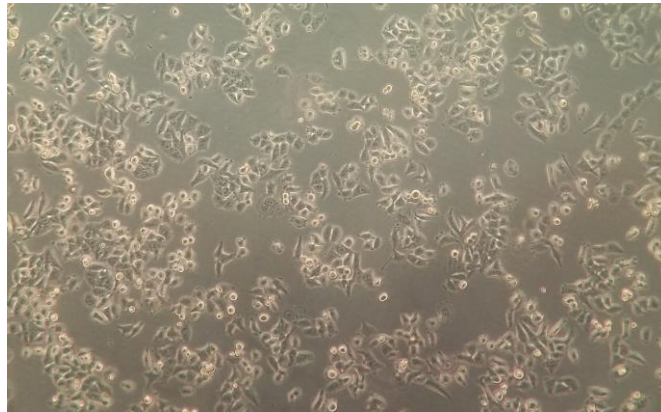


Figure 6: KB-3-1 cells as seen under the microscope.

3.3.3 Determination of the cell count

It is necessary to determine the cell count for the experiments described below. Therefore, cells were detached from the surface of the culture flask as described before and transferred to a 15 ml conical tube. After centrifuging at 1000 rpm for 5 minutes, the supernatant was removed in order to eliminate the dissociation reagent. The cell pellet was now resuspended in 5 ml medium. A sample of 10 μ l of cell suspension was mixed with 10 μ l of a 0,4% solution of trypan blue (purchased from Sigma-Aldrich, Austria) by pipetting. Trypan blue is an acid stain which can easily bind to proteins due to its anionic character. It is commonly used for assessment of cell viability, since it cannot pass through the membrane of a living cell, whereas it is absorbed by a dead cell, which appears entirely blue under the microscope (Gstraunthaler and Lindl, 2013).

10 μ l of this suspension were added to a hemocytometer. This counting chamber is divided into 3 x 3 squares (Fig. 7).

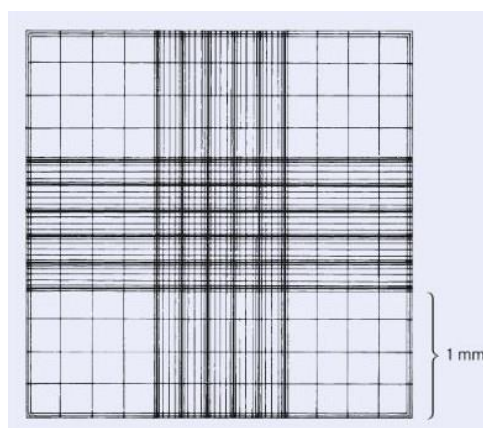


Figure 7: Arrangement of a “Neubauer improved” hemocytometer (Gstraunthaler and Lindl, 2013).

[illegible]

Cells that took up trypan blue must not be counted, as they are considered non-viable. The average of these four values was multiplied by 2, since the cell suspension had been diluted with trypan blue solution before. The value received multiplied by 10^4 makes the estimated number of cells per ml.

3.4 EZ4U cytotoxicity assay

3.4.1 Principle of the EZ4U assay

The EZ4U assay is a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, thus a nonradioactive cell proliferation and cytotoxicity assay. It is based on the reduction of uncoloured tetrazolium salts into intensely coloured formazan derivatives as an indicator of cell viability. As this process can only be done by intact mitochondria, this assay is an easy method to distinguish living from dead cells (Biomedica Immunoassays, 2016).

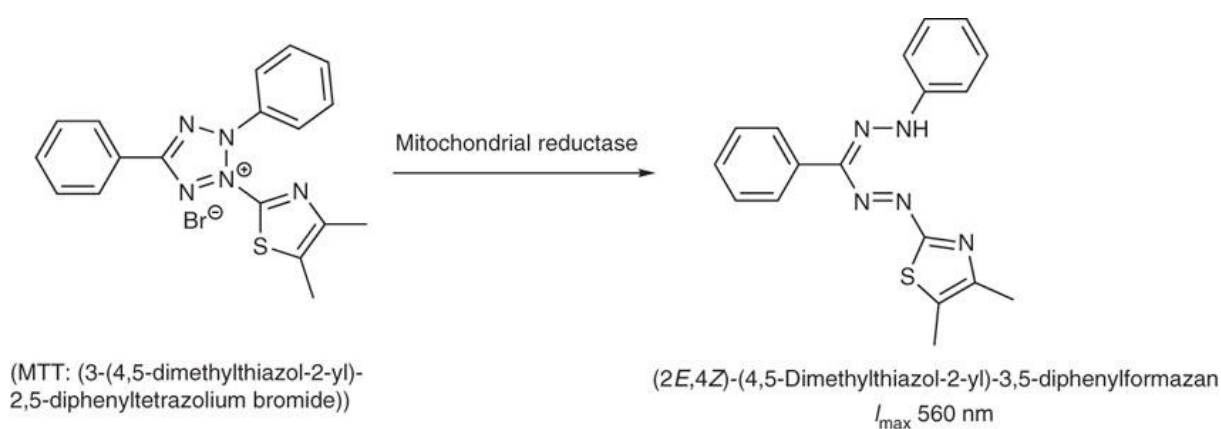


Figure 9: Principle of MTT assay (Ebada et al., 2008).

3.4.2 Procedure

Seeding cells and adding test substances:

A cell suspension with a density of 2×10^4 cells per ml was seeded in a tissue culture treated 96-well plate, pipetting 100 μ l of the homogenized suspension into each well. Only the exterior wells were filled with just medium. The plate was incubated overnight so that cells could adhere to the surface of the plate again.

The next day, the stock solutions of the test substances were diluted in order to achieve the desired concentrations and were added to the wells in a volume of 100 μ l thereafter. Thereby, cells were preincubated with Ca^{2+} level-affecting substances for 30 minutes before adding the mycotoxins. It had to be considered that the solutions would get diluted at the rate of 1:2 during this process, since each well already contained 100 μ l of cell suspension.

The first row of wells served as the control, hence only medium was added here. The different concentrations of mycotoxins respectively their combinations with TMB-8, Thaps or

Rya were tested in triplicates. Subsequently, cells were incubated for 24 respectively 48 hours.

Adding EZ4U reagent:

The EZ4U kit (purchased from Biomedica, Austria) contains vials filled with the same amount of lyophilised substrate each. The content of one vial was solved in 2,5 ml activator solution and diluted with medium at the rate of 1:10 thereafter. After the used medium and thereby also the test substances were removed from the plate, 100 µl of reagent solution were added to each well that contained cells, but also to 4 wells that were used as substrate blanks containing medium without cells. During this process, the occurrence of air bubbles had to be avoided. Afterwards, an incubation time of 2 hours followed.

3.4.3 Test evaluation

Already visible to the naked eye, a colour change occurred equivalently to the concentrations of the cytotoxic substances.

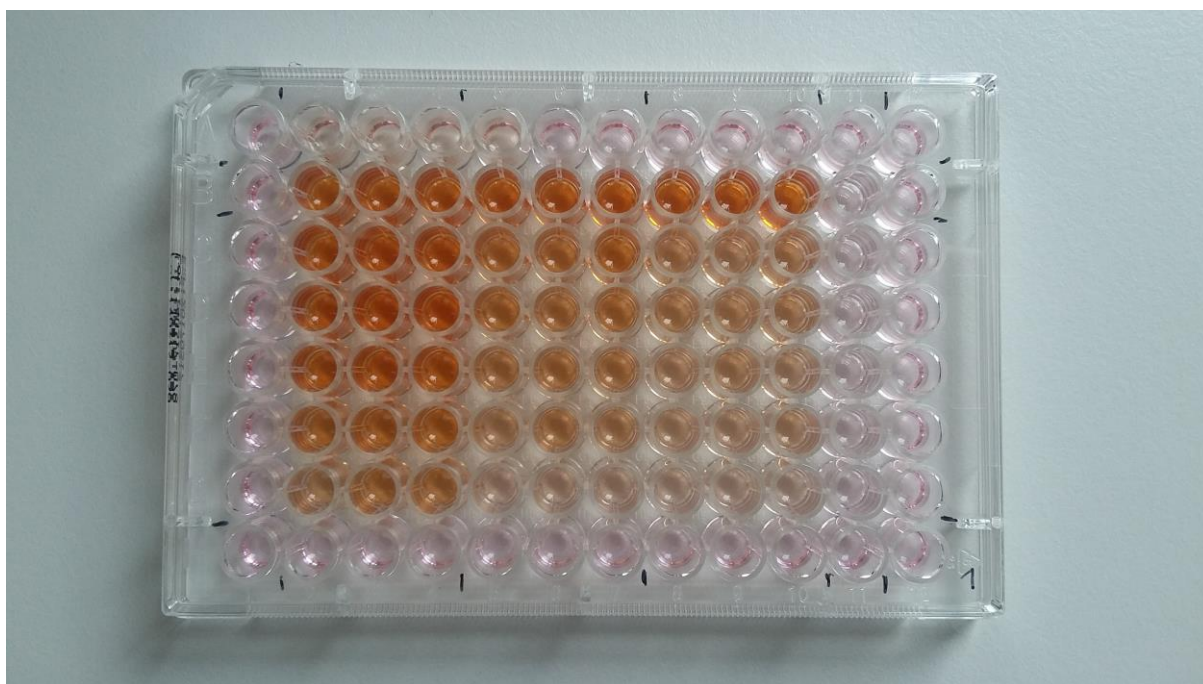


Figure 10: Colour change induced by EZ4U shows varying cytotoxicity.

The intensity of the colouring was measured in the form of absorbance by a microplate-reader set at 450 nm with 620 nm as a reference. The result was displayed in an Excel sheet. The average blank value was subtracted from all the other values, so that the

colouring of the reagent solution itself was not included in the result. The average of each triplicate was now calculated as a percentage of the control mean. The results were illustrated using SigmaPlot 9.0.

3.5 DAPI staining

3.5.1 Principle of DAPI staining

4',6-Diamidino-2-phenylindole (DAPI) is a nuclear and chromosome counterstain which was first synthesized by Dann et al. (1971), binds reversibly and preferentially to AT-rich regions of DNA (Williamson and Fennell, 1975) and thus emits blue fluorescence. DAPI and DNA interact in a non-intercalative way at two binding sites; more precisely, DAPI is located in the grooves, with the minor groove being the high-affinity site (Kubista et al., 1987).

This dye allows visualization of the nucleus in interphase and apoptotic cells and chromosomes in mitotic cells. The applications of DAPI include several types of cells (Williamson and Fennell, 1975).

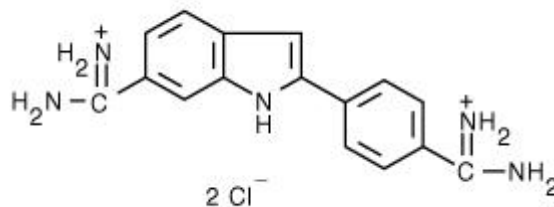


Figure 11: Structure of DAPI (Thermo Fisher Scientific, 2016).

3.5.2 Procedure

After placing a sterile cover slip into each well of a 6-well plate, 2 ml of a cell suspension with a density of 3×10^5 cells per ml were added into each well. The next day, the test substances were added and an incubation time of 24 hours followed.

After removing the medium and washing in phosphate buffered saline (PBS) with calcium and magnesium, cells were fixed on the cover slips by a 3,7% formaldehyde solution (received by diluting a 37% solution with PBS (Ca+Mg) at the rate of 1:10). Subsequently, each cover slip was placed on a drop of DAPI solution (purchased from Sigma-Aldrich, Austria) on a microscope slide, cells downwards, and fixed with transparent nail polish.

Experiments with DAPI always have to be performed in a darkroom, as the substance is light sensitive.

3.5.3 Imaging cells

Untreated controls as well as drug-exposed cells were examined under the fluorescence microscope (Olympus BX51). To ensure comparability, the cells of every sample were counted for 15 minutes and classified into interphase, mitotic and apoptotic cells. Microscopy images were taken using a 40x oil immersion objective.

3.5.4 Morphological alterations in cell nuclei during mitosis and apoptosis

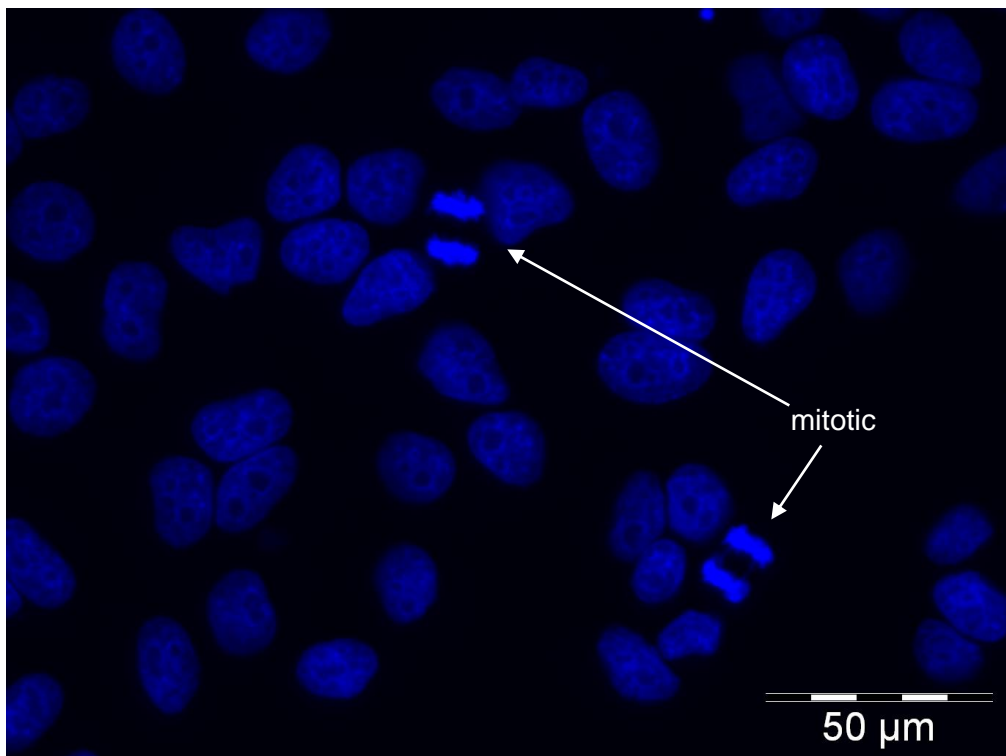


Figure 12: DAPI-stained interphase and mitotic nuclei (control).

The shape of interphase nuclei is mostly oval, and they are pictured clearly separated from each other. Chromatin is evenly distributed within the nuclei. The small, DAPI-negative “circles” within the nuclei are nucleoli, which are involved in the synthesis of ribosomes.

Mitosis is divided into several phases. In *prophase*, chromosomes densify, nuclear membrane dissolves, and the centrosomes move to the opposite poles of the cell. In *metaphase*, centrosomes form the mitotic spindle, which cleaves the two sister chromatids in *anaphase*, as seen in Fig. 12. The new nuclear envelopes are formed in *telophase*, and *cytokinesis* forms two completed daughter cells and thereby finalizes the process of mitosis (Müller-Esterl, 2011).

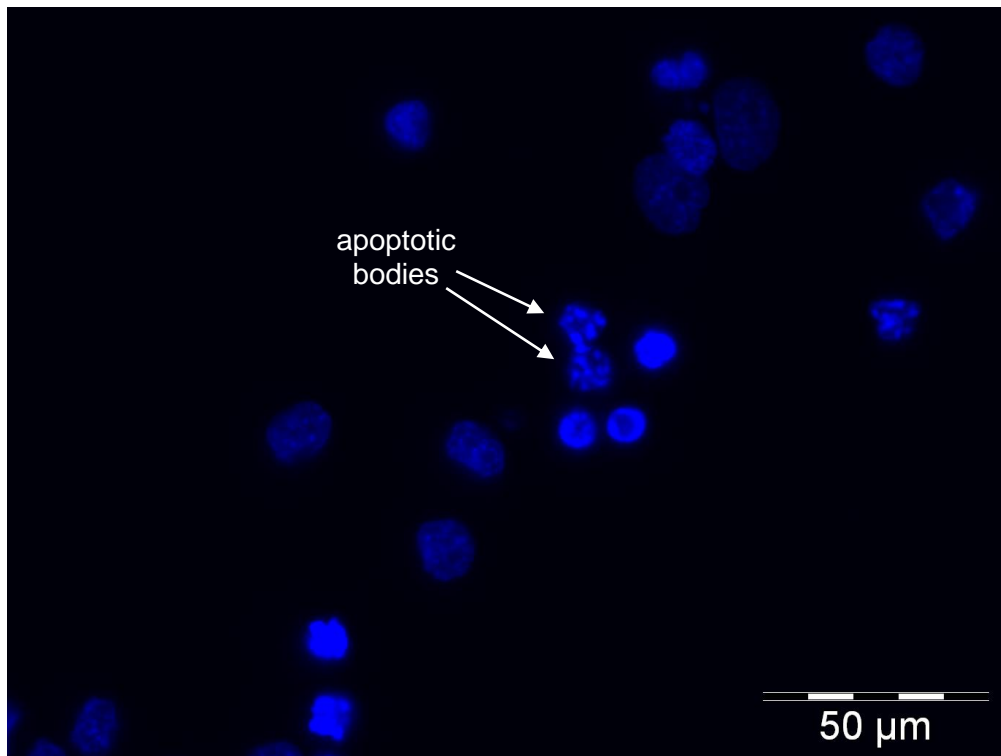


Figure 13: DAPI-stained apoptotic nuclei (Bea 5 μM + TMB-8 25 μM).

The morphological changes of apoptosis are classified into three phases. At first, there is condensation of chromatin and reduction in nuclear size and total cell volume. In phase 2, protuberances arise on the cell surface. Both nucleus and cytoplasm are separated into multiple, membrane-enclosed fragments, termed apoptotic bodies. These cell remnants are phagocytosed by neighbouring cells or macrophages. In phase 3, residual nuclear and cytoplasmic structures are degraded (Arends et al., 1990; Kerr et al., 1972).

Apoptotic bodies are well observable by DAPI staining. This fluorescence-based method enables to detect nuclear DNA condensation typical of apoptosis.

4 Results

4.1 Results of EZ4U assay

KB-3-1 cells were exposed to different concentrations of mycotoxins and Ca^{2+} level-affecting substances. The compounds were tested as single agents and in combination. Cytotoxicity was determined after 24 and 48 hours of incubation. Concentration-response curves were established in order to compare the effects of different drug concentrations and combinations.

At a mycotoxin concentration of 0 μM , the value of TMB-8, Thaps or Rya administered as single agents was plotted.

4.1.1 Impact of TMB-8

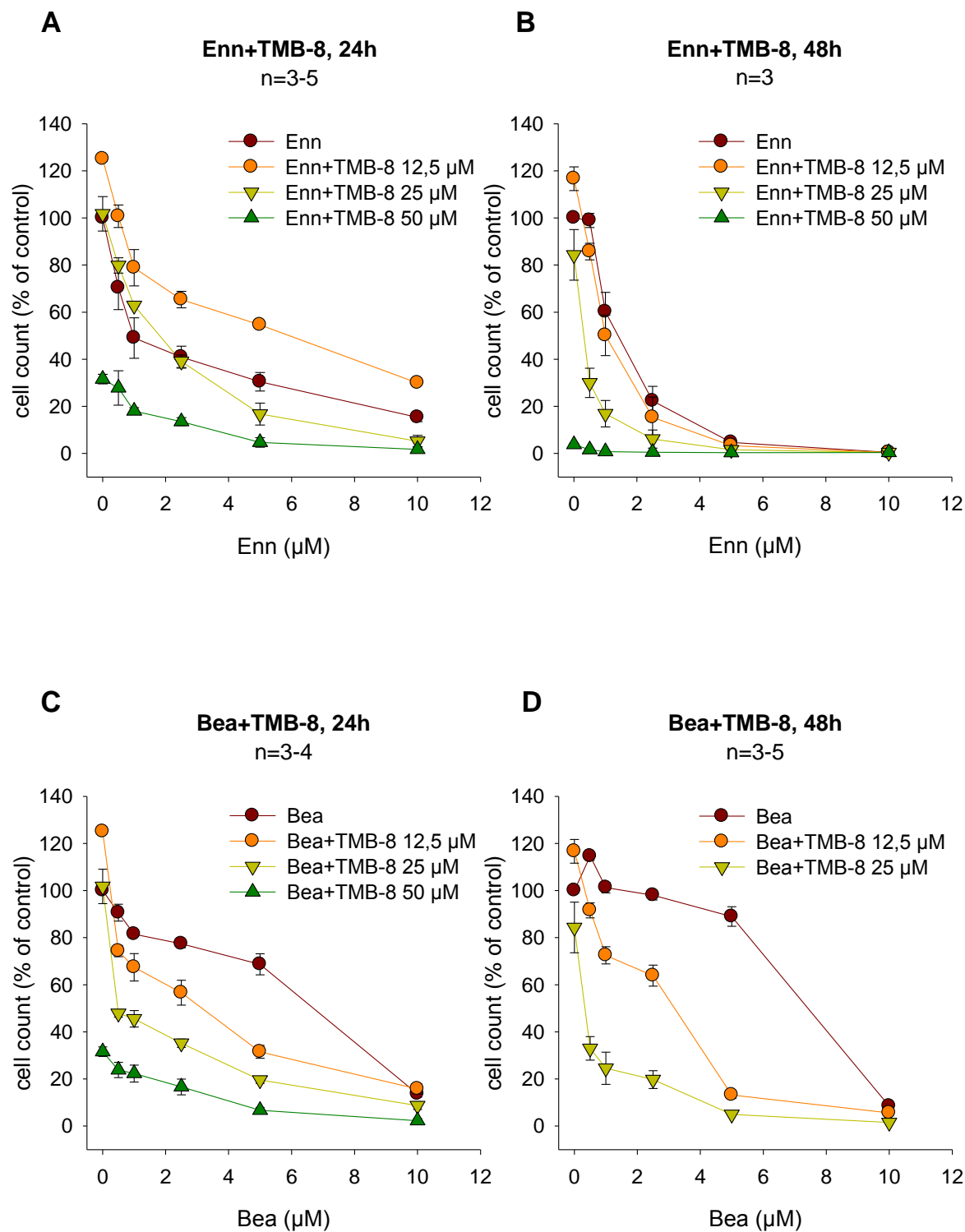


Figure 14: Concentration and time dependency of the impact of TMB-8 on the cytotoxicity of Enn and Bea.

Although Bea monotreatment ($IC_{50} > 5 \mu M$) (Fig. 14C+D) was shown to be less effective than Enn monotreatment ($IC_{50} \approx 1 \mu M$) (Fig. 14A+B), the combination of these mycotoxins with TMB-8 led to similar results. Higher dosage and longer treatment caused progressive enhancement of the cytotoxic effects of Enn and Bea. Only the co-administration of Enn and TMB-8 in the lowest concentration tested ($12,5 \mu M$) for 24 hours attenuated the effect compared to Enn monotreatment (Fig. 14A).

After 48 hours, the highest mycotoxin concentration that was tested ($10 \mu M$) reached the same effect as any combination with TMB-8, namely a cell survival rate tending towards zero (Fig. 14B+D), whereas there were still observable differences after 24 hours (Fig. 14A+C).

The combination with $50 \mu M$ TMB-8 evoked a very profound cytotoxicity especially after 48 h treatment (Fig. 14B), with a survival rate of under 1% already at a mycotoxin concentration of $1 \mu M$. Hence, this combination was not included in the following experiments any more.

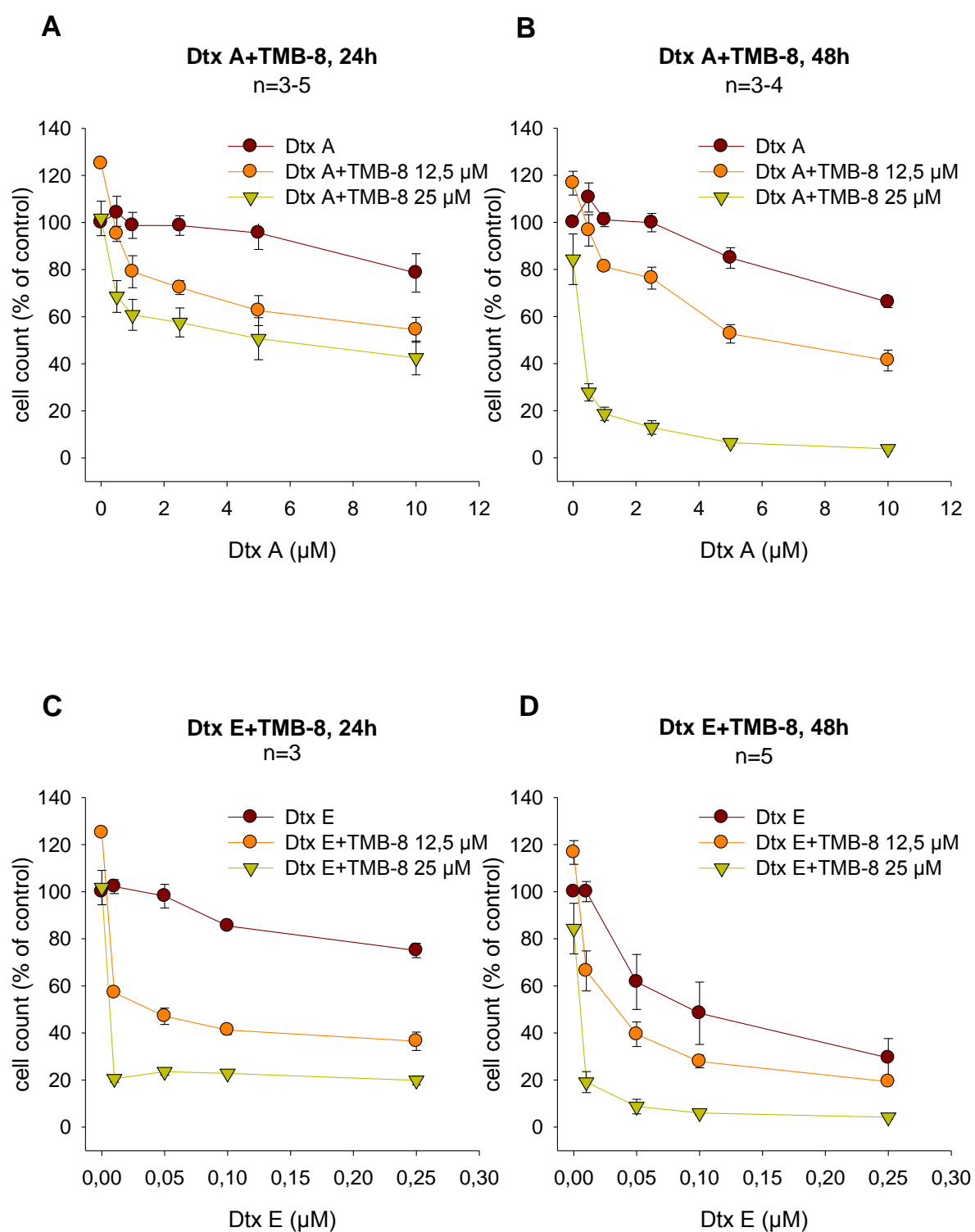


Figure 15: Concentration and time dependency of the impact of TMB-8 on the cytotoxicity of Dtx A and Dtx E.

Since Dtx E is a lot more potent than Dtx A, lower Dtx E concentrations that correlate with those of Dtx A were chosen.

KB-3-1 cells were only weakly affected by Dtxs after 24 h treatment, with approximately 80% of the cells being able to survive at the highest concentration that was tested (Fig. 15A+C). TMB-8 potentiated their cytotoxic activities in a concentration-dependent manner, however, killing all the cells was only accomplished after 48 h treatment and by administering the highest TMB-8 concentration tested (25 μ M) (Fig. 15B+D).

Co-treatment with TMB-8 was more effective with Dtx E than with Dtx A already after 24 hours. For example, treatment with 0,01 μ M Dtx E and 25 μ M TMB-8 reduced cell count to 20% (Fig. 15C), whereas the correlative combination of 0,5 μ M Dtx A and 25 μ M TMB-8 resulted in a survival rate of nearly 70% (Fig. 15A).

Compared to Enn and Bea, TMB-8 was not as potent with Dtx A and Dtx E. For instance, combination of 10 μ M Enn or Bea with 12,5 μ M TMB-8 provoked total cell death after 48 h treatment, whereas the co-administration with Dtx A or Dtx E only killed up to 60% and 80%, respectively.

4.1.2 Impact of thapsigargin

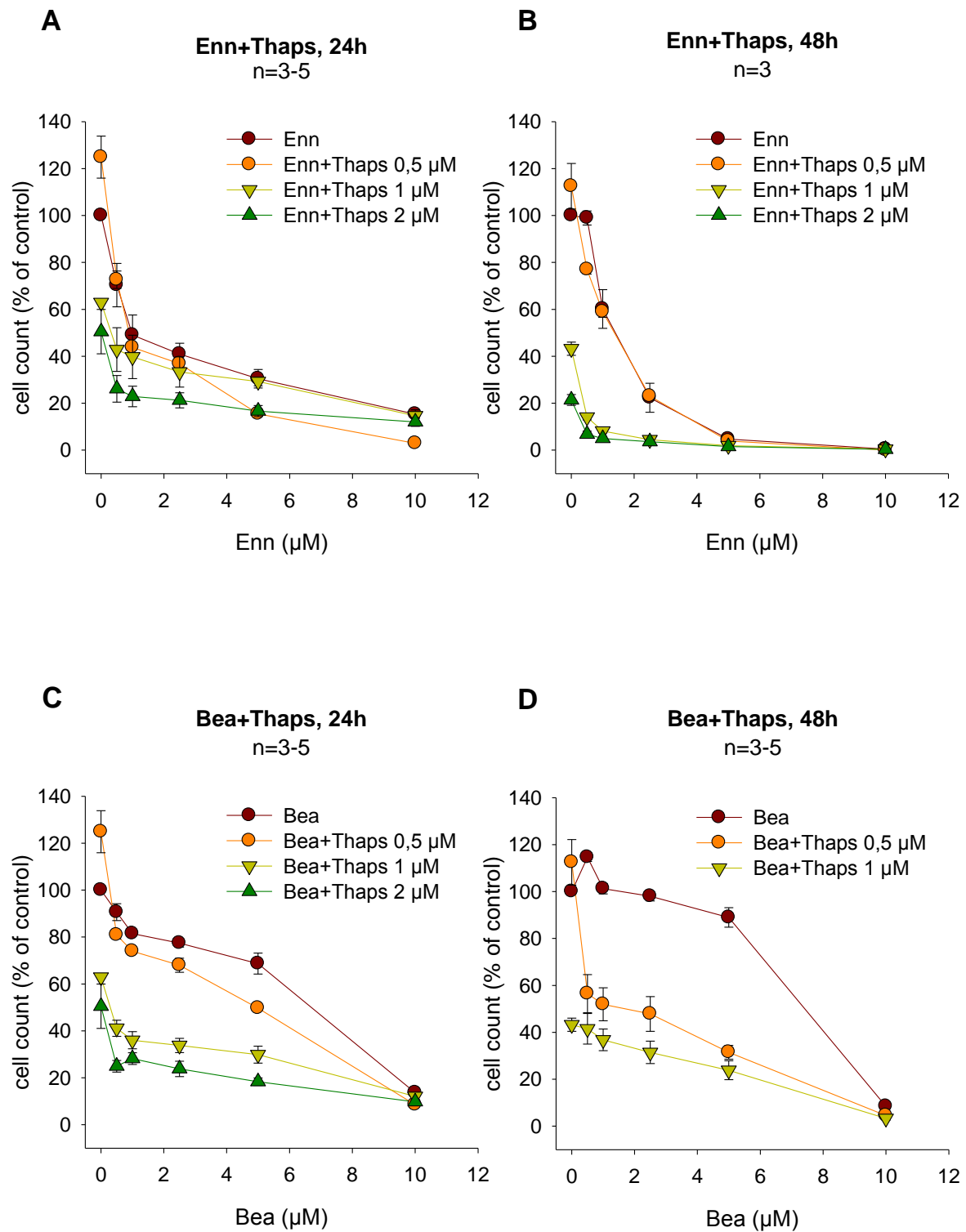


Figure 16: Concentration and time dependency of the impact of Thaps on the cytotoxicity of Enn and Bea.

Thaps increased the cytotoxic activities of Enn and Bea very similarly to TMB-8 (Fig. 16). The observed potentiation was greater with Bea (Fig. 16C+D). The administration of 10 μ M Enn/Bea led to comparable results regardless of Thaps concentration, however, lower Enn/Bea concentrations enabled a partially marked increase in cytotoxicity when combined with Thaps.

The enhancement of the cytotoxic effect mediated by Thaps was of dose-dependent nature, however, the lowest Thaps concentration tested (0,5 μ M) elicited a significant potentiation only in combination with Bea after 48 h treatment.

Similarly to TMB-8, the highest Thaps concentration tested (2 μ M) evoked such profound cytotoxicity (Fig. 16B) that only lower concentrations were included in the following experiments.

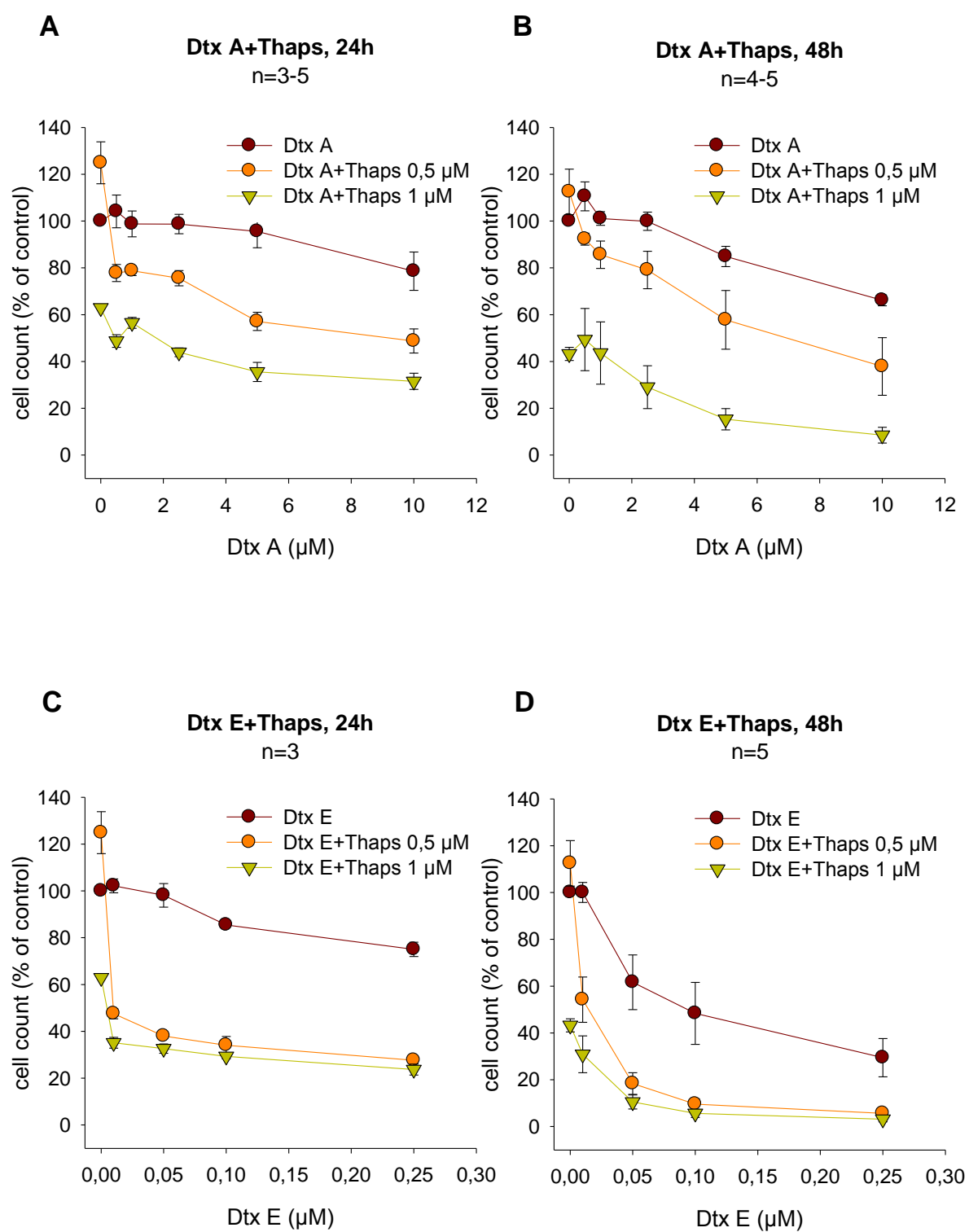


Figure 17: Concentration and time dependency of the impact of Thaps on the cytotoxicity of Dtx A and Dtx E.

Thaps also intensified the activity of Dtx A in a dose- and time-dependent manner (Fig. 17A+B): The combination with 0,5 μ M Thaps killed up to 60% of cells, whereas the double dose even increased this effect up to 90%.

However, the impact of Thaps on the cytotoxicity of Dtx E seemed to be less dependent on the used concentration. Although the co-administration of 0,5 μ M Thaps already killed up to 90% of cells and was thereby more effective than the combination with Dtx A, this activity could not be further potentiated by administering a higher concentration (Fig. 17C+D).

As with Enn and Bea, the influence of Thaps is comparable to TMB-8 to a large extent.

4.1.3 Impact of ryanodine

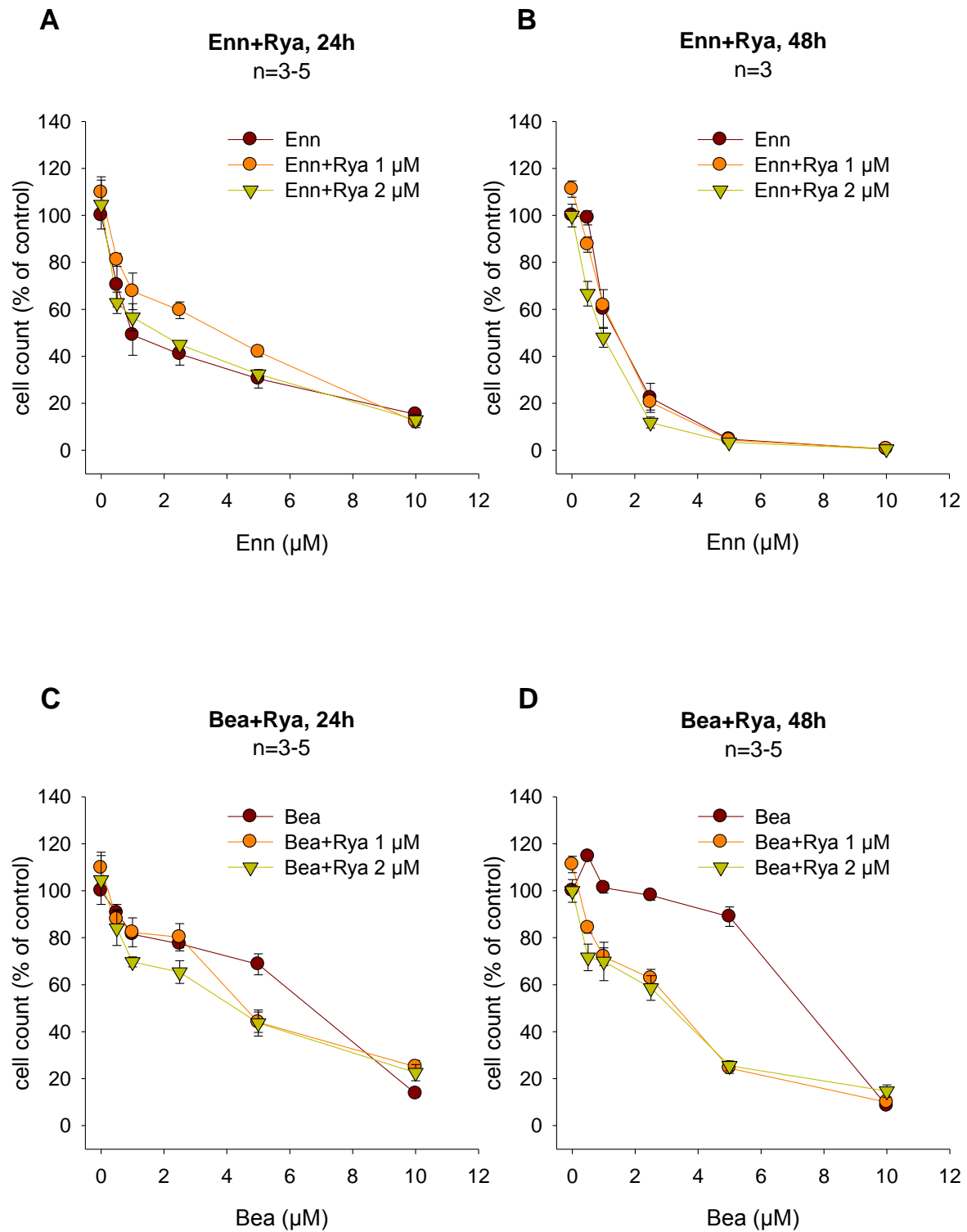


Figure 18: Concentration and time dependency of the impact of Rya on the cytotoxicity of Enn and Bea.

Rya exerted an influence on Enn and Bea that is a lot less notable than TMB-8 or Thaps. No potentiating effect was observed in combination with Enn after 24 h treatment; 1 μ M Rya even seemed to weakly attenuate the cytotoxic activity (Fig. 18A). Also after long-term treatment of 48 hours, the three concentration-response curves overlap nearly completely (Fig. 18B).

However, significant enhancement of the cytotoxic activity was observable after 48 h treatment with Bea and Rya, with no noticeable difference between the administration of 1 or 2 μ M Rya (Fig. 18D). After 24 h treatment, potentiation was only detectable at a Bea concentration of 5 μ M (Fig. 18C).

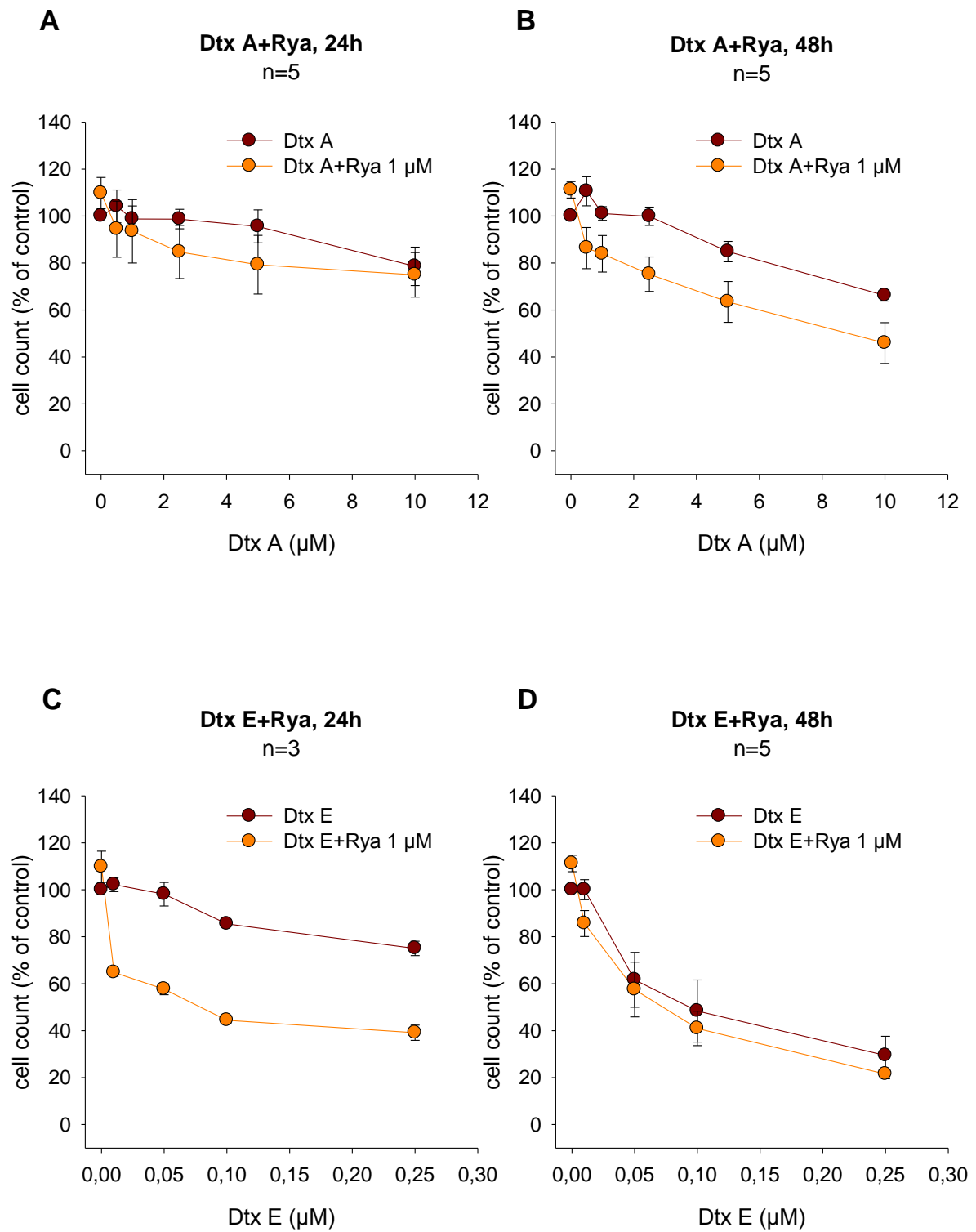


Figure 19: Concentration and time dependency of the impact of Rya on the cytotoxicity of Dtx A and Dtx E.

Since there was shown hardly a difference between the effects of Rya in a concentration of 1 or 2 μM in earlier experiments, only 1 μM Rya was tested in combination with Dtx A and Dtx E. Cytotoxic activity potentiation was found to be not as profound as with TMB-8 or Thaps, but Rya affected cells in concomitant administration with Dtxs (Fig. 19) more than with Enn or Bea.

The effect of Rya was most remarkable in combination with Dtx E after 24 h treatment, where the number of killed cells was doubled (Fig. 19C). However, all of the other investigations including Rya did not show a noteworthy potentiation of the cytotoxicity exerted by Dtxs (Fig. 19A+B+D), making Rya the least promising concomitant agent of the three substances that were tested.

4.2 Results of DAPI staining

4.2.1 Cytotoxicity of mycotoxins

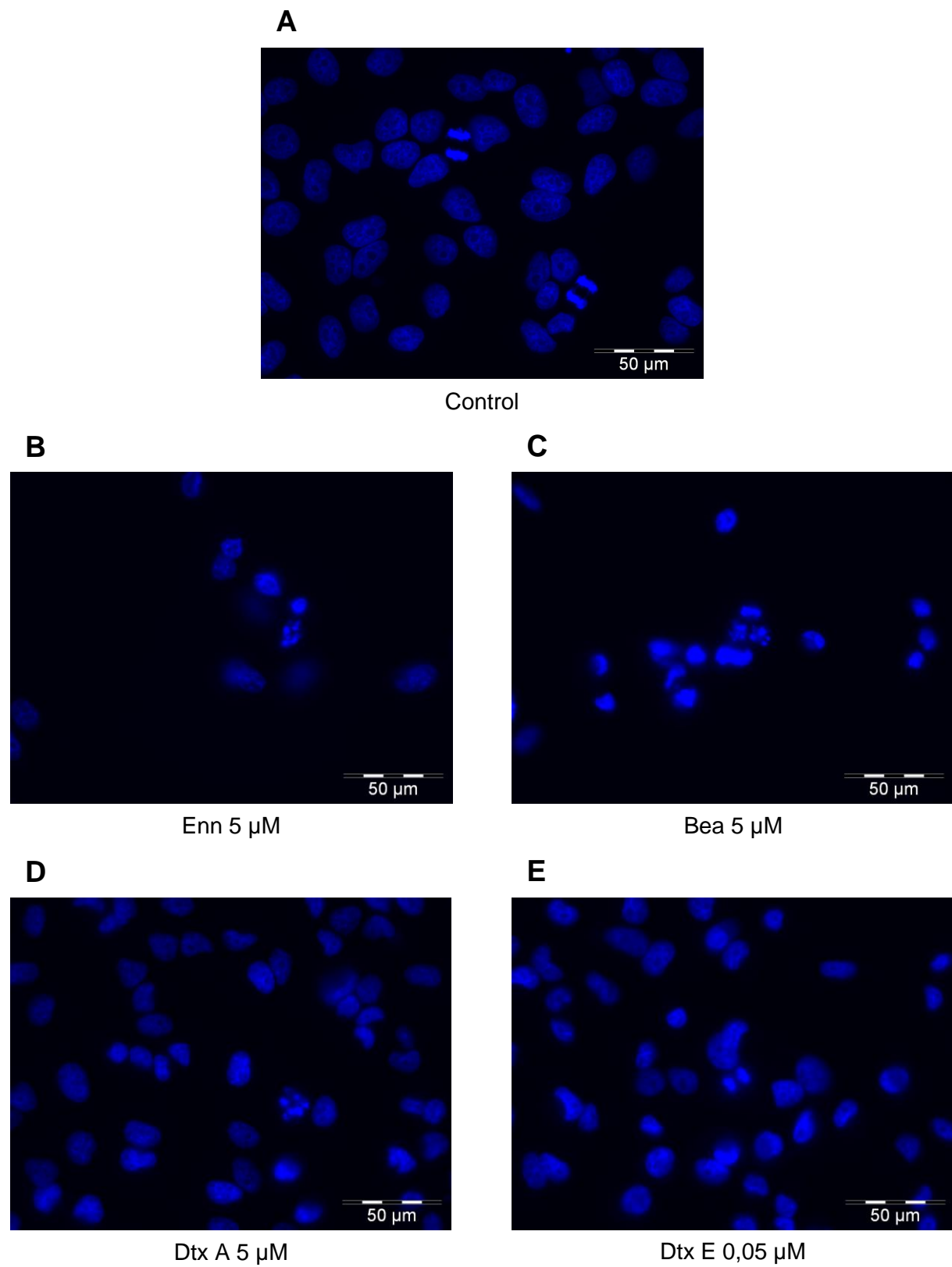


Figure 20: Morphological changes of nuclei after 24 h treatment with Enn (**B**), Bea (**C**), Dtx A (**D**) and Dtx E (**E**) compared to controls (**A**).

Fig. 20 shows DAPI-stained nuclei of controls and after 24 h treatment with Enn, Bea, Dtx A and Dtx E. In controls, almost exclusively interphase and mitotic nuclei were found, whereas apoptotic bodies were clearly visible when observing drug-exposed nuclei.

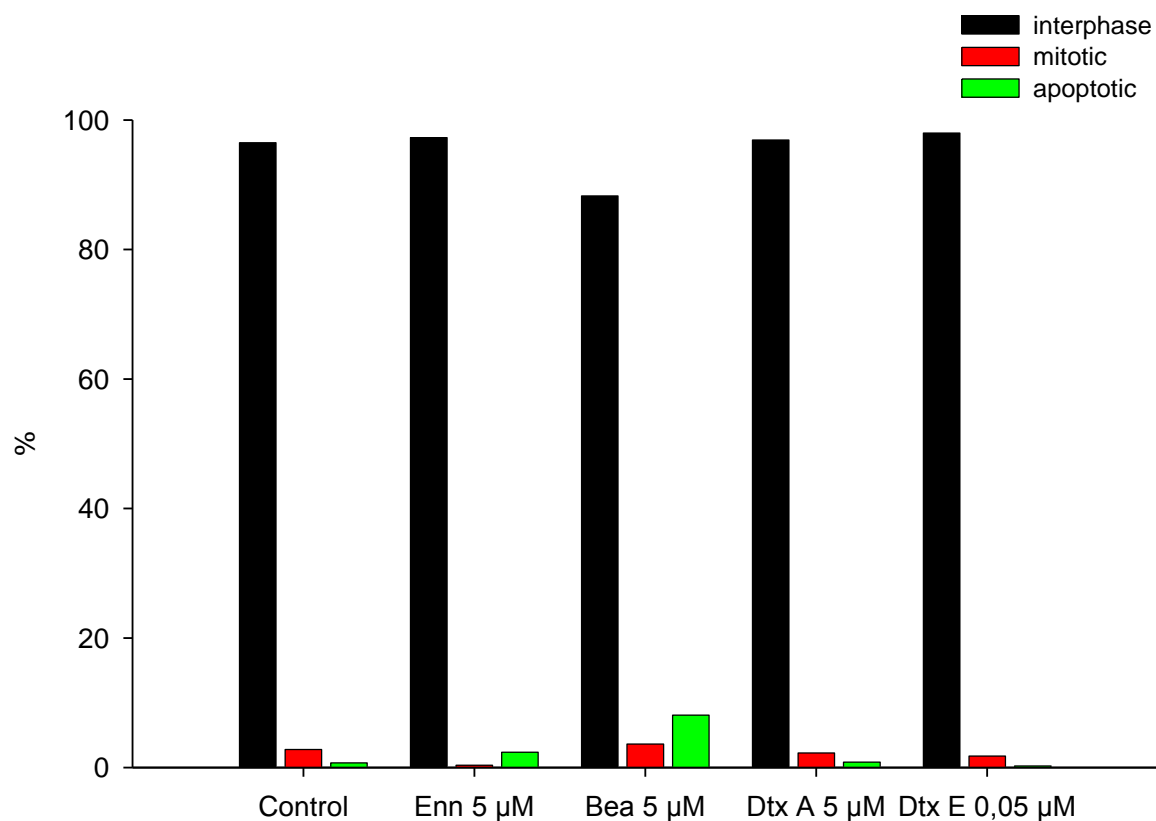


Figure 21: DAPI-stained nuclei were counted and classified into interphase, mitotic and apoptotic in an untreated control and after treatment with mycotoxins for 24 hours.

Fig. 21 illustrates that Bea evoked the highest percentage of apoptotic nuclei, namely 8,1%, compared to 0,73% in controls. Mitotic cells were found in all samples in a similar amount.

4.2.2 Impact of TMB-8

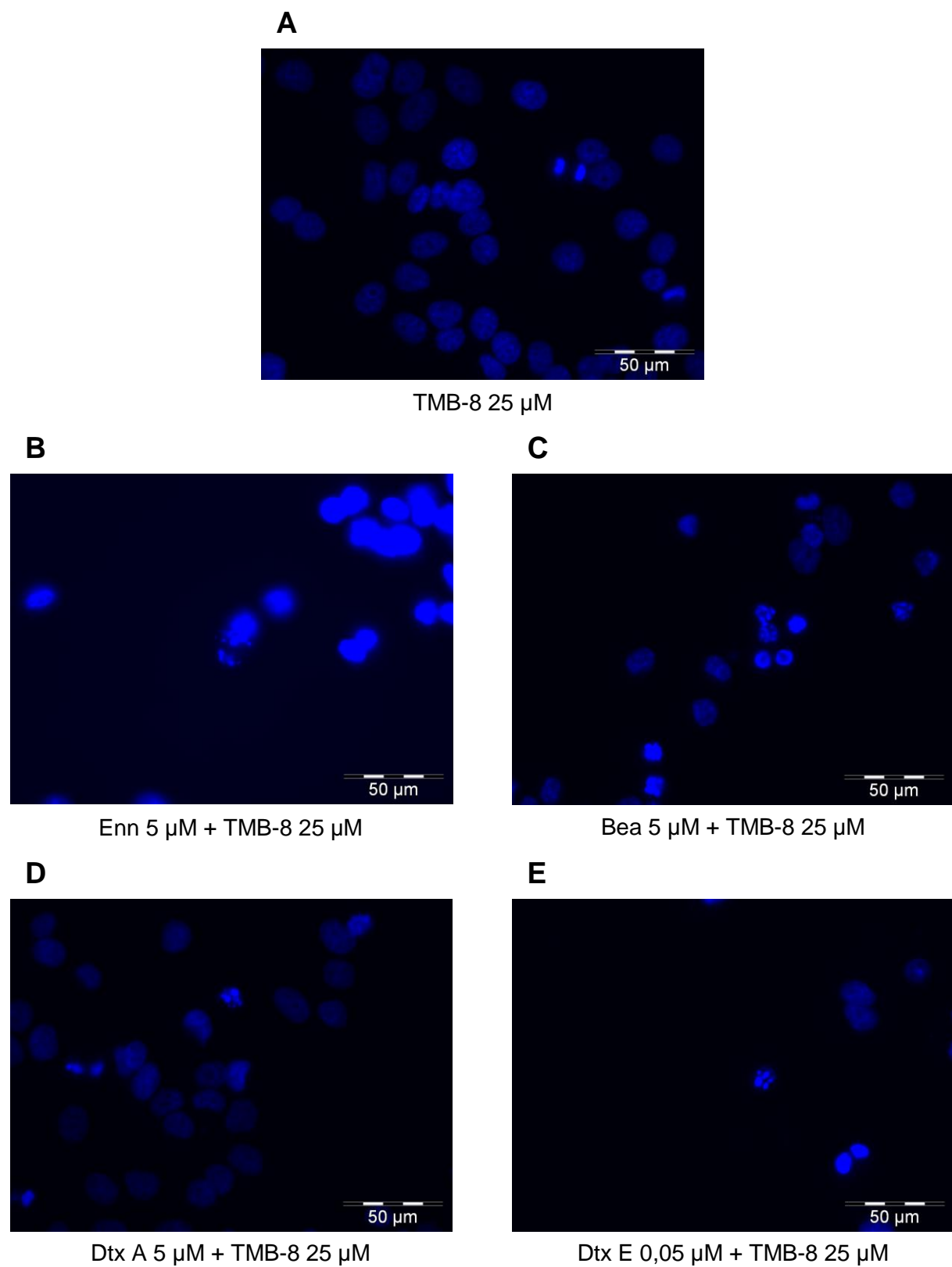


Figure 22: Morphological changes of nuclei after 24 h treatment with TMB-8 (**A**) and in combination with Enn (**B**), Bea (**C**), Dtx A (**D**) and Dtx E (**E**).

Characteristics of programmed cell death such as apoptotic body formation or chromatin condensation were found in all DAPI preparations of cells treated with TMB-8 and combinations (Fig. 22).

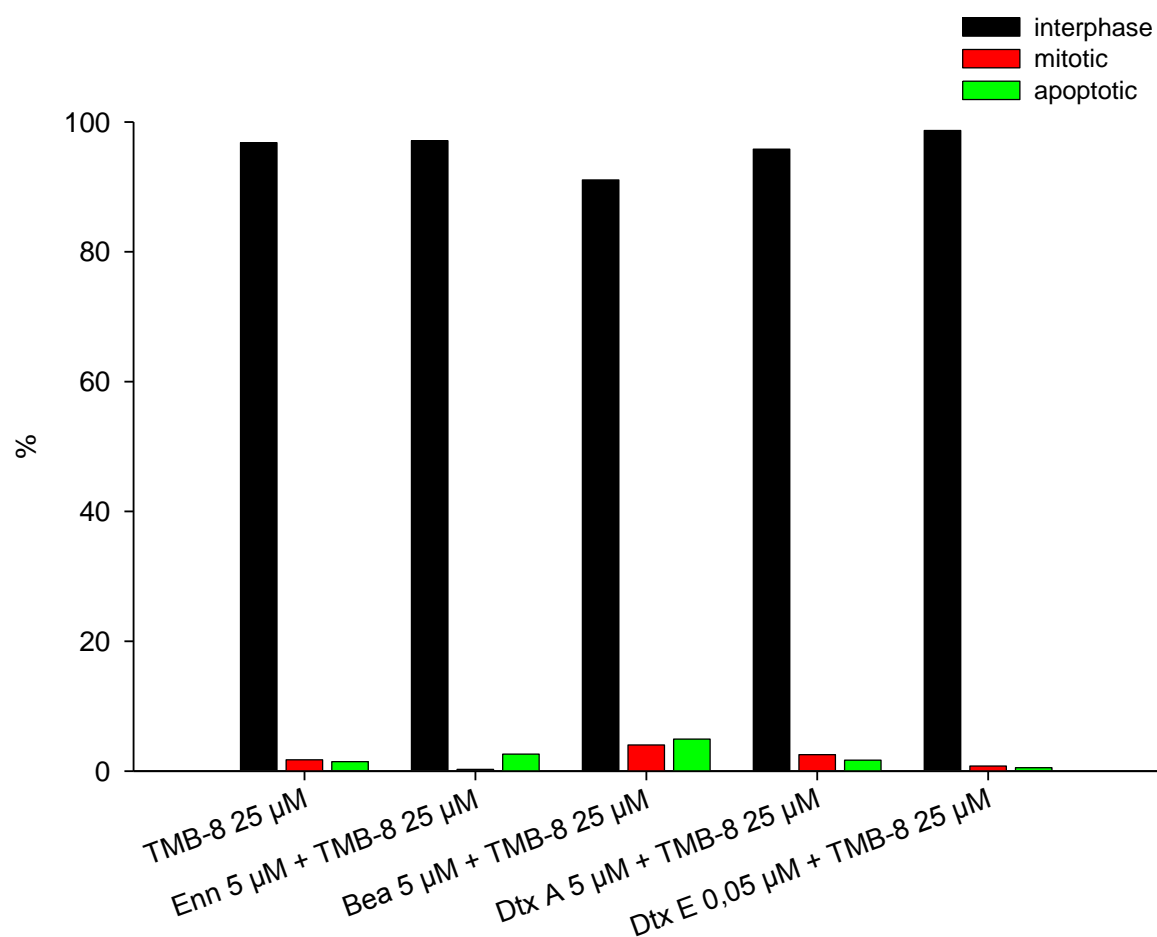


Figure 23: DAPI-stained nuclei were counted and classified into interphase, mitotic and apoptotic after treatment with mycotoxins combined with TMB-8 for 24 hours.

The distribution of interphase, mitotic and apoptotic nuclei in Fig. 23 strongly resembles Fig. 22, with Bea producing the highest proportion of apoptotic nuclei again. However, this amount was not potentiated by TMB-8; it even decreased to 4,92%.

4.2.3 Impact of thapsigargin

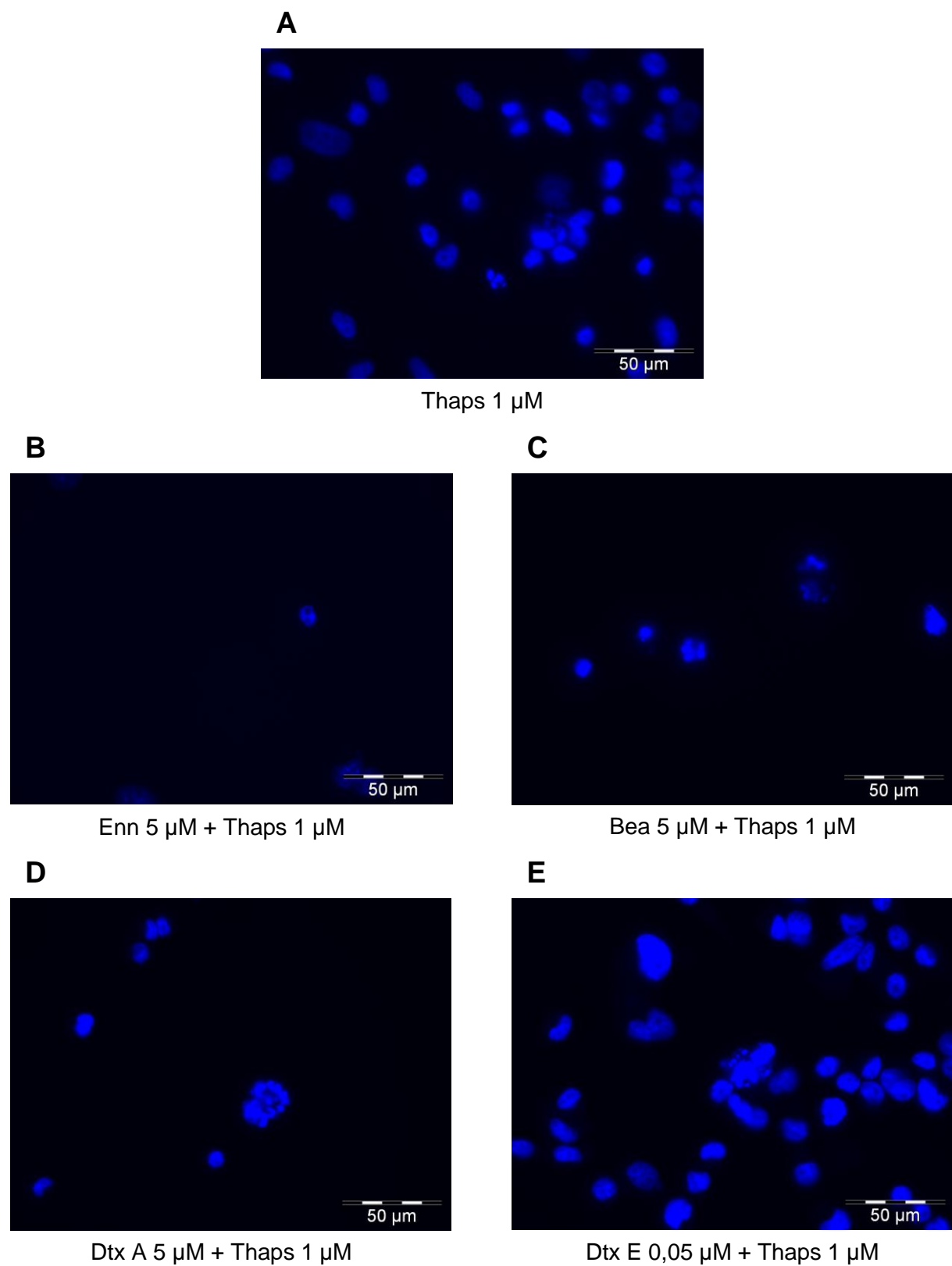


Figure 24: Morphological changes of nuclei after 24 h treatment with Thaps (A) and in combination with Enn (B), Bea (C), Dtx A (D) and Dtx E (E).

Apoptotic body formation was again well observable with cells treated with Thaps and combinations (Fig. 24).

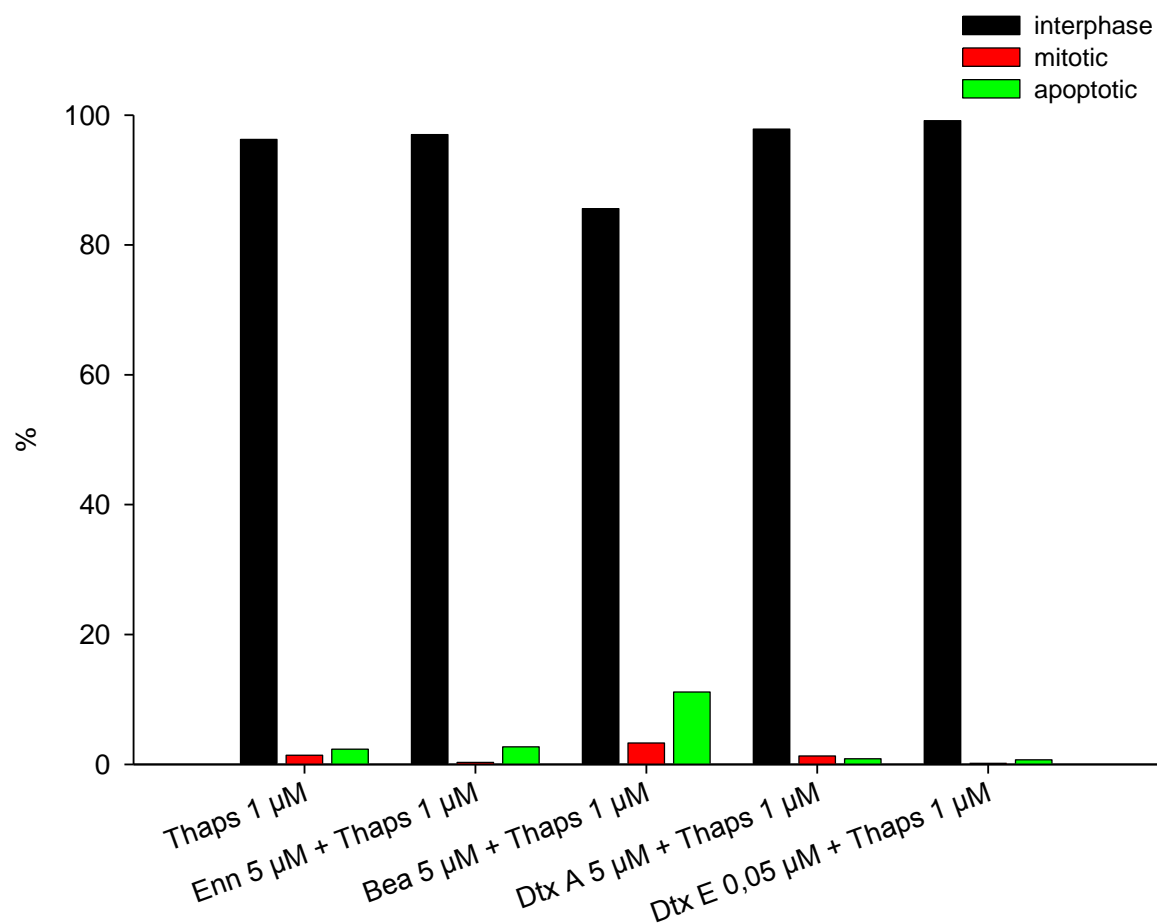


Figure 25: DAPI-stained nuclei were counted and classified into interphase, mitotic and apoptotic after treatment with mycotoxins combined with Thaps for 24 hours.

Whereas TMB-8 had no enhancing effect on Bea-induced apoptotic body formation, Thaps raised the percentage to 11,14% (Fig. 25). The activities of the other mycotoxins were again not affected concerning the proportion of apoptotic to interphase cells.

4.2.4 Impact of ryanodine

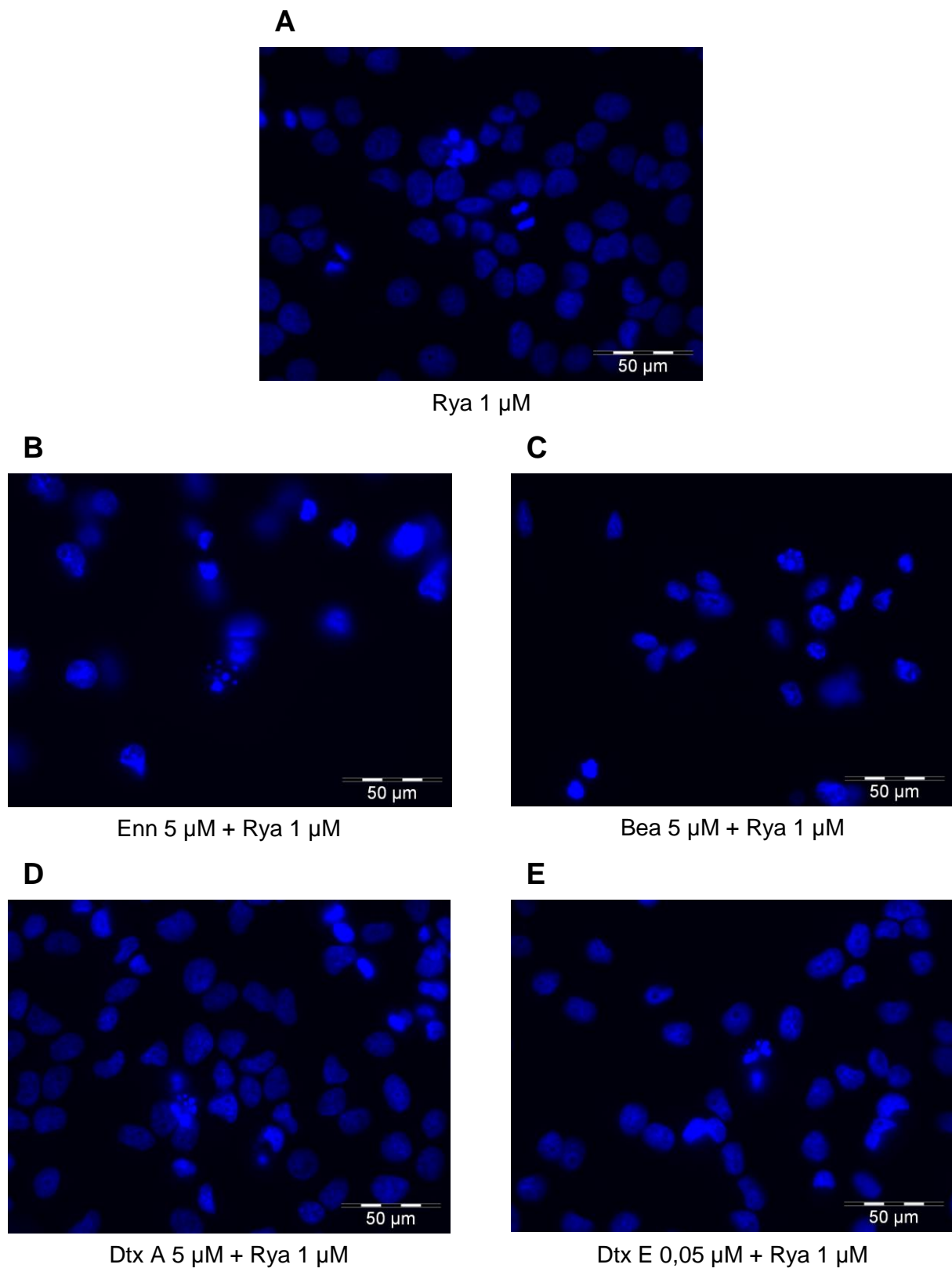


Figure 26: Morphological changes of nuclei after 24 h treatment with Rya (**A**) and in combination with Enn (**B**), Bea (**C**), Dtx A (**D**) and Dtx E (**E**).

Apoptotic bodies were also observed with nuclei treated with Rya (Fig. 26), however, not to the same extent as with TMB-8 and Thaps treated cells.

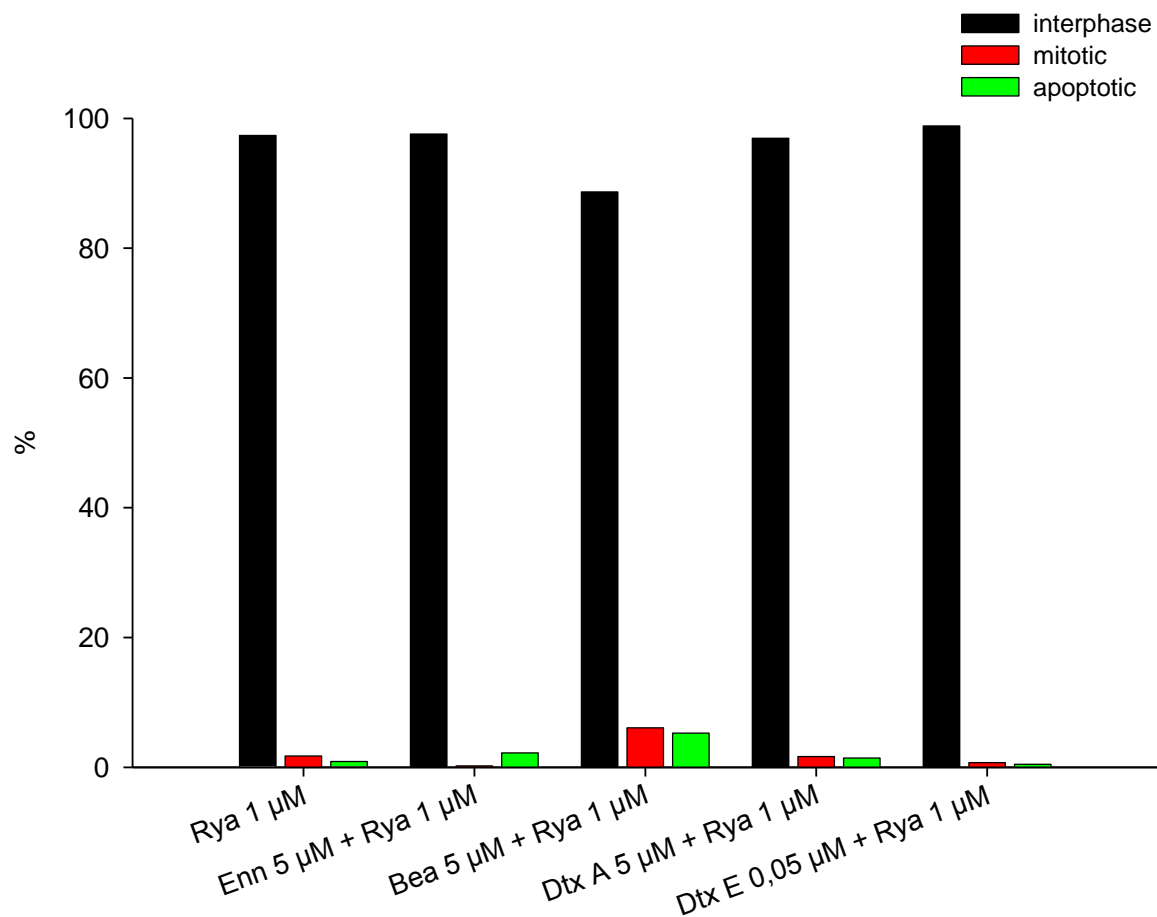


Figure 27: DAPI-stained nuclei were counted and classified into interphase, mitotic and apoptotic after treatment with mycotoxins combined with Rya for 24 hours.

Fig. 27 shows that apoptotic bodies did not occur as much as in earlier experiments, and that mitotic cells even outweighed apoptotic cells after 24 h treatment with Bea and Rya.

5 Discussion

Cancer diseases are still on the rise worldwide and make up one of the most frequent causes of death (World Health Organization, 2016). Current therapy strategies often have several side effects as a consequence of lacking selectivity and thus affect the patient's quality of life severely. Drug resistance is also an emerging issue. Furthermore, many current treatments do not always lead to complete recovery but are merely life-prolonging measurements. Hence, new approaches in cancer therapy are eagerly needed.

Researchers are facing challenges especially concerning cervical cancer. First-line therapy is a cisplatin-based treatment, which does not achieve the desired success with response rates ranging only from 20% to 30% and a survival of less than 10 months (Bonomi et al., 1985; Burnett et al., 2000; Long et al., 2005; Rose et al., 1999). Therefore, the development of alternative agents with more promising prospects for the treatment of cervical cancer is of particular importance.

Cyclohexadepsipeptides have long been known for their multifaceted characteristics. In recent years, they have come into focus of cancer research due to their cytotoxic potential. The finding of this activity has prompted the search for ways of using them as a part of cancer therapy.

In order to verify unpublished data that suggest a connection between enhanced anticancer activity of cyclohexadepsipeptides and a decreased Ca^{2+} level, Enn, Bea, Dtx A and Dtx E were tested in combination with TMB-8, Thaps and Rya in the cervical cancer cell line KB-3-1. The cytotoxicity was determined by an MTT assay.

TMB-8 and Thaps potentiated the cytotoxic activity similarly, although they exert opposite effects on the cell's Ca^{2+} level. In contrast, Rya induced no significant enhancement in spite of its ability to raise the intracellular Ca^{2+} level at the concentrations used. Therefore, no conclusions can be drawn about a possible Ca^{2+} dependency of the cytotoxicity mediated by the analysed cyclohexadepsipeptides. This proves that other modes of action are responsible for the potentiation.

TMB-8 and Thaps also provoked cytotoxicity when applied as single agents. These observations suggest that the enhancement of the actions of Enn, Bea, Dtx A and Dtx E is rather based on additive than synergistic effects. The fact that Rya monotreatment did not reduce cell survival supports the idea that the manipulation of intracellular Ca^{2+} level alone is not sufficient to affect anticancer activities of the mycotoxins.

Previous studies also indicate that TMB-8 and Thaps do not influence the effects of these cyclohexadepsipeptides. For example, Chen et al. (2006) showed that Bea-induced increase of intracellular Ca^{2+} concentration is affected neither by Thaps nor by the Ca^{2+} channel blocker nimodipine, but by adding or removing Ca^{2+} from the bathing medium. These findings suggest that rather extracellular than intracellular Ca^{2+} level is decisive regarding Bea-mediated cytotoxicity, and that Bea acts as a Ca^{2+} mobilizer by stimulating extracellular Ca^{2+} influx.

Similar results were obtained with Dtx A. TMB-8 does not interfere with its ability to inhibit fluid secretion rate by the Malpighian tubules of *Rhodnius prolixus* (Ruiz-Sanchez et al., 2010a), or to provoke contractions of insect visceral muscles of *Locusta migratoria* (Ruiz-Sanchez et al., 2010b). However, these findings are not necessarily related to the impact on Dtx-mediated cytotoxicity, as TMB-8, as mentioned above, exerts many paradoxical effects that also depend on the cell line. The latter study also supports the idea of extracellular Ca^{2+} concentration being the crucial factor.

Kouri et al. (2005) found that Rya even partially inhibits the increase of intracellular Ca^{2+} level mediated by Bea, while Ca^{2+} -buffering in the bathing solution again reduces the rise more effectively.

Besides their effect on the Ca^{2+} level, TMB-8 and Thaps have an impact on numerous other intracellular processes as described in chapter 1.5. Still, the mechanisms that form the basis of TMB-8-mediated cytotoxicity are not entirely understood. However, it has to be considered that not only elevation of intracellular Ca^{2+} concentration, but also a pronounced decrease could be fatal for a cell's viability, since Ca^{2+} also acts as a second messenger and therefore is essential for many signaling cascades that are necessary to sustain normal cell function. The anticancer activities of Thaps have been studied widely and its potential as a part of chemotherapy has already been recognized in recent trials (ClinicalTrials.gov, 2016).

Thus, the amplification of the effects of cyclohexadepsipeptides is not necessarily a result of the altered intracellular Ca^{2+} concentration, but rather a consequence of the effects of the single agents summing up.

DAPI staining was not consistent with the results of the MTT assay. Although the characteristics typical of apoptosis, such as nuclear condensation and fragmentation, were found with increased amount in drug-exposed nuclei, the percentage did not go beyond 12%. Other studies have shown a much greater effect. Dornetshuber et al. (2007) reported the

occurrence of 30% of apoptotic nuclei after 24 h treatment of KB-3-1 cells with 5 μ M Enn, whereas the correspondent value is only 2,37% in the investigations on hand.

Similar experiments were performed with Dtxs in HCT116 cells (Dornetshuber-Fleiss et al., 2013). Confirming the results of this thesis, there was no effect after 24 h treatment. However, 48 h Dtx exposure increased the occurrence of apoptotic signs compared to controls (approximately 11-fold at 10 μ M Dtx A and 17-fold at 0,5 μ M Dtx E).

However, it has to be considered that apoptosis is the only cell death modality that can be visualized by DAPI. Other forms of cell death might also contribute to mycotoxin-mediated cytotoxicity. In fact, treatment with Enn was shown to lead predominantly to necrotic rather than apoptotic cell death in Caco-2 cells (Ivanova et al., 2012). Prosperini et al. (2013) observed necrotic events particularly after long-term exposure (72h) to Enn, with the depolarization of the mitochondrial membrane playing a role in both types of cell death. In addition, a Bea-mediated increase of lactate dehydrogenase release was observed in CCRF-CEM cells, indicating an involvement of the necrotic pathway (Jow et al., 2004).

6 Conclusion and future prospects

Taken together, these data reveal that there is no distinct connection between the cytotoxic effects of cyclohexadepsipeptides and an intracellular Ca^{2+} concentration that was altered by TMB-8, Thaps or Rya. MTT assays showed that TMB-8 and Thaps induce enhancement in a similar manner, although they produce opposite effects on the cell's Ca^{2+} level, whereas Rya exerts no mentionable influence. However, many studies prove the existence of a Ca^{2+} -dependency. They do not only act as ionophores (Chen et al., 2006; Hinaje et al., 2002; Kamyar et al., 2004; Kouri et al., 2003) and thereby change the cell's ionic homeostasis themselves, but their effects are also influenced by manipulators of the Ca^{2+} concentration, such as Ca^{2+} chelators, Ca^{2+} channel inhibitors (e.g. nifedipine, nimodipine, CdCl_2), or by adding or buffering Ca^{2+} in the bathing medium (Chen et al., 2006; Dumas et al., 1996b; Jow et al., 2004; Kouri et al., 2005; Samuels et al., 1988).

This strongly supports the idea that Enn, Bea, Dtx A and Dtx E act in a Ca^{2+} -dependent manner only to a certain extent. Extracellular Ca^{2+} level seems to play a greater role than intracellular, and many factors such as the used cell line or concentrations might diversify the obtained results.

Further studies could complement these findings by measurement of intracellular Ca^{2+} concentration by fluorescence imaging, especially concerning TMB-8, as its effect is not completely elucidated. Fura-2 is a fluorescent dye which binds to free intracellular Ca^{2+} (Gryniewicz et al., 1985) and can therefore be used to detect and characterize alterations of the Ca^{2+} level. Moreover, the Chou-Talalay method could be used to calculate the combination index and thereby distinguish between additive and synergistic effects (Chou, 2006).

DAPI staining only demonstrated a slight increase in apoptotic nuclei, most pronounced after Bea treatment. Higher concentrations might be needed to provoke more significant results. Previous studies indicate that necrotic cell death is also involved in mycotoxin-mediated cytotoxicity (Ivanova et al., 2012; Jow et al., 2004; Prosperini et al., 2013). Double staining using fluorescein isothiocyanate (FITC)-labelled Annexin V and propidium iodide (PI) could be performed in order to differentiate between apoptotic and necrotic cells (Vermes et al., 1995) and therefore elucidate the preferred cell death modality.

Finally, assessment of the activities of these drug combinations in other cell lines, also non-cancer cell lines, is eagerly needed. Lacking selectivity is a common problem in current therapy strategies, as non-malignant cells with a high division rate often get affected as well, resulting in severe side effects. Dornetshuber et al. (2007) have already reported a

comparatively low sensitivity of normal human fibroblasts (WI-38) and endothelial cells (HUVEC) to Enn-mediated cytotoxicity. These observations have to be confirmed for the other mycotoxins in order to evaluate whether they are suitable for cancer therapy.

In conclusion, the cyclohexadepsipeptides Enn, Bea, Dtx A and Dtx E are potential anticancer agents that might exert synergistic effects with other cytotoxic drugs. Thaps and derivative substances have already been tested in trials with promising results (ClinicalTrials.gov, 2016). Their combination with one of the fungal secondary metabolites might increase their potential as future parts of the treatment of malignancies.

7 Abstract

Enniatin B (Enn), beauvericin (Bea), destruxin A (Dtx A) and destruxin E (Dtx E) are emerging mycotoxins which belong to the family of cyclohexadepsipeptides. They are known for their multifaceted properties, such as insecticidal, antibacterial, phytotoxic and ionophoric effects. They frequently contaminate grain worldwide and thereby are able to enter the food chain. In the last years, their cytotoxic potential has come into focus. Current chemotherapy strategies carry the risk of severe side effects, and drug resistance is on the rise. Thus, novel approaches in cancer treatment are eagerly needed.

This thesis aims to verify the assumption that the cytotoxic effects of these secondary metabolites underlie a Ca^{2+} dependency. Therefore, they were combined with 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), thapsigargin (Thaps) or ryanodine (Rya), which are well-known manipulators of intracellular Ca^{2+} concentration. Cytotoxicity in KB-3-1 cells was determined by an MTT assay after 24 h and 48 h treatment. Although TMB-8 and Thaps exert opposite effects on the Ca^{2+} level, they induced similar enhancement of the cytotoxicity of mycotoxins, whereas Rya showed no significant impact. Hence, extracellular might play a bigger role than intracellular Ca^{2+} , and the enhancement was rather of additive than of synergistic nature.

DAPI staining was performed in order to investigate morphological changes of drug-exposed nuclei. Only a slight increase in apoptotic bodies was observed; other cell death modalities might be involved as well.

Taken together, these data reveal that, in spite of a certain Ca^{2+} dependency that was proven by other studies, there is no definite connection between the cytotoxicity of Enn, Bea, Dtx A and Dtx E and the intracellular Ca^{2+} concentration. Further studies are necessary to evaluate their potential as anticancer drugs.

Enniatin B (Enn), Beauvericin (Bea), Destruxin A (Dtx A) und Destruxin E (Dtx E) sind Mykotoxine, die zur Familie der Cyclohexadepsipeptide gehören und denen vermehrt Bedeutung beigemessen wird. Sie sind für ihre vielfältigen Eigenschaften bekannt, wie zum Beispiel insektizide, antibakterielle, phytotoxische und ionophore Wirkungen. Weltweit kontaminieren sie häufig Getreide und sind dadurch fähig, in die Nahrungskette zu gelangen. In den letzten Jahren wurde ihr zytotoxisches Potenzial in den Fokus gerückt. Derzeitige Strategien in der Chemotherapie bergen das Risiko von schwerwiegenden Nebenwirkungen, und Arzneimittel-Resistenzen treten gehäuft auf. Neue Ansätze in der Krebstherapie werden daher dringend benötigt.

Die vorliegende Arbeit versucht die Annahme, dass die zytotoxischen Effekte dieser sekundären Metabolite einer Ca^{2+} -Abhängigkeit unterliegen, zu überprüfen. Daher wurden sie mit 8-(Diethylamino)octyl-3,4,5-trimethoxybenzoat (TMB-8), Thapsigargin (Thaps) oder Ryanodin (Rya) kombiniert, welche allesamt bekannte Manipulatoren der intrazellulären Ca^{2+} -Konzentration sind. Die Zytotoxizität auf KB-3-1 Zellen wurde nach 24- und 48-stündiger Behandlung mithilfe eines MTT-Tests bestimmt. Obwohl TMB-8 und Thaps gegenteilige Effekte auf den Ca^{2+} -Spiegel ausüben, verursachten sie eine ähnliche Verstärkung der Zytotoxizität der Mykotoxine, wohingegen Rya keinen wesentlichen Einfluss zeigte. Extrazelluläres Ca^{2+} spielt daher womöglich eine größere Rolle als intrazelluläres, und die Verstärkung war eher von additiver als von synergistischer Natur.

DAPI-Färbung wurde durchgeführt, um morphologische Veränderungen von behandelten Zellkernen zu untersuchen. Nur eine geringe Zunahme an apoptotischen Körperchen wurde beobachtet; andere Formen von Zelltod sind möglicherweise auch beteiligt.

Zusammengefasst ergeben diese Daten, dass trotz einer gewissen Ca^{2+} -Abhängigkeit, die durch andere Studien bewiesen wurde, keine eindeutige Verbindung zwischen der Zytotoxizität von Enn, Bea, Dtx A sowie Dtx E und der intrazellulären Ca^{2+} -Konzentration zu erkennen ist. Weitere Studien sind notwendig, um ihr Potenzial für die Behandlung von Tumoren zu beurteilen.

8 References

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

9 List of figures

Figure 1: Regulation of cytosolic Ca^{2+} concentration (Orrenius et al., 2003).	5
Figure 2: Intracellular targets in Ca^{2+} -mediated cytotoxicity (Zhivotovsky and Orrenius, 2011).	6
Figure 3: The role of Ca^{2+} as a messenger in apoptosis (Mattson and Chan, 2003).	7
Figure 4: Structures of Enn, Bea, Dtx A and Dtx E (based on ChemSpider, 2015).	13
Figure 5: Structures of TMB-8, Thaps and Rya (ChemSpider, 2015).	14
Figure 6: KB-3-1 cells as seen under the microscope.	16
Figure 7: Arrangement of a “Neubauer improved” hemocytometer (Gstraunthaler and Lindl, 2013).	16
Figure 8: Counting cells in a hemocytometer (Gstraunthaler and Lindl, 2013).	17
Figure 9: Principle of MTT assay (Ebada et al., 2008).	18
Figure 10: Colour change induced by EZ4U shows varying cytotoxicity.	19
Figure 11: Structure of DAPI (Thermo Fisher Scientific, 2016).	21
Figure 12: DAPI-stained interphase and mitotic nuclei (control).	22
Figure 13: DAPI-stained apoptotic nuclei (Bea 5 μM + TMB-8 25 μM).	23
Figure 14: Concentration and time dependency of the impact of TMB-8 on the cytotoxicity of Enn and Bea.	25
Figure 15: Concentration and time dependency of the impact of TMB-8 on the cytotoxicity of Dtx A and Dtx E.	27
Figure 16: Concentration and time dependency of the impact of Thaps on the cytotoxicity of Enn and Bea.	29
Figure 17: Concentration and time dependency of the impact of Thaps on the cytotoxicity of Dtx A and Dtx E.	31
Figure 18: Concentration and time dependency of the impact of Rya on the cytotoxicity of Enn and Bea.	33
Figure 19: Concentration and time dependency of the impact of Rya on the cytotoxicity of Dtx A and Dtx E.	35
Figure 20: Morphological changes of nuclei after 24 h treatment with Enn (B), Bea (C), Dtx A (D) and Dtx E (E) compared to controls (A).	37
Figure 21: DAPI-stained nuclei were counted and classified into interphase, mitotic and apoptotic in an untreated control and after treatment with mycotoxins for 24 hours.	38
Figure 22: Morphological changes of nuclei after 24 h treatment with TMB-8 (A) and in combination with Enn (B), Bea (C), Dtx A (D) and Dtx E (E).	39
Figure 23: DAPI-stained nuclei were counted and classified into interphase, mitotic and apoptotic after treatment with mycotoxins combined with TMB-8 for 24 hours.	40

Figure 24: Morphological changes of nuclei after 24 h treatment with Thaps (A) and in combination with Enn (B), Bea (C), Dtx A (D) and Dtx E (E).	41
Figure 25: DAPI-stained nuclei were counted and classified into interphase, mitotic and apoptotic after treatment with mycotoxins combined with Thaps for 24 hours.	42
Figure 26: Morphological changes of nuclei after 24 h treatment with Rya (A) and in combination with Enn (B), Bea (C), Dtx A (D) and Dtx E (E).	43
Figure 27: DAPI-stained nuclei were counted and classified into interphase, mitotic and apoptotic after treatment with mycotoxins combined with Rya for 24 hours.	44

10 List of abbreviations

AIF	apoptosis-inducing factor
ATP	adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-xL	B-cell lymphoma-extra large
Bcl-2	B-cell lymphoma 2
Bea	beauvericin
Ca ²⁺	calcium
CdCl ₂	cadmium chloride
CO ₂	carbon dioxide
Cyt c	cytochrome c
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dtx A	destruxin A
Dtx E	destruxin E
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia = for example
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Enn	enniatin B
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FasL	Fas ligand
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G6PD	glucose-6-phosphate dehydrogenase
h	hour
IC ₅₀	half maximal inhibitory concentration
Ins(1,4,5)P ₃	inositol-1,4,5-trisphosphate
IP ₃ R	inositol-1,4,5-trisphosphate receptor
MAPK	mitogen-activated protein kinase
ml	milliliter

mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
μl	microliter
μM	micromolar
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometer
NMDA	N-methyl-D-aspartic acid
NO	nitric oxide
PBS	phosphate buffered saline
PI	propidium iodide
PLCγ	phospholipase C-γ
PMCA	plasma membrane Ca ²⁺ -ATPase
PS	phosphatidylserine
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen
PtdIns(4,5)P ₂	phosphatidylinositol-4,5-bisphosphate
PTP	permeability transition pore
rpm	revolutions per minute
Rya	ryanodine
RyR	ryanodine receptor
SERCA	sarco/endoplasmic reticulum Ca ²⁺ -ATPase
SR	sarcoplasmic reticulum
Thaps	thapsigargin
TMB-8	8-(diethylamino)octyl-3,4,5-trimethoxybenzoate
V-ATPase	vacuolar-type H ⁺ -ATPase