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„Exploring the role of autophagy inhibition in the response plasticity of bladder cells "

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Abstract

Bladder cells face a challenging environment due to exposure to urine, which contains a constantly changing mixture of chemicals. These cells must adjust because of the mechanical strain and shear stress caused by the bladder's stretching and fluid movement during the filling and urinating cycles. Our findings indicate that bladder cells have unique biomechanical characteristics that, when combined with their detoxifying processes, allow them to adapt to these circumstances. The cellular “recycling process” known as autophagy is essential for sustaining cellular quality control and adapting to stress. We used compounds that inhibited autophagy (bafilomycin [1-10nM] and wortmannin [0.1-1µM]) in our experiments, and we observed changes in the morphology of the cells. We exposed bladder cells to temporary (3 hours) shear stress to simulate real-life conditions. Interestingly this resulted in a similar morphology for both control cells and cells treated with autophagy inhibitors. However, inhibiting autophagy may compromise the cells' ability to defend against external chemical stressors, particularly those targeting mitochondria. We examined the effects of fusaric acid (0.1-0.5-1mM) and deoxynivalenol (0.1-1-10µM) on cell viability and toxicity in T24 bladder cancer cells. Autophagy inhibition amplified the harmful effects of these substances, leading to mitochondrial damages, changes in autophagic flux, and modifications to the cell membrane. Our research demonstrates the complex interactions between chemical and physical factors that modulate bladder cells' capacity for adaptation. Autophagy inhibition significantly reduces these abilities, which may have a major impact on bladder toxicological profile, especially with connection to mycotoxin effects.

Zusammenfassung

Blasenzellen sind einer schwierigen Umgebung ausgesetzt, da sie dem Urin mit seiner wechselnden Mischung von Chemikalien ausgesetzt sind. Diese Zellen müssen sich an die mechanischen Belastungen und Scherbelastungen anpassen, die durch die Dehnung der Blase und die Flüssigkeitsbewegung während des Füllungs- und Urinierzyklus verursacht werden. Unsere Ergebnisse deuten darauf hin, dass Blasenzellen über einzigartige biomechanische Eigenschaften verfügen, die es ihnen in Verbindung mit ihren Entgiftungsprozessen ermöglichen, sich an diese Umstände anzupassen. Der als Autophagie bekannte zelluläre Recyclingprozess ist für die Aufrechterhaltung der zellulären Qualitätskontrolle und die Anpassung an Stress wesentlich. In unseren Experimenten verwendeten wir Verbindungen, die die Autophagie hemmen (Bafilomycin [1-10 nM] und Wortmannin [0,1-1 µM]), und wir beobachteten Veränderungen in der Morphologie der Zellen. Wir setzten die Blasenzellen vorübergehend (3 Stunden) einer Scherbelastung aus, um reale Bedingungen zu simulieren. Überraschenderweise verringerte diese zusätzliche Exposition die Empfindlichkeit der Zellen gegenüber Autophagie-Inhibitoren. Die Hemmung der Autophagie kann jedoch die Fähigkeit der Zellen beeinträchtigen, sich gegen externe chemische Stressfaktoren zu verteidigen, insbesondere gegen solche, die auf Mitochondrien wirken. Wir haben die Auswirkungen von Fusarinsäure (0,1-0,5-1mM) und Deoxynivalenol (0,1-1-10µM) auf die Überlebensfähigkeit und Toxizität der Zellen in T24-Blasenkrebszellen untersucht. Durch die Hemmung der Autophagie werden diese Fähigkeiten erheblich eingeschränkt, was sich erheblich auf das toxikologische Profil der Blase auswirken kann, insbesondere im Zusammenhang mit Mykotoxinwirkungen.

Acknowledgements

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Abbreviations

APOBEC	Apolipoprotein B catalytic polypeptide-like protein family
ATP	Adenosine triphosphate
Atg	Autophagy-related genes
BAFI	Bafilomycin
BC	Bladder cancer
Ba/Sq	Basal/squamous
CDKN	Cyclin dependent kinase
CG	Cisplatin, Gemcitabine
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DON	Deoxynivalenol
ERCC	Excision repair protein
EGFR	Epidermal growth factor receptor
ELF	E74-like factor
FA	Fusaric acid
FGFR3	Fibroblast growth factor receptor
FOXA1	Forkhead box protein A1
HER-2	Human epidermal growth factor receptor 2
KDM	Histone lysine demethylases
KRT	Keratin
LC3	Light chain 3
LumNS	Luminal non-specified
LumP	Luminal papillary
LumU	Luminal unstable
MAP kinase	Mitogen-activated protein kinase
MIBC	Muscle-invasive bladder cancer
mRNA	Messenger RNA
MS	Mechanosensitive
MSC	Mechanosensitive channels
mTOR	Mammalian target of rapamycin
M-VAC	Methotrexate, Vinblastine, Doxorubicin, Cisplatin
NE-like	Neuroendocrine-like
NMIBC	Non-muscle-invasive bladder cancer
PAS	Pre-autophagosomal structure
PINK1	PTEN-induced putative kinase protein 1
PI3K	Phosphoinositide 3-kinases
PPARG	Peroxisome proliferator activated receptor gamma
RB	Retinoblastoma protein
SERCA	Sarco(endo)plasmic reticulum Ca2+ ATPase
TERT	Telomerases reverse transcriptase
TP53	Tumor protein p53
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

ULK	Unc-51-like autophagy-activating kinases
VPS	Vacuolar protein sorting
WIPI1	WD repeat domain phosphoinositide-interacting protein 1
WORT	Wortmannin

Inhaltsverzeichnis

1 Introduction

The bladder is a hollow muscular organ and is in the lower abdomen. The bladder functions as a reservoir and is responsible for urine excretion. As the bladder fills with urine, sensory nerves travelling back to the central nervous system communicate with efferent somatic and autonomic nerves to control the release of urine by stimulating the bladder muscle and relaxing the urethral sphincters[1], [2].

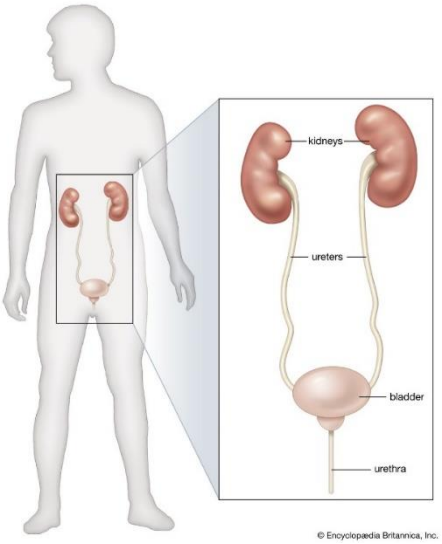

Figure 1 Organ of the renal system [2]

1.1 Bladder cancer

Bladder cancer is ranked as ninth most frequently diagnosed cancer types worldwide and has reported 430 000 cases in 2012. According to global bladder cancer incidence and mortality statistics it is shown that incidence rates are consistently lower in women than men, although sex differences varied substantially between countries[3]. Risk factors include geography, age, and exposure to carcinogens such as cigarette smoking. The most significant symptom is the presence of blood in the urine[4].

1.1.1 Characteristic

Bladder cancer can be differentiated as non-muscle-invasive bladder cancer (NMIBC), muscle-invasive bladder cancer (MIBC), or metastatic bladder cancer. Each subtype has different molecular drivers, disease progression and treatment approaches[5].

Tumor staging is based on the depth of bladder wall invasion. Stages Ta and T1 are considered non-muscle-invasive bladder cancer (NMIBC), in which the tumor is confined to the urothelium and lamina propria, respectively, and stages T2, T3, and T4, in which the muscle has invaded, are referred to as muscle-invasive bladder cancer (MIBC)[6].

By understanding the biology, gene expression and sequencing of bladder cancer cells effective immunotherapy can be developed. Bladder cancer is one of the most frequently mutated human cancers. It is shown that approximately 70-80% of the patients with bladder cancer has mutations in the promotor of the gene encoding telomerases reverse transcriptase (TERT), which can lead to better therapeutic approaches[5].

Non-muscle-invasive molecular subtypes (NMIBC)

Around 80% of the diagnosed bladder cancer belongs to the subtypes NMIBC with a survival rate of 5 years[7]. Multiple research projects have characterized DNA and RNA alteration in NMIBC like deletions in chromosome 9 (which contains the CDKN2A gene), mutations in genes encoding fibroblast growth factor receptor 3 (FGFR3) and PI3K and the promoter of the gene encoding TERT. These alterations were identified in early stages of urothelial malignancy[8]–[11].

The UROMOL study identified non-muscle-invasive molecular into three classes. Class 1 (luminal-like signature) tumors shows enrichments in early cell cycle genes. Class 2 (epithelial–mesenchymal transition) tumors were associated with high expression of late cell cycle. These two classes of NMIMS show high expression of uroplakins, which are the markers of bladder luminal/ umbrella cells. They were associated with lower rates of survival compared to class 1 or class 3 tumors. Class 3 (basal-like signature) tumors reveal high expression of KRT5 and KRT15, markers of undifferentiated or basal cells. These tumors also show elevated expression levels of long non-coding RNA[12].

Muscle-invasive molecular subtypes

This tumor subtype is advanced beyond epithelial layer and into muscle. MIBS is classified in six subgroups: luminal papillary (LumP), luminal non-specified (LumNS), luminal unstable (LumU), stroma-rich, basal/squamous (Ba/Sq) and neuroendocrine-like (NE-like) subtypes[13].

Luminal tumors (LumP, LumNS, and LumU) have enhanced urothelial differentiation, active PPARG and GATA3 regulons, and FGFR3 genetic altercation such as a mutation, fusion, or genomic amplification). The LumP subtype was frequently related with FGFR3 mutations and increased FGFR3 regulatory activity, implying that FGFR3 inhibitors could be beneficial[14], [15]. LumP tumors also had higher KDM6A mutations and CDKN2A deletions, although they still had wild-type TP53. LumNS are associated with carcinoma *in situ*, a non- muscle-invasive illness that can progress to muscle-invasive disease. PPARG amplification, overexpression of PPARG downstream target genes, and ELF3 mutations were found in LumNS tumors. LumU tumors were significantly more prone to genomic instability and copy number alterations. They also showed an increase in mutations in the mRNA editing genes for apolipoprotein B catalytic polypeptide-like protein family (APOBEC). Furthermore, LumU tumors had the highest amounts alterations in TP53 and ERCC2, the latter of which is linked with sensitivity to

chemotherapeutic drugs[5]. The stroma-rich subtype has intermediate levels of urothelial differentiation and is characterized by prominent stromal cell infiltration and high levels of smooth muscle, endothelial, fibroblast, and myofibroblast gene signatures. T and B cell markers were found to be more abundant in tumors with a stromal component. Ba/Sq subtype tumors have high expression levels of basal cell marker genes KRT14, KRT5 and KRT6 and loss of luminal cell marker genes s GATA3 and FOXA1. They are aggressive with poor prognosis and are often found in female patients and at higher stages. The most frequently mutated genes in Ba/Sq tumors are TP53 and RB1[16]. The NE-like subtype is the most homogenous subtype, with tumors displaying neuroendocrine histological features and immune infiltration. TP53 mutations are ubiquitously present in NE-like tumors, along with RB1 mutations and deletion[17].

Molecular subtyping of MIBC has primarily focused on mRNA expression and is becoming increasingly important in the clinical setting as it provides valuable information for personalized treatment approaches[5].

1.1.2 Metastasis

Tumor metastasis is the leading cause of death in cancer patients. It involves the process of tumor cells leaving the primary tumor, traveling through the circulatory system, and establishing secondary tumors in distant sites. Although the genetic basis of tumorigenesis can vary greatly, the steps for formation for metastasis are similar for all tumor cells[18]. Before becoming a clinically identifiable lesion, a cancer cell must successfully complete a several steps to colonize a secondary site[19].

These steps include:

1. Angiogenesis: The primary tumor generates blood vessels to support its growth, increasing the chance for tumor cells to enter the bloodstream and spread to secondary sites[20], [21].
2. Cell adhesion: Tumor cells need to attach to other cells and/or matrix. Adhesion molecules play a crucial role in this process[22].
3. Invasion: Tumor cells translocate across extracellular matrix barriers to invade surrounding tissues[23]. This requires the lysis of matrix proteins by specific proteinases[24].
4. Migration: Factors, such as matrix components, stimulate tumor cell migration, allowing them to move towards secondary sites[25], [26].

5. Proliferation: Once at the secondary site, tumor cells need to proliferate to establish a new tumor. Growth factors that stimulate metastatic cells to proliferate have been identified[27].

It is important to note that bone is a common site for metastasis, particularly for breast, prostate, and lung cancers. An estimated 40% of bladder cancer (BC) patients experience bone metastases, which can be extremely painful and drastically impact their quality of life. Furthermore, there have been no effective treatments developed that specifically target metastases to bone [18].

1.1.3 Therapy of bladder cancer

Systemic chemotherapy is used to treat metastatic tumors, whereas surgery and external radiation therapy are treatments for localized tumors. However, targeted radionuclide therapy and other alternative or complementary therapies, such as chemotherapy are typically not curative for diffuse urinary bladder tumors[28].

Until recent years, M-VAC (methotrexate, vinblastine, doxorubicin, cisplatin) has been regarded as the standard chemotherapy regimen for treating metastatic bladder carcinoma. It has consistently given overall response rates >70%, with 35% of patients obtaining a full response[29].

Over the past few years, the use of gemcitabine in metastatic bladder cancer has grown rapidly, especially in combination with cisplatin (CG). A phase III trial that compared M-VAC and CG has shown similar efficacy and lower toxicity for CG. CG has now become the new standard of care for patients with metastatic bladder cancer[30]–[32].

HER-2 and EGFR are both receptors that are overexpressed in bladder carcinoma metastases and corresponding primary tumors[33]. The addition of gefitinib, a small molecule inhibitor of EGFR, to standard chemotherapy did not improve response rates or time to progression. This suggests that other factors besides EGFR mutations may influence the response to gefitinib[30]. Trastuzumab, a monoclonal antibody against HER-2, in combination with paclitaxel, carboplatin, and gemcitabine has shown promising results in combination with chemotherapy in patients with advanced HER-2 positive bladder cancer[34]. Lapatinib, an EGFR and HER-2 tyrosine kinases inhibitor, has also shown activity in second-line therapy for metastatic bladder carcinoma[35].

There are variety of compounds like Farnesyl transferase inhibitors, such as lonafarnib and tipifarnib Sorafenib, a multikinase inhibitor, and angiogenesis inhibitors and Proteasome

inhibitors, such as Bortezomib are beginning studied for advanced bladder carcinoma. Further research is needed to determine their efficacy and potential use in clinical practice[36], [37].

1.2 Mechanotransduction

Mechanotransduction is a process where physical forces are converted into biochemical signals and incorporating these signals into biological response[38].

Mechanosensation plays a crucial role in our ability to perceive various physical stimuli such as skin contact, gravity, proprioception, sound waves, food texture, muscle stretch, and air flow[39]. Mechanical stimuli regulate almost all aspects of cell function, including growth, differentiation, migration, gene expression, protein synthesis, and apoptosis. Mechanical forces directly influence the form and function of tissues, the impact on compression on bone and cartilage and tension on muscle and skin[40]. Different proteins, including cytoskeletons, molecules associated with focal adhesions, G-protein-coupled receptors, and ion channels, are also responsible for mediating the sensation and response to mechanical forces[41].

Mechanosensitive (MS) ion channels can be activated by mechanical stresses applied to the lipid bilayer or its associated nonmembrane components. MS channels are involved in transforming physical signals into electrical stimuli so that sensory cells can respond quickly. MS channels play a crucial role in converting physical stimuli into electrical signals so that sensory cells respond quickly [39]. The urinary system has the vital function of regulating water and salt levels, maintaining acid-base balance, and producing hormones. Within the urinary system, MSCs (mechanosensitive channels) serve as essential mechanotransducers, which respond to mechanical stimuli like shear stress, stretching of the bladder wall, and urine flow. This activation of MSCs leads to a wide range of biological effects in the urinary system. It was discovered that there are two members of the piezo family, Piezo 1 and Piezo 2, a family of MSCs[41].

There is evidence suggesting that Piezo 2 plays a significant functional role in human urination. Piezo1 and Piezo2 are membrane proteins with numerous transmembrane passes, indicating their potential involvement in mechanotransduction. Piezo1 is capable of detecting and responding to different types of mechanical stimuli, such as poking, stretching, shear stress, and alterations in local membrane tension. In contrast, Piezo2 exhibits a lower level of sensitivity to changes in local membrane tension. Both Piezo1 and Piezo2 moderate the entry of cations, with calcium (Ca^{2+}) having the highest permeability, followed by potassium (K^{+}), sodium (Na^{+}), and magnesium (Mg^{2+}). They also generate a mechanically activated (MA) current.

Additionally, Piezo1 is involved in sensing mechanical stimuli in the kidney and regulating urinary osmolarity[42].

In the bladder urothelium, which serves as a physical barrier, Piezo1 plays an important role in detecting mechanical stretching of the bladder wall, which triggers the influx of calcium ions and the subsequent release of adenosine triphosphate (ATP). ATP acts as a stimulant for the sensory nerves connected to the bladder, communicating information about bladder filling, initiating the micturition reflex, and promoting voiding[43], [44]. Recent research has reported that in mouse models, Piezo2 plays a critical role as a mechanoreceptor in urinary function, turning the lower urinary tract to be responsive to stretching, triggering a well-timed micturition reflex [42]. Overall, ATP release mediated by Piezo1 and Piezo2 in the urothelium is crucial for normal urination[45].

The activity of Piezo channels has an impact on urological cancer, as they are involved in calcium signal transduction and electrochemical gradients maintenance. These channels play a significant role in multiple aspects associated with tumor formation, which include cell proliferation, angiogenesis, migration, extracellular matrix remodeling, and the tumor microenvironment[46]–[49].

It was found that in bladder carcinoma tissues, the mRNA expression of Piezo1 and Piezo 2 both in significantly increased. Moreover, the presence of Piezo1 is associated with tumor stage, grade, and size. On the other hand, Piezo2 expression specifically correlates with tumor [50].

Piezo1 is thought to be required for urothelial repair and regeneration in bladder carcinoma and therefore Piezo1 may be a promising target for early prevention and treatment of bladder carcinoma[51], [52].

1.3 Autophagy

Autophagy is a cellular process in which cells degrade and recycle their own components. It breaks down the cells its own structures to gain nutrients and energy. Autophagy is regarded as a housekeeping mechanism because it helps maintain cellular homeostasis by preventing the accumulation of protein aggregates and defective cellular structures[53].

Various environmental cues and intracellular stressors can activate signaling pathways that increase autophagy. These include starvation, high temperature, low oxygen levels, hormonal stimulation, damaged organelles, accumulation of mutant proteins, and microbial invasion. When these signals are detected, the cell initiates autophagy to remove and recycle damaged cells, providing the cell with the necessary resources for survival and adaptation[53], [54].

Efficient sequestration and clearance of unnecessary or damaged cellular or non-self-components is essential for cell survival and function because dysfunction of autophagy contributes to several illnesses, including cancer, neurodegeneration, cardiovascular problems, and microbial infection[55].

1.3.1 Molecular Machinery of Autophagy

Autophagy is a self-degradative process in cells. This process is composed of several closely related steps, including the induction of autophagy, assembly, and formation of the autophagosome, autophagosome docking and fusion with lysosomal membranes, and degradation and recirculation of intra-autophagosomal contents in the autophagolyosome[56], [57] (Figure 2).

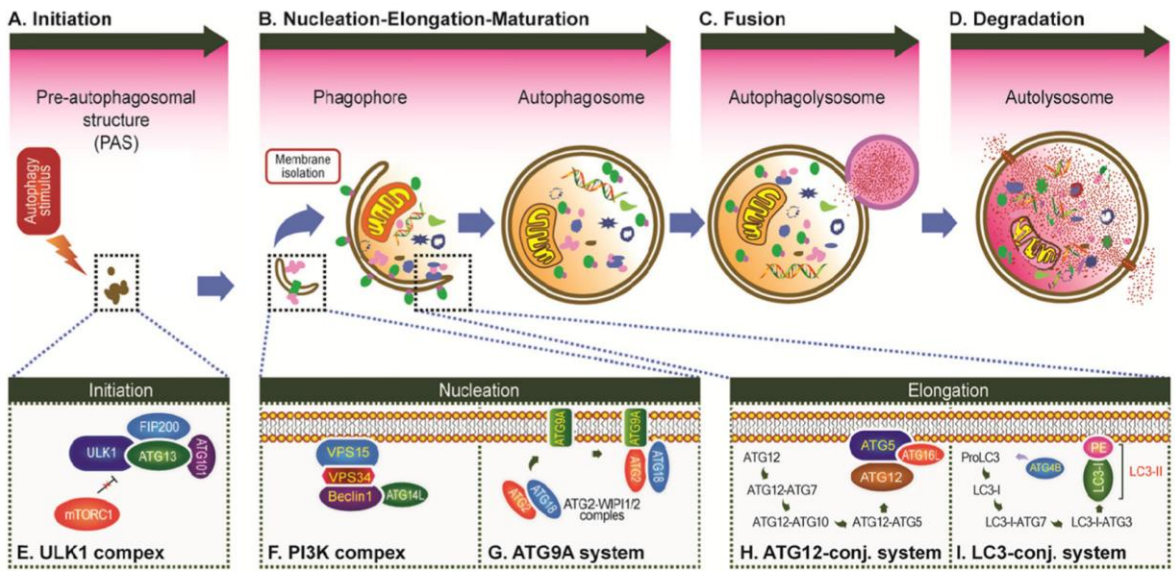

Figure 2 The mechanism of autophagy [58]

The enzyme called TOR kinase is a sensor of nutrient statue and regulate cell growth[53]. Under nutrient-rich condition, the active mTOR- kinase hyperphosphorylates Atg13 and prevents the interaction with ULK1. In response to intracellular and extracellular stimulus for example starvation or oxidative stress induces autophagy by inhibiting MTOR to recycle damaged organelles (e. g. mitochondria)[58]. Rapamycin, a MTOR-inhibitor (TOR), mediates the induction of autophagy by nutrient starvation.[59]In order to this process, Atg13 is no longer phosphorylated by TOR, and it can interact with and activate Atg1 (ULK1)[60]to leading to recruitment of FIP200[60][61].

The protein ATG13 binds ULK1 to a pre-autophagosomal structure (PAS). The PAS is involved in the assembly of various autophagy related (Atg) proteins and is essential for the formation of autophagosomes, which are involved in the process of autophagy. During the induction of autophagy, the ULK1/Atg1 complex (including ULK1, ATG13, FIP200, and ATG101) acts as the initiation complex. Once ATG13 and ULK1 are targeted to the PAS, other ATG proteins are recruited and localized to the PAS, marking the initiation of autophagy[59], [62], [63] (Figure 2A).

The final formation of a mature autophagosome involves multiple steps and the coordination of various Atg proteins[64], [65]. The nucleation of Atg proteins at the pre-autophagosomal structure (PAS) leads to the formation of a phagophore or isolation membrane. The ULK1/Atg1 complex initiates the nucleation process, and the PI3K complex with Beclin1, VPS34, VPS15, and ATG14L is recruited to the PAS to assist in phagophore formation. Additionally, ATG9A positive membrane vesicles associate with the ATG2-WIPI complex (Atg2-Atg18 complex) and are tethered to the PAS[66].

Once the first small ATG9A positive vesicles fuse at the PAS, the membrane elongates and engulfs portions of cytoplasm and organelles. This process is mediated by two ubiquitin-like ATG conjugation pathways: the Atg12-Atg5 conjugation system and the Atg8/LC3 conjugation system (Figure 2B). The isolation membrane forms a closed bilayer membrane structure, resulting in a mature autophagosome with an inner and outer membrane[67]. The process of autophagosome docking and fusion with lysosomal membranes involves the transportation of mature autophagosomes to the perinuclear region[68] (Figure 2C).

Autophagosomes can be formed throughout the cytoplasm, while lysosomes are found primarily in the perinuclear region. Once the mature autophagosomes are formed, they need to be moved to the perinuclear region where they dock and fuse with lysosomes, forming

autophagolysosomes[69] and lysosomal enzymes degrade the inner membrane of the autophagosome and the cytoplasmic components, such as proteins and organelles, into amino acids or peptides for reuse by the cell[70] (Figure 2D).

1.3.2 Mitophagy

Mitochondria are double-membrane-bound subcellular compartments that play a crucial role in various cellular processes such as ATP production, phospholipid biosynthesis/transport, calcium signaling, and iron homeostasis. They also act as platforms for events like apoptosis, innate immune response, and cell differentiation. However, mitochondria are constantly challenged by oxidative stress, which can lead to structural and functional failure. To maintain mitochondrial fitness, cells rely on diverse pathways, DNA repair, protein refolding/degradation, mitochondrial fusion, fission, and mitophagy. Mitophagy is a selective form of autophagy that degrades and removes damaged or excess mitochondria from the cell[71].

Studies have shown that mitophagy is preceded by mitochondrial fission, which divides elongated mitochondria into manageable sizes for encapsulation and selective removal of damaged material. Mitophagy is not only involved in quality control but also plays a role in adjusting mitochondrial numbers to changing metabolic requirements and during specialized developmental stages in mammalian cells. Recently identified pathways that mediate mitophagy include the involvement of the kinase PTEN-induced putative kinase protein 1 (PINK1) and the E3 ubiquitin ligase parkin. These proteins are crucial for the regulation of mitophagy in mammalian cells[72].

PINK1-parkin-induced mitophagy

The process of removing damaged mitochondria, are linked to Parkinson's disease[73]. Two genes, PINK1 and parkin, have been found to play a role in suppressing mitochondrial damage[74]. In mammalian cells, parkin is translocated to damaged mitochondria, and this process is dysregulated in Parkinson's disease. Overexpression of parkin selectively eliminates mitochondria with deleterious mutations, enriching cells with healthy mitochondria. PINK1 is responsible for the recruitment of parkin to damaged mitochondria. Overexpression of PINK1 or its accumulation on mitochondria is sufficient to induce parkin translocation and mitophagy, even in the absence of mitochondrial uncoupling[75]–[78].

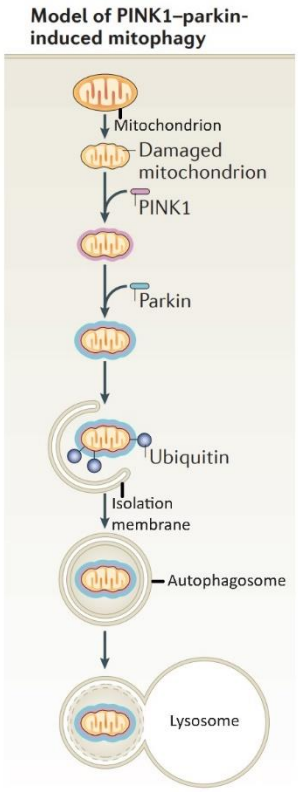

Figure 3 Mechanism of mitophagy [72] *(modified)*

Defects in mitophagy have been associated with various pathological conditions such as neurodegeneration, Parkinson´s disease, heart failure, cancer, and aging. Therefore, understanding the molecular mechanisms of mitophagy is important for developing therapeutic strategies for these diseases[71][72].

1.3.3 Autophagy and cancer

The role of autophagy in cancer is complex because it can function as a tumor suppressor during cancer development but also contribute to tumor cell survival[79], [80]. Tumor cells have the ability to use autophagy as a defense mechanism against cancer treatments. Cancer cells, with their higher metabolic demands and stress, may rely more on autophagy for survival[81].

Studies has shown that Inhibition of autophagy increased the therapeutic effectiveness of cancer therapies[82], [83]. However, the induction of autophagic cell death has also been shown to be a promising method of killing certain types of cancer cells. Autophagy regulation in cancer is still not fully understood but offers potential as a target for cancer treatment[84].

Insufficiencies in autophagy can result in the accumulation of damaged macromolecules and organelles (especially mitochondria) and cause oxidative stress, DNA damage, and chromatin instability[82], [85]. These defects in autophagy are considered to be related with the accumulation of oncogenic mutations and enhanced susceptibility to tumors[86], [87]. The

deletion of the gene Beclin 1, which is involved in autophagy, has been consistently observed in numerous types of cancer[88]–[90]. Activation of the PI3K-Akt-mTOR pathway suppresses autophagy and promotes tumor growth and survival. Several tumor suppressor proteins have been found to promote autophagy[91], [92]. In established cancer cells, metabolic stress can induce autophagy as a way for the cells to find alternative sources of energy and metabolites. Inducing autophagy can also increase chemoresistance and cell survival[81]. Inhibiting autophagy has been shown to sensitize cancer cells to numerous anti-cancer therapies[84], [93].

However, the concept of "autophagic cell death" has been controversial and is prone to misinterpretation. It is recommended to use the term "autophagic cell death" specifically a type of programmed cell death that can be suppressed by inhibiting autophagy[94].There is only a little amount of evidence for autophagic cell death in mammals[95].

Recent studies have shown that many tumors do not respond well to traditional cancer treatments such as chemotherapy and radiation[83] Autophagy is often increased in tumor and normal cells when treated with cancer therapies. However, tumor cells rely more heavily on autophagy for survival, presenting a potential therapeutic opportunity[96]. Inhibition of autophagy has been found to sensitize cancer cells to various anti-cancer drugs and therapies. Modulating autophagy pharmacologically has the potential to improve the efficacy of current anticancer treatments and contribute to cancer eradication in the future[81], [83]. Many solid tumor cells have apoptotic defects, which can make cancer patients more resistant to traditional cancer treatments[97]. Therefore, targeting other mechanisms of cell death, such as autophagy, offers a tempting strategy to improve the efficiency of anti-tumor therapy[84]. While some agents have been shown to trigger autophagic cell death in cancer cells in vitro, the determination of true autophagic cell death can be challenging. However, there are actual cases of autophagic cell death in response to specific compounds and the inhibition or knockdown of key regulators of autophagy might increase cell survival[79].

It has been demonstrated that certain chemotherapeutic agents and phytochemicals van cause autophagic cell death in particular cancer cell types. Additionally, sodium selenite has been found to selectively induce mitophagic cell death in malignant glioma cells. There is evidence that certain cell types, genetic background-, and stimulus-specific ways seem induce autophagic cell death. To determine the potential clinical effectiveness of autophagic cell death in cancer cells, more research is necessary[98], [99].

1.4 Autophagy Inhibitors

1.4.1 Bafilomycin

Bafilomycin (including A1, B, C and D), macrolide antibiotics, were isolated originally from the mycelium of *Streptomyces gresius*. Bafilomycin AI (Figure 4) is a specific Inhibitor of Vacuolar-type H^+-ATPase and induce accumulation of autophagosomes or apoptotic death[100], [101]. Lysosomal proton pump V-ATPases are involved in lysosomal acidification, activation of lysosomal enzymes and autophagy-specific cargo degradation. In principle, bafilomycin A1 inhibits autophagosome-lysosome fusion and blocks lysosomal acidification by prohibiting the passage of protons into the lysosomal lumen[102].

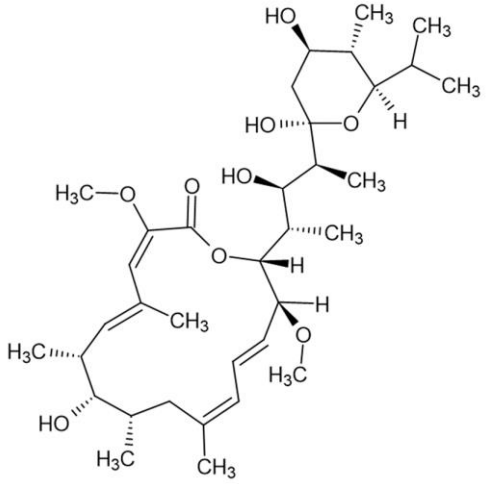

Figure 4 Chemical Structure of Bafilomycin

A study shows that bafilomycin A1 also serves as an inhibitor of Ca-P60A/SERCA activity. There is evidence for a significant enhancement of cytosolic calcium concentration in cells that are treated with bafilomycin, like the efficacy of Thapsigargin, an inhibitor of the Ca2C-transporting ATPase ATP2A/SERCA. Ca-P60A/ATP2A/SERCA stimulates vesicle fusion, and the nature of intracellular Ca^{2+} pools is important for autophagosome-lysosome fusion. The study also shows that abnormally increased Ca^{2+} concentration can inhibit fusion. Bafilomycin A1 also inhibits CaP60A/SERCA to disrupt autophagosome-lysosome fusion, which together results in a robust blockage of autophagic flux[103] (Figure 5).

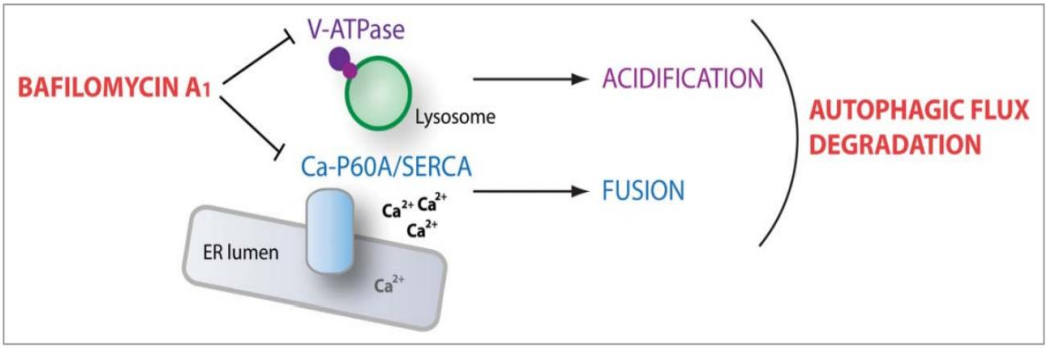

Figure 5 Targets of Bafilomycin[103] *(modified)*

EGFR inhibitors are used to treat bladder cancer. A study observed that cell viability and clonal proliferation in bladder cancer cell lines T24 of bladder cancer were significantly reduced when EGFR inhibitors (lapatinib and gefitinib) and autophagy inhibitors (bafilomycin A1 (BFA1), were combined. The apoptotic effect of EGFR receptors was also enhanced by autophagy inhibitor Bafilomycin[104].

1.4.2 Wortmannin

Wortmannin (Figure 6) is a natural product that was isolated in 1957 from *Penicillium wortmanni Klocker*[105].

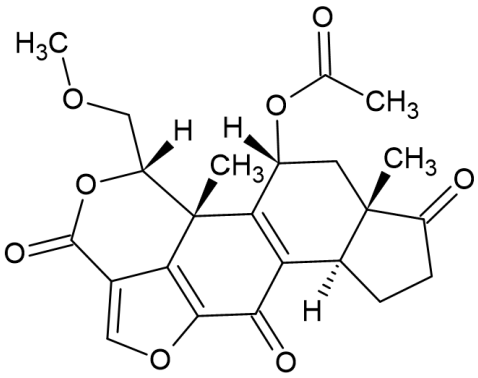

Figure 6 Chemical structure of Wortmannin

Wortmannin is a potent and specific inhibitor of a specific inhibitor of phosphatidyl inositol 3-kinase, and it results to inhibition of autophagosome formation[106], [107]. PI-3 kinase is an enzyme that plays a crucial role in cell growth and differentiation. Due to its potent antiproliferative properties, wortmannin is considered an effective agent in inhibiting cell proliferation[105]. In addition, wortmannin at higher concentrations can also inhibit mTOR, DNA-dependent protein kinase (DNA-PK), myosin light chain kinase, and mitogen-activated protein kinase (MAP kinase)[108] [109]. It has immunosuppressive [110] and potent anti-inflammatory effects[111].

1.5 Toxins

1.5.1 Deoxynivalenol

Deoxynivalenol (DON) (Figure 7) is a mycotoxin and metabolite of *F. graminearum and F. culmorum*. DON is mostly found as contaminant in grain-based foods worldwide. It changes normal cell function by inhibiting protein synthesis through binding to the ribosome and activating critical cell kinases involved in signaling pathways related to proliferation, differentiation, and apoptosis. Although DON is the least toxic trichothecene it can cause symptoms like abdominal distress, increased salivation, malaise, diarrhea, and emesis and anorexia. Different species shows the different sensitivity to DON, to rank order by pig, rodents, dogs, cats, poultry, and ruminants[112]**.**

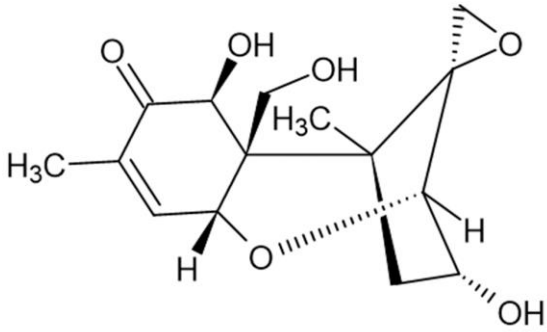

Figure 7 Chemical structure of Deoxynivalenol

According to earlier research DON also shows effects like immunosuppression, oxidative damage, and apoptosis. It has been noted that DON's pro-oxidant properties are the primary cause of immunological damage. DON generates oxidative stress in the body by increasing the amounts of reactive oxygen species (ROS), which inhibits the activity of antioxidant enzymes and accelerates up lipid peroxidation. DON is eliminated with urine and feces after being converted in the liver to the less harmful de-epoxy-DON. Numerous investigations have shown that various dosages of DON may result in acute or chronic liver damage[113]. DON has reproductive effects and crosses the blood brain barrier which causes neurotoxicity and disrupts mitochondrial function [114], [115].

There are currently many studies where DON is also used for treatment on different cancer cell lines.

Colon cancer: The impact of DON on colon cancer cells was observed in terms of cellular morphology and key regulators of cell apoptosis. This included the examination of cytochrome c, caspase-9, caspase-3, Bcl-2, Bax, and Bid. HT-29 cells were used as a sensitive cell line for

the mechanistic investigation. DON treatment inhibited cell proliferation and caused mitochondrial dysfunction and subsequent release of cytochrome c into the cytoplasm and successive activation of caspases, and this was likely regulated by Bcl-2 family proteins 1[115].

Prostate cancer: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potential antiprostate cancer target which triggers the triggers apoptotic signaling through death receptors (DRs). However, some cancer cells are resistant to the cytotoxicity that TRAIL causes. DON pretreatment increased TRAIL-induced apoptosis in androgen-dependent LNCaP and androgen-independent DU-145 cell lines, through oxidative stress and DNA damage. Additionally, DON and DON+TRAIL treatment induced the expression of DR4, a death receptor associated with apoptosis. In *vivo* experiment no significant effects on tumor mass could be found, despite this DON and DON + TRAIL had a larger effect that the TRAIL treatment alone. But furthermore, research is needed to explore the potential efficacy of DON[116].

Interestingly, there is also a study that shows when incubated in the nanomolar range, DON stimulated cell proliferation in the cancerous cells[117].

1.5.2 Fusaric acid

Fusaric acid (Figure 8) is a fungal toxin synthesized by *Fusarium* species that infect cereal grains and other agricultural commodities[118].

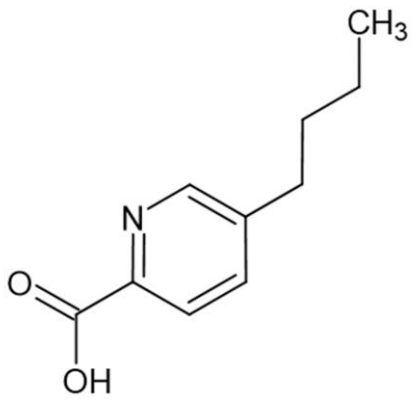

Figure 8 Chemical structure of Fusaric acid

Fusaric acid has been found to have neurochemical effects in the brain and pineal gland. Fusaric acid increases the secretion of melatonin in a dose-dependent manner. It inhibits dopamine beta-hydroxylase[119], which converts dopamine to noradrenaline and the inhibition lowered the blood pressure[120]. Fusaric acid has the potential to enhance the overall toxicity of other

mycotoxins. This means that its main significance in animal toxicity lies in its ability to interact synergistically with other naturally occurring mycotoxins. Two surveys conducted on various cereal grains, mixed livestock, and poultry feed have shown that fusaric acid is a natural contaminant in these food and feed grains. Additionally, several toxic mixed feeds and corn samples have been found to contain fusaric acid along with other mycotoxins[121].

FA can be toxic to humans. When exposed to FA, it can increase oxidative stress within cells, leading to mitochondrial energy dysfunctions, DNA damage, and apoptotic cell death [122].

A study has demonstrated that FA downregulates the mRNA expression of genes involved in the Toll-like receptor (TLR) pathway and inhibits the antiproliferative activity in Ishikawa endometrial cancer cells. These findings suggest that FA could potentially be an effective and safe natural compound for the treatment of endometrial cancer[123].

Another study evaluated tumoristatic properties of fusaric acid an *in vitro* model of HNSCC (Head and Neck Squamous Cell Carcinoma). Its mechanism of action offers a potential alternative approach to current therapies. Paclitaxel, a drug used in clinical trials for HNSCC treatment, has demonstrated significant tumoricidal effects in vitro. Fusaric acid combined with paclitaxel and have displayed synergistic and tumoricidal effects against HNSCC in vitro. However, before considering a clinical trial with the combination of fusaric acid and paclitaxel, further investigation is needed to understand the mechanism, toxicity, and tumor response in an in vivo model [124].

1.6 Aim of this Study

Bladder cancer cells have developed sophisticated adaptive capabilities to survive in the challenging environment of the bladder, which is constantly exposed to a complex chemical mixture and shear stress during the filling and emptying cycles. To evaluate the potential of using natural toxins as treatments, but also to shed light on factors contributing to tumor aggressiveness, it is central to have a solid knowledge about the mechanism of these toxins, and how they interact with the other stressors faced by the bladder epithelium. This study specifically focuses on understanding the role of autophagy in the adaptiveness of bladder cells to the physical challenge of shear stress and the toxins DON and FA, by inhibiting autophagy in the T24 cancer cells. Through this, enhancing our understanding of how bladder cells respond to secondary metabolites and whether this response can impact important characteristics of bladder cancer aggressiveness, such as the ability to withstand mechanical stimuli.

2 Materials and Methods

2.1 Materials

Table 1 List of reagents and material used in the study

Name	Supplier	Reference ID	Additional Information
Solutions			
McCoy's 5A Medium	Gibco	22330-021	
Fetal calf serum	Gibco	10270-106	
Penicillin/Streptomycin	Gibco	15140-122	
Trypsin	Gibco	-	
DPBS	Gibco	14190-094	
Crystal Violet	Fluka	548-62-9	0,1% dissolved in EtOH
DMEM	Gibco	21063-023	
CellTiterBlue reagent	Promega		1:10
Treatments			
Bafilomycin A1	Sigma-Aldrich	SML1661	
Wortmannin	Sigma-Aldrich	W3144	
Fusaric acid	Sigma-Aldrich	F6513	
Deoxynivalenol	Romer Labs		
Live cell imaging			
Live cell imaging solution	Invitrogen by Thermo Fisher Scientific	A14291DJ	
Mitotracker ™	Invitrogen by Thermo Fisher Scientific	M7514	1:1000
Lysotracker ™	Life technologies	L7528	1:1000

Cellmask Deepred™	Invitrogen by Thermo Fisher Scientific	C10046	1:2000
Plates/Flasks			
TC-Flasks	Sarstedt	83.3911.002	
µ-Slide I $^{0.4}$ Luer Collagen	Ibidi GmbH	80172	
TC-Platte 96 Well	Sarstedt	83.3924	
ELISA plate black Med. Bind. F	Sarstedt AG Co.	82.1581.120	CTB- ASSAY
µ-Slide 8 Well ibiTreat	Ibidi GmbH	80826	Live cell imaging

2.2 Methods

2.2.1 Cell Culture

The urinary bladder carcinoma cell line T24 (ATCC® HTB4™) was purchased from ATCC. The cells are cultivated in flasks in an incubator at 37°C and 5% CO_2. Cells were regularly passaged three times per week.

Media composition

Agent	Quantity
McCoy's 5A Medium	89 %
Fetal Calf Serum	10 %
Penicillin/Streptomycin Solution	1 %

T24 cultures and all incubations was performed using the medium in TC-Flasks T75. After removal of the medium and a washing step with Dulbeccos Phosphate Buffered Saline (DPBS, gibco) cells were trypsinised for 2 min with Trypsin solution. Cells were counted with a Neugebauer Counting chamber[125].

Cell Counting

After dilution the cell suspension with Trypan blue 1:5, measurement of cells counts per mL was determined. Since the cell membrane becomes more permeable after cell death the dye can penetrate and the cell appears blue, living cells with an intact cell membrane continue to be

transparent through the exclusion of the dye. A Neubauer counting chamber was filled with 10 L of the solution, and the solution was counted using an Olympus SC50 (10x magnification) microscope from Tokyo, Japan. Equation 1 was employed to calculate the concentration of live cells. The viable cells/mL number is used for further calculations.

$$\frac{\text{cells}}{\text{mL}} = \frac{\text{mean living cells} * \text{dilution factor}}{\text{camber depth} * \text{counted area}}$$

2.2.2 Treatment list

Table 2 List of single treatment

Bafilomycin 1nM (BAFI 1nM)	Wortmannin 0,1 µM (WORT 0,1µM)	Deoxynivalenol 0,1 µM (DON 0,1 µM)	Fusaric acid 0,1mM (FA 0.1mM)
Bafilomycin 10nM (BAFI 10nM)	Wortmannin 1 µM (WORT 0,1µM)	Deoxynivalenol 1 µM (DON 1 µM)	Fusaric acid 0,5mM (FA 0.5mM)
		Deoxynivalenol 10 µM (DON 10 µM)	Fusaric acid 1 mM (FA 1mM)

Table 3 list of combined treatment

BAFI 1nM + DON 0,1 µM BAFI 1nM+ DON 1 µM BAFI 1nM+ DON 10 µM	BAFI 1nM + FA 0.1mM BAFI 1nM+ FA 0.5mM BAFI 1nM+ FA 1mM
BAFI 10nM+ DON 0,1 µM BAFI 10nM+ DON 1 µM BAFI 10nM+ DON 10 µM	BAFI 10nM+ FA 0.1mM BAFI 10nM+ FA 0.5mM BAFI 10nM+ FA 1mM
WORT 0,1µM+ DON 0,1 µM WORT 0,1µM+ DON 1 µM WORT 0,1µM+ DON 10 µM	WORT 0,1µM+ FA 0.1mM WORT 0,1µM+ FA 0.5mM WORT 0,1µM+ FA 1mM

2.2.3 Quantification of cell morphology:

For the image analysis, data were used that were previously generated in the frame of another study. As previously described, cells ware seeded in µ-Slide I 0.4 Luer Collagen IV. Cells were exposed to shear stress for three hours at 2.7 dyn/cm^2 and were compared to static controls[126].

Static controls and cells subjected to shear stress (2.7 dyn/cm^2 for 3 hours) were compared in 22000 T24 Cells planted in -Slide I 0.4 Luer Collagen IV slides. Using an inverted Olympus microscope (CKX53SF, OLYMPUS, Tokyo, Japan), brightfield pictures were captured. A software named imageJ was applied for the quantification, cell area was selected using the "Polygon selection" tool and the parameters circularity ($4*\pi*(\text{area}/\text{perimeter}^2)$), aspect ratio (major axis/minor axis), solidity (area/convex area) and roundness ($4*\text{area}/(\pi*(\text{major axis})^2)$) were measured[127]. A total number of 15 cells per condition from at least 3 different independent cell preparations (biological replicates)[126].

2.2.4 Cell viability

Cell viability was determined by using the cell titer blue assay (CTB) assay and crystal violet assay.

2.2.4.1 CellTiter-Blue (CTB) assay

CellTiter-Blue assay is cell viability assay which contains highly purified resazurin. This assay is based on reduction of resazurin (dark blue) to fluorescent product resorufin, which is pink[128]. Nonviable cells quickly lose this ability through lacking metabolic capabilities, but viable cells can metabolize the dye. The resorufin fuses into the surrounding culture medium even if the actual decrease occurs inside the cell. Resorufin exhibits a substantial rise in fluorescence upon reduction, and this increase is inversely correlated with the number of viable cells. In culture medium including serum, the resorufin excitation and emission peaks are roughly 579 nm and 584 nm, respectively, allowing for fluorescence measurements. In addition, when resazurin is converted to resorufin, there is an absorbance shift from 605nm to 573nm[129].

The cells were cultivated for 24 hours and afterwards the treatments were applied to the cells. After incubating with the treatment for 24 hours, the medium was removed and incubated with a solution of the cell titer-blue reagent (1:10 in DMEM phenol red free) for 2 hours. The supernatant was transferred into an ELISA plate black Med. Bind. F. A multi-detector microplate reader, the Synergy H1 Hybrid (BioTek, Bad Friedrichshall, Germany), was used to measure the supernatant at 560/590 nm[130].

2.2.4.2 Crystal violet assay

During cell death, adherent cells detach from cell culture plates. This characteristic can be used to evaluate changes in cell proliferation and to indirectly quantify cell death. Crystal violet dye, which binds to proteins and DNA, can be used to stain adherent cells to identify cells that have retained their adhesion. The amount of crystal violet staining bound decreases because of cells undergoing cell death and their subsequent removal. This method shows that of the impact of chemotherapeutics or other compounds on cell survival and growth inhibition[131].

For the determination of cell viability based on cell biomass, cells were rinsed with pre-warmed D-PBS and fixed for 10 min with ice-cold EtOH (99%)[126]. Afterward, cells were stained for 5 min with Crystal Violet solution (0.1%) and rinsed four times with autoclaved dH2O. As the last step, cells were lysed with a mixture of 99% cold EtOH and 1% of acetic acid (10 min, shaking incubation 500 U/Min), and absorbance was measured (λ = 595 nm) with a Cytation 3 multi-mode plate reader (BioTek Instruments, Winooski, VT, United States). Every dataset resulted from the analysis of minimum three independent cell preparations, and measurements were performed in technical triplicates. The absorbance was determined at 595 nm by using a Synergy H1 Hybrid multi-detector microplate reader (BioTek, Bad Friedrichshall, Germany)[126].

2.2.5 Live cell imaging:

For the live cell imaging 20000 cells were seeded in µ-Slide 8 Well ibiTreat. The cells were incubated with the treatment and solvent control for 24 hours. At first the treated cells were washed with the live cell imaging solution and stained with a solution of the dyes in life cell imaging solution (1x) HEPES buffered physiological saline for 15 minutes at 37°C. Mitotracker (1:1000), Lysotracker (1:1000) and cell mask DeepRed PM (1:2000), were used to locate mitochondria, lysosomes, and cell membrane respectively[126], [132].

After the cells were incubated for 15 min with the staining solution, the washing process with LCIS is repeated 2 more times and the cells were imaged in LCIS, with a Zeiss LSM710 laser scanning confocal microscope (ELYRA PS.1 system) using a 63x/1.46 Plan-Apochromat oil immersion objective (Zeiss Microscopy GmbH, Germany). The program ZEN 2021 Black Edition (Zeiss Microscopy GmbH, Germany) was used for analysis and quantification[126].

2.3 Statistical evaluation

The data was statistically evaluated, and all the graphs were generated with OriginPro, Version 2022 SR1 (OriginLab Corporation, Northampton, MA, USA). Student's t-tests ($n > 40$) and ANOVA tests ($n > 3$, dosage dependent data sets) were used for the experiments. P values < 0.05 were considered as significant, and at least three separate biological replicates (cell preparations) were performed for each experiment.

3 Results

3.1 Morphological adaption to shear stress and autophagy inhibition

Autophagy plays a significant role in the mechanosensory apparatus of cells. It is known that the bladder cells are constantly subject to physical stimulation, such as through shear stress. In this study, we examined the effects of two autophagy inhibitors, bafilomycin and wortmannin, by applying a 3-hour physical stimulus to T24 bladder cancer cells. Our goal was to test the cells ability to adapt to fluid shear stress (2.78 dyn/cm^2) and verify the effectiveness of these inhibitors in simulated *in vivo* conditions (Figure 9).

To quantify the changes in cell morphology, we evaluated important parameters including roundness, circularity, solidity, and aspect ratio (Figure 10). In static conditions, bafilomycin at a concentration of 10 nM caused a significant decrease in circularity and solidity (Figure 10a, c), while Wortmannin at a concentration of 1 µM increased roundness and decreased aspect ratio compared to the control (Figure 10b, d). When comparing the static condition to the shear stress condition, no changes were observed in the control group. Bafilomycin increased circularity, roundness, and solidity but decreased aspect ratio, while wortmannin only effected circularity (Figure 10a-d).

Interestingly, in presence of shear stress, the effects of Bafilomycin on cell morphology observed in static conditions were reversed. Both Bafilomycin and Wortmannin led to a decrease in aspect ratio compared to the control (Figure 10d). Overall, it seems that shear stress eliminates any significant differences in the morphological profile of the cells. This suggests that mechanotransduction can override certain chemical signals, resulting in cells that are more rounded and less protruding, indicating that the physical signal is dominant over the chemical one.

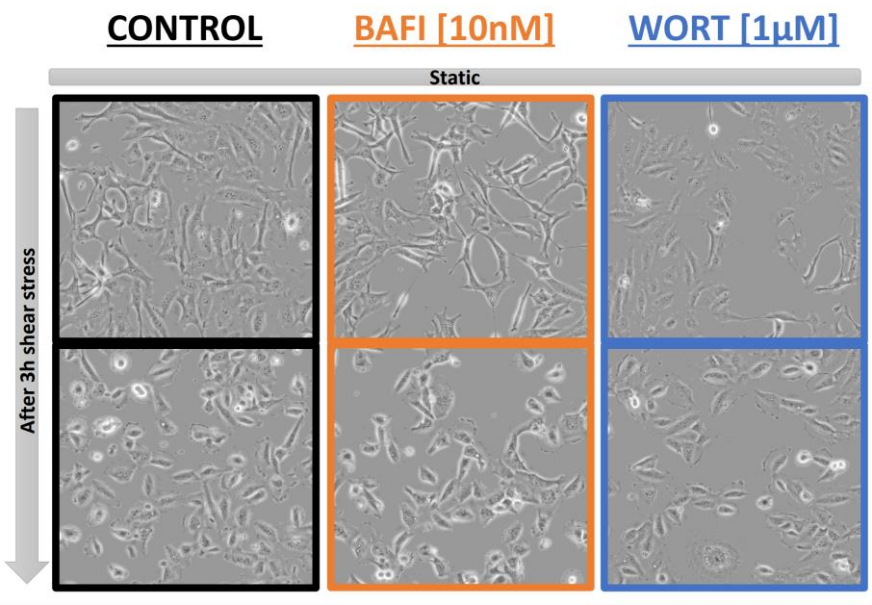

Figure 9 Images of the morphological response after the incubated T24 cells were under static condition and after 3-hour shear stress (3h, 2,7 dyn/cm²) conditions (scale bar: 100µm)

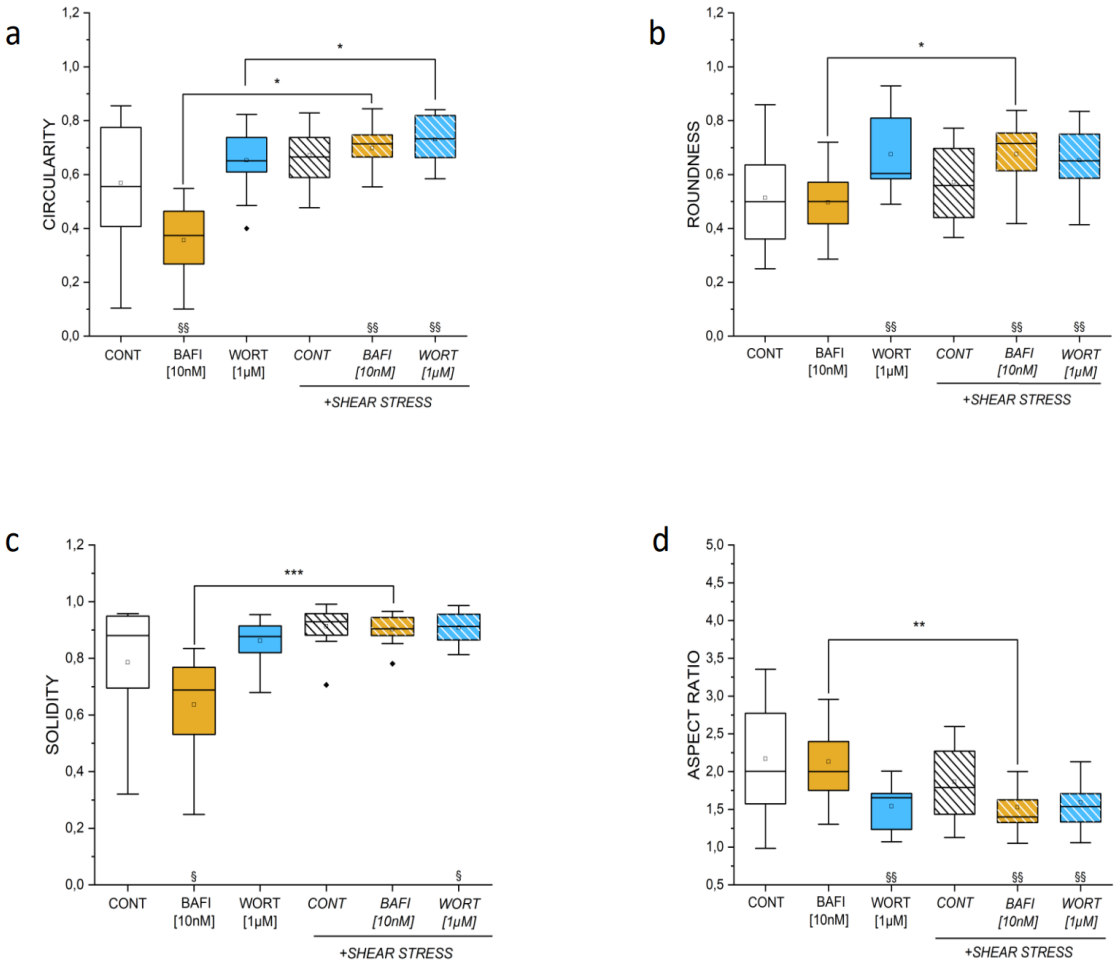

*Figure 10 Quantification of morphological parameters: circularity, roundness, solidity and aspect ratio, the following symbols indicate statistical significance for the student's t-test: * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).*

3.2 Autophagy modulates cytotoxicity of DON and FA

Autophagy plays a crucial role in the cellular stress response, especially when dealing with oxidative stress. It helps the cell adapt and maintain metabolic function by degrading damaged mitochondria. Due to this, it was hypothesized that cells lacking functional autophagy might be more sensitive to toxins that target mitochondria, resulting in increased toxicity.

To test this hypothesis, CellTiter-Blue (CTB) assays were conducted to measure cell viability, which is indicative of mitochondrial function. Additionally, crystal violet assays were performed to assess the total cell mass and investigate cytotoxic effects.

The findings showed that the autophagy modulators, bafilomycin and wortmannin, had limited impact on cell viability or only minor effects on cell mass. However, higher concentrations of Bafilomycin led to a decrease in cell mass (Figure 11, Figure 13). In contrast, wortmannin at a concentration of 0.1 µm showed higher cell viability and cell mass, while no significant changes were observed at other concentrations (Figure 12, Figure 14). DON shows concentration-dependent effects on cell viability and toxicity and as well as combined with BAFI and WORT (Figure 11, Figure 12).

Cells incubated with DON [0,1µM] had higher viability yet this effect changed with the higher concentrations [1-10µM] (Figure 11a, Figure 12a). This shows that the concentration dependent toxicity of DON. DON, with a concentration of 10µM, exhibits a high level of toxicity and similar effects of DON are also seen when it is combined with BAFI [1nM] and WORT [0,1µM]. When autophagy is inhibited using BAFI [10nM] and WORT [1µM], the effect of DON at concentrations of 0.1µM and 1µM on cell viability and toxicity increases compared to when DON at concentrations of 0.1µM and 1µM is combined with a lower concentration of the Autophagy inhibitors (Figure 11, Figure 12).

FA [0.1mM] significantly increases cell viability, but fusaric acid has been shown to increase toxicity at higher concentrations. As with DON, the effects of FA were also partly enhanced when combined with BAFI and WORT. There are no significant changes in toxicity between FA [1mM] and FA [1mM] in combination with an autophagy inhibitor (Figure 13, Figure 14). The toxicity of FA [0.5mM] increases when combined with increasing concentrations of WORT (Figure 14b).

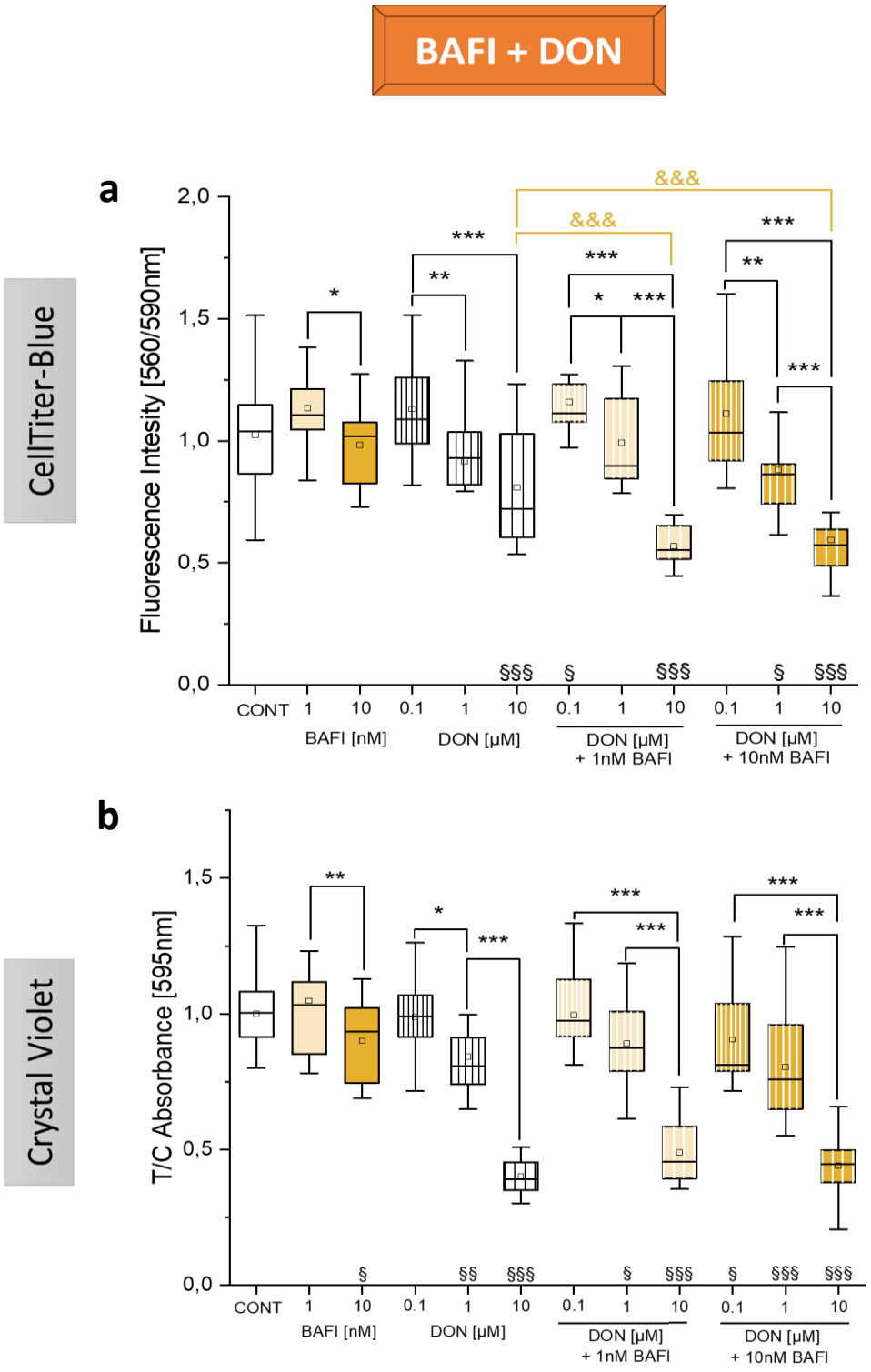

*Figure 11 Changes in cell viability and cytotoxicity in T24 cells response to autophagy inhibitors Bafilomycin (BAFI; 1-10nM), mycotoxin Deoxynivalenol (DON; 0.1-1-10µM) and DON combined with BAFI. . The graphics a shows the result of the Cell-Titer Blue (CTB) assays, which was measured in fluorescent intesity [560/590nm]. The graphic b is the results of Crystal violet assays and these results are presented as the mean treatment/control absorbance [595nm]. According to the one-way ANOVA Test and Fisher LSD Means Comparison, statistical significance is displayed. The symbol & shows significant changes compared to the control, , & shows changes caused by autopagy inhibiton and * denotes significance changes between all other conditions. §/&/*, §§ /&&/**, and §§§/&&&/*** ($p < 0.05$, 0.01, and 0.001, respectively).*

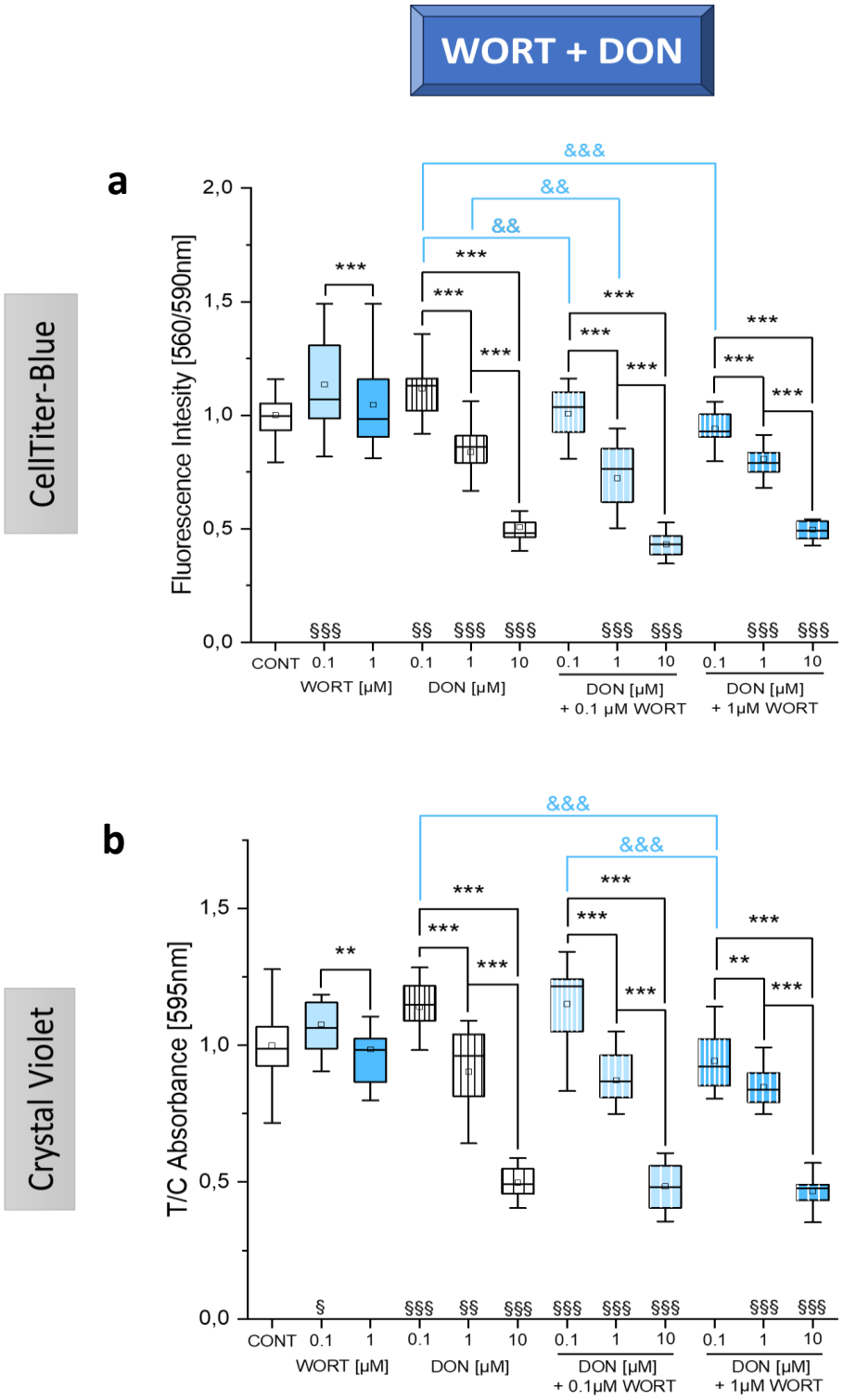

*Figure 12 Changes in cell viability and cytotoxicity in T24 cells response to autophagy inhibitors Wortmannin (WORT; 0.1-1µM), mycotoxin Deoxynivalenol (DON; 0.1-1-10µM) and DON combined with WORT. The graphics a shows the result of the Cell-Titer Blue (CTB) assays, which was measured in fluorescent intesity [560/590nm]. The graphic b is the results of Crystal violet assays and these results are presented as the mean treatment/control absorbance [595nm]. According to the one-way ANOVA Test and Fisher LSD Means Comparison, statistical significance is displayed. The symbol & shows significant changes compared to the control, , & shows changes caused by autopagy inhibiton and * denotes significance changes between all other conditions. §/&/*, §§ /&&/**, and §§§/&&&/*** ($p < 0.05$, 0.01, and 0.001, respectively).*

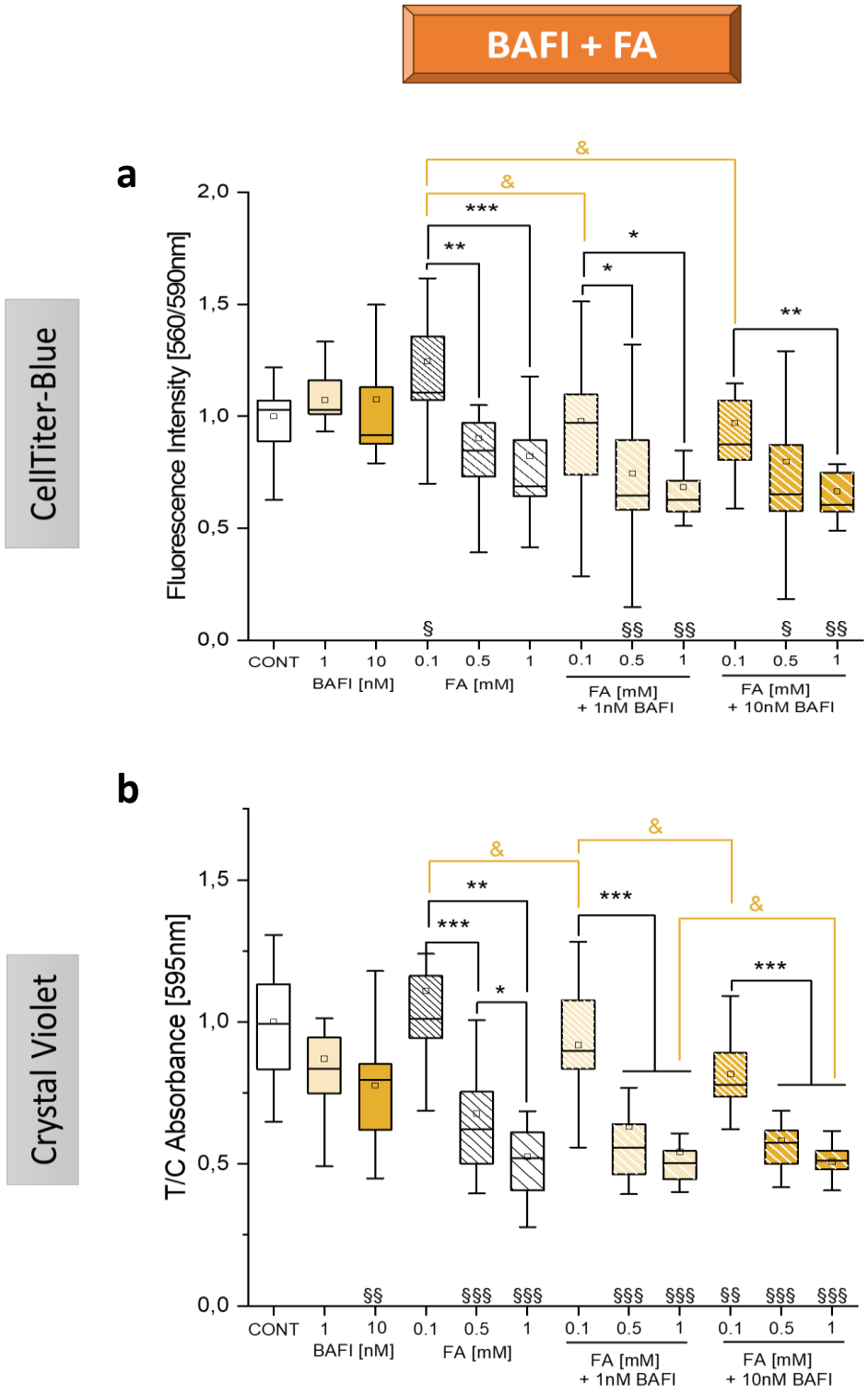

*Figure 13 Changes in cell viability and cytotoxicity in T24 cells response to autophagy inhibitors Bafilomycin (BAFI; 1-10nM), mycotoxin Fusaric acid (FA; 0.1-0,5-1µM) and FA combined with BAFI. . The graphics a shows the result of the Cell-Titer Blue (CTB) assays, which was measured in fluorescent intesity [560/590nm]. The graphic b is the results of Crystal violet assays and these results are presented as the mean treatment/control absorbance [595nm]. According to the one-way ANOVA Test and Fisher LSD Means Comparison, statistical significance is displayed. The symbol & shows significant changes compared to the control, , & shows changes caused by autopagy inhibiton and * denotes significance changes between all other conditions. §/&/*, §§ /&&/**, and §§§/&&&/*** (p < 0.05, 0.01, and 0.001, respectively).*

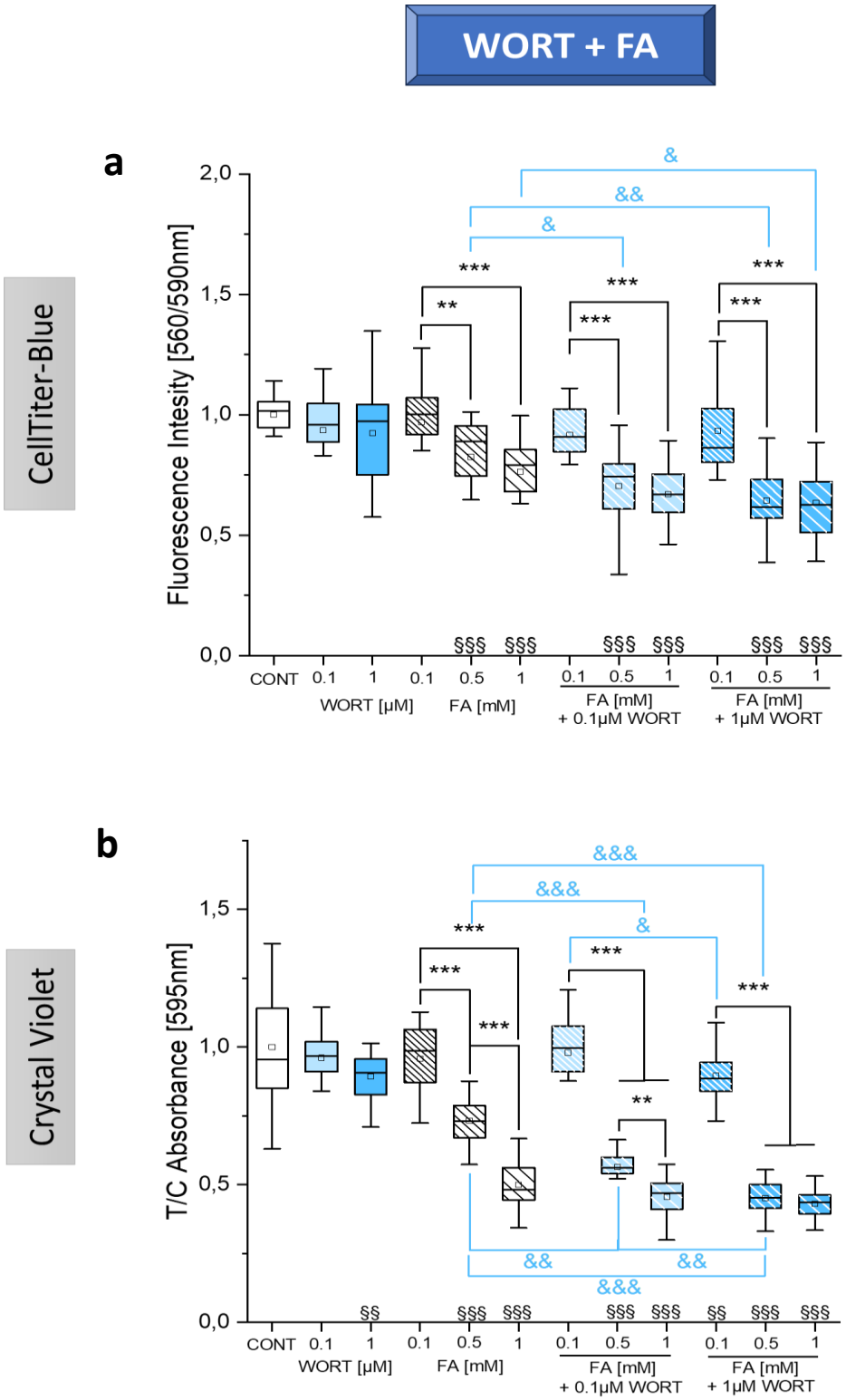

*Figure 14 Changes in cell viability and cytotoxicity in T24 cells response to autophagy inhibitors Wortmannin (WORT; 0.1-1µM), mycotoxin Fusaric acid (FA; 0.1-0,5-1µM) and FA combined with WORT. The graphics a shows the result of the Cell-Titer Blue (CTB) assays, which was measured in fluorescent intesity [560/590nm]. The graphic b is the results of Crystal violet assays and these results are presented as the mean treatment/control absorbance [595nm]. According to the one-way ANOVA Test and Fisher LSD Means Comparison, statistical significance is displayed. The symbol & shows significant changes compared to the control, , & shows changes caused by autopagy inhibiton and * denotes significance changes between all other conditions. § /&/*, §§ /&&/**, and §§§/&&&/*** (p < 0.05, 0.01, and 0.001, respectively).*

3.3 Live cell imaging

To clarify the underlying molecular mechanisms of our treatments, we first determined the fate of the involved organelles) (Figure 15). Building upon the previous findings, our research was specifically focused on the mitochondria [125]. As a first step we measured the mitochondrial signal in areas where it was colocalized with lysosomes and in areas without lysosomes, which were mostly found in the periphery of the cell.

In order to obtain a more comprehensive understanding, we also examined the lysosomes, the role in autophagy. Additionally, considering the cell membrane as a potential downstream target of mitochondrial disruption[133], it was also investigated in our study.

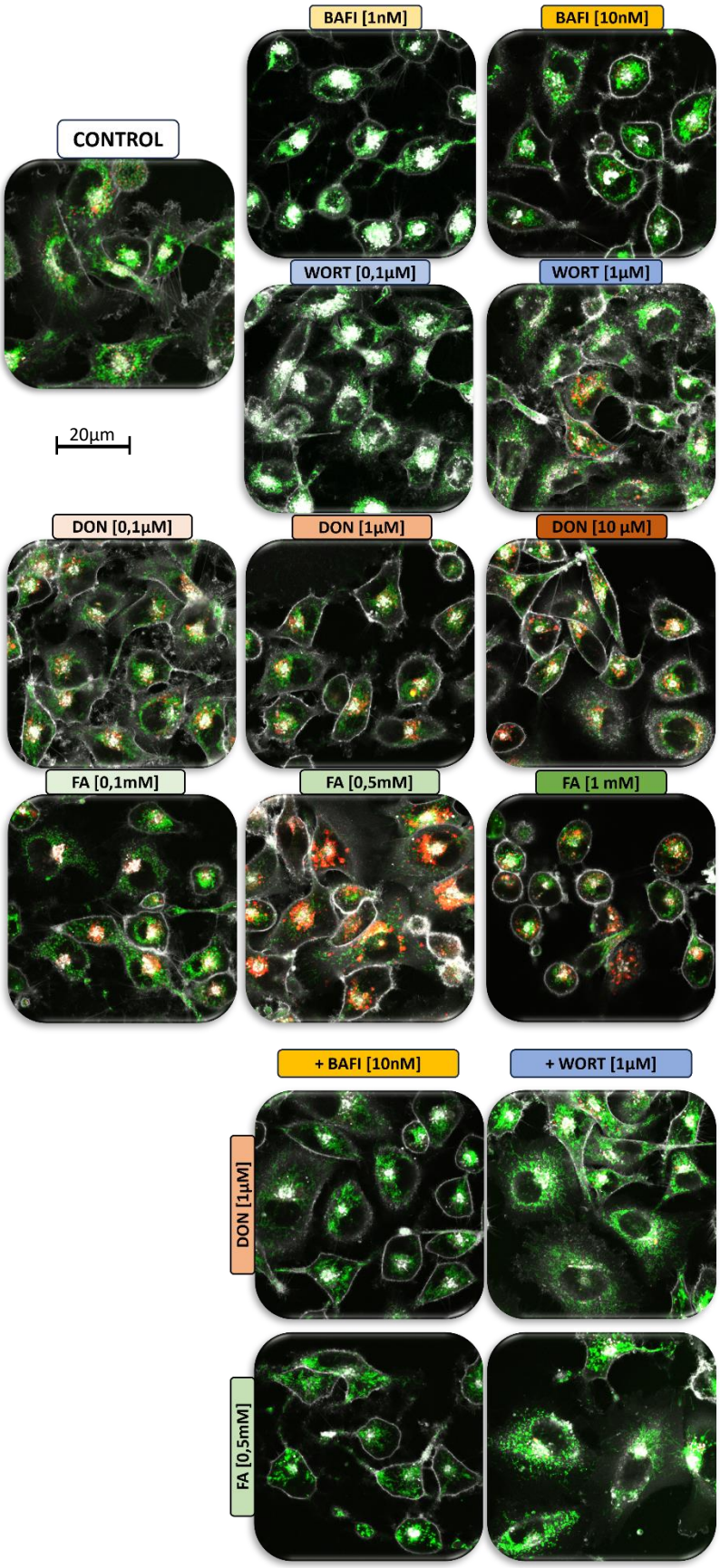

Figure 15 Representative images of the staining for the conditions, at least n >30 cells per condition were evaluated from at least three independent cell preparations (biological replicates), Mitochondria are depicted in green, membrane in gray, lysosomes in red.

3.3.1 Effects on mitochondria

Parallel to the investigation of the other two organelles mitochondrial signal was examined in two regions of interest (ROIs): lysosomal, which was typically found in the perinuclear region, and for the second ROIS, areas were chosen where lysosomes were not present, primarily located in the peripheral regions.

The overall mitochondrial signal was higher in the lysosomal regions of the cell. In the lysosomal area, cells exposed to BAFI [1nM] showed an increased signal, and WORT [1µM] decreased the signal of mitochondria. When DON [1µM] and FA [0.5mM] both combined with autophagy inhibitor WORT [1µM], it displayed a reduction in the mitochondrial signal (Figure 16a).

We observed that the mitochondrial signal in the periphery of the cells, which were treated with BAFI [1nM] and in combination with FA [0.5mM], increased the signal. The combination of WORT and DON also increased the signal. Furthermore, for FA at a concentration of [0.1mM], cells displayed a decrease in the mitochondrial signal, which turned into a significant decrease at higher concentrations [0.5-1mM] (Figure 16b).

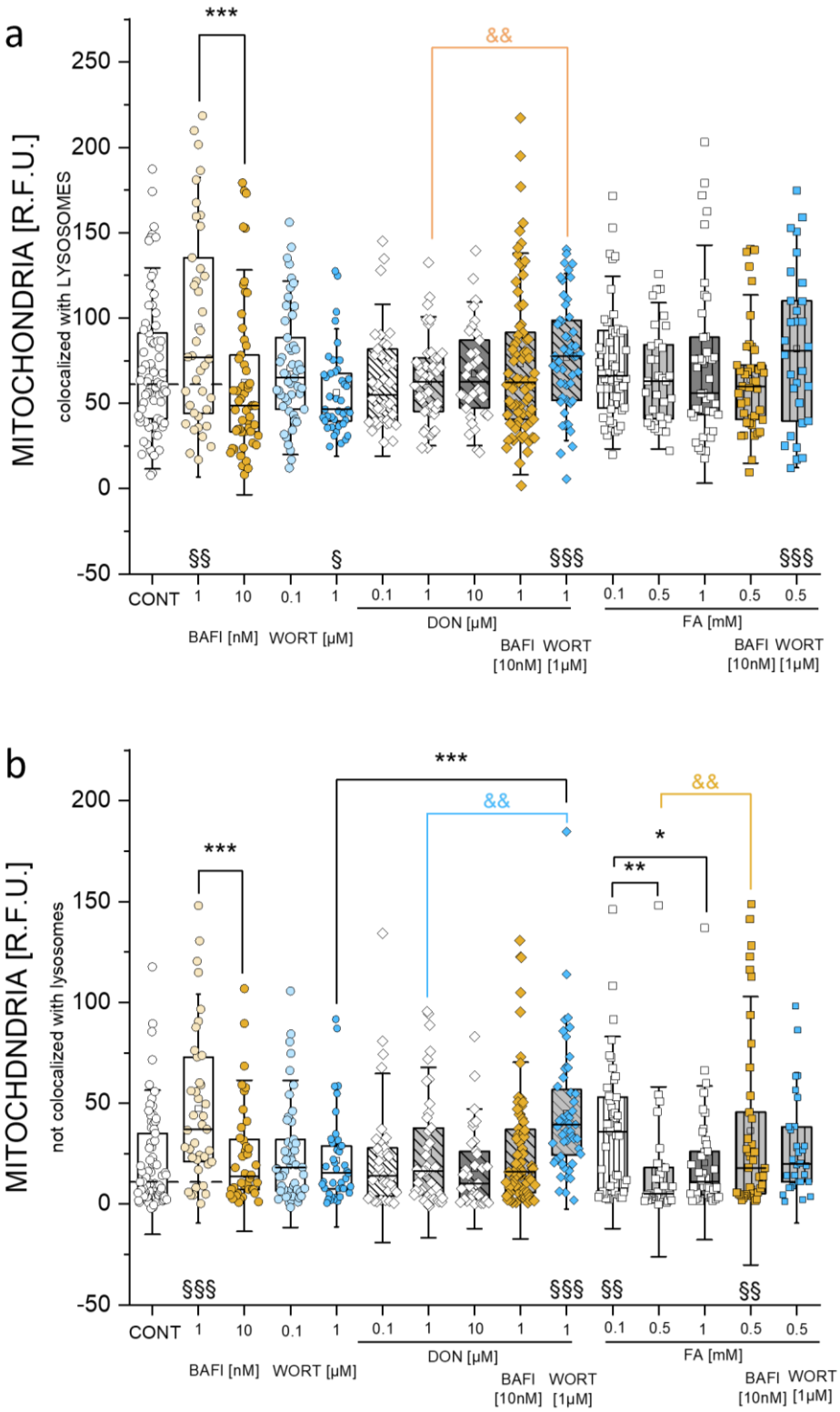

*Figure 16 Quantification of the mitochondrial signal, **a** in areas co-localized with lysosomes, and **b** areas without lysosomes. Live cell imaging of T24 cell response to autophagy inhibition bafilomycin (BAFI; 10nM) or wortmannin (WORT; 0.1µM), in combination with the mycotoxins fusaric acid (FA; 0.1-0.5-1mM) and deoxynivalenol (DON; 0.1-1-10µM). According to the one-way ANOVA Test and Fisher LSD Means Comparison, statistical significance is displayed. The symbol & shows significant changes compared to the control, , & shows changes caused by autopagy inhibiton and * denotes significance changes between all other conditions. §/&/* ($p < 0.05$), §§/&&/** ($p < 0.01$) and §§§/&&&/*** ($p < 0.01$).*

3.3.2 Effects on lysosome

The lysosomal signal represents the correct function of the autophagy inhibitors, for bafilomycin, we found the signal significantly reduced fitting previous studies using the lysotracker as dye. Bafilomycin inhibits the acidification of the autophagosome, thereby preventing the detection of the pH-dependent dye and resulting in a very low lysosome signal for BAFI[134]. Similar effects were seen when BAFI was co incubated with the toxins. for example, BAFI reversed the increased signal of cells treated with FA [0.5mM].

After incubation with a higher concentration of wortmannin [1µM], we observed an accumulation of lysosomes, which is consistent with the inhibition of autophagosome formation. The toxins mostly decreased the lysosomal signal, except for FA [0.5mM], which could be attributed to an increased demand for autophagy to repair damaged compounds. Similarly, with DON and FA incubated cells, along with the inhibitor WORT, resulted in a decreased lysosomal signal compared to controls and WORT alone (Figure 17).

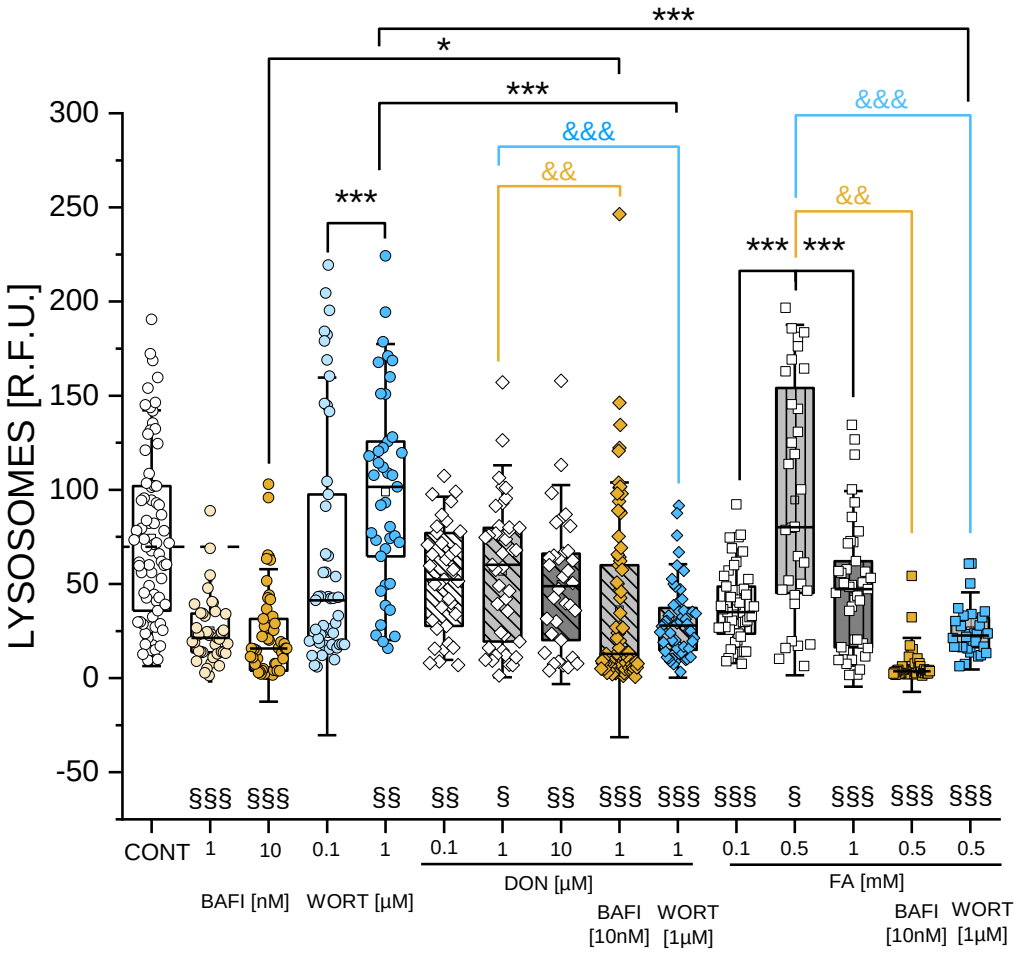

*Figure 17 Signal of the lysosomes. Live cell imaging of T24 cell response to autophagy inhibition bafilomycin (BAFI; 10nM) or wortmannin (WORT; 0.1µM), in combination with the mycotoxins fusaric acid (FA; 0.1-0.5-1mM) and deoxynivalenol (DON; 0.1-1-10µM). According to the one-way ANOVA Test and Fisher LSD Means Comparison, statistical significance is displayed. The symbol & shows significant changes compared to the control, & shows changes caused by autophagy inhibition and * denotes significance changes between all other conditions. §/&/* ($p < 0.05$), §§/&&/** ($p < 0.01$) and §§§/&&&/*** ($p < 0.01$).*

3.3.3 Effect on cell membrane

Cells incubated with autophagy inhibitors BAFI shows an increased membrane signal when incubated at lower concentrations, but a decreased signal at higher concentrations. WORT treated cells displayed increased membrane signal at both tested concentration in comparison to control. Interestingly the effects of the lower concentration (WORT [0,1µM]) are more significantly higher than WORT[1µM].

DON does not exhibit any changes compared to the control. FA at a higher concentration displays a lower signal. However, when both toxins are combined with BAFI, the signal significantly decreases, whereas the combination with WORT increases the membrane staining dye (Figure 18).

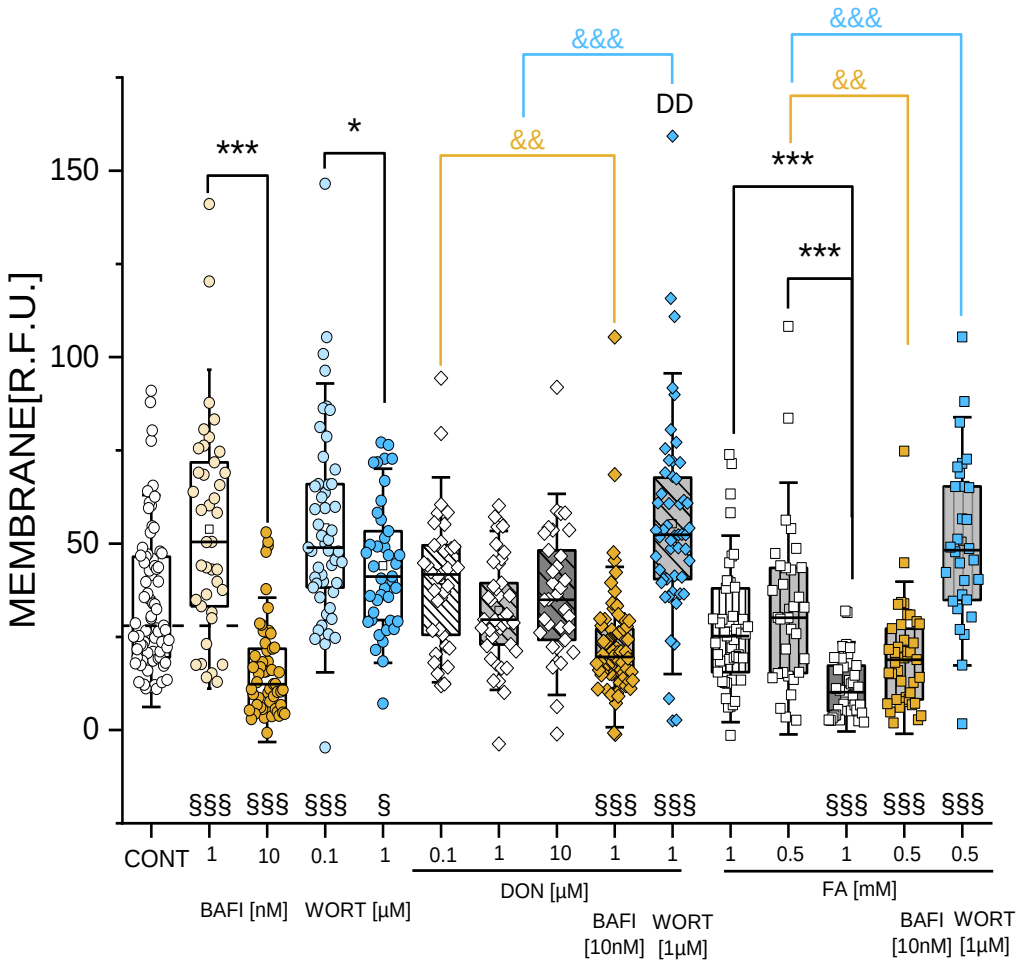

*Figure 18 Quantification of the membrane intensity Live cell imaging of T24 cell response to autophagy inhibition bafilomycin (BAFI; 10nM) or wortmannin (WORT; 0.1µM), in combination with the mycotoxins fusaric acid (FA; 0.1-0.5-1mM) and deoxynivalenol (DON; 0.1-1-10µM). According to the one-way ANOVA Test and Fisher LSD Means Comparison, statistical significance is displayed. The symbol & shows significant changes compared to the control, , & shows changes caused by autopagy inhibiton and * denotes significance changes between all other conditions. §/&/* ($p < 0.05$), §§/&&/** ($p < 0.01$) and §§§/&&&/*** ($p < 0.01$).*

4 Discussion

Bladder cells are consistently exposed to shear stress originating from urine or interstitial fluids as well as the accumulation of xenobiotics and bioactive metabolites due to the excretion of numerous compounds through urine[125], [126]. Many industrial and environmental toxins that seem to be easily tolerated at low doses may cause renal damage at higher doses. Furthermore, some toxic substances that are carelessly used despite being poorly tolerated at any dose[135]. The bladder urothelium, which acts as a permeability barrier for urine, senses temperature, pH, fullness, and infection. The organ possesses a significant number of receptors and channels that to detect changes in the luminal environment and release multiple types of mediators in response. Many diseases are thought to result from abnormalities in the urothelium's sensing or signal transduction. Similarly, toxins may also modulate the signaling capabilities of the tissue. For example, DON and FA, activating MAPKs which involved in inflammation and immunotoxicity, may and change the expression of important parts of the signaling cascade. Along this line, cancer is one of the most prevalent diseases affecting the bladder[136].

Autophagy plays a critical role in the cellular elimination of unfolded proteins and damaged or unnecessary organelles, including mitochondria, peroxisomes, ribosomes, endoplasmic reticulum, and endosomes. Autophagy may be induced as a stress response and/or starvation in order to recycle cell components to maintain cellular homeostasis. This means that autophagy is considered of as a stress-related mechanism for cell survival[137]–[139].

Although autophagy as a whole is largely investigated, many downstream processes of its induction or inhibition remain unexplored. Hence, it plays a dual role as a double-edged sword in numerous diseases[140].The survival of tumor cells is significantly influenced by autophagy. While some cancer cells rely on autophagy to survive, others inhibit autophagy as a survival strategy. However, the impact of autophagy on bladder cancer cells is an open and interesting research question[79]–[83]. Numerous studies have shown that inhibiting autophagy can enhance the effectiveness of cancer therapies. Therefore, this study aims to further contribute to this ongoing evaluation of autophagy as a possible oncological target, through the investigation of autophagy inhibitors bafilomycin and wortmannin in T24 bladder cancer cells.

Initially, we observed the effect of BAFI and WORT under the mechanical stimuli on the cell morphology of T24 cells. Cancer cells can sense the stiffness of their surrounding microenvironment allows them to respond by pulling on the extracellular matrix (ECM), which can alter the cytoskeletal structure and appearance of the cells[141], especially important for the T24 because of the physiological niche inhabited by bladder cells. In an *in vitro* model under

static conditions, BAFI reduced cell circularity and solidity, while WORT increased roundness and decreased aspect ratio. However as soon as we included shear stress to our treatments, the differences disappeared, and the cells showed the identical characteristic regardless of the chemical treatment. Under shear stress, both BAFI and WORT increased roundness circularity compared to the control but reduced aspect ratio (Figure 10). Kadandale et al. reported that autophagy, particularly selective autophagy, regulates cell spreading and protrusion extension. This study conducted on Drosophila macrophages, known as hemocytes, found that selective autophagy, possibly through p62-mediated degradation of a regulator of the Rho1 pathway, is consistently necessary for dynamic F-actin-based protrusions[142]. Although our *in vitro* model showed that autophagy inhibition with BAFI resulted in more protrusions and less circularity compared to the control, *in vivo* under shear stress, BAFI and WORT increased circularity and roundness in cells (Figure 9, Figure 10a, b). This suggests that autophagy inhibitors might influence F-actin-based protrusions and changes the cell morphology.

It is also interesting to note that the T24 cells, with WORT treated, demonstrate higher solidity under shear stress (Figure 10c).

Solidity refers to the measure the density of an object (describing the similarity to a perfectly convex area)[143]. Only BAFI [10nM] in static condition changed this parameter (describing this way a less uniform cell with more irregular protrusion). Changes in morphology are part of the cell's adaptive response to chemical cues[144], this is for example already used to develop and screen for new bioactive small molecules[145], assess differential processes [146] or investigate gene function [147].

However, the connection of the actual morphometric phenotypes and the underlying biological causes has not been fully established. Similarly, in the experiments with the T24 cells a clear connection of the changing morphology and both chemical/physical cues could be established. Yet, both the up- and downstream effects of these changes remain an open research question.

Considering these results we can conclude, cells, that both physical cues i.e., shear stress, and chemical signals, i.e., autophagy inhibitors such as bafilomycin and wortmannin can result changes in cytoskeletal structure and cell morphology. Additionally, the response to physical signaling seems to overtake the changes induced by the chemical signal, this might have important consequences for the interplay between mechanical cues, i.e., changes in morphology induced by substrates, and drug responsiveness.

The role of autophagy inhibition in toxicology is frequently underappreciated. While it may be harmful if mixed with toxins, where additive effects may occur through the combined exposure. But on the other hand, this may be exploited when autophagy inhibition may be used to sensitize cancer cells against when natural toxins. The fusarium mycotoxins fusaric acid and deoxynivalenol are known contaminants of grain-based food[112], [118]. DON and FA both induce oxidative stress by causing the accumulation of reactive oxygen species (ROS). Mitochondria regulate the production of ROS, and they are also targeted by ROS, which can result in mitochondrial damage. Numerous disorders such as diabetes, atherosclerosis, Parkinson, and Alzheimer are associated with a decrease in mitochondrial activity. [148]–[150]. Therefore, we proceeded to investigate the viability and toxicity of the individual compounds as well as when autophagy inhibitors and toxins both were combined, taking mitochondrial impairment as an important mode of action. To assess the status of the organelles directly, live cell imaging was performed to quantify the intensity of lysosomes, mitochondria, and membranes.

Bafilomycin, a vacuolar H+ ATPase inhibitor, prevents the acidification of the lysosome and protein degradation in the lysosome[134]. Our results show that BAFI with a higher concentration shows increased toxicity (Figure 11, Figure 13), which can be confirmed by other studies. Redmann et al. demonstrate that BAFI increases mitochondrial DNA damage or accumulation of damaged mitochondria and decreases mitochondrial quality[151]. The accumulation of damaged mitochondria could also be a reason why there is a higher signal of mitochondria seen in live cell imaging when the cells are treated with BAFI [1nM]. Similarly, BAFI [1nM] also increases the signal of the cell membrane and decreases the signal with a higher concentration (Figure 18). There is no such evidence of a correlation between mitochondria and cell membrane but mitochondria and membrane both have components such as in sterols, sphingolipids, and phosphatidylserine but in different percentages. This might be the reason why the mitochondria and membranes show similar intensity in signals[152].

The specific phosphoinositide 3-kinase (PI3K) inhibitor wortmannin with the higher concentration induce toxicity in the cells (Figure 12, Figure 14) and the mitochondrial signal was significantly decreased in the lysosomal area (Figure 16a). WORT with the lower concentration shows an increased membrane signal but a decreased signal at a higher concentration (Figure 18). Wortmannin also shows an increased lysosomal signal with a higher concentration (Figure 17). Since WORT inhibits autophagy, the lysosomes can accumulate. This means that the

lysosomes are not used up and the signals increase. It is still unclear how wortmannin affects the cell organelle.

Deoxynivalenol shows concentration-dependent toxicity, for both cell viability and cell mass. When DON is combined with BAFI and WORT, toxicity is amplified (Figure 11, Figure 12). DON damages the mitochondria and induces mitochondrial autophagy[148]. It might be the reason that if BAFI and WORT are involved, the damaged mitochondria cannot be degraded, which leads to an excessive accumulation of ROS, produced by the malfunction organelles. Although cells treated with DON and DON combined with BAFI show no changes in mitochondrial signal, DON combined with WORT shows increased mitochondrial signal, indicating this accumulation (Figure 16).

DON reduces the signal of the lysosome as well. Li et al. (2021) demonstrated that DON reduces protein stability and enhances endocytosis and tight junction protein degradation in the lysosome of swine jejunal epithelial cells [153]. The signal reduction of the lysosome is amplified if cells are treated with DON and WORT (Figure 17).

The analysis of the membranes signals reveals that the cells treated with DON combined with BAFI present decreased signals such as BAFI, while DON combined with WORT presents increased signals, like WORT (Figure 18).

Similar to DON, FA also indicates concentration dependent toxicity, and this effect was amplified by the autophagy inhibitors BAFI and WORT (Figure 13b, Figure 14b). There are many studies that shows the cytotoxic effect of FA in the cancer cells and affect mitochondrial polarity. A study shows that response to FA exposure increased MAPK signaling and p38 MAPK, part of MAPK, reduces the autophagy[139], [149], [154]. Our results shows that FA with the concentration of 1mM decreases the mitochondrial signal. Although FA in combination with BAFI reduces the mitochondrial signal but combined with WORT induced signal is visible (Figure 16). This can probably be explained by the fact that BAFI has a specific effect on mitochondrial dysfunction, but WORT inhibits IPK3 and therefore does not have such a toxic effect on mitochondria. It has also been observed that when the signals in mitochondria were reduced, the reduced signals in membranes were also noticeable. This suggests that the mitochondria might be responsible for the reduced signals in the membrane.

FA as single treatment and as combination treatment with autophagy inhibitors reduces the lysosomal signal (Figure 17). FA also decreases the membrane signal. Like DON, FA combined with BAFI present decreased signals, while FA combined with WORT presents increased

signals (Figure 18). FA, due to lipophilic properties, can freely enter the cell membrane and cause damage[149]. There is already a study that has investigated the effect of FA on mitochondria and membrane signals in other cancer cells and have found analogous results[155].

Along this line, autophagy inhibition is already being investigated for its potential use in combination with traditional chemotherapy regimens.

5 Conclusion

Overall, the results show that both mycotoxins, FA, and DON, had significant effects on cytotoxicity and these effects are partly amplified by autophagy inhibition. Autophagy inhibitors rarely induced cytotoxicity, yet they had a significant impact on organelles like mitochondria, lysosome, and cell membrane at non cytotoxicity concentration. It can also be demonstrated that autophagy inhibitors and physical stimulation play a major role in cell shape, and that through mechanotransduction the chemical signaling could be modulated. Furthermore, inhibition of autophagy increased the cytotoxic effect of the mycotoxins on the T24 cells in multiple conditions, indicating a potential interaction between food contaminants and autophagy modulators. However, further studies are needed to find out the exact mechanisms of action of the compounds. It is still uncertain whether the component has the same effect on healthy bladder cells.

Declaration

Part of the data generated in this master's thesis were included in a peer-review paper titled "Autophagy modulation changes mechano-chemical sensitivity of T24 bladder cancer cells" by Maximilian Jobst, Maliha Hossain, Endre Kiss, Janice Bergen, Doris Marko, Giorgia Del Favero which is published in “Biomedicine and Pharmacotherapy” (DOI: 10.1016/j.biopha.2023.115942)[156].

All the chemical structures were created using the software ChemSketch.

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*Ich habe mich bemüht, sämtliche Inhaber*innen 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.*