Effects of the marine natural products tropodithietic acid and dimethylsulphoniopropionate on neuronal and oligodendroglial cells as well as *Caenorhabditis elegans*

Auswirkungen der marinen Naturstoffe Tropodithietsäure und Dimethylsulfoniopropionat auf neuronale und oligodendrogliale Zellen sowie *Caenorhabditis elegans*

zur Erlangung des Grades und Titels eines Doktors der Naturwissenschaften

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Für meinen Sohn Nicolas

The planet`s hope and salvation lies in the adaption of

revolutionary new knowledge being revealed

at the frontiers of science.

Bruce Lipton

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Zusammenfassung

Seit Jahrtausenden ist der Menschheit die Nutzung von Wirkstoffen aus der Natur vertraut. Wie alle Lebewesen produzieren auch marine Organismen eine Vielzahl an metabolischen Verbindungen mit pharmakologischem Potenzial. Sekundärmetabolite von marinen Organismen sind bereits zur Behandlung einer Reihe menschlicher Krankheiten wie z.B. Krebs, AIDS und Malaria Gegenstand der Forschung. Der Schwerpunkt lag hierbei bisher hauptsächlich auf Anti-Krebs-Aktivitäten, wo u. a. der inhibitorische Effekt diverser Sekundärmetabolite in entsprechenden Zelllinien festgestellt wurde. Weniger ist dagegen über neuroaktive marine Naturstoffe bekannt, wobei dieses Forschungsgebiet durch unsere alternde Gesellschaft und den damit verbundenen Anstieg von neurologischen und neurodegenerativen Krankheiten von wachsender Bedeutung ist. Während der letzten Jahre sind (marine) Naturstoffe, welche auf das zentrale Nervensystem abzielen, in den Fokus der Forschung gelangt. In der vorliegenden Doktorarbeit wurde das pharmakologische Potenzial der marinen Naturstoffe Tropodithietsäure (TDA) und Dimethylsulfoniopropionat (DMSP) untersucht. Um eine größeren Überblick der Auswirkungen von TDA und DMSP auf das Gehirn von Säugetieren zu bekommen, wurden N2a-Zellen, als Modell für Neuronen und OLN-93 als Modell für Gliazellen gewählt, um zytotoxische oder protektive Effekte auf der zellulären Ebene zu untersuchen. Ergänzend wurden Studien im mehrzelligen Modellorganismus *Caenorhabditis elegans* durchgeführt. Die hier gezeigte Zytotoxizität von TDA auf neuronale und oligodendrogliale Zellen sowie auf *C. elegans* waren inkonsistent mit vorherigen Studien, worin keine zytotoxische Wirkung beschrieben wurde. Die hierin detektierte toxische Wirkung beinhaltete die Beeinflussung zellulärer Mechanismen, einschließlich Depolarisation der mitochondrialen Membran, dem Anstieg des intrazellulärem Calciums, Hochregulierung des Hitzeschockproteins 32 (Hsp32), Aktivierung der extrazellulären signalregulierten Kinase 1/2 (ERK1/2) und Reorganisation der Mikrofilamente. Ergänzend konnte gezeigt werden, dass DMSP die Ausbildung von Zellfortsätzen induziert, und die Organisation der Mikrotubuli beeinflusst. Des Weiteren unterdrückte die Vorbehandlung mit DMSP den toxischen Effekt von TDA, welches auf eine potenzielle antioxidative Abwehr in neuralen Zelllinien hinweist. Ergänzend wurde ein modifizierter *C. elegans* killing Assay unter der Verwendung des auxotrophen Bakteriums *E. coli* ST18 etabliert, mithilfe dessen die

antithelminitische Wirkung der beschriebenen Naturstoffe untersucht wurde. Durch Vergleich der Tötungseffizienz von TDA in lebendem oder devitalisiertem *E. coli* ST18 konnte gezeigt werden, wie wichtig das Abtöten der Futterbakterien ist, um Diskrepanzen in der Aufnahme der Testsubstanzen zu verhindern.

Summary

Nature provides a plethora of natural products from plants, animals and microbes, which have been historically used to treat diseases and are to date the source of most of the active ingredients in medicine. The marine environment harbors a variety of organisms producing a high diversity of natural products with promising pharmacological potential. These substances show a broad spectrum of biological activities, including anti-cancer, antimicrobial, antifungal and antifouling effects with some being in the focus as possible treatments for neurological diseases. Along with the increase of life expectancy, the prevalence of neurological and neurodegenerative diseases is constantly rising, underlining the need for novel drugs targeting the central nervous system (CNS). This thesis addresses the pharmacological potential of the marine natural products tropodithietic acid (TDA) and dimethylsulphoniopropionate (DMSP). For a broader overview of the possible effects of TDA and DMSP on mammalian brain cells, we have chosen N2a cells as a model for neurons and OLN-93 as a model for glial cells to determine their cytotoxic or protective capabilities on the cellular level. In addition, the whole model organism *Caenorhabditis elegans* was investigated, particularly in view for further biomedical applications. The herein shown toxicity of TDA on mammalian clonal cell lines of neuronal and glial origin, as well as on *C. elegans,* were inconsistent with previous studies, where no cytotoxicity could be determined. Toxic effects of TDA were linked to various cellular mechanisms, including mitochondrial membrane depolarization, increased intracellular Ca^{2+}-levels, an upregulation of heat shock protein 32 (Hsp32), activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) and microfilament reorganization. Additionally, the outgrowth inducing effect, microtubule reorganization and protective capabilities of DMSP on N2a and OLN-93 cells was analyzed. Preincubation with DMSP prohibited cytotoxic effects of TDA, indicating that this compound provides an antioxidant defense in neural cell lines. Furthermore, the herein established improved *C. elegans* killing assay using auxotrophic *E. coli* ST18 was used to determine the antithelmintic effect of TDA, sigillin A and DMSP. Comparing the killing efficiency of TDA in living or devitalized *E. coli* ST18 showed the importance of the devitalization process to avoid discrepancies in the test compound uptake.

List of publications

This thesis includes three manuscripts of which one is submitted for publication, one is close to submission and, in addition, one chapter on establishment of a modified *C. elegans* killing assay, where the manuscript is in preparation.
My contribution to the manuscripts is outlined in the following.

Cytotoxic effect of tropodithietic acid on mammalian clonal cell lines of neuronal and glial origin

Heidi Wichmann, Farina Vocke, Thorsten Brinkhoff, Meinhard Simon and Christiane Richter-Landsberg

HW: Experimental work, concept of study, first draft of manuscript. FV: Fura-2 AM calcium imaging. TB: revision of manuscript. MS: revision of manuscript. CRL: concept of study, supervised the experimental work, help with drafting of manuscript, revision of manuscript.

Neuroprotective and outgrowth inducing effects of dimethylsulphoniopropionate on mammalian clonal cell lines of neuronal and glial origin

Heidi Wichmann, Thorsten Brinkhoff, Meinhard Simon and Christiane Richter-Landsberg

HW: Experimental work, concept of study, first draft of manuscript. TB: revision of manuscript. MS: revision of manuscript. CRL: concept of study, supervised the experimental work, help with drafting of manuscript, revision of manuscript.

A modified *C. elegans* killing assay for drug screening using auxotrophic *E. coli* ST18 to test for antithelmintic effects of tropodithietic acid, sigillin A and dimethylsulphoniopropionate

Heidi Wichmann, Suhelen Egan, Tristan Schneider, Stefan Schulz, Martine Berger, Meinhard Simon and Thorsten Brinkhoff

HW: concept of study, supervised the experimental work, first draft of manuscript. SE: concept of study, help with drafting of manuscript, revision of manuscript. TS: experimental work. SS: revision of manuscript. MB: concept of study. MS: revision of manuscript. TB: concept of study, supervised the experimental work, help with drafting of manuscript, revision of manuscript.

Presentation of my work at scientific meetings:

Wichmann, Heidi, Brinkhoff, Thorsten, Simon, Meinhard, and Richter-Landsberg, Christiane: Dimethylsulphoniopropionate mimics neurotrophic factors and protects against cytotoxic effects of tropodithietic acid in OLN-93 and Neuro2a cells. 5th International HIPS Symposium, Saarbrücken, Germany, 02.07.2015 (book of abstracts, poster)

Abbreviations

AHL	*N*-Acyl-homoserine lactones
CNS	Central nervous system
DHA	Docohexaeonic acid
DMS	Dimethylsulphide
DMSO	Dimethylsulphoxide
DMSP	Dimethylsulphoniopropionate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HSP	Heat shock protein
DMEM	Dulbecco's modified Eagle medium
EPA	Eicosapentaenoic acid
ERK 1/2	Extracellular regulated kinase 1/2
FBS	Fetal bovine serum
HRP- conjugated antibodies	Horseradish peroxidase- conjugated antibodies
MAP	Microtubule associated protein
MAPK	Mitogen-activated protein kinases
MPT	Mitochondrial permeability transition
MT	Microtubule
PBS	Phosphate buffered saline
PLL	Poly-L-Lysine
TBS-T	Tris buffered saline - with Tween
TDA	Tropodithietic acid

Chapter 1: Introduction

Natural products and secondary metabolites

Plants, animals and microorganisms are chemically rich entities that produce a variety of substances, including primary (i.e. essential) and secondary metabolites. The latter are compounds that are not directly needed for the organisms' survival, but can confer an advantage by e.g. mediating chemical interactions. The producer organisms are able to grow and survive without these secondary metabolites and produce them in response to environmental cues (Molinari, 2009). In addition, natural products play an important role in drug discovery, particularly in the areas of cancer and infectious diseases. In fact, more than 60% of the approved drugs are of natural origin (Harvey, 2008; Molinari, 2009). Bioactive natural products are mostly low-molecular weight organic compounds (Molinari, 2009). Due to the potent bioactivities, secondary metabolites have long been served as important resource for development of small-molecule therapeutics (Joyner and Cichewicz, 2011). The marine environment represents a promising resource of bioactive molecules, which can be developed as nutraceuticals and pharmaceuticals (Fan et al., 2014), even for largely undescribed targets, such as neurological disorders (Grosso et al., 2014; Pangestuti and Kim, 2011). Along with the increase of life expectancy, the prevalence of neurodegenerative and neurological diseases is rising, and marine natural products are increasingly investigated for targeting the central nervous system (CNS) (Bradbury, 2011; Grosso et al., 2014; Song and Zhao, 2007). Studies with marine compounds affecting the CNS involve areas of neuropharmacology, such as stimulation of neurogenesis or neuroprotective abilities (Essa et al., 2012; Grosso et al., 2014; Palyanova et al., 2013). The present study aimed at the characterization of bioactive natural products from marine organisms for their effects on neural cell lines, interactions and other bioactivities. The analysis focused on secondary metabolites from marine algae and bacteria, including members of the *Roseobacter* clade (Brinkhoff et al., 2004; Sagar et al., 2013), which are known to produce biologically active compounds with vast pharmacological and nutraceutical potential (Brinkhoff et al., 2004; de Jesus Raposo et al., 2013; Pangestuti and Kim, 2011; Sagar et al., 2013).

Algae as source of bioactive natural products

Although global marine algae utilization is a multibillion dollar industry, their bioactive potential is still underexploited. For centuries, the medicinal properties of algae were limited and only recently plastics, cosmetics, pharmaceutical and food industries started to focus their attention on the discovery and development of compounds from marine algae (Barbosa et al., 2014). One target of bioactive compounds are neurodegenerative diseases, which are estimated to surpass cancer as the second most common cause of death among elderly by the 2040s, requiring the development of safe and effective neuroprotective agents (Pangestuti and Kim, 2011). Many classes of natural and synthetic neuroprotective agents have been reported, while synthetic neuroprotective agents are believed to have certain side effects. Hence, there is a high interest in using natural bioactive compounds as neuroprotective agents. Marine algae are thought to have promising potential as source for such agents (Pangestuti and Kim, 2011). In this context it is important to consider the role of malnutrition in health and disease. Malnutrition is a condition that results from eating a diet in which nutrients are either not enough or are too much so that the diet causes health problems. It is associated with many adverse outcomes including depression of the immune system, impaired wound healing, muscle wasting, extended hospital stay, higher treatment costs and increased mortality (Barker et al., 2011). A growing body of evidence also suggests that nutrition play an important role in neurodegenerative diseases (Seidl et al., 2014). For instance, patients with Alzheimer disease (AD) showed a worse nutritional status and was reported to support the progression of AD (Hu et al., 2013). Many studies support the important role of antioxidants in the prevention of AD as this disease is closely related to the occurrence of oxidative stress (Hu et al., 2013; Vina et al., 2011). Algae have been considered as a rich source of natural antioxidants due to the presence of various secondary metabolites with antioxidative effects (Fan et al., 2014; Pangestuti and Kim, 2011). However, more research is needed to elucidate the potential of algae and algae derived compounds to reach its full potential.

Dimethylsulphoniopropionate

Dimethylsulphoniopropionate (DMSP), produced by several algae, corals and higher plants, has multifunctional roles in the ocean (Alison Buchan, 2005; Raina et al., 2013). It is an important component of the marine sulphur cycle (Raina et al., 2013). This compound occurs at high concentrations in algae and serves as osmolyte, cryoprotectant, and antioxidant (Raina et al., 2013; Sunda et al., 2002). Built in enormous amounts of worldwide ~ 10^9 tonnes annually, this compound is degraded by bacteria, such as the *Roseobacter* clade, by two general pathways (Moran et al., 2012). The demethylation pathway leads to the synthesis of methylmercaptopropionate and the cleavage pathway yields dimethyl sulphide (DMS) and either acrylate or 3-hydroxypropionate. The emitted DMS can be further metabolized by DMS-consuming bacteria or released into the atmosphere, where it can be converted to dimethyl sulphoxide or sulphate aerosols. Here, they can act as cloud condensation nuclei impacting the amount of sunlight reflected back into space (albedo). The return of these compounds back to the Earth`s surface, via rain or snow, is a major step in the global sulphur cycle and is transferring the element from sea to land. The resulting volatile DMS is also very important as a chemoattractant for zooplankton, seabirds and marine mammals (Curson et al., 2011). In addition to its role in natural biogeochemical processes, DMSP has also been found as a promising bioactive compound for biomedical research. Beside its beneficial effects on stressed fish and crustaceans, significant healing effects in rodents on a wide range of diseases such as cancer, stress-induced gastric ulcers and neurodegenerative disorders could be determined and are discussed especially in context of the nutraceutical potential of algae (Nakajima, 1996, 2015). The purpose of the present thesis was to investigate effects of DMSP on mammalian neural cell lines, particularly in view of potential neuroprotective and outgrowth inducing effects. Here, we investigated the influence of DMSP on neural cell lines.

Tropodithietic acid

Tropodithietic acid (TDA) is a broad spectrum antibiotic produced by several *Roseobacters* like *Phaeobacter inhibens* 17395 or *Ruegeria mobilis*, whose TDA production make them potential candidates as probiotics in aquacultures (Porsby et al., 2011). The *Roseobacter* clade belongs to the *Alphaproteobacteria* and is one of the most intensively studied groups of marine bacteria. They are broadly distributed, from coastal regions to deep-dea sediments, have been detected in different marine habitats and can constitute up to 25% of the bacterioplankton community (Brinkhoff et al., 2008; Selje et al., 2004), and are considered as major players in the sulphur cycle of the ocean by degrading DMSP (Alison Buchan, 2005; Gonzalez et al., 2000; Penesyan et al., 2011; Raina et al., 2013). Members of the *Roseobacter* clade were found as free-living pelagic microorganisms or in biofilms, but more often associated with phytoplankton blooms (Rooney-Varga et al., 2005; West et al., 2008) and diverse marine eukaryotes such as invertebrates, vertebrates and algae (Buchan et al., 2005). The production of diverse secondary metabolites, such as TDA and various *N*-acyl-homoserine lactones (AHLs) may be one reason for the common occurrence and abundance of these bacteria in different habitats, in particular in association with living and detrital surfaces, e.g. by providing a competitive benefit over other species (Brinkhoff et al., 2004; Cude et al., 2013; Martens et al., 2007). Previous studies suggested that AHL mediated quorum sensing (QS) is one of the most common cell-to-cell communication mechanism in roseobacters (Cude et al., 2013) and one important factor for TDA production (Berger et al., 2011). TDA is a sulphur containing compound with a molecular weight of ~ 212 Dalton. It is able to inhibit a broad spectrum of both Gram-positive and -negative bacteria, including clinical pathogens, fungi and microalgae (Brinkhoff et al., 2004; Geng and Belas, 2010; Harrington et al., 2014; Liang et al., 2003), but resembles more the characteristics of antimicrobial peptides or biocides with regard to the mechanism of action than antibiotics (Porsby et al., 2011). TDA showed no toxicity against the multicellular eukaryotic model organism *Artemia sp.* and no negative effect of TDA-producing bacteria on *C. elegans* was determined (Neu et al., 2014). Additionally, no noteworthy anti-cancer effect was detectable (Liang et al., 2003) and the molecular mechanism has not been elucidated to date. Despite the various ecological roles and bioactivities of marine natural products, such as DMSP and TDA, still little is known about their potential interactions with neural cell lines.

The central nervous system

The nervous system consists of two parts, the central nervous system (CNS) and the peripheral nervous system (PNS). The PNS consist of the nerves and ganglia outside the brain and spinal cord, which are part of the CNS, and serves to connect the CNS to organs and limbs. In terms of the CNS, the cells of the brain can broadly be divided into two main categories: neurons and glias. Neurons are electrically excitable cells that transmit and process informations through electrical and chemical signals (Alberts et al., 2002; Purves et al., 2001). Glial cells are basically divided in three categories: astrocytes, oligodendrocytes and microglia, which are often referred to be supporters of neurons (Fig.1).

Neurons

Neurons, the grey matter of the brain, are electrically excitable cells that process and transmit information through electrical and chemical signals via specialized connections (synapses) by sending those signals even over long distances to the specilized target cells. These signals occur between neurons and can connect to each other to form neural networks. Neurons consist of a soma, dendrites and an axon.The definition neurite describes either a dendrite or an axon, particularly in its undifferentiated stage. Dendrites, arising from the soma, are thin constructions which often spread out and branch multiple times, forming the dendritic tree. Although the neuron can build multiple dendritic branches there is no more than one axon which is build at the axon hillock (Alberts et al., 2002; Purves et al., 2001).

Neuroglial cells

Neuroglial cells, also refered as glia cells, are more numerous than neurons in the brain. Opposed to neurons, glia cells are not directly involved in synaptic interactions and electric signaling, although their supportive function helps to maintain synaptic contacts and signaling abilities of neurons (Purves et al., 2001). There are three different types of glial cells in the CNS: astrocytes, oligodendrocytes and microglia.

Astrocytes are restricted to the brain and spinal cord and form a starlike appearance due to their local processes. A major function of astrocytes is to maintain an appropriate environment

for neuronal signaling and to provide biochemical support of the endothelial cells which build the blood-brain-barrier (Abbott et al., 2006; Purves et al., 2001).

Oligodendrocytes, which are limited to the CNS, provide support and insulation of axons by building the myelin shealth (white matter), equivalent to the role of Schwann cells in the PNS. Depending on the region of the CNS, oligodendrocytes are able to myelinate up to 50 axonal segments (Baumann and Pham-Dinh, 2001; Purves et al., 2001).

Microglia act as resident macrophages in the CNS. They share many properties with macrophages in other tissues and primarily remove cellular debris from sites of injury or normal cell turnover. Additionally, they secrete signaling molecules in particularly a wide range of cytokines that are also produced by cells of the immune system. Microglia constantly scavenge the CNS for plaques, damaged neurons and infectious agents (Gehrmann et al., 1995; Purves et al., 2001).

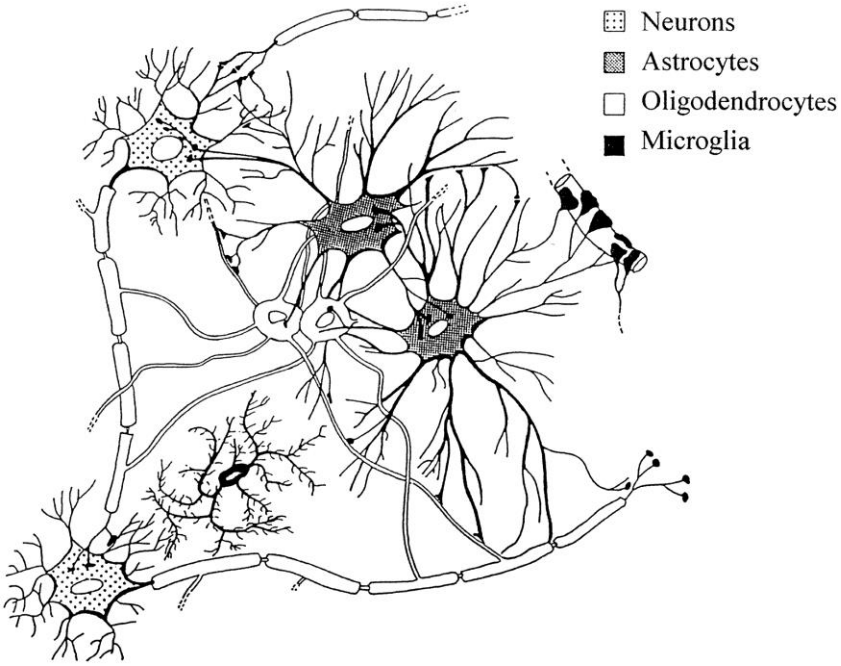

Figure 1. Scheme of different types of glial cells in the CNS and their interactions among themselves and neurons. Astrocytes are stellate cells with numerous processes contacting several cell types in the CNS: soma, dendrites and axons of neurons, soma and processes of oligodendrocytes, as well as other astrocytes; astrocytic feet additionally enshealth endothelial cells around blood capillaries forming the blood-brain barrier. Oligodendrocytes are myelinating neurons and are able to myelinate up to 50 axonal segments depending on the region of the CNS (Baumann and Pham-Dinh, 2001).

The cytoskeleton

The cytoplasm of eukaryotic cells harbours a cytoskeleton network constructed of various proteins. These filamentous structures are highly dynamic and vital for cell stabilization, morphology, movements, and intracellular transport (Huber et al., 2013). Cytoskeleton filaments are divided into three different classes, each being formed from different proteins: actin filaments, intermediate filaments and microtubules.

Actin, also known as microfilament, is the most abundant protein in most eukaryotic cells. It is highly conserved and participates in more protein-protein interactions than any known protein. These properties, along with its ability to transition between monomeric (G-actin) and filamentous (F-actin) states under the control of ATP hydrolysis, ions, and a large number of actin-binding proteins, make actin a critical player in many cellular functions such as cell motility, the maintenance of cell shape, and the regulation of transcription. Moreover, the interaction of filamentous actin with myosin forms the basis of muscle contraction (Dominguez and Holmes, 2011; Holmes et al., 1990).

Intermediate filaments are also important for the mechanical stability of the cell. They are termed this way because they are, due to their diameter of about 10 nm, exactly in between the size of actin filaments (7nm) and microtubules (20-30nm). In contrast to actin filaments and microtubules they are not directly involved in cell movements. Instead, they appear to play basically a structural role by providing strength to cells and tissues (Cooper, 2000).

Microtubules (MTs) are present in all eukaryotic cells. Being a part of the cytoskeleton, they are known to be generally involved in different cellular processes including mitosis, cell motility, intracellular transport, secretion, cell shape and polarization (Janke and Kneussel, 2010). They provide platforms for intracellular transport, including the movement of secretory vesicles, organelles and intracellular macromolecular assemblies. Additionally, they are involved in chromosome separation and are the major constituent of mitotic spindles. There are many proteins that bind to microtubules, including the motor protein dynein and kinesin (Howard and Hyman, 2007; Vale, 2003). MTs are heterogeneous in length and highly dynamic in vivo and in vitro, undergoing cycles of polymerization and rapid depolymerization. This “dynamic instability” property is a feature that is crucial to many microtubule functions and modulated by

interactions with other proteins, microtubule motor proteins and non-motor microtubule-associated proteins (MAPs) (Janke and Bulinski, 2011). Microtubules are nucleated and organized by the microtubule organization centers (MTOCs) such as the centrosome. MTs are dynamically assembled polymers of α- and β-tubulin, growing from the (-) end away from the MTOC in the (+) direction. These α/β-tubulin dimers are able to bind two molecules of guanosine triphosphate (GTP), where one of those can be hydrolyzed subsequently for assembly. Although most MTs have a half-life of 5-10 min, certain MTs can remain stable for hours (Infante et al., 2000). These stabilized MTs accumulate post-translational modifications of tubulin subunits. These modifications include detyrosination and acetylation, which will be of subject in this work. Other posttranslational modification of tubulin are polyglutamylation, polyglycylation, delta2, phosphorylation, ubiquitination, sumoylation and palmitoylation (Janke and Bulinski, 2011). MTs can undergo posttranslational modifications (PTMs), including acetylation and detyrosination, which are crucial for controlling the interaction with other cellular components such as MAPs (Howes et al., 2014; Song and Brady, 2015). Tubulin acetylation, by the acetylation of α-tubulin on the amino group of lysine (Lys40), is considered as an indicator of MT stability but not strictly associated with stable (long-lived) MTs (Janke and Bulinski, 2011). Detyrosination of α-tubulin (detyr- tub) stabilizes indirectly MTs and is an indicator of enhanced MT stability while dynamic MTs contain tyrosinated tubulin (Janke and Bulinski, 2011). The tyrosination–detyrosination cycle is initiated by the removal of a tyrosin (Tyr) functional group (detyrosination), whereas re-addition of Tyr (tyrosination) returns tubulin to its nascent state and is more found in dynamic MTs (Janke and Bulinski, 2011).

Neural cell and whole organism models

The mouse neural crest-derived cell line **Neuro 2A** (N2a) has been extensively used to study neuronal differentation, axonal growth and signaling pathways. N2a cells have the advantage of responding quickly to serum deprivation and other stimuli in their environment by expressing signaling molecules that lead to neuronal differentiation and neurite outgrowth (Tremblay et al., 2010). Due to this ability to differentiate into neurons within a few days, N2a cells are an

excellent model for studying neurons and are therefore used in this work for investigating the effects of TDA and DMSP.

OLN-93 are derived from spontaneously transformed cells in primary rat brain glial cultures, and resemble morphological and antigenic features like oligodendrocytes (Richter-Landsberg and Heinrich, 1996). Thus, this cell line provides a useful model system for investigating the effect of TDA and DMSP on oligodendrocytes.

The nematode ***Caenorhabditis elegans*** is a free living, non-parasitic, transparent nematode with a length of about 1mm and broad occurence in soil and sediment. It is a well suited *in vivo* model organism for various scientific questions and has developed into an important model for biomedical research (Jorgensen and Mango, 2002) due to the cellular complexity and strong conservation of cellular pathways between *C. elegans* and higher organisms such as mammals (Jorgensen and Mango, 2002; Kaletta and Hengartner, 2006). Subsequent comparison between the human and *C. elegans* genome confirmed that the majoritiy of human disease genes and pathways also exist in *C. elegans.* It is also used as an "user friendly" model organism for parasitic worms and has been the direct use for anthelmintic and nematicide discovery programmes (Ballestriero et al., 2014; Holden-Dye and Walker, 2005). For instance, the reliance on only few chemotherapeutics has resulted in nematode resistance and the search for new compounds with antinematode activity are of great interest. Also, there is a crucial requirement for new antithelmintics that can be developed into new chemotherapeutic drugs (Ballestriero et al., 2014). Furthermore, *C. elegans* is very interesting for ecologists and ecotoxicologists (Menzel et al., 2002) due to the existence of pollution-inducible genes and its high sensitivity to environmental pollutants, which can be consumed in the diet as well as through its thin cuticule (Saul, 2011; Traunspurger et al., 1995). Due to the simplicity and cost-effectiveness of its cultivation, *C. elegans* is an effective *in vivo* model for whole-organism compound screens (Kaletta and Hengartner, 2006) for the search of new drugs with various biomedical applications.

Thesis outline

The aim of this study was to investigate the effect of the marine natural products tropodithietic acid and dimethylsulphoniopropionate on mammalian clonal cell lines of neuronal and glial origin, as well as on the eukaryotic model organism *C. elegans*.

The first part of this study was focused on the effect of TDA on OLN-93 as well as N2a cells. The shown adverse effects of TDA on cell viability and morphology, protein expression, mitochondrial membrane potential and changes in the intracellular Ca^{2+} concentration were inconsistent with previous studies. Here, no adverse effects on *Artemia* health or *C. elegans* (Neu et al., 2014), and neither found noticeable cytotoxic activity against different cancer cell lines (Liang et al., 2003). In the second part of this study the influence of DMSP on process outgrowth, morphological changes and protective characteristics on the cellular level, by analyzing cell viability, morphology, microtubule organization as well as protein expression was examined. In addition, the protective effects of DMSP were studied in the presence of TDA. The third part of this work extended the bioactivity screening against cell lines by testing the effect of DMSP and TDA in *C. elegans.* For this part a method modification was established to control for the influence of test compounds and possible cross-reactions with the bacterial food source (Brenner, 1974; Dimeski, 2008). In this study, we show the importance of the devitalization process and avoidance of growth of possibly survived food bacteria using auxotrophic *E. coli* ST18 (Thoma and Schobert, 2009) to ensure the target organism incorporates the concentration of interest. Additionally, the modified assay was used to investigate antithelmintic effects of TDA and DMSP.

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Chapter 2: Cytotoxic effects of tropodithietic acid on mammalian cell lines of neuronal and glial origin

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Keywords: nerve cells; oligodendrocytes; cytoskeleton; mitochondria; oxidative stress; MAPkinases; *Roseobacter* clade bacteria

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Article

Cytotoxic effects of tropodithietic acid on mammalian clonal cell lines of neuronal and glial origin

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Abstract: The marine metabolite tropodithietic acid (TDA), produced by several *Roseobacter* clade bacteria, is known for its broad antimicrobial activities. It is of interest not only as a probiotic in aquaculture, but may also be of use as an antibacterial agent in non marine or non aquatic environments, and thus potentially cytotoxic influences on eukaryotic cells need to be evaluated. The present study was undertaken to investigate its effects on cells of the mammalian nervous system, i.e. neuronal N2a cells and OLN-93 cells as model systems for nerve cells and glia. The data show that in both cell lines TDA exerted morphological changes and cytotoxic effects at a concentration of 0.3-05µg/ml. Furthermore, TDA caused a breakdown of the mitochondrial membrane potential, the activation of extracellular signal-regulated kinases ERK1/2, and the induction of the small heat shock protein HSP32/HO-1, which is considered as a sensor of oxidative stress. The cytotoxic effects were accompanied by an increase in intracellular Ca^{2+} levels, the disturbance of the microtubule network and the reorganization of the microfilament

system. Hence, mammalian cells are a sensitive target for the action of TDA and react by the activation of a stress response resulting in cell death.

Keywords: nerve cells; oligodendrocytes; cytoskeleton; mitochondria; oxidative stress; MAPkinases; *Roseobacter* clade bacteria

1. Introduction

The marine environment harbors a variety of organisms producing a high diversity of structurally unique natural products. These substances show a broad spectrum of biological activities, including anti-cancer, antimicrobial, antifungal, antifouling effects and some are even able to alter mammalian neurological activity [1-3]. Marine bacteria represent a promising resource for novel secondary metabolites with largely undescribed targets, including the central nervous system (CNS). A plethora of marine bacteria are known to produce biologically active compounds, such as members of the *Roseobacter* clade [4-6]. The *Roseobacter* clade belongs to *Alphaproteobacteria* and represents a major fraction of bacterial communities in different marine habitats [7-9]. They are considered as major players in the sulphur cycle of the ocean by degrading the climate relevant algal and coral metabolite dimethylsulphoniopropionate (DMSP) [7, 10-13]. The production of diverse secondary metabolites may be one reason for the common occurrence and abundance of these bacteria in different habitats, for instance by providing a competitive benefit over other species [6, 14].

Tropodithietic acid (TDA), firstly described in 2004 [6, 15], is a broad spectrum antibiotic produced by several *Roseobacters* like *Phaeobacter inhibens* 17395 or *Ruegeria mobilis*, whose TDA production make them potential candidates as probiotics in aquacultures [16]. It is able to inhibit a broad spectrum of both Gram-positive and -negative bacteria, including clinical pathogens, fungi and microalgae [6, 15, 17, 18], but no toxicity against the multicellular eukaryotic model organisms *Artemia sp.* and *Caenorhabditis elegans* was observed [19]. However, not much is known about the interaction of TDA with eukaryotic cells and its effects on neural cell lines so far have not been investigated.

The purpose of the present study was to investigate the effects of TDA on cells of the mammalian nervous system. Towards this two cell lines representing the main types of the nervous system, i.e. nerve cells and glia, were used, namely mouse neuroblastoma N2a cells as a model for neuronal cells [20] and rat oligodendroglial OLN-93 cells as a model for the myelin forming cells of the CNS [21]. The effects of TDA on cell viability and cell morphology were evaluated. Furthermore, mitochondrial integrity and the impact on Ca^{2+} influx were determined.

2. Results and Discussion

2.1. TDA induced cytotoxicity, upregulation of Hsp32 and ERK1/2 activation

To investigate the cytotoxic potential of TDA cells were incubated for 24 h with increasing concentrations. Fig. 1A shows that in both cell lines morphological changes occurred at 0.3 µg/ml, cell density was decreased and cellular processes retracted. At 0.5 µg/ml severe cytotoxic effects were observed (Fig. 1A).

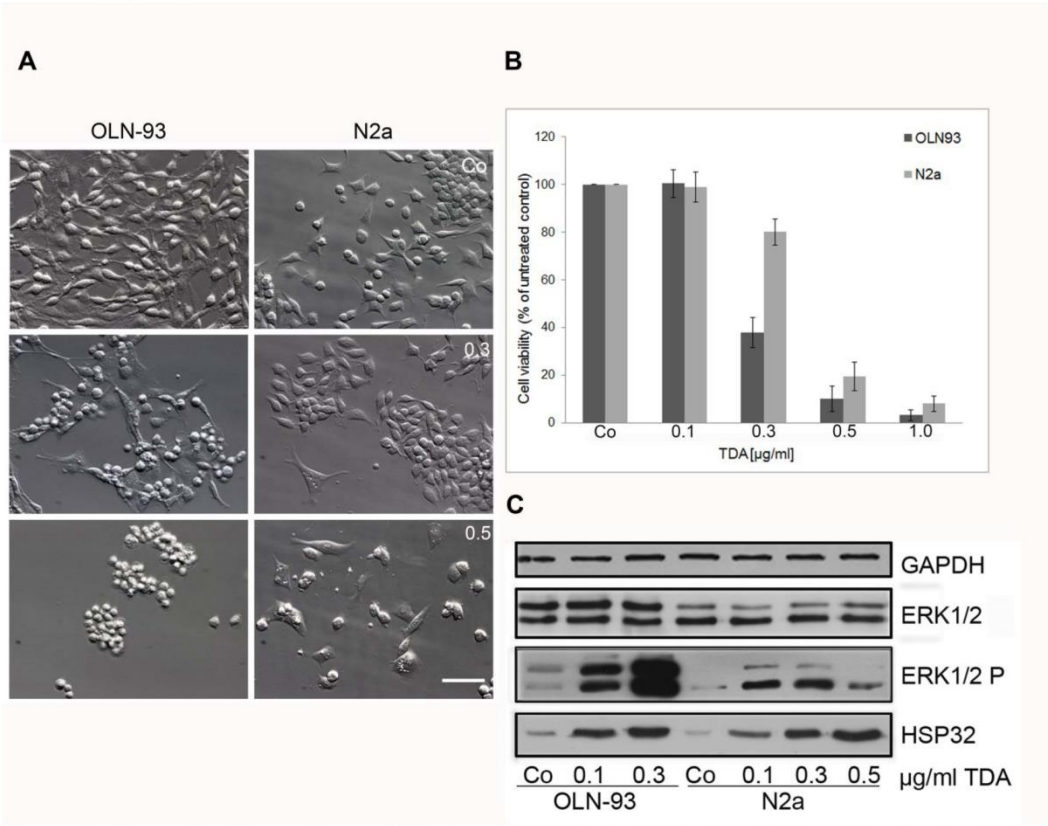

Figure 1. Cytotoxic effects of tropodithietic acid (TDA) in OLN-93 and N2a cells after incubation with TDA for 24 h. A: Effect of TDA on cell morphology. Hoffman modulation contrast images are shown. OLN-93 and N2a cells were either treated with DMSO as negative control or subjected to 0.3 µg/ml or 0.5 µg/ml TDA. Scale bar 50 µm. B: MTT assay. Cells were incubated with increasing TDA concentrations (0.1 µg/ml – 1 µg/ml, as indicated) (OLN-93 dark grey bars, N2a cells light grey bars). C: Western blot analysis. Cell lysates of OLN-93 and N2a cells were prepared and subjected to immunoblot analysis using antibodies as indicated on the right.

To quantitatively assess the cytotoxic effects, an MTT viability assay was carried out, which revealed that at a concentration of 0.3 µg/ml cell viability was decreased to 40% in oligodendroglial OLN-93 cells and to 80% in neuronal N2a cells (Fig. 1B). After incubation with 0.5 µg/ml of TDA only 10% of OLN-93 cells and 20% of N2a cells were viable, and the treatment with 1.0 µg/ml resulted in cell death of more than 90% of the cells (Fig. 1B). Hence, OLN-93 cells were more sensitive to the treatment than N2a cells, and in both cell lines TDA exerted cytotoxic effects at a much lower concentration than previously observed in MCF7 cells (breast adenocarcinoma), HM02 cells (gastric adenocarcinoma) and HEPG2 cells (hepatocellular carcinoma). In these cells cytotoxicity occurred only at concentrations above 8 µg/ml [15]. This indicates that neuronal and glial cells display a higher sensitivity to TDA than cell lines derived from other organs than the brain.

To further study the molecular events underlying the cytotoxic effects of TDA, immunoblot analysis was carried out. Cells were incubated for 24 h with TDA as indicated, cell lysates were prepared and the activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) and the induction of heat shock protein 32 (HSP32) or so-called heme oxygenase 1 (HO-1) was investigated. ERK1/2, which belongs to the family of MAP Kinases, has been connected to protective mechanisms in a number of studies, but also death promoting activity is likely [22]. HO-1 interacts with the MAPK cascade and involved in the regulation of oxidative stress [23, 24]. Immunoblot analysis using antibodies recognizing the dually phosphorylated forms of ERK1/2 (ERK1/2P) and antibodies directed against total ERK1/2, and antibodies against HSP32 was carried out. GAPDH was used as a loading control. Fig. 1C demonstrates that TDA at 0.1-0.3 µg/ml caused the activation of ERK1/2 with no concomitant changes in the amount of total ERK1/2. Also, a concentration dependent induction of HSP32 was observable (Fig. 1C). Thus, treatment with TDA exerted a stress response in neural cells. Upregulation of HSP32/HO-1 initially after the stress is considered a protective means, but at later chronic stages has pathological consequences. As shown before, OLN-93 cells and primary oligodendrocytes respond to oxidative stress exerted by hydrogen peroxide by upregulation of HSP32/HO-1, the activation of ERK1/2, and the onset of programmed cell death [24, 25]. HSP32/HO-1 may be considered as sensor of oxidative stress. The stress regulated kinases ERK1/2 have been shown to be activated under similar conditions that induce HO-1 transcription [26], and it has been suggested that the rapid and transient activation of ERK1/2 enhances the survival capabilities of cells, while a delayed response participates in the regulation of cell death [25, 27, 28]. The present data indicate that in neural cells the cytotoxic effects of TDA are associated with the induction of oxidative stress.

2.2 TDA affects microtubule organization and causes mitochondrial impairment

To test whether morphological changes and cell death is causally related to the impaiment of microtubule organization and mitochondrial damage, mitochondria were stained with MitoTrackerRed, followed by indirect immunofluorescence using antibodies against α-tubulin. MitoTrackerRed is binding only to intact mitochondria and is a measure to assess the integrity of the membrane potential. Thus, the fluorescent signal is more prominent in healthy mitochondria. Cells were incubated with TDA for 24 h as indicated and then analyzed. Fig. 2 demonstrates that in both cell lines the microtubule network is disorganized and a decrease in mitochondrial staining was observed, indicating a loss of the mitochondrial membrane potential. To further assess mitochondrial integrity and distribution, cells treated under the same conditions were labeled with MitoTrackerGreen, which is taken up by mitochondria regardless of their functional state, and heat shock protein 60 (Hsp60), which is constitutively expressed in the cells and localized in the mitochondria. It is associated with the mitochondrial matrix and involved in the folding and assembly of transported protein into the mitochondrium [29, 30]. As depicted in Fig. 3 after treatment with TDA mitochondria appeared smaller and more condensed, however, although the membrane potential was impaired, they remained distributed throughout the cytoplasm and within the cellular processes. Furthermore, Hsp60 remained associated with the mitochondria and was not released into the cytoplasm.

Oxidative stress and a variety of chemicals and Ca^{2+} influx have been connected to mitochondrial pore opening, which causes mitochondrial membrane depolarization and thus may lead to necrotic orer apoptotic cell death [31-33]. Our data indicate that TDA similarly leads to a breakdown of the mitochondrial membrane potential, yet without impacting mitochondrial distribution. Under the present conditions necrotic rather than apoptotic cell death occurred, since cellular nuclei were not pyknotic and no DNA fragmentation was detectable (data not shown). It may be possible that the extent of damage was too severe and the cells die due to inadequate energy production [34]. Taken together, mitochondrial and cytoskeletal alterations are involved in the cytotoxic effects exerted by TDA.

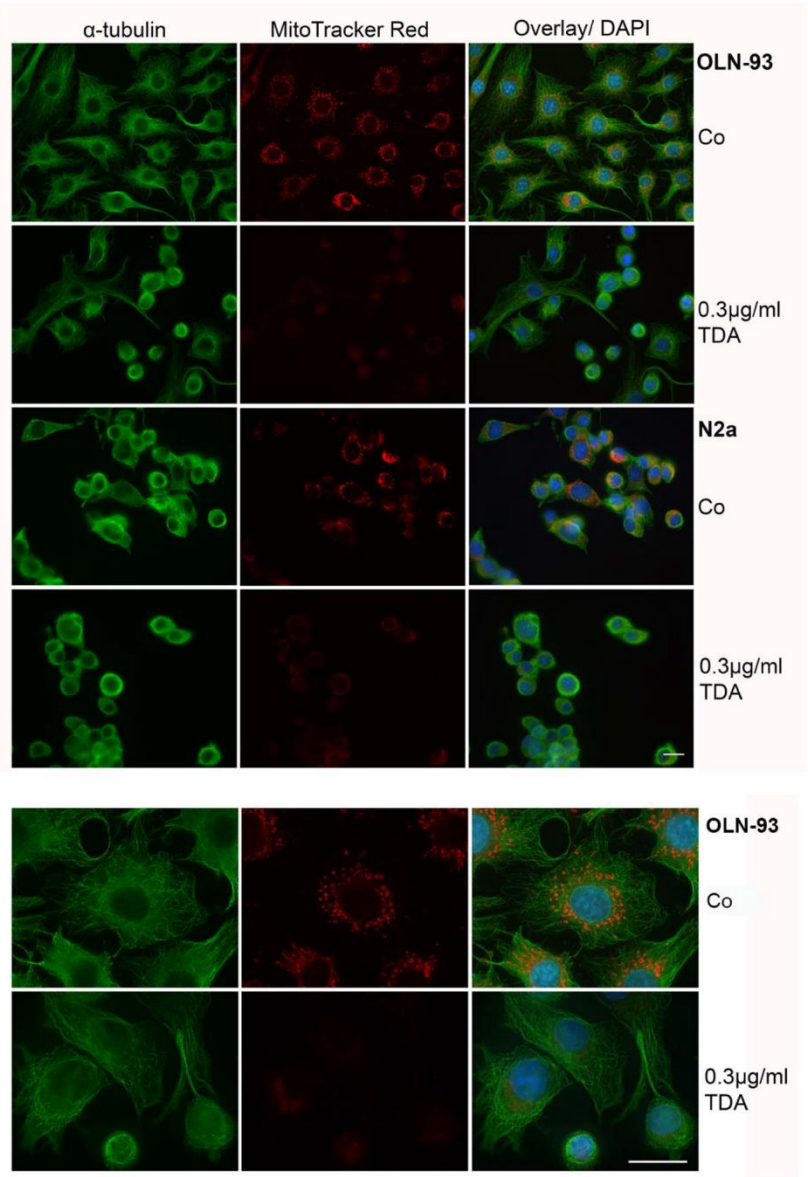

Figure 2: Effect of TDA in microtubule organization and mitochondrial integrity. Cells were subjected to 0.3 µl/ml TDA for 24 h. Subsequently, cells were incubated for 30 min with MitoTrackerRed, fixed with methanol and subjected to indirect immunofluorescence using antibodies against α-tubulin (green). Nuclei were stained with DAPI (blue). Co, untreated control incubated with the solvent DMSO only. Lower panel, OLN-93 cells shown at a higher magnification. Scale bars 20 µm.

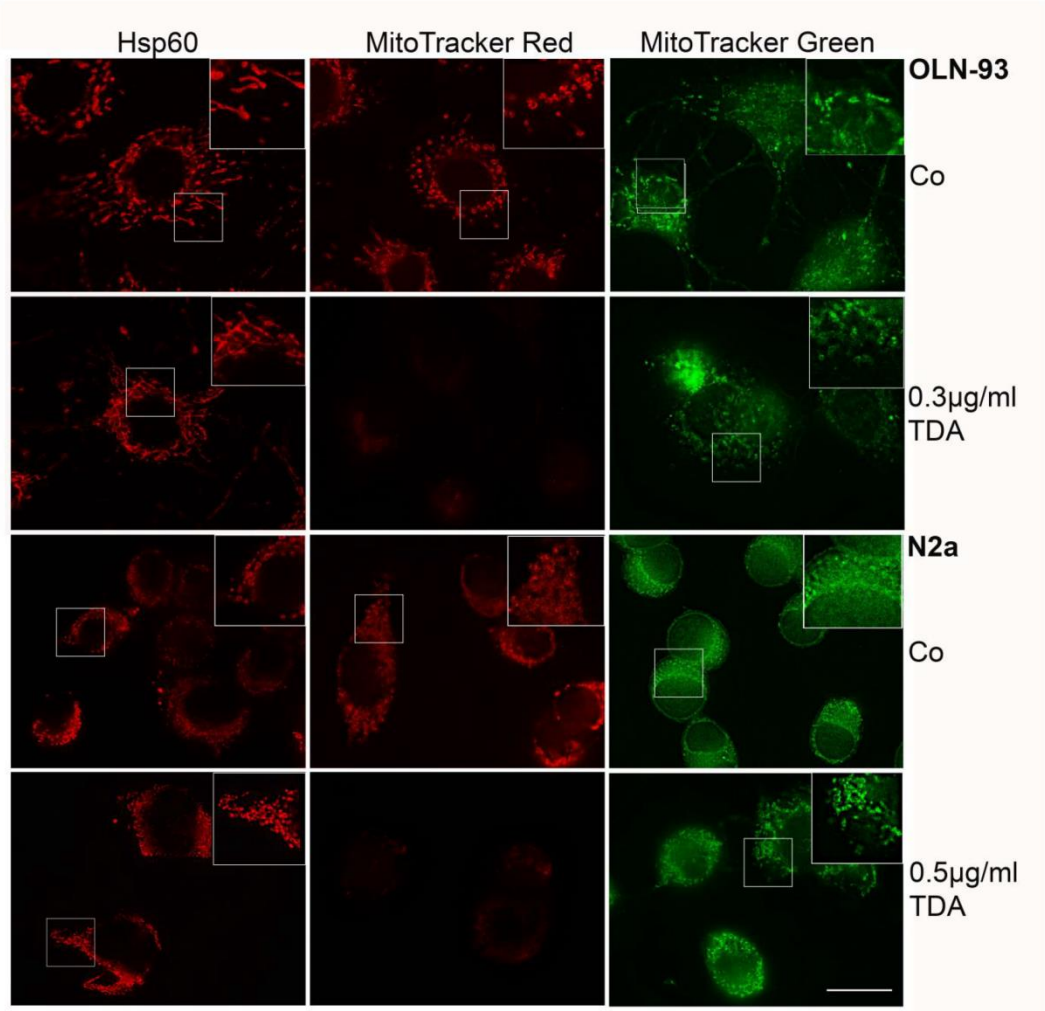

Figure 3: Effect of TDA on mitochondrial integrity. Cells were subjected to TDA for 24 h as indicated. Thereafter, cells were subjected to indirect immunofluorescence using antibodies against heat shock protein 60 (Hsp60), or incubated for 30 min with MitoTrackerRed or MitoTrackerGreen. Co, untreated control incubated with the solvent DMSO only. Scale bar 20 µm.

2.3 Intracellular Ca^{2+} levels are increased after supplementing TDA

A disturbance of the intracellular calcium homeostasis contributes to mitochondrial damage, cytoskeletal disorganization and cell death [35]. To explore whether TDA may influence the Ca^{2+} concentration within the cells, we measured the intracellular Ca^{2+} concentration using Fura-2 AM calcium imaging using N2a cells (Fig. 4).

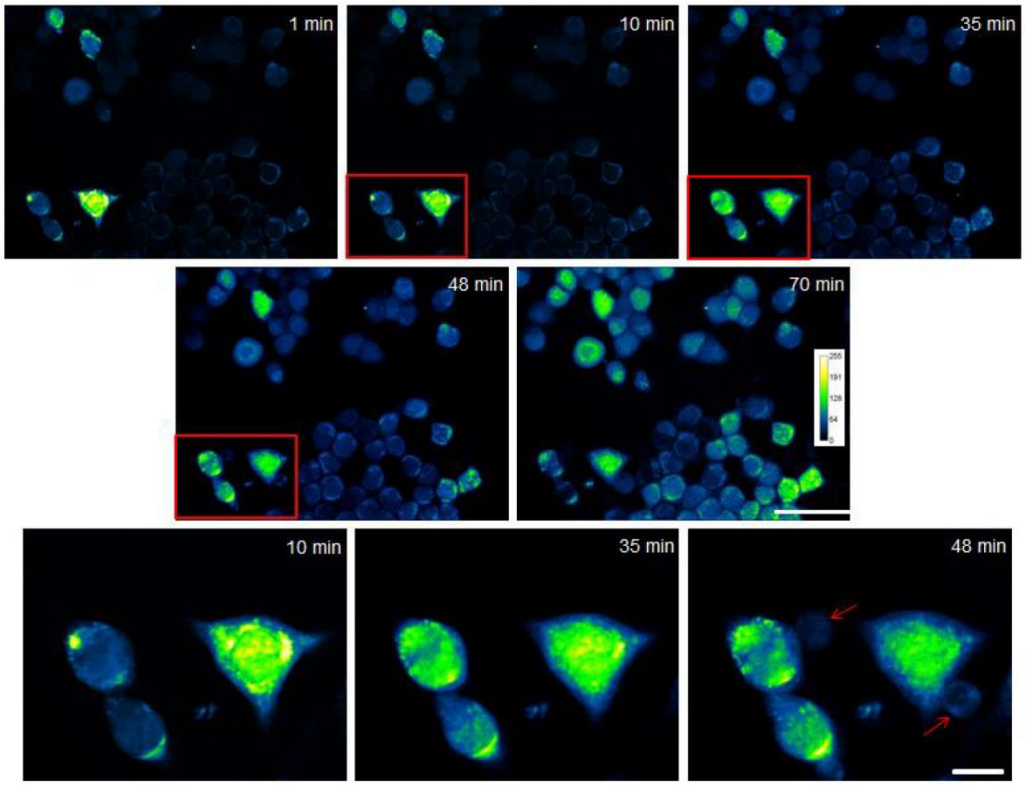

Figure 4: Fura-2 AM calcium imaging. TDA induces an increase of intracellular Ca^{2+} in N2a cells. Cells were incubated with Fura-2-AM solution at 37 °C in the dark. Subsequently, 0.1 µg/ml TDA was added and cells were monitored after 10 min, 35 min, 48 min and 70 min, as indicated. Lower panel: enlargement of the insets depicted in the upper panels (red arrows: necrotic cells). Scale bars: 50 µm; 5 µm.

The Fura-2 AM calcium imaging was recorded on video and representative images are shown (Fig. 4). The amount of cells with augmented Ca_i^{2+} concentration increased over time, cell swelling occurred and cell lysis was observed after 48 h (Fig. 4 lower right, red arrows).

A number of chemical substances and toxic agents are known to cause an imbalance of Ca^{2+} homeostasis and a lethal influx of Ca^{2+} into cells [32, 35]. Ca^{2+} is a mediator of necrosis, whose core event is bioenergetics failure and rapid loss of plasma membrane integrity [36]. Also, oxidative stress can promote cell death and mitochondria are sensitive targets. Induction of mitochondrial permeability transition (MPT), which results in opening large conductance permeability transition pores and making the inner membrane permeable, allows Ca^{2+} to leave the mitochondrion. Additionally, depolarization of the $\Delta\psi_m$ results in an uncontrolled flow of protons and some molecules across the outer membrane. Recently, investigations raised the possibility that mitochondria might act as large and dynamic physiological Ca^{2+} buffers [32, 33]. The exact molecular mechanisms underlying TDA induced cell death need to be further investigated.

2.4 Influence of TDA on microfilament organization

Local cell adhesion and attachment to the extracellular matrix is connected to local Ca^{2+} signals, and the cytoskeleton, in particular the sensitive microfilament system, reacts to oxidative stress and elevation of free Ca^{2+} [37]. To study the effect of TDA on microfilament organization, OLN-93 cells were treated with 0.1 µg/ml TDA for 24 h, and filamentous actin (F-actin) was stained by phalloidin-green. Fig. 5 demonstrates that TDA severely affected F-actin organization.

While in control cells an actin rich network is prominently expressed and clearly observed at the outer boundaries of the cells, in TDA treated cells it appears more diffuse, diminished and retracted. This might be causally related to the observed changes in the intracelluar Ca^{2+} concentration and/or to a possible oxidative insult exerted by TDA. ATP deprivation, potentially induced by TDA, due to mitochondrial Ca^{2+} overload, the loss of $\Delta\psi_m$ and mitochondrial impairment, or other indirect or direct mechanisms, may cause dramatic actin cytoskeletal reorganization [34, 38, 39]. A similar unusual actin relocalization was described in a study by Hrouzek et. al [38]. These authors showed that cyanobacterial derived compounds induced necrosis in HeLa cells via membrane permeabilization and an increase of Ca^{2+}-influx. Also, the involvement of cell adhesion molecules is rather likely.

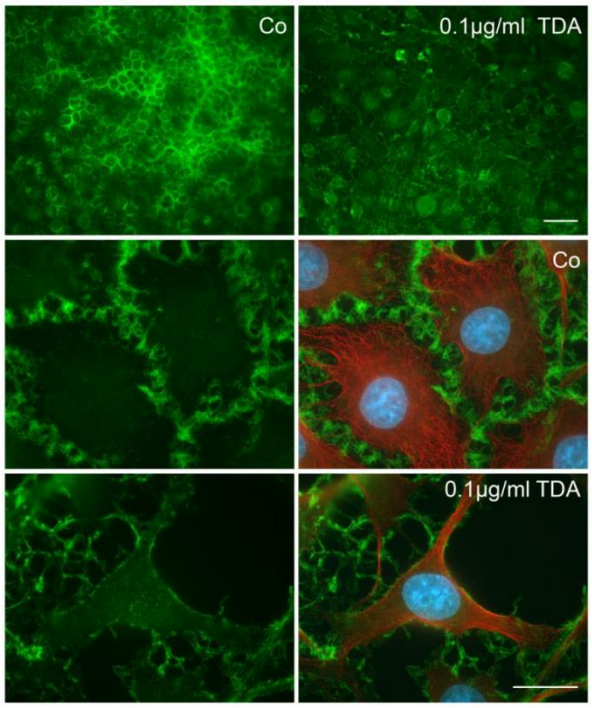

Figure 5: Effect of TDA on F-actin cytoskeleton in OLN-93 cells. Cells were subjected to 0.1 µl/ml TDA for 24 h. Subsequently, cells were fixed with 3% paraformaldehyde, incubated with phalloidin green and indirect immunofluorescence using antibodies against α-tubulin (red) was carried out. Nuclei were stained with DAPI (blue). Co, untreated control. Scale bars: 50 µm (upper images, 200x magnification), 20 µm (1000x magnification).

The present data indicate that the microfilament network is a sensitive target for TDA induced cytotoxicity. However, further studies are needed to elucidate the molecular mechanisms and signal transduction pathways underlying these effects. This is in particular interesting in the context of energy metabolism and influence on cell adhesion processes.

3. Experimental Section

3.1 Materials and Antibodies

Cell culture media were purchased from Gibco/BRL (Grand Island, NY,USA). Poly-L-lysine (PLL), from Sigma (Munich, Germany). Tropodithietic acid (TDA) was purchased from Bioviotica Naturstoffe GmbH, Göttingen, dissolved in DMSO and stored in dark at -20 °C.

For Western blot analysis, the following antibodies were used, working dilutions are given in brackets: mouse monoclonal antibody (mAb) anti-α-tubulin (1:1000) anti-extracellular regulated kinase 1,2 (ERK1,2; 1:2000) and mouse mAb ERK1,2-P (1:1000) from Sigma (Munich, Germany), mouse mAb anti-GAPDH (1:1000) from Sigma-Aldrich (St. Louis, MO).Monoclonal antibody anti-HSP32 (1:1:000) from Enzo Lifesciences (Lörrach, Germany).

3.2 Cell Culture

In this study, OLN-93 cells, an oligodendroglial cell line derived from rat brain glial cultures [21] and N2a (wt) cells, a mouse derived neuroblastoma cell line [20], were used. Cells were kept in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) for OLN-93 cells and 0.5% FCS for N2a cells, 2mM Glutamine, 50 U/ml penicillin (P), and 50 µg/ml Streptomycin (S) at 37 °C and 10% CO_2 [21]. In all subsequent experiments DMSO was added to control cultures. All experiments were carried out at least three times with similar results. Cells were monitored by Hoffman modulation contrast microscopy.

3.3 Immunoblot Analysis

Cellular monolayers of control and treated cells were washed once with PBS, scraped off in sample buffer containing 1% SDS, and boiled for 10 min. The protein contents were determined according to Neuhoff et al. [40]. For immunoblotting, total cellular extracts (10–20 µg protein per lane) were separated by one-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 8.75–10% polyacrylamide gels and blotted onto nitrocellulose membranes (Whatman, Dassel, Germany; 0.2 µm). The blots were saturated with TBS (20 mM Tris, 136.8 mM NaCl, pH 7.5) containing 5 % dry milk and incubated with the individual antibodies overnight at 4 °C. After washing with Tris-buffered saline (TBS) with 0.1 % v/v Tween 20 (TBS-T), blots were incubated with HRP-conjugated anti-mouse (1:10000) or anti-rabbit (1:10000) antibodies for 1 h at RT. After washing with TBS-T, blots were

visualized by the enhanced chemiluminescence procedure as described by the manufacturer (Thermo Scientific, Rockford, IL, USA). All experiments were carried out at least three times with similar results.

3.4 Mitochondrial Staining

OLN-93 cells were grown on PLL-coated glass cover slips, N2a cells were grown without PLL and incubated with MitoTracker Red (100 nM) or MitoTracker Green (150 nM) (Molecular Probes, Oregon, USA) for 30 min, washed twice with PBS and fixed with ice-cold methanol for 7 min (MitoTracker Green) or with 3% paraformaldehyde (MitoTracker Red). The latter were permeabilized with 0.1% Triton X-100. Thereafter, indirect immunofluorescence staining was carried out as described below.

3.5 Indirect Immunofluorescence

OLN-93 cells (3.5×10^5 cells/10 cm dish) were cultured on PLL-coated glass coverslips for 24 h in DMEM/1 % FBS and subjected to treatment as indicated. N2a cells (1.2×10^6 cells/10 cm dish) were cultured on HCl treated glass coverslips without PLL for 24 h in DMEM/0.5% FBS. Cells were incubated overnight at 4 °C with the following antibodies (the working dilutions are given in brackets): mouse mAb anti-α-tubulin (1:250) (Sigma, Munich, Germany). Actin Staining was performed with Phalloidin labeled with FITC (Phalloidin Green, 1 µM) from Sigma (Munich, Germany) and mouse mAb anti-HSP60 (1:1000) from Enzo Lifesciences. After washing with PBS, cells were incubated for 1 h with Dylight (488 or 594) conjugated (1:400; Thermoscientific, Rockford, IL) and FITC-conjugated (1:100) secondary antibodies (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) washed with PBS and mounted. Nuclei were stained by 4, 6-diamidino-2-phenylindole (DAPI) (1.5 mg/mL) included in the mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Fluorescent labeling was studied using a Zeiss epifluorescence microscope (Oberkochen, Germany) equipped with a digital camera using a plan neofluar objective (40x magnification for overview images, 100x magnification for detailed images).

3.6 MTT-Viability assay

To assess the cytotoxic potential of TDA the MTT (Tetrazolium) assay was carried out as described before [41]. Briefly, OLN93 or N2a cells were prepared as described above, plated on (PLL-coated)

96-microwell cell culture plates (3500 or 12000 cells per well) incubated for 24 h. The growth medium was removed and fresh medium (100 µl/well) was added. Cells were then incubated with 0.1– 1 µg/mL TDA and incubated for 24 h. 10 µl of MTT solution (5 mg/mL in PBS) were added to the wells (each containing 100 µl medium) and the plates were incubated for 2 h. 100 µl of a solubilization solution (10% sodium dodecyl sulfate in 0.01 mol/L HCl) was added and incubated overnight to dissolve the water-insoluble formazan salt. Quantification was carried out with an ELISA reader at 595 nm using a 655-nm filter as a reference. Data are expressed as percentage of the untreated controls, with each value representing the mean ^ SD of eight microwells from three independent experiments (n=24).

3.7 Fura-2 AM staining

The procedure was carried out as described by the manufacturer (Molecular Probes, Oregon, USA). Briefly, chamber slides were coated with 0.1 mg/ml poly-l-lysine. After washing with PBS, cells (about $1x10^5$/well) were incubated in DMEM/10% FCS for 2 days at 37 °C and 5% CO_2. The cells were washed two times with PBS and incubated for 1 h in 5 µM Fura-2-AM solution at 37 °C in the dark. Two additional washing steps followed and cells were further incubated for 30 min in PBS at 37 °C in the dark to allow the reaction to complete. For fluorescence imaging, the cells were washed again with PBS and covered with 200 µl PBS and focused under a fluorescence microscope (Olympus IX81; LUCP PlanFi 40x/0.60 objective). The fluorescence was detected every minute at 340 and 380 nm by an excitation of 280 nm for 10 min. Thereafter, 0.1 µg/ml TDA was added, and fluorescence was documented for further 60 min. The evaluation was carried out with the xcellence software (Olympus) and the freeware program Fiji imagej.

4. Conclusion

The marine metabolite TDA, which is known for its broad antimicrobial activity, has previously been suggested to be non-hazardous to multicellular eukaryotic model organisms. Here we show that TDA exerts severe cytotoxic effects on mammalian neuronal and oligodendroglial cell lines in culture. These effects include mitochondrial damage, disorganization of the cytoskeleton, i.e. the microtubule and microfilament network, an influx of intracellular calcium, and the upregulation of the heat shock protein Hsp32/HO-1 and the stress activated MAP kinases ERK1/2. Hence, cells respond to the treatment with TDA by activation of a stress response, which cannot counteract the cytotoxic effects and protect the cells. The observed cytotoxic effects occur at rather low concentrations in the range of

0.3-0.5 µM. TDA is produced in aquacultures and in marine environments by several bacteria of the *Roseobacter* clade. It may be used as an antibacterial agent in other settings, since it also shows actions against human pathogenic bacteria [16]. The question arises whether its potential accumulation in fish or marine organisms may endanger human organisms. The present results may add new aspects to the use of TDA and TDA-producing bacteria and calls for a reconsideration for its use in aquacultures [16].

It needs to be tested whether and how TDA may be transmitted from e.g. *P. inhibens* dominated biofilms into higher organisms. Nonetheless, it has to be considered that although the observed effects occurred at rather low concentrations, the expected concentrations of this compound are likely even lower in the natural environment. Its light sensitivity and the subsequent loss of its activity points to an only weak hazardous potential of TDA for e.g. humans or fish.

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Authors Contributions

CRL, HW, TB, conceived and designed the experiments. FV, designed and performed the Fura-2 AM staining. HW, performed all other experiments. CRL, HW, analyzed the data. CRL, TB, MS, contributed reagents, materials and analysis tools. CRL, HW, MS, TB wrote the paper.

Conflict of Interest

The authors declare no conflict of interest.

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Chapter 3: Neuroprotective and outgrowth inducing effects of dimethylsulphoniopropionate on mammalian cell lines of neuronal and glial origin

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Neuroprotective and outgrowth inducing effects of dimethylsulphonio-propionate on mammalian cell lines of neuronal and glial origin

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Abstract: The marine environment harbors a plethora of bioactive substances, including drug candidates of potential value in the field of neuroscience. Only little is known about the influence of dimethylsulphoniopropionate (DMSP), produced by several algae, corals and higher plants, on neural cell lines. In this study, we analyzed the outgrowth inducing and microtubule reorganization effects of DMSP as well as protective capabilities on representative cell lines of the central nervous system (CNS), Neuro2a and OLN-93 cells. Preincubation with DMSP prohibited cytotoxic effects of tropodithietic acid (TDA), which included a decreased mitochondrial membrane potential ($\Delta\psi_m$) as well as upregulation of heat shock protein 32 (Hsp32) and phosphorylated extracellular signal-regulated kinase 1/2 (ERK 1/2). The neuroprotective effect of DMSP indicates that this natural product potentially provides an antioxidant defense in neural cell lines.

Keywords: dimethylsulphoniopropionate; tropodithietic acid; neurotrophins; process outgrowth; neuroprotection, OLN-93 and N2a cells

1. Introduction

Marine organisms produce a high diversity of structurally unique natural products with a broad spectrum of biological activities, including anti-cancer or antimicrobial effects and showed a promising potential as drugs in the field of neuroscience [1-5]. Natural products of marine algae, derived from the algae itself or their bacterial associates, are a focal point in drug discovery programs due to anti-cancer and various other biological activities [6]. However, more research on the pharmaceutical relevance of algal natural products especially regarding its positive effects against various neurodegenerative diseases is needed to ensure their full biomedical potential [1, 7].

The algal compound dimethylsulphoniopropionate (DMSP) has multifunctional roles in the ocean, being precursor for the climate relevant volatile dimethylsulphide (DMS), substrate for marine bacteria such as *Roseobacter* [8], and an important component of the marine sulphur cycle [9]. In algae, DMSP occurs at high concentrations and serves as osmolyte, cryoprotectant, and antioxidant [9, 10]. In addition to its role in natural biogeochemical processes, DMSP has also been found as a promising bioactive compound for biomedical research. It showed beneficial effects on stressed fish and crustaceans as well as diseased terrestrial animals with ameliorating effects on diseases such as Parkinson, breast cancer, and induced diabetes [11, 12].

The search for novel compounds which mimic neurotrophic factors, like neurotrophins, is a promising goal as they are essential for development, differentiation, survival and functional maintenance of neurons in the central and peripheral system [13-15]. Neurotrophins promote the initial growth and development of neurons [16]. It has been suggested that reconstruction of the neuronal and synaptic networks in the injured brain is necessary for the recovery of brain functions [17]. Stimulatory substances such as the neurotrophin nerve growth factor (NGF) assist in the regeneration of damaged neurons [16], which makes the discovery of neurotrophin-mimicking compounds of great interest [18].

The purpose of the present study was to investigate effects of DMSP on mammalian neural cells, particularly in view of potential neuroprotective and outgrowth inducing effects. Here, we

investigated the influence of DMSP on neural cell lines. The cells of the brain can generally be divided into two main categories: glia and neurons. Hence, for a broader overview of the possible effects of TDA on mammalian brain cells, we have chosen N2a cells as a model for neurons [19] and OLN-93 as a model for glial cells [20]. This allowed the investigation of process outgrowth, morphological changes and protective characteristics of DMSP on the cellular level, by analyzing cell viability, morphology, microtubule organization as well as protein expression. In addition, the effects of DMSP were studied in combination with the marine secondary metabolite tropodithietic acid (TDA). TDA is a broad spectrum antibiotic produced by several *Roseobacter* clade bacteria, such as *Phaeobacter inhibens* 17395 [21, 22]. It is able to inhibit a broad spectrum of both Gram-positive and -negative bacteria, including clinical pathogens, fungi and microalgae [21, 23-25]. The data demonstrates that TDA induces influx of Ca^{2+} -ions, mitochondrial depolarization and upregulation of heat shock protein 32 (Hsp32) and and the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) which results in cell death. The neuroprotective effects of DMSP in presence of TDA, including process outgrowth, microtubule organization and protection of the mitochondrial membrane potential, suggested that DMSP has a range of unknown bioactivities with potential pharmacological relevance.

2. Results and Discussion

2.1. Neurotrophin-like effect of DMSP

Compounds with neurotrophin-like activities are promising therapeutics to treat neurodegenerative diseases [5, 13]. Such effects are of great interest, as neurotrophins are essential for development, differentiation, survival [13, 15] and neuron rehabilitation [16, 18].

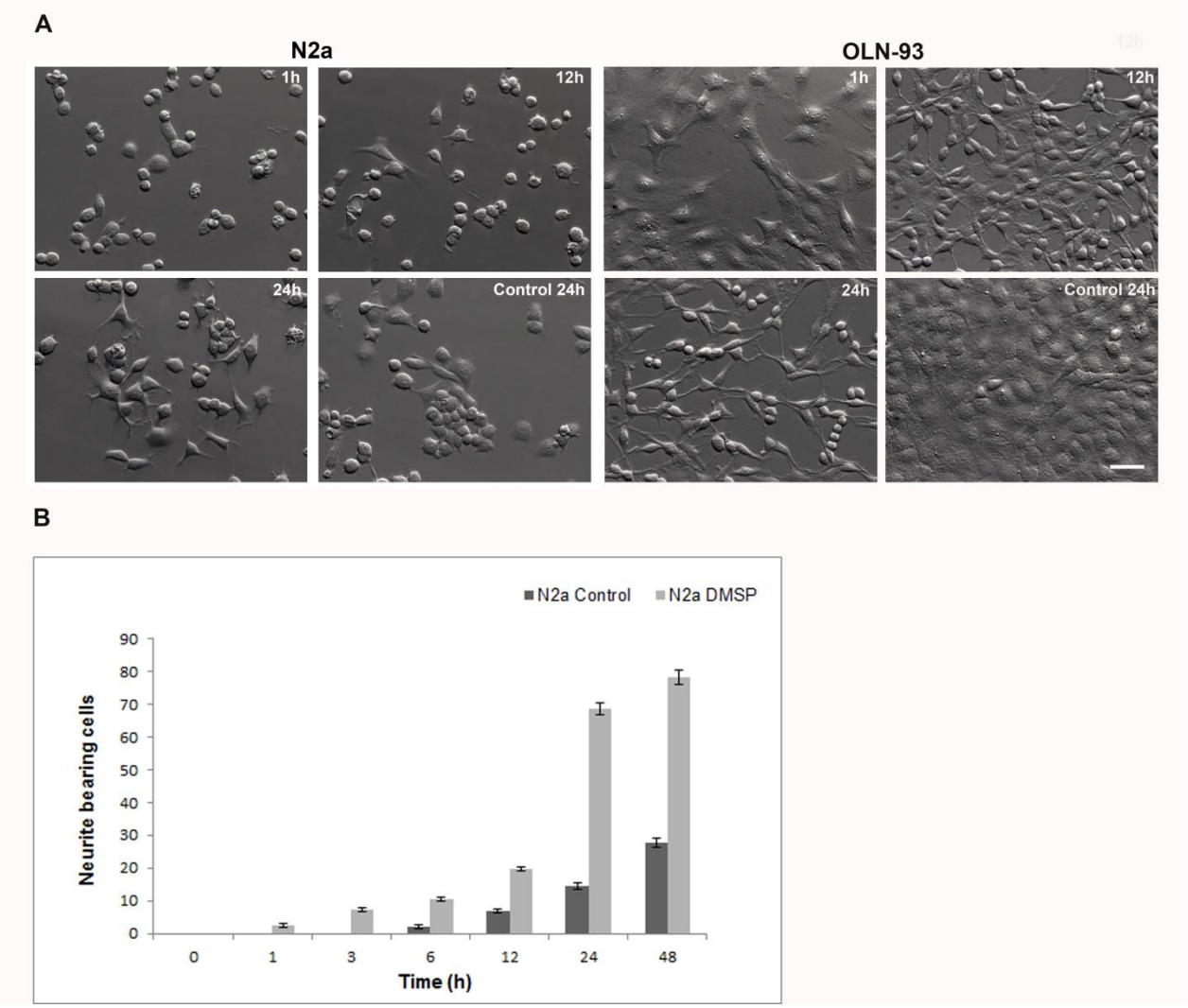

Figure 1. Effect of DMSP on cell morphology. A: DMSP process outgrowth in N2a and OLN-93 cells. Cells were incubated with 1mg/ml DMSP for the indicated times. Hoffman modulation contrast images are shown. Scale bar: 20µm. B: Effect of DMSP on N2a cells. Cells were incubated with 1mg/ml DMSP for the indicated times and process bearing cells were quantitatively evaluated.

To investigate the effect of DMSP on process outgrowth, N2a and OLN-93 cells were used. Treatment with 1mg/ml DMSP resulted in a notably increased process outgrowth in both N2a and OLN-93 cells after 12h (Fig. 1A). Quantitative evaluation revealed in N2a cells a 3-fold higher increase of neurite- bearing cells (number of cells bearing neurites longer than two cell diameters or bearing more than two processes) after incubation with 1mg/ml DMSP for 24h. DMSP did not exert cytotoxicity in both cell lines at concentrations up to 5mg/ml as determined by MTT survival assay (data not shown).

2.2. DMSP induces microtubule reorganization and bundling

Microtubules (MTs) are dynamically assembled polymers of α- and β-tubulin present in all eukaryotic cells. Being a part of the cytoskeleton, MTs are essential for various cellular processes, including mitosis, cell motility, intracellular transport, secretion, cell shape and polarization [26]. MTs are heterogeneous in length and highly dynamic in vivo and in vitro, undergoing cycles of polymerization and rapid depolymerization. This “dynamic instability” property is a feature that is crucial to many microtubule functions and modulated by interactions with other proteins, microtubule motor proteins and non-motor microtubule-associated proteins (MAPs) [27]. MTs can undergo posttranslational modifications (PTMs), such as acetylation and detyrosination, which are crucial for controlling MT stability and dynamics, as well as the interaction with other cellular components such as MAPs [28, 29]. Tubulin acetylation, at α-tubulin on Lys40, is considered as an indicator of MT stability [27], and determined here to further assess the influence of DMSP on MT organization. Indirect immunofluorescence staining with antibodies against α-tubulin (α-tub) and acetylated (ac-tub)tubulin was performed on both OLN-93 and N2a cells (Fig.2,3).

In comparison to control cells, incubation with DMSP resulted in a recruitment of MTs to the cellular extensions of OLN-93 cells (Fig. 2) and neuronal N2a cells (Fig. 3). Elongated processes were characterized by a dense MT-network and MT appeared bundled. Ac-tub was specifically prominent in the long cellular extensions (Figs. 2, 3).

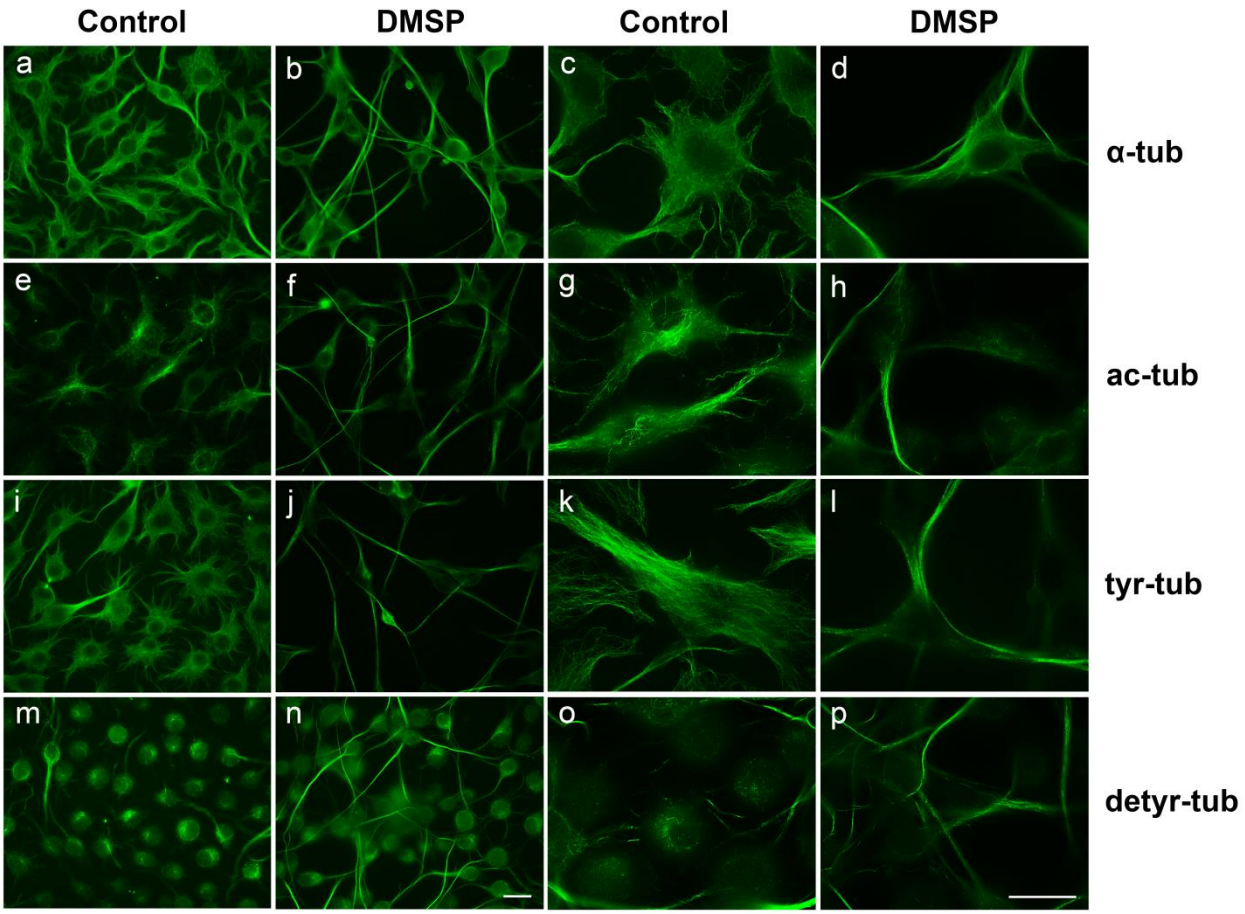

Figure 2. Microtubule bundling and reorganization after DMSP treatment: OLN-93 cells were incubated with DMSO only (Control)or 1mg/ml DMSP for 48h. Indirect immunofluorescence staining with antibodies against α-tubulin (a-d), acetylated tubulin (e-h), tyrosinated tubulin (i-l) and detyrosinated tubulin (m-p) as indicated on the right. Scale bars: 20μm.

Since tubulin acetylation is not strictly associated with stable (long-lived) MTs, we also determined the tyrosination state of tubulin by using antibodies against tyrosinated(tyr- tub) and detyrosinated tubulin (detyr.tub).Detyrosination of α-tubulin stabilizes indirectly MTs and is an indicator of enhanced MT stability while dynamic MTs contain tyrosinated tubulin [27]. The tyrosination–detyrosination cycle is initiated by the removal of a tyrosin (Tyr) functional group (detyrosination), whereas re-addition of Tyr (tyrosination) returns tubulin to its nascent state and is more found in dynamic MTs [27]. In control cells tyr- tub was distributed throughout the cell soma and processes (Figs. 2, 3 i, k), while detyr- tub was mainly expressed in the cell soma

(Figs. 2, 3 m, o). After DMSP treatment tyr. tub (Fig. 2, 3j, l) as well as detyr- tub (Fig. 2, 3 n, p) were concentrated mainly within the long processes and hardly visible within the soma.

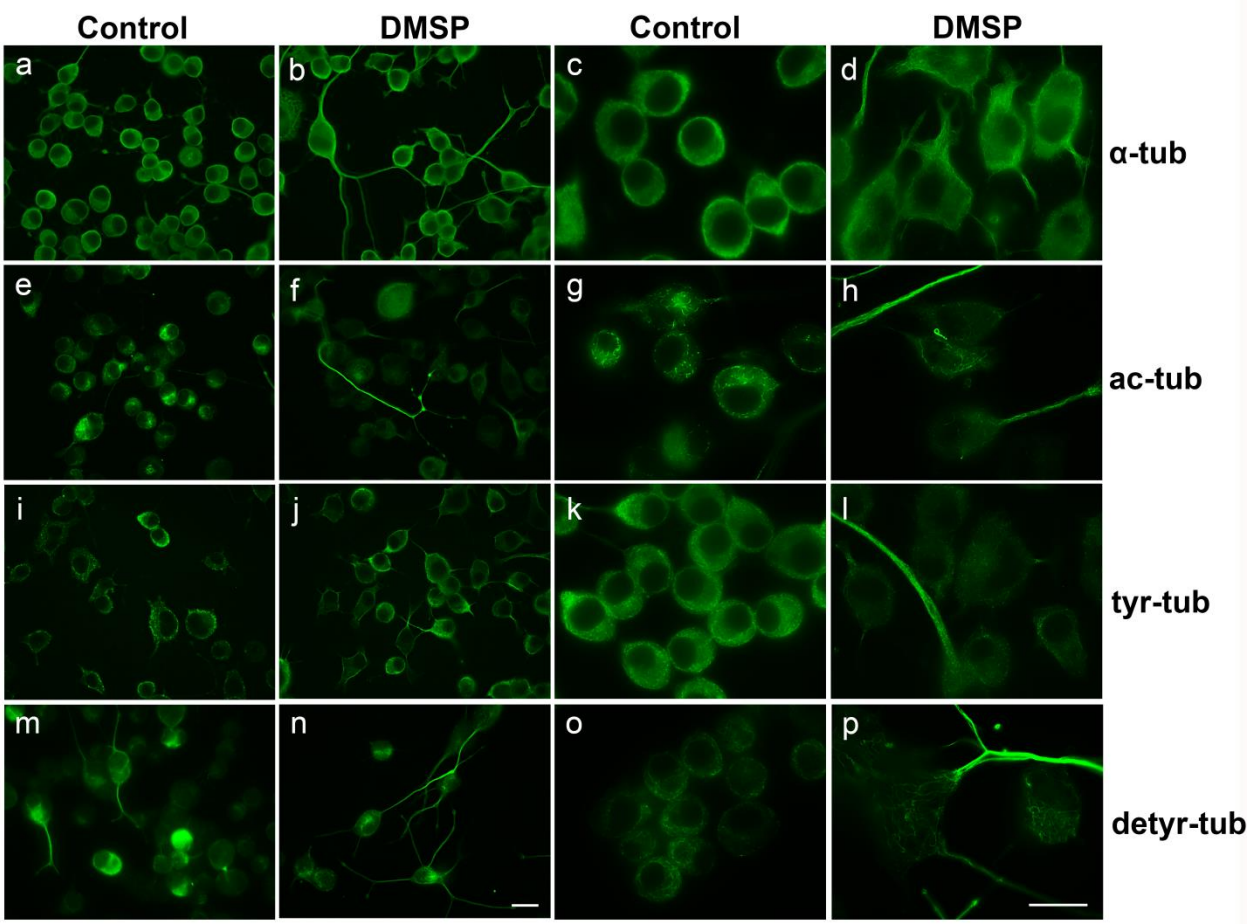

Figure 3. Microtubule bundling and reorganization after DMSP treatment: N2a cells were incubated with DMSO only (Control) or 1mg/ml DMSP for 48h. Indirect immunofluorescence staining with antibodies against α-tubulin (a-d), acetylated tubulin (e-h), tyrosinated tubulin (i-l) and detyrosinated tubulin (m-p) as indicated on the right. Scale bars: 20µm.

In addition to immunostaining methods, the influence of DMSP was analyzed on the protein level. Immunoblot analyses of cell extracts revealed slightly alterations but no significant changes on the posttranslational modifications of tubulin (Fig. 4).

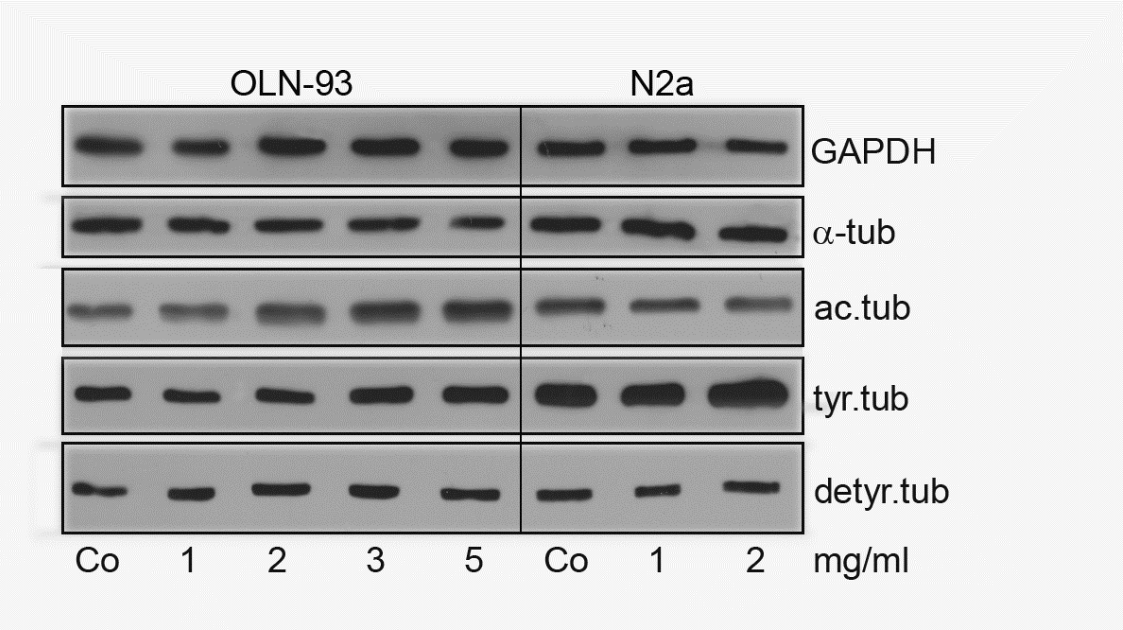

Figure 4. Western blot of OLN-93 and N2a cell lysates after treatment with 1 to 5mg/ml DMSP for 48h prepared and subjected to immunoblot analysis using antibodies as indicated on the right.

These findings showed that DMSP treatment results only in reorganization and recruitment of ac. tub to cellular extensionsin both cell lines (Fig. 2, 3). Increased posttranslational tubulin modifications, found in primary cultures which undergo neuronal development, can also be mimicked by NGF-induced outgrowth of neurites in PC12 cells [30, 31].This may point to the conclusion that DMSP exerts similar neurotrophic effects.

2.2. Protective effect of DMSP against toxic concentrations of tropodithietic acid

Previously studies of our laboratory demonstrated that tropodithietic acid (TDA) exerts cytotoxic effects of (TDA) on OLN-93 and N2a cells. These neurotoxic effects included an influx of Ca^{2+} -ions, mitochondrial depolarization, and upregulation of proteins such as Hsp32, resulting in serious cell damage. To acess whether DMSP is capable to protect cells the effect of TDA on OLN-93 cells with and without 24h DMSP- preincubation and subsequent treatment with TDA for 24h on cell morphology was determined using Hoffman modulation contrast microscopy. Fig. 5 shows the protective effect of DMSP on OLN-93. Cells were preincubated

with DMSP (24h) followed by TDA (24h). After preincubation with DMSP cell morphology was preserved (Fig. 5) and cell viability was promoted as indicated by a higher density of cells and MTT viability assay (Fig. 6A).

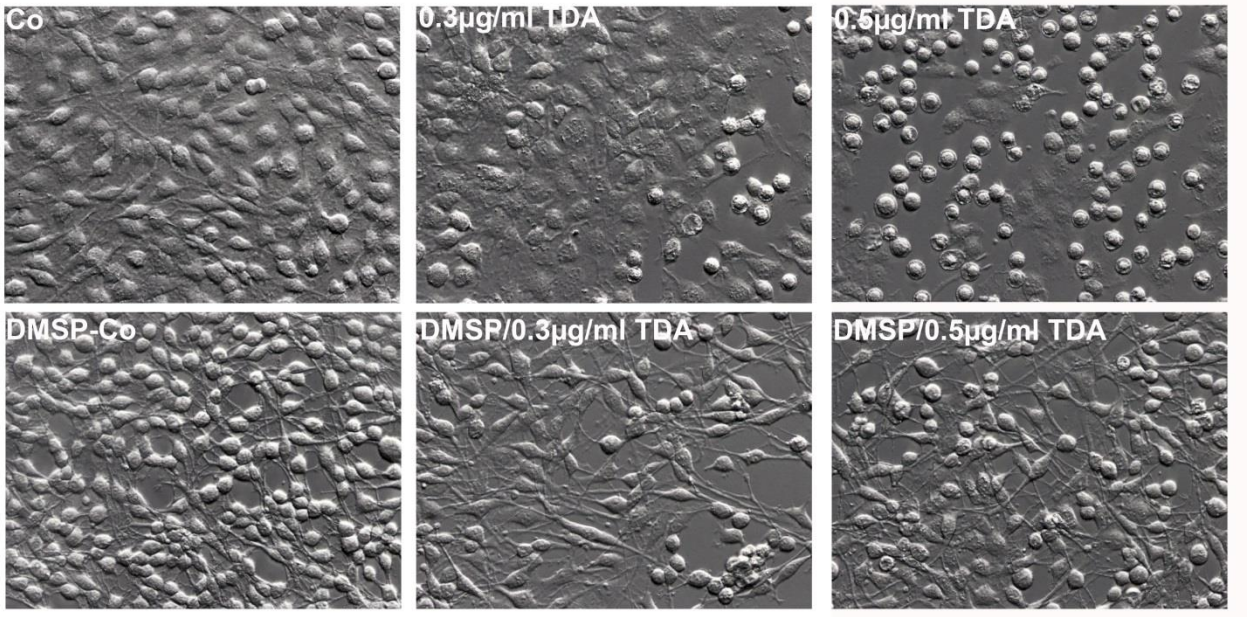

Figure 5. Protective effect of DMSP in presence of TDA. Effect of TDA on OLN-93 with or without DMSP- preincubation (24h) and subsequent treatment with different TDA concentrations (24h) on cell morphology. Hoffman modulation contrast images are shown. Scale bar 50µm.

Preincubation for 24h with (DMSP/TDA, light green/- light blue) or without 1mg/ml DMSP (TDA, dark green/ dark blue) and subsequent exposure to 0.1µg/ml - 1µg/ml TDA for 24h yielded in an approximately 20% higher survival rate compared to non- DMSP preincubated cells (Fig. 6 A).

Immunoblot analysis (Fig. 6B) revealed that TDA induces the upregulation of heat shock protein 32 (Hsp32) and the activation of extracellular signal-regulated kinase 1/2 (ERK1/2 P, Fig. 6). Hsp32 is presumably essential for regulation of oxidative stress, inflammation, as well as cell survival and proliferation [32, 33], indicating that TDA induces oxidative stress [32, 34]. ERK1/2 are widely expressed protein kinases that are involved in the regulation of cell death and survival [35-37]. A slight activation of ERK1/2 after treatment with DMSP was recognizable,

which is also induced by neurotrophins [38] and therefore substantiate the neurotrophin-like effect of DMSP. Pretreatment with DMSP suppressed the TDA-induced induction of Hsp32.

Additionally, DMSP pretreatment combined with subsequent exposure to TDA suppressed the ERK 1/2 activation (Fig. 6 B). Comparable effects could be observed with the polyunsaturated fatty acid docohexaenic acid (DHA), an essential constituent of both glial and neuronal cell membranes [39-41]. DHA, known for its antioxidative capabilities, induced neuritogenesis, upregulation of Hsp32 and ERK1/2 activation [40]. On the other hand, DHA, in high amounts, has pro-oxidant properties and can act as target for reactive oxygen species. Combined with the antioxidant and vitamin E analogue α-tocopherol Hsp32 upregulation was inhibited [39]. Here, comparable effects could be demonstrated in our study with DMSP and TDA, suggesting that DMSP may not only work as an efficient antioxidant system in algae [10] but also in the cell models.

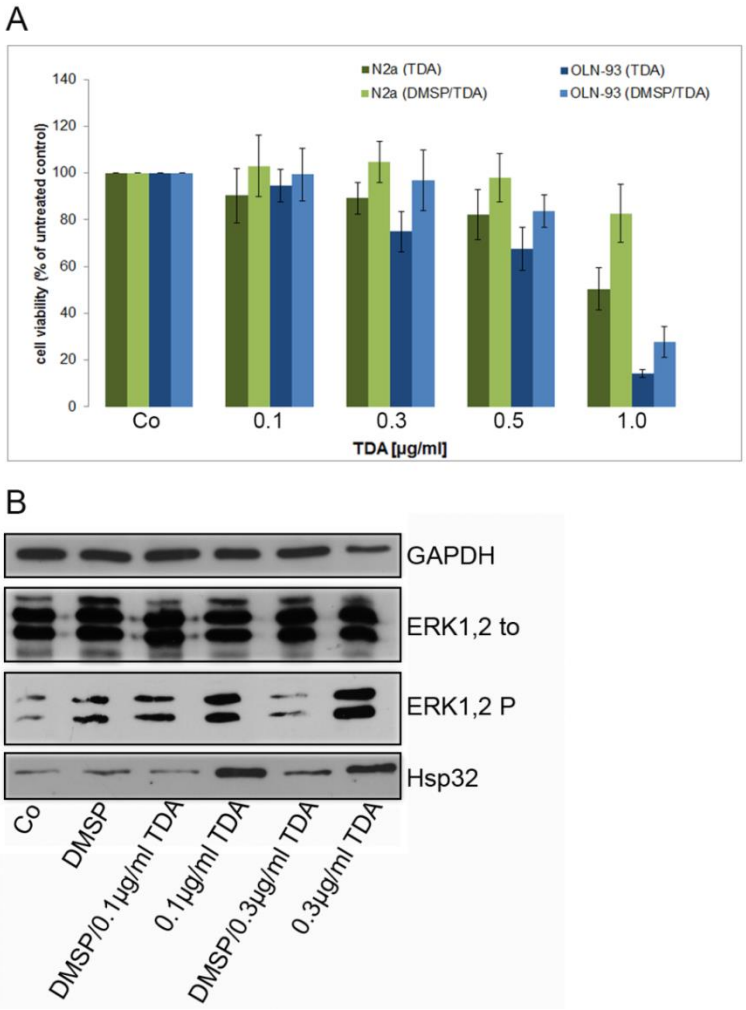

Figure 6. Protective effect of DMSP in the presence of TDA. A: MTT assay (OLN-93 blue bars, N2a cells green bars) for assessing cell viability with 1mg/ml DMSP - preincubation for 24h (DMSP/TDA, light green/- light blue) or without (TDA, dark green/ dark blue) and subsequent exposure to 0.1 - 1µg/mL TDA for 24h. B: Western blot analysis. Cell lysates of OLN-93 cells were prepared and subjected to immunoblot analysis using antibodies as indicated on the right. DMSP = preincubation with 1mg/ml DMSP for 24h and subsequent exposure to TDA for 24h as indicated below.

The effect on mitochondrial membrane potential ($\Delta\psi_m$) was tested by staining cells with MitoTracker Red, whose accumulation in living cells is dependent upon membrane potential. The fluorescence signal is brighter in active mitochondria than in mitochondria with depolarized membranes.Given that TDA depolarizes the mitochondrial membrane potential ($\Delta\psi_m$), we

studied the protective effect of DMSP on the possible protection of mitochondria, by staining cells with MitoTracker Red (Fig. 7, 8, red). DMSP preincubation resulted in a preserved $\Delta\psi_m$, as illustrated by stronger MitoTracker Red fluorescence compared to non - DMSP incubated cells.

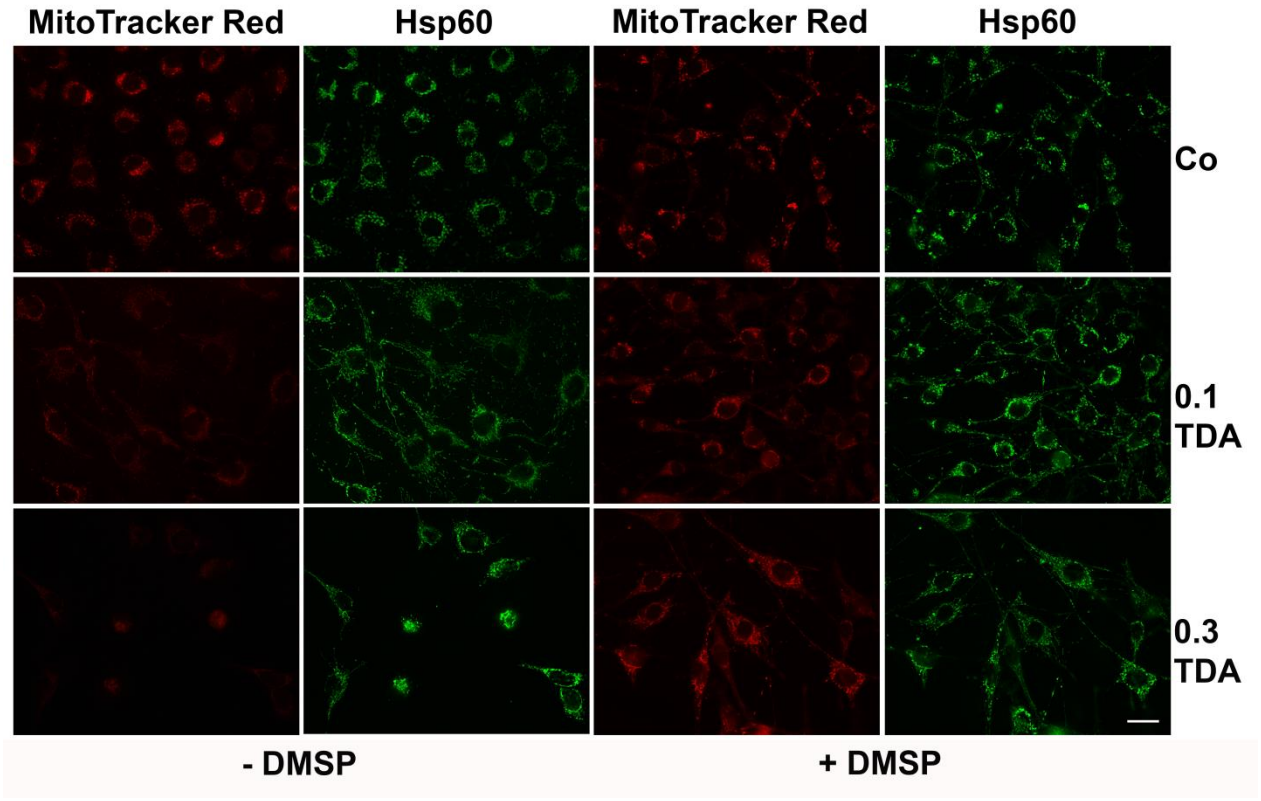

Figure 7: Protective effect of DMSP in presence of TDA-toxin in OLN-93 cells on mitochondrial morphology and membrane potential ($\Delta\psi_m$). Staining of heat shock protein 60 (Hsp60, green) and mitochondrial membrane potential (MitoTracker Red). Cells were treated with DMSO only (- DMSP) or preincubated with 1mg/ml DMSP for 24h (+ DMSP), subsequently, 0.1 - 0.3µl/ml TDA was added for 24h. Afterwards, cells were stained for 30min with MitoTracker Red, fixed with methanol and subjected to indirect immunofluorescence using antibodies against HSP60. Magnification: 400x. Scale bar 20µm.

Mitochondrial morphology was studied using indirect immunofluorescence with antibodies against heat shock protein 60 (Hsp60, Fig. 6, 7, green), a mitochondrial chaperone which is also involved in protein import. Hsp60 has been suggested to play a role in pro-survival or pro-apoptotic pathways [42, 43] and its distribution can show the influence on the mitochondrial

morphology and stress-induced changes such as mitochondrial fusion and fission. Treatment with TDA, respectively, caused a depolarized mitochondrial membrane potential in OLN-93 (Fig. 7, 8, red) and the morphology appeared smaller compared to the mitochondria in the DMSP pretreated samples (Fig. 8, green). Therefore, DMSP-preincubation resulted in a protection of the mitochondria in occurrence of toxic amounts of TDA. Additionally, no depolarization of $\Delta\psi_m$ was detectable.

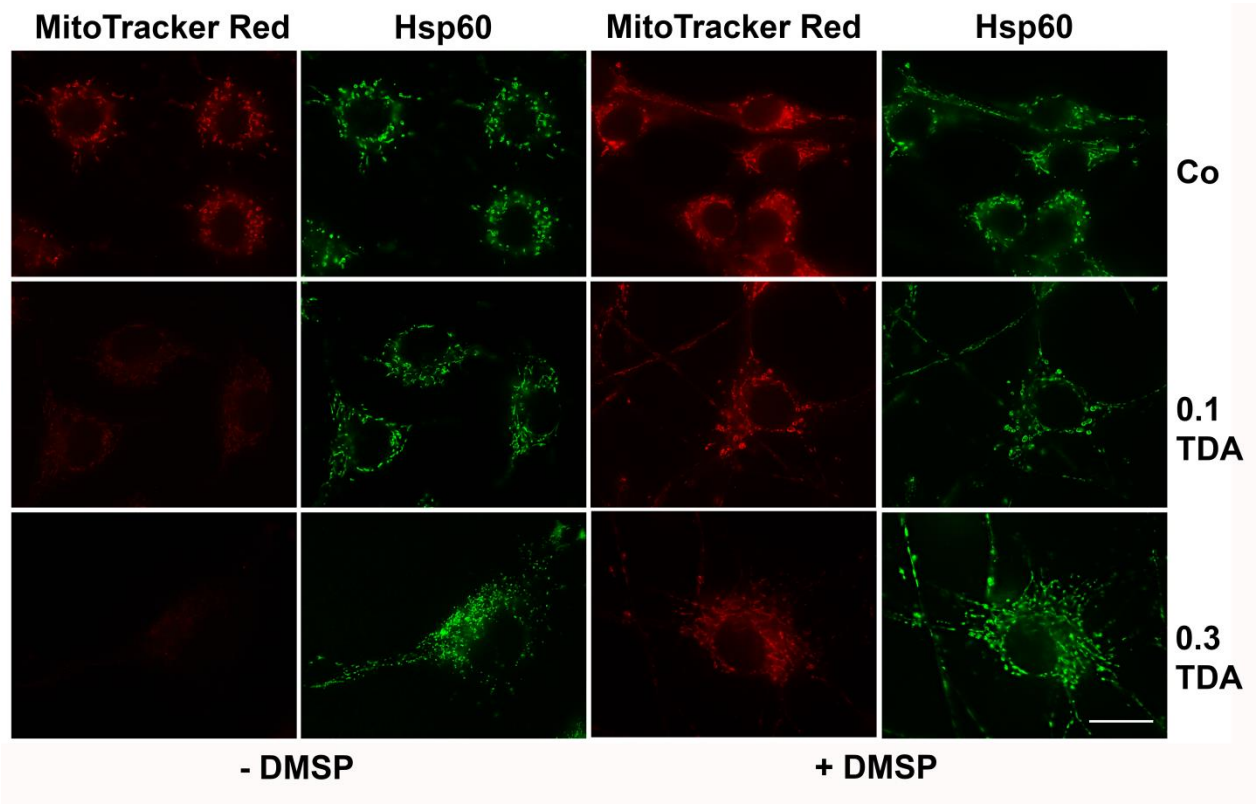

Figure 8: Protective effect of DMSP in presence of TDA-toxin in OLN-93 cells on mitochondrial morphology and membrane potential ($\Delta\psi_m$). Staining of heat shock protein 60 (Hsp60, green) and mitochondrial membrane potential (MitoTracker Red). Cells were treated with DMSO only (- DMSP) or preincubated with 1mg/ml DMSP for 24h (+ DMSP), subsequently, 0.1 - 0.3µl/ml TDA was added for 24h. Afterwards, cells were stained for 30min with MitoTracker Red, fixed with methanol and subjected to indirect immunofluorescence using antibodies against HSP60. Magnification: 1000x. Scale bar 20µm.

It is known that DMSP and its enzymatic cleavage products DMS and acrylate are effective cellular scavengers of hydroxyl radicals and constitute an highly effective antioxidant system in algae [10]. These molecules constitute an antioxidant system, which could be in high DMSP containing algae more effective than other well-recognized antioxidants, such as ascorbate and glutathione [10]. They also work as antioxidant defense in animals [44], for instance in the CNS of mammals [45, 46]. These similarities are raising the question of the potential role of DMSP as an antioxidant in mammalian neural systems. The main focus of research in this context lies on DHA and EPA, which not only have antioxidative properties, but also stimulate neural development, promote neurogenesis and accumulate as the most abundant fatty acids in the brain [47, 48]. The origin of DHA and eicosapentoenic acid (EPA) in aquatic ecosystems are algae. Certain microalgae produce high levels of DHA or EPA and are, for instance, used as supplementary vegetarian source for these essential fatty acids [49] The potential role of DMSP in this context is rarely understood. The shown outgrowth inducing and neuroprotective effects of DMSP assume that not only DHA and EPA play important roles e.g. in the environment. DMSP may also provide an antioxidant defense in mammalian neural cell lines. However, the exact mechanism of DMSP interactions with mammalian neural cell lines, including whether DMSP itself or its breakdown products cause the protective effect on cell survival, will be subject of future studies.

3. Experimental Section

3.1 Materials and Antibodies

Cell culture media were purchased from Gibco/BRL (Grand Island, NY,USA). Poly-L-lysine (PLL), from Sigma (Munich, Germany). Dimethylsulphoniopropionate (DMSP) and tropodithietic acid were purchased from Bioviotica Naturstoffe GmbH, Göttingen, dissolved in DMSO and stored at -20°C.

For Western blot analysis, the following antibodies were used, working dilutions are given in brackets: mouse monoclonal antibody (mAb) anti-α-tubulin (1:1000), and mouse mAb anti acetylated α-tubulin (1:1,000), anti-extracellular regulated kinase 1,2 (ERK1,2; 1:2000) and

mouse mAb ERK1,2-P (1:1000) from Sigma (Munich, Germany), mouse mAb anti-GAPDH (1:1000) from Sigma-Aldrich (St. Louis, MO). Monoclonal antibody anti-HSP32 (1:1:000) from Enzo Lifesciences (Lörrach, Germany). Rabbit polyclonal antibody (pAb) anti detyrosinated α-tubulin (1:1:000) was from Merck Millipore (Darmstadt, Germany) and rat mAb anti tyrosinated α-tubulin clone YL1/2 (1:1:000) was from Santa Cruz (Dallas, TX, USA).

3.2 Cell Culture

In this study, OLN-93 cells, an oligodendroglial cell line derived from rat brain glial cultures [20] and N2a (wt) cells, a mouse derived neuroblastoma cell line [19], were used. Cells were kept in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % heat inactivated fetal bovine serum (FBS) for OLN-93 cells and 0.5% FBS for N2a cells, 2mM Glutamine, 50 U/ml penicillin (P), and 50 µg/ml Streptomycin (S) at 37°C and 10% CO_2 [20]. In all subsequent experiments DMSO was added to control cultures. All experiments were carried out at least three times with similar results.

3.3 Immunoblot Analysis

Cellular monolayers of control and treated cells were washed once with PBS, scraped off in sample buffer containing 1 % SDS, and boiled for 10 min. The protein contents were determined according to Neuhoff et al. [50]. For immunoblotting, total cellular extracts (10–20 µg protein per lane) were separated by one-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 8.75–10% polyacrylamide gels and blotted onto nitrocellulose membranes (Whatman, Dassel, Germany; 0.2 µm). The blots were saturated with TBS (20 mM Tris, 136.8 mM NaCl, pH 7.5) containing 5 % dry milk and incubated with the individual antibodies overnight at 4 °C. After washing with Tris-buffered saline (TBS) with 0.1 % v/v Tween 20 (TBS-T), blots were incubated with HRP-conjugated anti-mouse (1:10000) or anti-rabbit (1:10000) antibodies for 1h at RT. After washing with TBS-T, blots were visualized by the enhanced chemiluminescence procedure as described by the manufacturer (Thermo Scientific, Rockford, IL, USA). All experiments were carried out at least three times with similar results.

3.4 Mitochondrial Staining

OLN-93 cells were grown on PLL-coated glass cover slips, N2a cells were grown without PLL and incubated with MitoTracker Red (150 nM) (Molecular Probes, Oregon, USA) for 30 min, washed twice with PBS and fixed with ice-cold methanol for 7 min or with 3 % paraformaldehyde (MitoTracker Red). The latter were permeabilized with 0.1 % Triton X-100. Thereafter, indirect immunofluorescence staining was carried out as described below.

3.5 Indirect Immunofluorescence

OLN-93 cells (3.5×10^5 cells/10 cm dish) were cultured on PLL-coated glass coverslips for 24 h in DMEM/10 % FBS and subjected to treatment as indicated. N2a cells (1.2×10^6 cells/10 cm dish) were cultured on HCl treated glass coverslips without PLL for 24h in DMEM/ 0.5% FBS. Cells were incubated overnight at 4 °C with the following antibodies (the working dilutions are given in brackets): mouse mAb anti-α-tubulin (1:250) (Sigma,Munich, Germany). Mouse mAb anti-HSP60 (1:1000) was from Enzo Lifesciences. After washing with PBS, cells were incubated for 1h with Dylight (488 as well as 594) conjugated (1:400; Thermoscientific, Rockford, IL) and FITC-conjugated (1:100) secondary antibodies (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) washed with PBS and mounted. Nuclei were stained by 4C, 6-diamidino-2-phenylindole (DAPI) (1.5 mg/mL) included in the mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Fluorescent labeling was studied using a Zeiss epifluorescence microscope (Oberkochen, Germany) equipped with a digital camera using a planneofluar objective (40x magnification for overview images, 100x magnification for detailed images).

3.6 MTT-Viability assay

To assess the cytotoxic potential of TDA and the protective effect of DMSP the MTT (Tetrazolium) assay was carried out as described before [51]. Briefly, OLN93 or N2a cells were prepared as described above, plated on (OLN-93 cells; PLL-coated) 96-microwell cell culture plates (3500 or 12000 cells per well) then grown for 24h. The growth medium was removed and fresh medium (100 µl/well) was added, some cells were preincubated with 1mg/mL DMSP for 24h. Growth medium was removed and fresh medium was added and subsequently 0.1µg/ml – 1µg/ml TDA was added for additional 24h. 10 µl of MTT solution (5 mg/ml in PBS) were added

to the wells (each containing 100 µl medium) and the plates were incubated for 2 h. 100 µl of a solubilization solution (10% sodium dodecyl sulfate in 0.01 mol/L HCl) was added and incubated overnight to dissolve the water-insoluble formazan salt. Quantification was carried out with an ELISA reader at 595 nm using a 655-nm filter as a reference. Data are expressed as percentage of the untreated controls, with each value representing the mean ^ SD of eight microwells from three independent experiments (n =24).

4. Conclusion

The natural product dimethylsulphoniopropionate (DMSP), which is produced by several algae, corals and higher plants and abundant in marine systems, is known to act as an antioxidant system in algae [10]. Our studies revealed neurotrophin-like abilities and microtubule reorganization as well as protective capabilities of DMSP on mammalian cell lines of the central nervous system, e.g. Neuro2a and OLN-93 cells. DMSP prevented cytotoxic effects by counteracting decreased mitochondrial membrane potential ($\Delta\psi_m$) and induction of Hsp32 as well as activation of ERK1/2 from treatment with tropodithietic acid (TDA), indicating that DMSP potentially provides an antioxidant defense in neural cell lines and has the potential to act as a neurotrophin.

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Author Contributions

CRL, HW, conceived and designed the experiments. HW, performed all experiments. CRL, HW, analyzed the data. CRL, TB and MS, contributed reagents, materials and analysis tools. CRL, HW, MS, TB wrote the paper.

Conflict of Interest

The authors declare no conflict of interest.

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Chapter 4: A modified *C. elegans* killing assay for drug screening using auxotrophic *E. coli* ST18 to test for antithelmintic effects of tropodithietic acid, sigillin A and dimethylsulphoniopropionate

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Keywords: antithelmintic, tropodithietic acid, dimethylsulphoniopropionate, sigillin A, cross-reaction

in preparation

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A modified *C. elegans* killing assay for drug screening using auxotrophic *E. coli* ST18 to test for antithelmintic effects of tropodithietic acid, sigillin A and dimethylsulphoniopropionate

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Abstract

The objective of this study was to establish an improved *C. elegans* killing assay using auxotrophic *E. coli* ST18 to determine the antithelmintic effect of tropodithietic acid (TDA), sigillin A and dimethylsulphoniopropionate (DMSP). Comparing the killing efficiency of TDA in living or devitalized *E. coli* ST18 showed the importance of the devitalization process to avoid discrepancies in the test compound uptake. The modified assay was used to demonstrate antithelmintic effects of TDA (>10 µg/ml) and sigillin A (>3 µg/ml). In contrast, DMSP was only toxic in high concentrations (>500 µg/ml) and therefore showed no notable antithelmintic effect. In conclusion, this study showed the importance of using a devitalized food source in antinematode tests as well as the antithelmintic effect of TDA and sigillin A.

Keywords: antithelmintic, tropodithietic acid, dimethylsulphoniopropionate, sigillin A

Introduction

Parasitic nematodes are a global problem causing disease in not only humans but also in agricultural, companion animals and wildlife thus they represent a major burden to both the medical and agricultural industries (Holden-Dye and Walker, 2005). The nematode *Caenorhabditis elegans* is a suitable model organism for various questions in biomedical research, particularly in the search of novel drugs. It is an effective *in vivo* model due to the conservation of disease pathways in *C. elegans* and higher organisms (Kaletta and Hengartner, 2006). Several cultivation assays are available, but there is still a lack of information on possible cross-reactions with the bacterial food source, such as the commonly used *E. coli* OP50 (Brenner, 1974; Dimeski, 2008). Additionally, antibiotics in the culture media can lead to cross-reactions with the bacterial food source and test compounds, which can affect the mitochondria of eukaryotic models (Moullan et al., 2015) and may alter assay results. To avoid these unwanted impacts *E. coli* has to be devitalized. Our experiences and different studies showed that a devitalized bacterial food source can be problematic by preventing larval development (Kaeberlein et al., 2006) or leading to unwanted effects like internal-hatching (Saul, 2011).

In the first part of this study the importance of the devitalization process and avoidance of growth of food bacteria during killing assays by using auxotrophic *E. coli* ST18 (Thoma and Schobert, 2009) was shown. Due to a preference for living cells, a divergence in the test compound uptake may occur, which requires the absence of living *E. coli* during compound tests. Here, we establish an *E. coli* mutant (strain ST18), auxotrophic for aminolevulinic acid (ALA) as total viability control and to ensure that no living bacteria remain that may interfere with the test compounds whereas the uracil-auxotrophy of *E. coli* OP50 is only growth inhibiting (Brenner, 1974). Another benefit is the cultivation of *C. elegans* with living *E. coli* ST18 by avoiding problems during larval development and potentially displaced cells are not able to grow on ALA-depleted test plates.

The herein established assay was subsequently used to test different substances for antithelmintic activity. Finding such compounds is vital to tackle the global problem of parasitic nematodes, which represent a major burden to both the medical and agricultural industries (Holden-Dye and Walker, 2005). Unfortunately, the reliance on only few chemotherapeutics

for the treatment of thelmintic diseases has resulted in an increased prevalence of nematode resistance (Ballestriero et al., 2014), clarifying the importance of the search for new antinematode compounds. Here, the applicability of the optimized method is shown by determining the antithelminthic effect of three natural products:

- the marine secondary metabolite tropodithietic acid (TDA), a broad spectrum antibiotic produced by several *Roseobacter* clade bacteria, including *Phaeobacter inhibens* 17395 or *Ruegeria mobilis*, whose TDA production make them potential candidates as probiotics in aquaculture settings (Porsby et al., 2011). TDA is able to inhibit a broad spectrum of both Gram-positive and -negative bacteria, clinical pathogens, fungi and microalgae (Brinkhoff et al., 2004; Geng and Belas, 2010; Harrington et al., 2014; Liang et al., 2003b). To date, TDA-producing bacteria or TDA itself showed no toxicity against the eukaryotic model organisms *Artemia* sp.and *Caenorhabditis elegans* (Neu et al., 2014).
- Sigillin A, an arthropod deterrent produced by *Collembola* (springtails) to repel predators, showed high activity in a bioassay against predatory ants (Schmidt et al., 2015).
- Dimethylsulphoniopropionate (DMSP), an environmentally important substance produced by several algae, corals and higher plants (Raina et al., 2013), is very abundant in marine waters and extremely attractive to bony fishes (Klimley, 2013). In algae, DMSP occurs at high concentrations and serves as osmolyte, cryoprotectant, and antioxidant (Raina et al., 2013; Sunda et al., 2002). In addition to its role in natural biogeochemical processes, DMSP has also been suggested as a promising bioactive compound for biomedical applications (Nakajima, 2015).

Material and methods

Strains and culture conditions

Auxotrophic *Escherichia coli* ST18 was used as bacterial food source for *C. elegans* cultures. *E. coli* ST18 was grown in liquid Lysogeny broth (LB) supplemented with 50 µl/ml of 5-aminolevulinic acid (ALA) and incubated at 25°C. *E. coli* ST18 is a hemA deletion mutant defective in tetrapyrrole biosynthesis. The hemA mutation can be complemented by addition of 5-aminolevulinic acid (Thoma and Schobert, 2009).

C. elegans strain N2 Bristol was provided by the *Caenorhabditis* Genetics Center, University of Minnesota(CGC). *C. elegans* cultures were grown on solid nematode growth medium (NGM) (Stiernagle 2006) and incubated at 25 °C. NGM was supplemented with 50µg/ml ALA for worm development. Compound tests were performed on NGM plates without ALA to avoid unwanted growth of *E. coli* ST18. Hundred millimeter (diameter) plates were used for the worm maintenance, 35mm plates for compound tests. Observation of worm survival was performed with a stereomicroscope (475002, Carl Zeiss Jena, Germany).

Heat killing of *E. coli* ST18

E. coli ST18 was devitalized at 70 °C for 3.5 hours. In order to confirm that heat killed bacteria were dead, samples of the bacterial lawns were streaked on LB and NGM agar plates before the nematode killing assay, incubated at 25 °C and subsequently checked for an absence of bacterial colonies for 30 days.

Test compounds

Tropodithietic acid and dimethylsulphoniopropionate were purchased from Bioviotica Naturstoffe GmbH, Göttingen, Germany. Sigillin A was provided by the laboratory of Prof. Dr. Stefan Schulz, Institute of Organic Chemistry, TU Braunschweig, Germany (Schmidt et al., 2015). All compounds were dissolved in DMSO and stored at -20°C in the dark.

Nematode killing assay

Twenty L4 stage larvae were transferred onto 35 mm NGM plates without ALA. The transfer process was carried out with a 100µl Eppendorf pipette. Triplicates were performed for every concentration of the test compounds. Compounds/DMSO stock solutions were added to 0,5ml of devitalized *E. coli* ST18 (OD>5). 100µl of each compound concentration was dropped on each test plate. DMSO was supplemented in control. The killing assays were performed for 24 h at 25 °C in the dark.

Statistics

Results are expressed as mean ± SD (percentage of survived worms) from three independent experiments of triplicates. Statistical evaluations were carried out by Student's t test: *p <0.05 significant.

Results and discussion

For the experiments we have exemplarily chosen the marine natural product tropodithietic acid (TDA), fed to the worms in living (Fig.1, dark grey bars) or devitalized *E. coli* ST18 (Fig.1 light grey bars) for 24h. Feeding TDA to *C. elegans* with living *E. coli* ST18 had negligible difference to the control group, whereas the same concentration fed with devitalized *E. coli* resulted in a decrease of ~ 50% (IC_{50}).

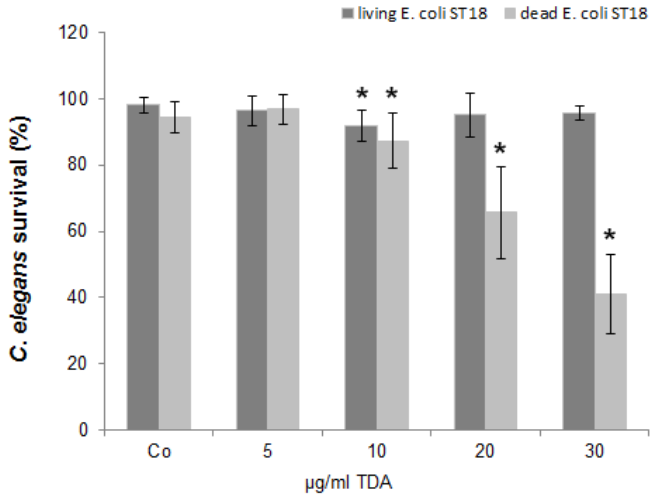

Figure 1: Antithelmintic effect (Dose respond curve) of different concentrations tropodithietic acid (TDA) on *C. elegans* survival fed with living or devitalized *E. coli* ST18 for 24h. Average number of survived worms,* $P < 0.05$.

One explanation is the uncontrolled growth of *E. coli* ST18, the worms preferred the living cells and avoid the TDA contaminated cells. Another reason is a potential cross reaction of the active bacteria and TDA by for instance metabolizing this compound and altering the antithelmintic properties. The devitalization process and prohibition of bacterial growth on test plates was identified as are very important to prevent a misinterpretation of the effectiveness of investigated compounds.

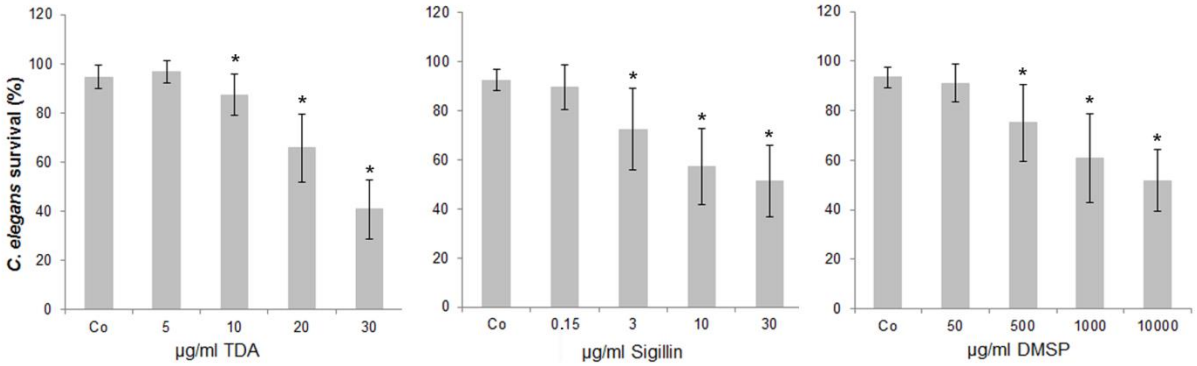

Figure 2: Antithelmintic effect of tropodithietic acid (TDA), sigillin A and dimethylsulphoniopropionate (DMSP) on *C. elegans*for 24h, supplemented concentrations as indicated below. Average number of survived worms,* $P < 0.05$.

Using the modified protocol with devitalized *E. coli* ST18, exposure to >10µg/ml TDA or >3µg/ml sigillin A, respectively, was shown to yield a significant decrease of *C. elegans* survival (Fig.2). In addition to its broad antimicrobial and neurotoxic effects, TDA was thus identified as a potent antithelmintic. Our observations were inconsistent with previous studies that did not find no toxicity against eukaryotic model organisms *C. elegans* and *Artemia sp.* (Neu et al., 2014) and the discovery of the antinematode activity adds to the growing list of biological activities of TDA.

Sigillin A, acting as a strong feeding deterrent against ants, is hypothesized to play an important role in the ecology of springtails (Schmidt et al., 2015). *C. elegans* showed a higher sensitivity for this compound compared to TDA. To our best knowledge this is the first report of sigillin A toxicity in nematodes, revealing a new bioactivity of this compound.

In contrast, DMSP was only toxic to *C. elegans* in high concentrations (>500µg/ml). Previous studies on cell lines of the central nervous system (Chapter 2) also showed no negative effect of this compound, underlining the non-cytotoxic effect of this metabolite.

Outlook

In this study we demonstrated the importance of the devitalization process as growing bacterial worm feed can result in a potential divergence of measured compound activities. However, more tests with known antithelmintics, such as violacein (Ballestriero et al., 2014), must be carried out to ensure that this observation is not restricted to TDA. Also, the applicability of the method for high throughput screening (HTS) of compound libraries by comparing hits of the old method (*E. coli* OP50) with the described new method (*E. coli* ST18) should be carried out to confirm the effectiveness of this modification.

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Chapter 5: Discussion

Marine natural products are a promising resource of secondary metabolites with biomedical potential. However, some bioactivities, including beneficial effects on neural cells, have received less attention to date. In this study, the effect of the marine natural products tropodithietic acid (TDA) and dimethylsulphoniopropionate (DMSP) on mammalian neural cell lines and *C. elegans* was studied to determine their cytotoxic or protective capabilities on the cellular level (Chaper 2 & 3), as well as in a whole organism (Chapter 4), particularly for further biomedical applications.

The marine metabolite TDA has broad antimicrobial activities, but no noteworthy toxic effect on different eukaryotic model systems has been detected to date (Liang et al., 2003; Neu et al., 2014). The herein shown toxicity of TDA on mammalian clonal cell lines of neuronal and glial origin, OLN-93 and N2a cells (Chapter 2), as well as on *C. elegans* (Chapter 4) were inconsistent with these previous studies. Toxic effects of TDA were linked to cellular mechanisms, including mitochondrial membrane depolarization, increased intracellular Ca^{2+}-levels, an upregulation of heat shock protein 32 (Hsp32), activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) and microfilament reorganization (Chapter 2). The found sensitivity of the used cell lines was contrary to further studies on MCF7 (breast adenocarcinoma), HM02 (gastric adenocarcinoma) and HEPG2 cells (hepatocellular carcinoma), which were less sensitive to TDA treatment and considerably higher concentrations were needed to induce cytotoxicity (Liang et al., 2003).

The demonstrated cytotoxicity adds new aspects to the use of TDA and TDA-producing bacteria and indicates a reconsideration of its use in aquacultures (Porsby et al., 2011). Our results (Chapter 2) indicate that it needs to be tested whether and how TDA may be transmitted from e.g. *P. inhibens* dominated biofilms into higher organisms. Nonetheless, it has to be considered that although these effects already occurred at very low concentrations, expected natural concentrations of this compound are likely even lower. Combined with its light sensitivity and following loss of activity there is hence possibly only a weak hazardous potential of TDA for e.g. humans or fish. Also, the ability of TDA to cross the blood-brain barrier (BBB) would be essential to induce the found neurotoxicity, but needs to be determined whether TDA is able to cross this highly selective permeability barrier. This aspect is also relevant from a

pharmacological point of view, as one of the challenges in finding drugs for treating brain disorders is the required ability to cross BBB, which prevents the uptake of most pharmaceuticals. Almost all drugs for the brain presently in clinical practice are lipid soluble small molecules with an MW < 400 Da and high lipid solubility, which is equivalent to low hydrogen bonding (< 8 hydrogen bonds). Very few small molecule drug candidates meet these dual requirements (Pardridge, 2012; van de Waterbeemd et al., 1998). With a molecular weight of ~ 212 Da and two hydrogen bonds TDA might be a candidate to cross the BBB.

Also, only little is known about the influence of DMSP, produced by several algae, corals and higher plants (Nakajima, 2015; Sunda et al., 2002), on the cellular level. In this study, the outgrowth inducing effect, microtubule reorganization and protective capabilities of DMSP on N2a and OLN-93 cells was analyzed. Preincubation with DMSP also prohibited cytotoxic effects of TDA, indicating that this natural product potentially provides an antioxidant defense in neural cell lines (Chapter 3). DMSP has multifunctional roles in the ocean, including osmolytic, cryoprotectant, and antioxidant function in algae and serving as bacterial metabolite which influences global biochemical cycles (Raina et al., 2013; Sunda et al., 2002). In contrast, little is known about specific chemical interactions of DMSP with eukaryotic organisms. In the present study we showed the outgroth inducing, microtubuli reorganization and protective capabilities on N2a and OLN-93 cells. DMSP also prohibited cytotoxic effects of TDA, indicating that this natural product potentially provides an antioxidant defense in neural cell lines.

DMSP and its enzymatic cleavage products DMS and acrylate are known effective cellular scavengers of hydroxyl radicals and constitute an highly effective antioxidant system in algae (Sunda et al., 2002). This antioxidant system, which could be in high DMSP containing algae more effective than other well-recognized antioxidants, such as ascorbate and glutathione (Sunda et al., 2002), which also work as antioxidant defense in animals (Meister, 1994), including in the CNS of mammals (May et al., 2012; Ross et al., 2012). These similarities suggest a potential role of DMSP as e.g. an antioxidant in mammalian neural systems.

The antioxidant ability of DMSP is also of interest regarding the use of algae as nutraceuticals, i.e. foods that are beneficial to the customers health. The major beneficial compounds from algae are so far docohexaenoic acid (DHA) and eicosapentoenoic acid (EPA)

(Doughman et al., 2007), which have antioxidative properties, and stimulate neural development, promote neurogenesis and accumulate as the most abundant fatty acids in the brain (Bradbury, 2011; Cao et al., 2005). DHA is also plays an important role in the prevention of neuropsychiatric and neurodegenerative disorders (Wu et al., 2009). Marine algae are considered as sea vegetables not only for consumption, but also as an alternative medicine since ancient times (Thomas and Kim, 2013) and the potential role of DMSP in this context is rarely understood. The shown outgrowth inducing and neuroprotective effects of DMSP (Chapter 3) and its non-toxicicity effects on *C. elegans* (Chapter 4) indicate the beneficial effect of algal-based food could also be attributed DMSP. Nakajima et al. (2015) showed various ameliorating effects of DMSP, administered both purified or within pulverized green sea algae (*Monosodium nitidum*), on rodents who suffered from different disorders, especially on free cell and solid cancers but also induced diabetes or Parkinson. The observed anti-cancer effects were linked to an up-regulation of the immune system, including the recruitment of macrophages, as well as repair mechanisms of damaged cells and tissues without side effects. Nakajima et al. suggest that the consumption of green sea algae invokes regeneration of inflamed and damaged cells and tissues in diseased animals and humans in Japan, who customarily eat green sea algae accompanied by activation of the innate immune system by DMSP. Hence, administering high and frequent doses of DMSP may treat suffering from acute and chronic diseases such as cancer without any side effects *in vivo,* suggesting that DMSP mitigates and heals different diseases different kind of animals (Nakajima, 2015). These results are consistent with the protective effect of DMSP on the cellular level (Chapter 3), suggesting that the beneficial effects on various diseases can also rely on its antioxidative capabilities. It remains determined which molecular mechanism DMSP induces that yields the protection of e.g. the mitochondria and process outgrowth, comparable to e.g. neurotrophins (Chaper 4). Compounds with neurotrophin-like activities are promising therapeutics to treat neurodegenerative diseases (Grosso et al., 2014; Palyanova et al., 2013), and further research is advised to elucidate the mode of action and biomedical applicability of DMSP. In addition to pharmacological considerations, the shown effects on the cell lines may also have a role in the environment. The found health supporting effects raises the question if DMSP has this influence on animals in the marine environment, such as marine mammals, and how DMSP potentially interacts with neural systems. DMSP is

built in enormous amounts, e.g. during phytoplankton blooms (Yoch, 2002), indicating that it also plays a chemical-ecological role in high-DMSP containing habitats and associated organisms.

Cell models are an important step towards the understanding of bioactivities and their modes of action were applied here to provide insights into toxicity and protective effects of TDA and DMSP. However, particularly in view of biomedical potential, whole organism models should be investigated to demonstrate the interactions of secondary metabolites in complex biological systems. The herein established improved *C. elegans* killing assay using auxotrophic *E. coli* ST18 was used to determine the antithelmintic effect of TDA, sigillin A and DMSP (Chapter 4). Comparing the killing efficiency of TDA in living or devitalized *E. coli* ST18 showed the importance of the devitalization process to avoid discrepancies in the test compound uptake.

Outlook

The high sensitivity of neural derived N2a and OLN-93 cells to TDA may indicate the potential of this substance as small molecule drug candidate for future research as drug for the brain. N2a cells also act as a model in finding anti-cancer compounds (Aydın et al., 2014), but so far performed tests with different cancer cell lines only showed effects at much higher concentrations (Liang et al., 2003). However, further investigations on the *in vivo* effect of TDA on higher organisms like rodents regarding its neurotoxicity or, on the other hand, its capability to selectively differentiate between healthy and cancerogenous tissue, is needed to ensure its pharmacological potential.

To unlock the biomedical potential of DMSP, detailed knowledge about its molecular mode of action and mechanisms of interaction with neural cells for example, is required. A following project will provide an in-depth characterization of the targets and neural networks DMSP acts and whether DMSP is able to protect neural cells from damage. One interesting aspect is the neuritogenic effect of DMSP by mimicking neurotrophic factors, which are of great interest, as neurotrophins are essential for development, differentiation, survival and rehabilitation of neurons (Larpthaveesarp et al., 2015; Palyanova et al., 2013). Low molecular weight compounds with neurotrophin-like activities are promising therapeutics to treat neurodegenerative diseases (Grosso et al., 2014; Palyanova et al., 2013), underlining the promising biomedical potential of this compound. Detailed information about potential targets will be presented in a following research proposal.

The demonstrated importance of the devitalization process in the improved *C. elegans* killing assay showed that growing bacterial worm feed can result in a divergence of measured compound activities. However, more tests with known antithelmintics, such as violacein, will be carried out to ensure that this observation is not restricted to TDA. Also, the applicability of the method for high throughput screening (HTS) of compound libraries by comparing hits of the old method (*E. coli* OP50) with the described new method (*E. coli* ST18) should be carried out to confirm the effectiveness of this modification.

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Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbstständig verfasst habe und keine anderen, als die hier angegebenen Hilfsmittel und Quellen benutzt habe. Zudem versichere ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat. Die Leitlinien guter wissenschaftlicher Praxis der Carl von Ossietzky Universität Oldenburg wurden befolgt.

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