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Neurotoxicity of deoxysphingolipids in an *in vitro* model of taxane induced peripheral neuropathy

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Neurotoxicidad de los deoxyesfingolipidos en un modelo in vitro de neuropatía periférica inducida por taxanos

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A mi familia, que, aunque físicamente nuestros caminos tomen un rumbo a parte, nuestros corazones siempre estarán conectados.

“La admiración que siente uno ante el prodigio de la naturaleza es muy grande; que algo tan pequeño tenga tanta información sobre la vida”

Ángela Restrepo Moreno

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Resumen:

Neurotoxicidad de los deoxyesfingolipidos en un modelo in vitro de neuropatía periférica inducida por taxanos

El uso de agentes quimioterapéuticos como taxanos, paclitaxel y docetaxel, puede causar neurotoxicidad que conlleva a un efecto secundario de neuropatía periférica inducida por taxanos (TIPN). Investigaciones anteriores mostraron que el metabolismo de los esfingolípidos (SL) estaba desregulado en la TIPN. Esta investigación mostró una sobreproducción de SL atípico llamado 1-deoxiesfingolípidos (deoxySL) como resultado del tratamiento con taxanos. Los deoxySL tienen una degradación más lenta en comparación con los SL canónicos y, cuando se producen en exceso, tienden a acumularse, lo que lleva a la neurotoxicidad.

El primer objetivo de esta investigación consistía en evaluar la neurotoxicidad de los deoxySL individuales. Probamos la toxicidad de los deoxySL individuales, en dos modelos de células de neuroblastoma, KCNR y Neuro2a mediante el ensayo de citotoxicidad LDH y midiendo los cambios morfológicos, como las hinchazones de neuritas y el redondeo celular. Nuestros resultados mostraron que la 1-deoxiesfinganina fue la más citotóxica de las bases 1-deoxiesfingoide para ambas líneas celulares de neuroblastoma, KCNR y N2a. El tratamiento con DeoxySL mostró cambios morfológicos. En células N2a diferenciadas, los deoxySL individuales indujeron a hinchazones de neuritas en diferentes momentos o concentraciones, lo que sugiere que las hinchazones de neuritas son probablemente un efecto neurotóxico transitorio del tratamiento con deoxySL. En las células de neuroblastoma KCNR, los efectos neurotóxicos de deoxySL se manifestaron en el redondeo de los cuerpos de las células. Las diferencias en la citotoxicidad y la hinchazón de las neuritas se evidenciaron en los efectos neurotóxicos de las bases 1-deoxiesfingoide y las 1-deoxiceramidas, también en los isómeros de doble enlace 4E y 14Z 1-deoxiesfingosinas y 1-deoxiceramidas. Además, para probar si los efectos neurotóxicos de deoxySL incluyen efectos sobre la organización de actina, utilizamos inmunocitoquímica e imágenes fluorescentes de células vivas para visualizar la arquitectura de actina celular. Nuestros resultados mostraron que en las células KCRN, deoxySL provocó la disrupción y reorganización de las fibras de estrés de actina en la corteza celular. En las neuronas DRG por sus siglas en inglés (Dorsal Root Ganglia), también se evidenciaron hinchazones de neuritas y distribución de actina de neuritas. Además, abordamos la cuestión de si la neurotoxicidad de deoxySL está mediada por los receptores de esfingosina-1-fosfato (S1PR). Utilizamos un antagonista funcional más amplio de S1PR, FTY720, en

combinación con los deoxySL para identificar si hay atenuación del daño neurítico causado por los deoxySL. Los resultados del tratamiento con FTY720 no mostraron atenuación del daño neurítico causado por los deoxySL, lo que sugiere que otros mecanismos, además de la señalización de S1P podrían interactuar o modular la toxicidad de los deoxySL.

En conclusión, la neurotoxicidad de los deoxySL resultó en citotoxicidad y cambios morfológicos tales como hinchazones de neuritas y células redondeadas en líneas celulares de neuroblastoma, incluida la reorganización de actina en células KCNR y neuronas DRG. La señalización de S1P y otros mecanismos que podrían estar implicados en la neurotoxicidad de los deoxySL deben continuar estudiándose.

Palabras clave: 1-deoxiesfingolípidos (deoxySL), 1-deoxiesfingosina, 1-deoxiesfinganine, 1-deoxiceramida, neuropatía periférica inducida por taxanos (TIPN), esfingosina-1-fosfato (S1P), receptores de esfingosina-1-fosfato (S1PR), FTY720.

Abstract

The use of chemotherapeutic agents such as taxanes, paclitaxel, and docetaxel, can cause neurotoxicity leading to Taxane-Induced Peripheral Neuropathy (TIPN) side effect. Previous research showed that sphingolipid (SL) metabolism was deregulated in TIPN. Specifically, the research showed an overproduction of atypical SL called 1-deoxysphingolipids (deoxySL) as a result of taxane treatment. The deoxySL have a slower degradation compared to the canonical SL, and when produced in excess, tend to accumulate, leading to neurotoxicity.

The first goal of this research aimed at evaluating the neurotoxicity of individual deoxySL. We tested the toxicity of the individual deoxySL, in two neuroblastoma cell model, KCNR and Neuro2a by LDH cytotoxicity assay and by measuring morphological changes such as neurite swellings and cell rounding. Our results showed that 1-deoxysphinganine was the most cytotoxic of the 1-deoxysphingoid bases for both neuroblastoma cell lines, KCNR and N2a. DeoxySL treatment showed morphological changes. In differentiated N2a cells, the individual deoxySL induced neurite swellings at different time points or concentrations, suggesting that neurite swellings are likely a transient neurotoxic effect of deoxySL treatment. In KCNR neuroblastoma cells, the neurotoxic effects of deoxySL manifested in rounding of the cells' bodies. Differences in cytotoxicity and neurite swellings were evidenced in the neurotoxic effects of the 1-deoxysphingoid bases and the 1-deoxyceramides, also in the double bond isomers 4E and 14Z 1-deoxysphingosines and 1-deoxyceramides. In addition, to test if the neurotoxic effects of deoxySL include effects on actin organization we used immunocytochemistry and live cell fluorescent imaging to visualize cellular actin architecture. Our results showed that in KCRN cells, deoxySL caused actin stress fibers disruption and re-organization to cell cortex. In primary dorsal root ganglia (DRG) neurons, neurite swellings were also evidenced, and neurite actin distribution. Additionally, we addressed the question if deoxySLs' neurotoxicity is mediated by the sphingosine-1-phosphate receptors (S1PRs) We utilized a broader functional antagonist of S1PRs, FTY720, in combination with deoxySL to identify if there is attenuation of neuritic damage caused by deoxySL. Results for the FTY720 treatment did not show attenuation of neuritic damage due to deoxySL, suggesting that other mechanisms than S1P signaling, might interact or modulate deoxySL toxicity.

In conclusion, neurotoxicity of deoxySL resulted in cytotoxicity, and morphological changes such as neurite swellings and rounding cells, in neuroblastoma cell lines, including actin re-

organization in KCNR cells and DRG neurons. S1P signaling and other mechanisms that might be implicated in deoxySL neurotoxicity must continue to be studied.

Keywords: 1-deoxysphingolipids (deoxySL), 1-deoxysphingosine, 1-deoxysphinganine, 1-deoxyceramide, taxane-induced peripheral neuropathy (TIPN), sphingosine-1-phosphate (S1P), sphingosine-1-phosphate receptors (S1PRs), FTY720.

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Abbreviations

Abbreviation	Term
deoxySL	1-deoxysphingolipids
TIPN	Taxane induced peripheral neuropathy
CIPN	Chemotherapy induced peripheral neuropathy
DRG	Dorsal root ganglia
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptors

Introduction

Taxanes, paclitaxel and docetaxel, are chemotherapeutic agents used in the treatment of various types of cancer such as breast, ovarian, lung, head and neck, prostate, among others. However, taxane chemotherapy has several side effects, including Taxane-Induced Peripheral Neuropathy or TIPN (Velasco & Bruna, 2015), (Costa et al., 2020). It is estimated that in patients treated with docetaxel, 57% develop TIPN and 83% for those treated with paclitaxel (Shahpar et al., 2018). The TIPN symptoms that these patients experience are shooting pain, tingling and dysesthesia in the extremities, sensory ataxia, and loss of fine-motor movements (Jankovic, 2022). Currently, the most used solutions to address TIPN are the reduction of the treatment dose or the cessation of the treatment, and the use of medications to alleviate the symptoms (Loprinzi et al., 2020).

In Dr. Spassieva's laboratory, it was shown that the plasma deoxySL levels in patients treated with paclitaxel, correlate with the incidence and severity of TIPN in these patients (Kramer et al., 2015). High levels of deoxySL were also found in the dorsal root ganglia of docetaxel-treated mice (Becker et al., 2020).

DeoxySL are produced when the first enzyme in the sphingolipid biosynthetic pathway, serine palmitoyltransferase (SPT), conjugates Palmitoyl-CoA with a less preferred substrate, L-alanine (Km>10mM), instead of it higher affinity L-serine substrate (Km~0.75mM), by which conventional lipid metabolites are produced (Gable et al., 2010), (Eichler et al., 2009). The deoxySL lack the hydroxyl group in the C1 position, therefore they cannot form complex sphingolipids or be degraded the same way as the L-serine derived SL (Goins & Spassieva, 2018), (Alecu, Othman, et al., 2017). Degradation of deoxySL is slower than canonical sphingolipids, and when produced in excess, the deoxySL accumulate, leading to neurotoxicity (Goins & Spassieva, 2018), (Kolter & Sandhoff, 1999), (Wilson et al., 2018), (Penno et al., 2010), (Becker et al., 2020).

Currently, the molecular mechanisms by which deoxySL contribute to neurotoxicity remain largely unknown. It is still not clear which individual deoxySL are involved, or if their mechanism of action and toxic effects are the same. This research aims to address these questions in neuroblastoma and primary DRG neuronal cell culture models. Previous research has suggested deoxySL as molecular intermediates of taxane neurotoxicity,

although, the exact molecular mechanism is currently not understood (Becker et al., 2020), (Kramer et al., 2015).

DeoxySLs toxic effects include axonal degradation, disruption of cytoskeleton structures, loss of dendritic complexity, fragmentation, retraction, and clusters in the neurites of neurons, as well as, reduction in number, length, and formation of the neurites (Andersen Hammond et al., 2019), (Carreira et al., 2019), (Penno et al., 2010), (Wilson et al., 2018), (Alecu, Tedeschi, et al., 2017), (Güntert et al., 2016).

Becker et al, showed *in vitro*, in DRG neurons that treatment with 1-deoxysphingosine caused neurite swellings. This toxic effect was shown to be rescued by the bioactive sphingolipid, sphingosine-1-phosphate (S1P) (Becker et al., 2020). S1P action is mediated by its receptors (S1PRs) (Mendelson, 2014). The present investigation will address the question if deoxySL neurotoxicity depends on S1PRs signaling.

1. Chapter 1

Chemotherapy-induced peripheral neuropathy (CIPN) is a side effect of chemotherapy treatment, such as taxanes, platinum based, vinca alkaloids, proteasome inhibitors, and others (Zhang, 2021), (Bae, 2021).

TIPN affects approximately 60% of patients undergoing chemotherapeutic treatments based on taxane-type drugs (Argyriou et al., 2008). It is estimated that in patients treated with Docetaxel, 57% develop TIPN and 83% for those treated with Paclitaxel (Shahpar et al., 2018). The symptoms of TIPN include pain, numbness and tingling in the feet and hands, dysesthesia in the extremities, areflexia, sensory ataxia, and motor neuropathies that lead to muscle weakness and difficulty with fine-motor movements, among which instability when walking, handling small objects, inability to perform precision tasks, hearing loss, etc. (Jankovic, 2022), (Blasco & Caballero, 2019), (Holmes et al., 1991). Additionally, these harmful effects can lead to patients losing physical independence, becoming disabled, which results in increased medical costs (Molassiotis et al., 2019).

It is estimated that the prevalence of TIPN side effect one month after cessation of treatment is 68%, after three months 60%, and from six months onwards 30% (Colvin, 2019). There are no effective treatment and with side effects, and no prevention of TIPN. Therefore, TIPN is addressed by limiting the treatment dose or in severe cases of TIPN, the treatment is terminated, which results in a decrease in the treatment effectiveness, affecting the survival rate of the patients and the life quality of both, patients who are in treatment and those who are in remission (Loprinzi et al., 2020), (Timmins et al., 2021), (Hertz et al., 2021).

So far, only symptomatic treatments are offered for severe neuropathic pain due to TIPN, such as analgesic drugs, antidepressants, anticonvulsants, among others, which have side effects of their own (Loprinzi et al., 2020). Vitamins, minerals, antioxidants, ion channel blockers have been tested, for neuroprotective effect and preventions of TIPN. However, no reliable results have been reported on their use in clinical trials (Jankovic, 2022), (Blasco & Caballero, 2019), (Velasco & Bruna, 2010), (Andersen Hammond et al., 2019). Alternative treatments have been proposed, like neuromodulation, magnetic stimulation, neurofeedback and Scrambler therapy, these techniques have shown some promise for reducing neuropathic pain in the extremities, and overall, the severity of the disease, however it requires further research to establish its effectiveness for treating TIPN (Much,

2017), (Smith et al., 2020), (Childs et al., 2021). Nevertheless, the American Society for Clinical Oncology has stated that there are still no agents that can prevent CIPN (Loprinzi et al., 2020).

In 2012, Pike et al, conducted a study comparing the costs associated with patients with CIPN with respect to patients with cancer but who did not show signs of neuropathy (Pike, 2012). The authors concluded that the annual monetary burden for CIPN patients was $17,000 per patient.

Studies on TIPN in Colombia are scarce. Martínez et al., presented a study in 2019 on the prevalence of CIPN in a Colombian population with cancer (Martínez et al., 2019). The study held between the years 2015 and 2016, included patients with different types of cancer, who underwent treatment with chemotherapeutic agents such as taxanes (paclitaxel and docetaxel), and others including, oxaliplatin, bortezomib and ixabepilone. The results of the study showed development of CIPN in 48% of patients treated with paclitaxel, 50% treated with docetaxel, 58% treated with oxaliplatin, 43% treated with bortezomib and 95% treated with ixabepilone. Of the chemotherapy drugs investigated in the study, paclitaxel is the most prescribed, and as a consequence, TIPN affects significant proportion of the cancer patients in Colombia treated with chemotherapy. Additionally, there was a predominance in women developing CIPN, possibly related to the fact that these drugs are highly prescribed in breast or ovarian cancer.

In 2010, an interdisciplinary group in Colombia conducted a comprehensive review of chemotherapeutic agents causing CIPN, CIPN diagnostic methods, and therapies. The study emphasizes that more research in the field is needed, because, due to the lack of efficacy in preventive and curative treatments, there is a high incidence of CIPN among cancer patients (Cardona et al., 2010).

Despite the identification in recent years of the pathophysiological effects of taxanes, such as distal axonal degeneration, the molecular mechanisms are poorly studied (Argyriou et al., 2008). TIPN cellular mechanisms including mitochondrial dysfunction, oxidative stress, and inflammatory processes have been proposed (Alecu, Tedeschi, et al., 2017), (Jaggi & Singh, 2012). The ongoing studies have been trying to elucidate the molecular mechanisms of TIPN, and despite the development in the proposed mechanisms of neurotoxicity, the results have not reached the translation into effective treatment, leaving the continued need

for the identification of new insights for the prevention or treatment of TIPN (Brewer et al., 2016), (Acevedo, 2009), (Martínez et al., 2019).

During the study of the cellular mechanisms involved in the TIPN neurotoxicity, an atypical class of sphingolipids, deoxySL, have been implicated. This deoxySL are produced when the first enzyme of the sphingolipid biosynthetic pathway, serine palmitoyltransferase (SPT), that catalyzes the *de novo* production of sphingoid bases, conjugates the fatty acid, usually palmitoyl CoA, with a less preferred amino acid L-Alanine (Km>10mM), instead L-Serine (Km~0.75mM), which generates the canonical sphingolipids **(Figure 1)** (Lone et al., 2020), (Wigger et al., 2019), (Gable et al., 2010), (Eichler et al., 2009). DeoxySLs lack the C1-OH functional group, which results in the inability to form complex SLs or be degraded by S1P lyase (Goins & Spassieva, 2018), (Merrill, 2011), (Alecu, Othman, et al., 2017).

DeoxySL accumulate when they are produced in excess, resulting in neurotoxicity, as it has been shown in hereditary sensory autonomic neuropathy type 1, diabetic neuropathy, and TIPN (Goins & Spassieva, 2018), (Kolter & Sandhoff, 1999), (Alaamery et al., 2021), (Bouscary et al., 2021), (Wilson et al., 2018), (Penno et al., 2010), (Zuellig et al., 2014).

A study with breast cancer patients treated with paclitaxel showed an association between the levels of deoxySL in patients' plasma and the incidence of TIPN. In this same study, *in vitro* cell culture models (U87 human astroglioma cells and human embryonic kidney (HEK) 293 cells) were treated with Paclitaxel, which showed that in a dose-dependent manner those treated with paclitaxel had increased levels of deoxySLs, such as very-long chain C_{24} and C_{22} 1-deoxyceramides and 1-deoxydihydroceramides (Kramer et al., 2015).

A subsequent study by our group, using a murine model of TIPN, evaluated the effects of docetaxel treatment on deoxySL metabolism in the peripheral nervous system (Becker et al., 2020). In the study, levels of deoxySLs in the dorsal root ganglion (DRG), sciatic nerve, and spinal cord of mice treated with docetaxel were analyzed and compared to levels of control mice, a group without treatment. This study demonstrated that the levels of deoxySL were elevated due to treatment with docetaxel, not only in plasma as previously demonstrated, but also in DRG. However, in the spinal cord there were no significant changes in the levels of deoxySLs. Reduced levels of some deoxySL species were found in sciatic nerve samples, concluding that the effects of docetaxel on deoxySL metabolism are not uniform in the nervous system (Becker et al., 2020). The authors suggest that it may be due to the protection of the spinal cord by the blood-brain barrier and the sciatic

nerve by the blood-nerve barrier, both of which are not permeable to taxanes. On the other hand, DRGs do not have this protection, so they are directly exposed to the toxic effects of systemic taxane treatments.

In addition to the study mentioned above, the neurotoxicity of deoxySLs have been studied in primary neuronal cultures and in other neuropathies (Wilson et al., 2018), (Penno et al., 2010). Güntert et al. (2016), observed that in neurons treated with the deoxySL,1-deoxysphinganine, mitochondrial activity decreased, cytoskeletal architecture was altered and cell death was induced. The neurotoxicity caused by deoxySL has been related to possible disruption of the cytoskeleton architecture, possibly through deregulation of actin, microtubules and neurofilaments dynamics, leading to toxicity (Fletcher & Mullins, 2010), (Penno et al., 2010), (Cuadros et al., 2000). In agreement with these studies, it has been shown in primary motor, dopaminergic, and DRG neurons, that the neurite number, length, and outgrowth, is reduced (Wilson et al., 2018), (Penno et al., 2010), (Alecu, Tedeschi, et al., 2017), (Martinez et al., 2012).

Becker et al (2020) showed that S1P achieved to attenuate the deoxySL neurotoxicity in DRG neurons. The mechanism of S1P rescue of deoxySL neurotoxicity is yet to be understood.

S1P is a signaling molecule involved in regulating cell proliferation, cell migration and cell survival processes (Bravo, 2022). In the nervous system, S1P has been shown to participate in the regulation of both, neurite extension and retraction by signaling through S1PR (O'Sullivan & Dev, 2013). Also, it has been suggested that S1P and its receptors are implicated in mechanisms associated with TIPN, neuroinflammatory responses, and neuropathic pain (Patel & Spassieva, 2018). Moreover, S1P signaling has been studied in neurorepair and neurogenesis, and their modulatory mechanisms continue to be a focus of studies in neuropathic diseases and other pathological conditions (Goins & Spassieva, 2018), (Tsai & Han, 2016), (Mendelson, 2014).

The molecular mechanism by which deoxySL causes neurotoxicity remains largely unknown. So far it is not well elucidated whether the toxicity is due to an individual specific deoxySL species or the deoxySL class as a whole. Another question that is yet to be addressed is deoxySL neurotoxic signaling is through S1PRs (Alecu et al., 2017), (Goins & Spassieva, 2018) (Becker et al., 2020).

To address those questions, we propose the following research question, what is the mechanism of deoxySL neurotoxicity? By addressing the mechanism of deoxySL neurotoxicity we will provide knowledge to facilitate the search of a possible treatment of TIPN.

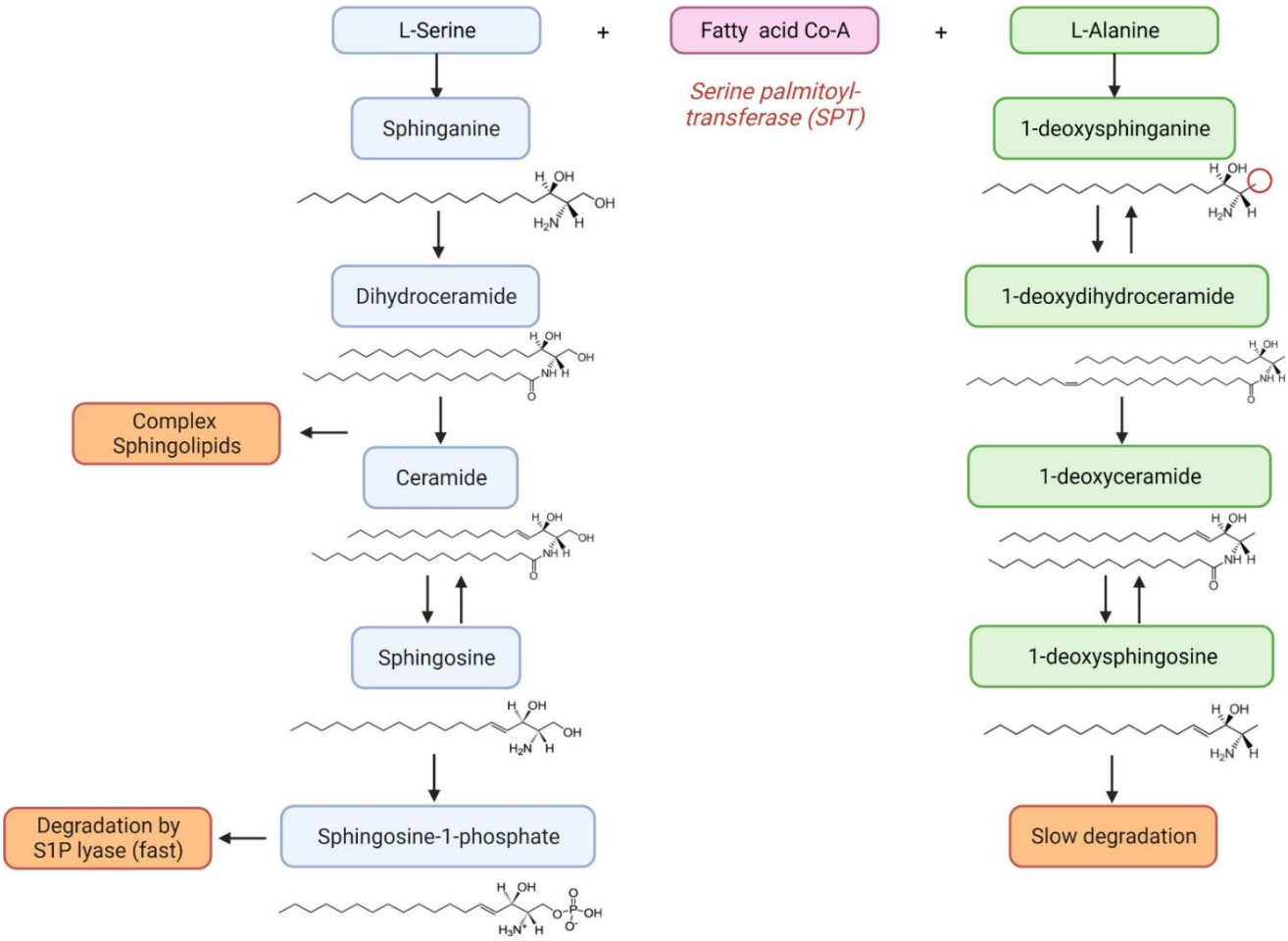


Figure 1. Schematic representation of the sphingolipid pathway. Red circle represents the lack of the hydroxyl group on the C1 position. Created with BioRender.com.

2. Chapter 2

The causes of peripheral neuropathy are related to a wide variety of factors, like genetics, hereditary disease, diabetes mellitus, toxin exposure, injury, alcohol abuse, another medical disorder such as autoimmune diseases or kidney and liver diseases, infections, or as a side effect of medications like chemotherapy drugs (Castelli, 2020), (NIH, 2023), (Clinic, 2022). The specific symptoms in the individual patients can differ, which makes early diagnosis of the disease quite complex, resulting in further development of patients' symptoms, in some cases allowing progression of neuropathy into chronic pain, which delays recovery and ultimately leads to decreasing the quality of life of patients (Maihöfner et al., 2021). Therefore, studying and understanding of the molecular and biological mechanisms involved in peripheral neuropathy is essential not only to understand the disease but also to propose standardized diagnostic methodologies, as well as explore possible treatments (Velasco & Bruna, 2010), (Martínez et al., 2019).

The identification of markers or factors that can help detect patients with a possible risk of developing peripheral neuropathy is necessary (Velasco & Bruna, 2010). It has been proposed that the levels of deoxySLs in plasma, including the levels of individual deoxySL metabolites, can serve as markers for identifying patients, which will develop TIPN (Kramer, 2015). Additionally, its identification of a particular deoxySL metabolite could define the biological point of action or target in a possible diagnostic, preventive and therapeutic treatment.

Current therapies for peripheral neuropathy are essentially symptomatic treatments with no clinically proven efficacy (Jankovic, 2022). They include pain medications, analgesics, or antidepressants, anticonvulsants, opioids, cannabinoids, among others (Maihöfner et al., 2021), (Blasco & Caballero, 2019). Medication-free therapies such as acupuncture and electrostimulation are applied to reduce the pain of patients. However, their clinical effectiveness is yet to be established (Ni, 2023), (Javeed, 2021), or they showed variable results and need to be standardized before implemented clinically (Smith et al., 2020), (Colvin, 2019). Due to the anticipation for the development of standardized and effective therapies, it has been shown the need to study different avenues, especially the understanding of the molecular mechanisms of peripheral

neuropathy that can lead into direct evidence-based therapies, which will be able to address the underlying causes of neuropathy.

S1P together with its receptors (S1PRs) have ignited a great interest due to its regulatory role in signaling pathways, such as cell proliferation, cell migration and cell survival processes, which have a high impact on pathophysiological processes (Stepanovska & Huwiler, 2020), (Bravo, 2022). The modulation of S1P through its receptors also has the potential to slow or stop disease progression and provide additional disease markers (Cohan et al., 2020). Thus, the S1P signaling through the S1PR has become a target to study and provide a possible therapy for different inflammatory and autoimmune diseases such as multiple sclerosis, respiratory infections, psoriasis, ovarian and cardiovascular diseases, bone regeneration, etc. (Stepanovska & Huwiler, 2020), (Gonzalez-Cabrera et al., 2014), (Tong et al., 2021), (Zhang et al., 2020), (Hernández-Coronado et al., 2019).

It has been showed that S1P can rescue the toxic effects of deoxySLs in primary DRG neurons treated with 1-deoxysphingosine (Becker et al., 2020), and, in addition of the fact that the deoxySL toxic pathway remains unknown, it has suggested that S1PRs might be involved in the downstream transmission of deoxySLs´ neurotoxic effects.

Over the last years, the study of deoxySLs has brought the attention of the scientific field, including studying their biophysical properties, molecular mechanisms of toxicity, and especially their role in the pathology of peripheral neuropathies. The neurotoxic effects of individual class of deoxySLs at a molecular level are poorly understood.

The development of a treatment for TIPN and other peripheral neuropathies where deoxySLs are implicated, would not only improve the quality of life of patients and the efficacy of taxane chemotherapy treatment, but it would also help to reduce the costs associated with the disease. A treatment based on the cellular mechanism of peripheral neuropathy would lead to a lower rate of hospitalization, less medical consultations, and a decreased use of ineffective drugs for the symptoms of peripheral neuropathy instead of addressing the cause of it. A treatment of peripheral neuropathy will also decrease depression, the need of muscle therapies, and the loss of employment of the patients (Molassiotis et al., 2019).

Finally, this work will contribute to the field of biotechnology by advancing the scientific knowledge on the molecular mechanism of deoxySL neurotoxicity.

In conclusion, the results of this research will not only provide a contribution to the scientific knowledge on deoxySL neurotoxicity, but also provide a basis to devise treatments to improve quality of life of patients who suffer from peripheral neuropathy.

3. Chapter 3

3.1 Cancer

Cancer is a disease which affects the process of the cell growth. Cancer causes an uncontrolled multiplication of cells, and their transformation into abnormal or tumor cells. Cancer cell can acquire the ability to spread throughout the body, causing metastasis and in the process, destroying normal tissues (*World Health Organization*, 2022), (*Instituto Nacional Del Cáncer*, 2021), (*Mayo Clinic*, 2019).

The causes of cancer are wide and varied. In general, it is caused by mutations in the DNA, which can be hereditary such as BRCA mutations in breast cancer or by external agents such as exposure to radiation, chemical substances, unhealthy sedentary lifestyle or smoking (*American Cancer Society*, 2020), (*Mayo Clinic*, 2019).

According to the World Health Organization, by 2020, cancer was still considered the leading cause of death in the world, being responsible for approximately 10 million deaths worldwide and 1.4 million in Americas (World Health Organization, 2022), (*Organización Panamericana de La Salud*, 2020).

In Colombia, a study from 2015 to 2018 showed that 275,348 people were diagnosed with cancer, of whom 19,814 died. Of the patients diagnosed with cancer, 173,494 were women with a mean age of 59 years and 101,854 were men with a mean age of 63 **(Figure 2)** (*Cuenta de Alto Costo*, 2020). Additionally, the Ministry of Health indicates that the incidence of cancer in Colombia is 182 people per 100,000 inhabitants and mortality is 84 per 100,000 inhabitants, giving Colombia sixth place in cancer mortality in Latin America (*Ministerio de Salud*, 2021).

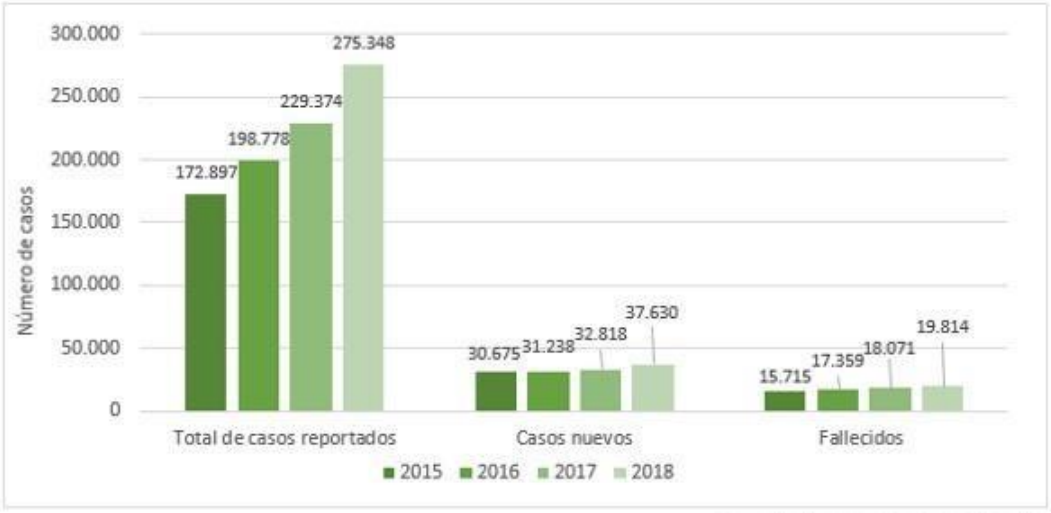


Figure 2. Bar chart of the number of cancer reported cases in Colombia in the years 2015, 2016, 2017 and 2018 (Cuenta de Alto Costo, 2020).

Currently there are more than 100 types of cancer in the world, the most common and contributing to patients dying from cancer in recent years are breast, lung, prostate, colorectal, and cervical cancer (*Instituto Nacional Del Cáncer*, 2021). For 2020, the World Health Organization registered the cases of the types of cancer (World Health Organization, 2022):

- breast (2.26 million cases), (685,000 deaths)
- lung (2.21 million cases), (1.8 million deaths)
- colorectal (1.93 million cases), (916,000 deaths)
- prostate (1.41 million cases);

Lung and colorectal cancer affect proportionally both women and men (Institute, 2020). Breast, cervical and pancreatic cancers are prevalent in women, while in men, prostate and bladder cancers are more commonly found (*Organización Panamericana de La Salud*, 2020), (Cuenta de Alto Costo, 2020).

Both organizations, the World Health Organization and the International Cancer Research Center have proposals to motivate scientific studies that can contribute to knowledge addressing the causes and mechanisms involved in the cancer disease process, as well

as, of the studies aimed in devising prevention strategies, improve diagnosis and the treatments for cancer (World Health Organization, 2022).

3.2 Cancer treatments

The appropriate treatment for an individual patient will depend mainly on the type of cancer, and its stage, along with other factors such as the patient's general health and medical history (National Institutes of Health, 2015).

Cancer treatments have as their main objective the elimination of cancer or cure of the patients. If this is not possible, the treatments also serve to reduce tumor size, and burden and delay the growth or spread of cancer (Mayo Clinic, 2020).

Currently, several types of cancer treatments are available. Some of those treatments are already available for some time, but continue to be effective, and improved with the advancement of technology. Some primary treatments are designed to remove the cancer, others such as the adjuvants, help the primary treatment or are used after treatment to reduce cancer recurrence. There are also palliative types of treatments that treat the symptoms and secondary morbidities caused by cancer. Depending on the doctor's recommendation, some cancer treatments can be used in combination (Mayo Clinic, 2020). According to the National Institute of Health (2015), these are the current cancer treatments:

- *Surgery*: A procedure in which cancer is removed from the body. It can be done by cutting with a scalpel, by cryosurgery with liquid nitrogen or argon gas, by laser cutting, by hypertermination with high temperatures, or by photodynamic therapy in which drugs that react with light are used. This type of treatment is used locally, with the intention of high precision in superficial parts of the body such as the skin or in other places such as the cervix, vagina, esophagus, lung, etc.

- *Radiotherapy*: is achieved by the use of radiation, high-energy rays to eliminate cancer cells and reduce tumors. Like most cancer treatments, it is intended to cause a DNA damage of the fast dividing cancer cells to stop their proliferation. The use of this type of therapy can be limited by the size of the tumor, its location and the patient's condition. It can be applied as internal or external radiation and is used in cancers of the head, breast, prostate, thyroid, neck, cervix, etc.

- *Immunotherapy:* it is a biological therapy that helps the immune system in its response against cancer. For immunotherapy, substances derived from living organisms are used, or drugs that increase the response of the cells of the immune system. Some of the strategies are the transfer of T cells, the use of monoclonal antibodies that allow the identification and destruction of cancer cells, or the use of vaccines, including nanovaccines.

- *Hormone therapy*: Hormone therapy prevents cancer cells from getting the hormones they use for growth, thereby stopping the growth of the cancer. This therapy can block the production of natural hormones in the body or modulate hormonal behavior in general. It is mainly used for prostate and breast cancer.

- *Targeted therapy*: This therapy involves precision medicine that aims to identify the factors responsible for the growth, division and spread of cancer cells, and targets them by use of micro modulating drugs and monoclonal antibodies. This type of therapy helps the immune system by destroying cancer cells, as well as preventing their uncontrolled growth.

- *Stem cell transplant*: This therapy allows the restoration of stem cells that were purged or destroyed during other cancer treatment such as radiotherapy or chemotherapy, by replacing bone marrow cells. This therapy is widely used in leukemia, lymphoma, neuroblastomas, multiple myeloma, etc.

- *Targeted therapy using biomarkers*: tests that use biomarkers, in order to identify genes, proteins and other biological markers to identify cancer behavior factors, allowing a good diagnosis and correct decision making in the treatment to be followed. It is used in solid tumors and blood cancers.

- *Chemotherapy*: This therapy relies on drugs that destroy cancer cells, stop and/or delay their division. It is used in a wide variety of cancers and can be administered orally, intravenously, intra-arterially, topically, etc.

- *New/future therapies currently tested in clinical trials*: they are research studies with people in which different novel and advanced treatments are evaluated, in which various molecular biology and nanotechnology techniques are analyzed, which include studies of gene therapies, nanoparticles, microRNAs, siRNA (RNA interference), viral and non-viral vectors, drug delivery through micelles, among many others (Zaimy et al., 2017).

3.3 Chemotherapy

Chemotherapeutic drugs vary in many aspects such as their composition, their efficiency, mode of action, or the side effects they cause. Most of those drugs are intended to block different phases of the cell cycle (Sun et al., 2021). For example, some chemotherapeutics are antimetabolites, which interfere with metabolism such as purine and pyrimidine antagonists, genotoxic agents that cause DNA damage, Others, such as taxanes and vinca alkaloids are mitotic spindle inhibitors interfering with the components of the cytoskeleton involved in cell division leading to cell cycle arrest and inhibition of cancer cells proliferation (Cancer Quest, 2022a).

In two extensive reviews held in 2010 by the groups of Cardona et al., and Velasco & Bruna, the following chemotherapy drugs are listed:

- Compounds derived from platinum: such as Cisplatin, Carboplatin, Oxaliplatin, which are genotoxic agents and alkylating agents that prevent cell reproduction.

- Vinca alkaloids: they inhibit the formation of microtubules or the mitotic spindle, therefore they are mitosis inhibitors, such as Vincristine, Vinblastine, vinorelbine, Vindesine, etc.

- Epothilones: there is Ixabepilone, they also interfere with the activity of microtubules.

- Bortezomib: is a proteasome inhibitor, associated as an immunomodulatory drug, along with other drugs such as Thalidomide and Lenalidomide.

- Alkylating agents: a large number of drugs fall into this category, they prevent cell reproduction in all phases of the cycle, one of the best known is Ifosfamide, which is a genotoxic agent and many others.

- Antimetabolites: Capecitabine, Gemcitabine, Fludarabine, Cytarabine, Dacarbazine, 5-Fluorouracil, Methotrexate, etc. are found.

- Taxanes: Paclitaxel and Docetaxel are cytotoxic agents, inhibit the mitotic spindle and induce apoptosis.

- Others: topoisomerase inhibitors, metalloids, Etoposide, anthracyclines, antitumor antibiotics, corticosteroids, among many others.

3.4 Secondary effects

Cancer treatments result in a wide range of side effects, this is because healthy cells are also affected by the treatment. These effects will vary depending on the treatment and the patient, since each one may even be different in intensity and duration (Cancer Quest, 2022), (CDC, 2021).

Some of the more common side effects encountered are hair loss, nausea and vomiting, anemia, blood clots, diarrhea, fatigue, infections, weight loss, sexual side effects, balance problems, anxiety, delirium, seizures, depression, cramps, pain, reasoning and memory problems, peripheral neuropathy (NIH, n.d.), (*American Cancer Society*, 2020).

3.5 Taxane-induced peripheral neuropathy

Peripheral neuropathy is a disease that arises as a side effect of some treatments used in cancer, as for taxane chemotherapy treatment, called TIPN, in which the peripheral nervous system is affected, presenting consequences of pain and motor, sensitive or sensory alterations (Barrell & Smith, 2019), (Velasco & Bruna, 2010).

The molecular and pathophysiological mechanisms of TIPN are still under study, however, there are several factors that are suggested to be attributed to the nerve damage. TIPN can

cause a direct damage to the cell bodies of dorsal root ganglion neurons, and it can have an effect on myelin or axonal components, such as neuronal fibers, mitochondria, ion channels, all of which are important in neurotransmission **(Figure 3)** (Maihöfner et al., 2021), (Canta et al., 2015).

Some studies propose that TIPN is due to an alteration in the axonal membrane, others to the axonal mitochondrial part, there is dysfunction of the axonal transport, because taxanes intervene directly in the microtubules and the entire mitotic spindle. There is axonal degradation and demyelination by nerve cells, leading neurons to dysfunction, and therefore to problems in sensory and motor actions that construct peripheral neuropathy (Molassiotis et al., 2019), (Jankovic, 2022), (Shahpar et al., 2018), (Velasco & Bruna, 2010), (Cardona et al., 2010).

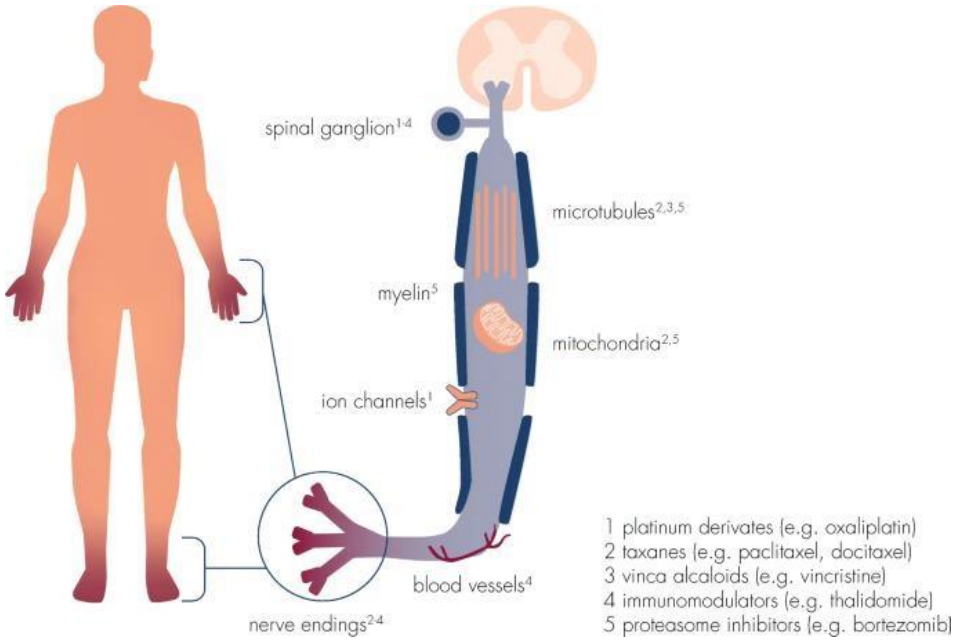


Figure 3. Illustration of nervous system structures or subcellular organelles affected in CIPN (Maihöfner et al., 2021).

TIPN affects sensory nerve endings (Maihöfner et al., 2021). The sensory nerve endings are structures which include neural growth cones and filipodia (**Figure 4**). Those structures are important in neuronal growth. Any disruption of cytoskeletal structures such as actin

fibers and microtubules strongly affect the dynamics, action, morphology, retraction, and advancement of peripheral nerves, affecting not only neuronal communication, conduction of nerve impulses, but it can also activate certain pathways of inflammation, ultimately generating the known characteristics of peripheral neuropathy, which is the appearance of pain and sensory loss (Andersen Hammond et al., 2019).

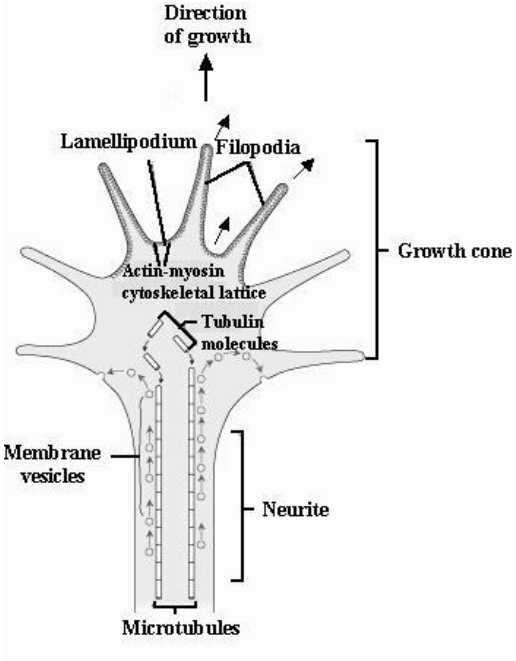


Figure 4. Schematic representation of the neural growth cone structure (Hopkins, 2019).

3.6 1-deoxysphingolipids

In recent years, the function of sphingolipids has been a topic of interest, not only because of their fundamental structural function in the membranes of eukaryotic cells, but also because of their signaling function. Sphingolipid signaling plays a role in, intra- and intercellular traffic, cell division and proliferation, resistance to stress, inflammation, and apoptosis (Gomez-Larrauri et al., 2020), (Hannun & Obeid, 2018), (Quinville et al., 2021). Sphingolipid metabolism is complex, encompassing multiple metabolites and enzymes (Clarke et al., 2020), (Quinville et al., 2021). It is more and more studied, with focus on any alteration or imbalance in the levels of sphingolipid metabolites, which can cause incorrect

cell functioning and, therefore, cause serious diseases (Lone et al., 2019), (Carreira et al., 2019).

De novo sphingolipid synthesis is localized in the endoplasmic reticulum (Tidhar & Futerman, 2013). The first step is catalyzed by the enzyme serine-palmitoyltransferase (SPT), which normally conjugates L-serine with palmitoyl-CoA, producing 3-keto-sphinganine that is converted to sphinganine (Merrill, 2002). Sphinganine is then metabolized to dihydroceramide, which is then converted to ceramide by a desaturase. Ceramide is the main sphingolipid metabolite used for the generation of complex sphingolipids such as sphingomyelin and gangliosides (Merrill & Jr., 2011). Ceramide can be degraded to sphingosine, which can be further degraded through S1P by S1P lyase or reuse to production of ceramide through the so called salvage pathway (**Figure 1**) (Stoffel, 1970), (Saba, 2019).

L-serine is SPT's preferred amino acid substrate (Gable et al., 2010), (Eichler et al., 2009). SPT, however, can use alternative amino acid substrates, L-alanine and glycine. L-alanine and glycine have lesser affinity to SPT then L-serine (Gable et al., 2010), (Eichler et al., 2009). This substrate shift generates atypical sphingolipids called deoxySLs (from L-alanine) (Fig. 1) or 1-deoxymethylsphingolipids (from glycine). Those atypical sphingolipids are structurally different from their canonical L-serine-derived equivalents because they lack the C1-OH functional group in the canonical sphingolipids. Due to this change, the deoxySLs and deoxymethylsphingolipids cannot be converted to complex sphingolipids or degraded in a conventional manner by S1P lyase (Lone et al., 2019).

Initially it was believed that since deoxySLs could not be degraded by the canonical pathway, they were dead paths, but it has been proved that deoxySLs can undergo a slow degradation by different degradation pathways, involving cytochrome P450 (CYP4F) enzymes (Alecu, Othman, et al., 2017). However, due to the non-specific pathways and their slow degradation, when deoxySLs are produced in excess, they accumulate (Lone et al., 2019), (Carreira et al., 2019).

The elevated levels of deoxySLs have been implicated in neurotoxicity and diseases (Gui et al., 2021), (Hube et al., 2017), (Bertea et al., 2010), (Penno et al., 2010), (Kramer et al., 2015), (Martinez et al., 2012). There are only few studies focused on the mechanism by which deoxySLs cause neurotoxicity, which as a result remains still poorly understood

(Loewith et al., 2019), (Hornemann, 2021), (Schwartz et al., 2019), (Penno et al., 2010), (Mwinyi et al., 2017), (Fridman et al., 2021), (Hornemann et al., 2018).

The neurotoxic effects of deoxySLs include morphological changes in the mitochondria and neurite abnormalities (Alecu, Tedeschi, et al., 2017), (Becker et al., 2020), (Wilson et al., 2018). In mitochondria, the deoxySLs were shown to induce fragmentation, clustering, taking a more spherical shape, abnormal distribution, and malfunctions (Alecu, Tedeschi, et al., 2017), (Wilson et al., 2018), (Galih Haribowo et al., 2019). The deoxySL effect on the neurites includes reduction, fragmentation and retraction, leading to decrease in their length (Penno et al., 2010), (Güntert et al., 2016), (Zuellig et al., 2014), (Alecu, Tedeschi, et al., 2017), (Wilson et al., 2018). DeoxySLs were shown to affect the cytoskeletal components, such as actin, neurofilaments, and microtubules (Cuadros et al., 2000), (Penno et al., 2010), (Güntert et al., 2016), (Galih Haribowo et al., 2019). Specifically, they were shown to cause a loss of actin filaments in DRG neurons, changes in the motility of neuronal growth cones, axonal migration, dendritic complexity. DeoxySLs also have molecular effects such as ATP reduction, endoplasmic reticulum stress, calcium flow alterations (Alecu, Tedeschi, et al., 2017).

These molecular and structural effects of deoxySLs are involved in different metabolic and neurodegenerative diseases. They have been implicated in diabetic sensory neuropathy, type 2 diabetes mellitus, hereditary sensory and autonomic neuropathy type 1, Fabry disease, Niemann-Pick disease, Guillain-Barré syndrome, multiple sclerosis, Sandhoff's disease, and associated with Tumor Necrosis Factor (TNF) treatment, etc. (Hornemann, 2021), (Goins & Spassieva, 2018), (Mwinyi et al., 2017), (Wilson et al., 2018), (Fridman et al., 2021), (Martinez et al., 2012). Additionally, high levels of 1-deoxySL have been found in peripheral neuropathy induced by chemotherapy, specifically by taxanes (Kramer et al., 2015), (Becker et al., 2020).

Thus, deoxySLs have become an important object of study because of their role in diseases that severely affect humans. DeoxySLs have potential as possible biomarkers and studying their metabolism can provide knowledge for discovering therapeutic agents for these diseases (Zuellig et al., 2014), (Mwinyi et al., 2017), (Boso et al., 2019). Currently, the lack of knowledge regarding to the mechanism of their neurotoxicity and many questions regarding their metabolism that need to be answered are challenges that needs to be addressed in future research (Hornemann, 2021), (Carreira et al., 2019), (Lone et al., 2019).

4. Chapter 4

4.1 Hypothesis

The variation in the dosage of three species of 1-deoxysphingolipids from the SPT interaction generates neuritic retraction and bulges at the ends of the neurons, allowing their toxicity to be evaluated. Furthermore, attenuation of sphingosine-1-phosphate (S1P) signaling by inhibiting its receptors decreases neurotoxic effects to elucidate the possible mechanism.

4.2 Objectives

4.2.1 General objective

Evaluate the neurotoxic effect induced by 1-deoxysphingolipid species, and their cell signaling mechanism, in an *in vitro* model of peripheral neuropathy induced by taxanes.

4.2.2 Specific objectives

- Evaluate the neuritic damage of the individual species of 1-deoxysphingolipids associated with TIPN, through morphological and immunocytochemical parameters.

- To determine the modulatory effect of Sphingosine-1-Phosphate receptors (S1PRs) in an in vitro neuronal culture model by using S1PR modulators.

5. Chapter 5

5.1 Materials

RPMI 16-40 medium (Corning), EMEM medium (BD), KCNR cell line, Neuro-2a cell line, fetal bovine serum (FBS) (Thermo Fisher), dialyzed fetal bovine serum (lipid-reduced), penicillin/ streptomycin (Thermo Fisher), poly-L-ornithine (Sigma Aldrich), laminin (Thermo Fisher), Sir Actin (Cytoskeleton, Inc), Huzzah © (Avanti Polar Lipids), 1-deoxysphinganine (Avanti Polar Lipids), 1-deoxysphingosine (4E) (Avanti Polar Lipids), 1-deoxysphingosine (14Z) (Avanti Polar Lipids), $C_{24:1}$ 1-deoxyceramide (4E) (Cayman Chemical), $C_{24:1}$ 1-deoxyceramide (14Z) (Cayman Chemical), C_{22} 1-deoxyceramide(14Z) (Cayman Chemical), medium F12 (VWR), and FTY720.

5.2 Methods

5.2.1 Neuroblastoma cell line cultures

SMS-KCNR cells were cultured in RPMI 16-40 medium. Neuro-2a cells were cultured in EMEM. Both media were supplemented with 10% bovine serum and 1 % penicillin/ streptomycin. Cells were incubated at 37°C and 5% CO_2. For neuronal differentiation of Neuro-2a cells, after reaching 80% confluence, cells were serum deprived for 2 or 3 days until they acquired a neuron-like morphology.

5.2.2 Preparation and culturing of primary DRG neurons

The isolation of mouse DRG neurons culture was carried out as previously described by (Malin, 2007). DRG neurons were isolated from the DRG at the lumbar vertebra of three female 10-week-old C57BL/6 mice were cultured for 48 hours at 37°C, 5% CO2, in Hams F12 medium, supplemented with 10% fetal bovine serum FBS, 1% penicillin/streptomycin on glass bottom plates coated with poly-L-ornithine and laminin.

The animal study had permission from the Institutional Animal Care and Use Committee of the University of Kentucky, (protocol # 2018-2952).

5.2.3 Actin fluorescence labeling in primary DRG neurons and in KCNR neuroblastoma cells

After 48 h of primary DRG neurons culture, media was replaced with Ham´s F12 culture medium without phenol red, supplemented with 10% dialyzed fetal bovine serum (lipid-reduced). 5h before deoxysphingolipid treatment, the SiR-Actin dye was added to the media at a concentration of 75nM (Becker et al., 2020).

For KCNR cell line, 3h before treatment with deoxysphingolipids, cells were labeled with 40nM SiR Actin.

5.2.4 1-deoxysphingolipids treatment

After reaching 80-90% confluency, KCNR culture media was replaced with RPMI 1640 supplemented with 10% lipid-reduced FBS. In differentiated Neuro2a cells, the treatment was performed in EMEM media with no serum. 1-deoxysphingolipids were prepared as huzzah:lipid conjugate at a 2:1 ratio, with a final working concentration of 120 µM, and later sonicated for 2 minutes. Both neuroblastoma cell lines, were treated with the following 1-deoxysphingolipids: 1-deoxysphinganine, 1-deoxysphingosine (4E), 1-deoxysphingosine (14Z), $C_{24:1}$ 1-deoxyceramide (4E), $C_{24:1}$ 1-deoxyceramide (14Z), C_{22} 1-deoxyceramide (14Z), at concentrations of 0.5 µM, 1 µM, and 1.5 µM, monitored after 3h, 7h, 15h and 24h after treatment for morphological changes, by microscopy (Nikon Instruments Inc.).

For the LDH Assay, the sphingoid bases were applied: 1-deoxysphinganine, 1-deoxysphingosine (4E), 1-deoxysphingosine (14Z), at concentrations of 0.5 µM, 1 µM, and 1.5 µM. For the KCNR treatment was performed for 16h and for the Neuro-2a for 7h, based on preliminary morphological changes test.

5.2.5 LDH Assay

A Lactate dehydrogenase (LDH) assay was performed, for both, KCNR and Neuro2a cell lines, Cells were seeded in a 96-well plate with a density of 1000 cells/well, cultured until 90% confluence was reached, and treated subsequently treated with deoxysphingolipids as described above. followed by differentiation for the Neuro2a cells were differentiated after reaching 90% confluency. LDH assay was performed following manufacturer's protocol.

5.2.6 Microscopy analysis

The evaluation of the morphological damage in the neuroblastoma cell lines KCNR and Neuro2a, and in the primary DRG neurons, such as neurite bulges and swellings (N2a and DRG neurons), and cell body rounding (KCNR), was performed by using fluorescence microscopy (Nikon Ti2E) at 3h, 7h, 15h and 24h after treatment with deoxysphingolipids or by time-lapse live-imaging. Increasing concentrations (0.5 µM, 1 µM, and 1.5 µM) of deoxysphingolipids were used. The analysis and quantification of the neurotoxic effects of the deoxySL was carried out using the Nikon Elements Imaging Software General analyses tool (Nikon). In the N2a cells, the number of neurite swellings in the individual images were normalized to the cell body area in the image. For KCNR cells, to quantify the rounding of the cell bodies the average circularity of the cell bodies was used as a criterium.

5.2.7 FTY720 treatment

Differentiated N2a cells were treated with 100nM of FTY720 for 2 hours, followed by addition of 0.5µM or 1µM 1-deoxysphinganine (m:18). The morphological changes of the neurites were monitored by live-imaging microscopy (Nikon Ti2E fluorescence microscope) for 7h.

5.2.8 Statistical analyses

To compare the toxic effect of each individual deoxySLs in the LDH cytotoxic assay and for the morphological changes, multiple comparison using an Analysis of Variance (ANOVA) and pairwise multiple comparison by different methods were used, analyzed with the software's, SigmaPlot 15.0 and STATISTICA 10.0. In both cell lines, KCNR and N2a, the statistical analyses were performed on treatment groups representing concentration, time point, or individual deoxySL tested.

6. Chapter 6

6.1 Results

6.2 1-deoxysphinganine is the most cytotoxic deoxysphingoid base

In order to address the question of which deoxysphingoid base is the most cytotoxic, an LDH assay was performed to evaluated plasma membrane damage as a marker of toxicity. We used two neuroblastoma cells lines, KCNR and differentiated N2a cells. For both, KCNR and N2a cell lines, the three deoxysphingoid bases (1-deoxysphinganine, 1-deoxysphingosine (4E), 1-deoxysphingosine (14Z)) were tested at increasing concentrations (0.5 µM, 1 µM and 2 µM). For KCNR cells, cytotoxicity was measured after 16h of treatment, and for N2a cells after 7h, based on preliminary morphological changes tests.

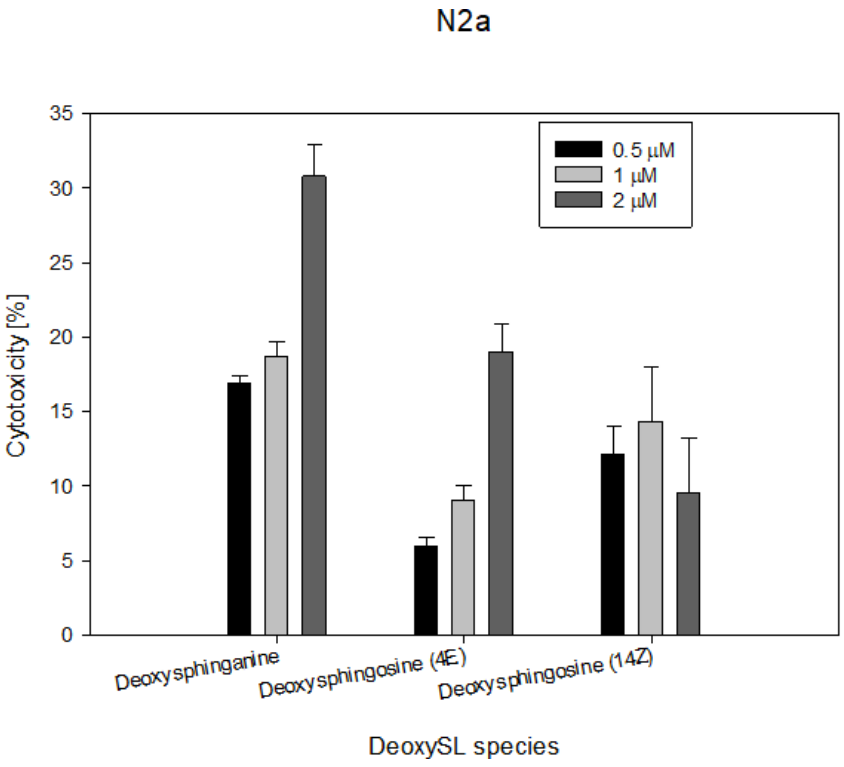
Based on the results obtain with LDH assay, the species 1-deoxysphinganine showed the highest cytotoxicity in both cell lines, KCRN and N2a **(Figure 5).**

In differentiated N2a cells, the cytotoxicity of the three 1-deoxysphingoid bases was tested after 7h of treatment (Fig 5 (A)). At all the concentrations tested, 1-deoxysphinganine showed to be significantly more toxic than the 1-deoxysphingosines ($P<0.03$). For the highest concentration tested, 2 µM, a dose response was seen for 1-deoxysphinganine and 1-deoxysphingosine(4E) but not for 1-deoxysphingosine(14Z). Differences in cytotoxicity between the positional isomers 1-deoxysphingosines were seen at different concentrations, as for 0.5 µM 1-deoxysphingosine(14Z) showed more toxicity ($P=0.012$), also for 1 µM, but not for 2 µM, where 1-deoxysphingosine(4E) showed the highest cytotoxicity of the two 1-deoxysphingosines.

In KCNR cells deoxySL cytotoxicity was tested after 16h of treatment (Fig 5 (B)). In KCNR cells, 1-deoxysphinganine (1 µM) also showed significantly higher cytotoxicity compared to both positional isomers of 1-deoxysphingosine ($P= <0.001$). Between the positional isomers 1-deoxysphingosines, showed differences when compared, although with no statistical significance. Initially at 0.5 µM, 1-deoxysphingosine(4E) showed a slightly higher cytotoxicity, then at 1 µM and 2 µM change, being 1-deoxysphingosine(14Z) more cytotoxic.

In KCNR, all the 1-deoxysphingoid base species showed reduced cytotoxicity at 2 µM, while showing a dose-response at the lower concentration (Fig.5B).

A)



B)

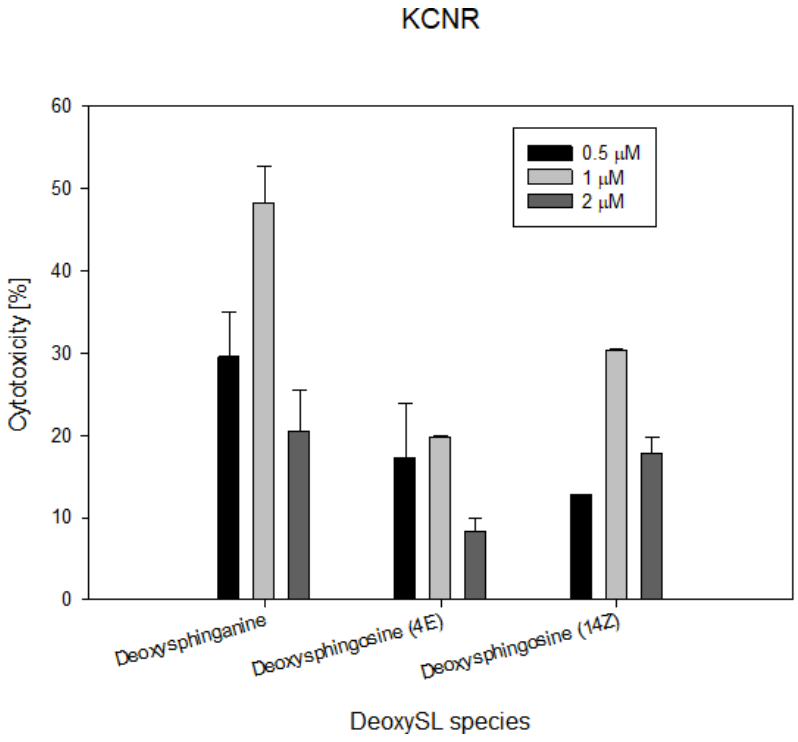


Figure 5. Deoxysphingoid bases cytotoxicity. The cytotoxicity was measured by LDH assay. A) Differentiated N2a cells were treated for 7 h with 1-deoxysphinganine,1-deoxysphingosine (4E), and 1-deoxysphingosine (14Z), at 0.5 µM, 1 µM, and 2 µM. B) KCNR cells were treated for 16 h with 1-deoxysphinganine,1-deoxysphingosine (4E), and 1-deoxysphingosine (14Z) at 0.5 µM, 1 µM, and 2 µM. The error bars, standing for standard deviation from two independent experiments. The statistical analyses were generated in Sigma plot 15.0 using multiple comparison by ANOVA and pairwise multiple comparison, using Holm-Sidak method.

6.3 Toxicity of 1-deoxysphingolipids, a transient response in differentiated N2a cells

To evaluate which deoxySL is the most toxic, a morphological followed up and analyzed after treatment with deoxySL was performed.

Unlike KCNR cell lines, N2a cells are characterized by neurite presence, especially when differentiated. The deoxySL treatment in differentiated N2a cells produced neurotoxic effects such as more robust appearance of neurites, losing more delicate look like branches, resulting in the loss of neurite complexity, and formation of neurite swellings **(Figure 6)**.

In differentiated N2a cells, we tested six deoxySL species (deoxysphinganine, deoxysphingosine (4E), deoxysphingosine (14Z), $C_{24:1}$ deoxyceramide (4E), $C_{24:1}$ deoxyceramide (14Z) and C_{22} deoxyceramide (14Z)), for their ability to trigger the formation of neurite swellings. We tested increasing concentrations (0.5 µM, 1 µM, and 1.5 µM) of the individual 1-deoxySL, at four time points (3h, 7h, 15h, 24h). The formation of neurite swellings was monitored by microscopy (Nikon). The quantification of neurite swellings was performed by the General analyses tool of the Nikon Analyses software as described in the methods and normalized to the number of cells in the analyzed images.

Neurite swellings results showed that, after 3h post treatment with deoxySL (Fig 7. (A)), 1-deoxysphinganine showed increased swellings for all the tested concentrations compared to control ($P<0.04$). Treatment with 1 µM 1-deoxysphingosine (4E), also increased the neurite swellings ($P<0.03$). The positional isomer 1-deoxysphingosine (14Z) did increase neurite swelling compared to the control at all three concentrations. However, no statistical

differences were found between deoxysphingosines positional isomers. Treatment with 1-deoxyceramides, $C_{24:1}$ deoxyceramide (4E) showed higher neurite swellings compared to the control at all concentrations specially at 1 µM ($P<0.003$). The positional isomer $C_{24:1}$ 1-deoxyceramide (14Z) showed no increase in the swellings formation at all tested concentrations compared to the control (**Figure 7).** For $C_{24:1}$ 1-deoxyceramide positional isomers, statistical differences were evidenced for the three concentrations ($P<0.03$). The treatment with C_{22} deoxyceramide, (14Z) showed a dose response, triggering the formation of the most neurite swellings at the highest concentration, 1.5 µM ($P<0.0003$).

7h after treatment with deoxySL (Fig 7. (B)), presented no significant difference in formation of neurite swellings compared to the control, neither for deoxysphinganine or deoxysphingosine (4E), contrary, deoxysphingosine (14Z) showed a dose response, presenting high swellings formation at 1 and 1.5 µM ($P<0.03$). For lower concentrations of C24:1 deoxyceramide (4E), increased swellings were found compared to control ($P<0.02$), specially at 1 µM ($P<0.002$), but for the positional isomer, C24:1 deoxyceramide (14Z), 0.5 µM had the most swellings compared to control ($P<0.003$), and for C22 deoxyceramide (14Z), all concentrations were increased compared to control ($P<0.05$).

At 15h (Fig 7. (C)), similar levels of swellings were seen for all the concentrations for each species, no dose-response was evidenced. Some results were similar to the 3h counting, rather the 7h. Deoxysphinganine showed high swellings at 1 µM compared to control ($P<0.02$), swellings for deoxysphingosine (4E) maintain low with no significant differences, deoxysphingosine (14Z) had high levels at all concentrations, but specially at 0.5 µM ($P<0.002$). C24:1 deoxyceramide (4E) showed increase swellings at all concentrations ($P<0.005$), followed by C24:1 deoxyceramide (14Z) at 0.5 µM ($P<0.001$), and lower swellings for C22 deoxyceramide (14Z) including at its higher concentration 1.5 µM ($P<0.04$).

Final time tested, 24h after treatment (Fig 7. (D)), swelling levels decreased for the majority of the species, compared to the control. Deoxysphinganine showed no increase in swellings compared to the control, otherwise, deoxysphingosine (4E) started showing more swellings, specially at 0.5 µM ($P<0.01$), the positional isomer, deoxysphingosine (14Z) decreased its swellings, showing no significant differences between the isomers. High levels at 1 µM were seen for C24:1 deoxyceramide (4E) ($P<0.003$). Low levels were evidenced for C24 and C22 deoxyceramides (14Z).

In summary, treatments with individual deoxySL in differentiated N2a cells, produced swelling formation as a neurotoxic effect, presenting a transient response to either concentration and time, As results showed above, where at the beginning, 3h after treatment, the deoxySL with more swellings formation was deoxysphingosine (4E) at 1 µM, later at 7h, 0.5 µM of C24 deoxyceramide (14Z) presented more swellings, for 15h 0.5 µM of deoxysphingosine (4E), C24 deoxyceramide (4E) and (14Z) had tight levels of swellings, and 24h after treatment, only deoxysphingosine (4E) and C24 deoxyceramide (4E) presented swelling formation compared to control.

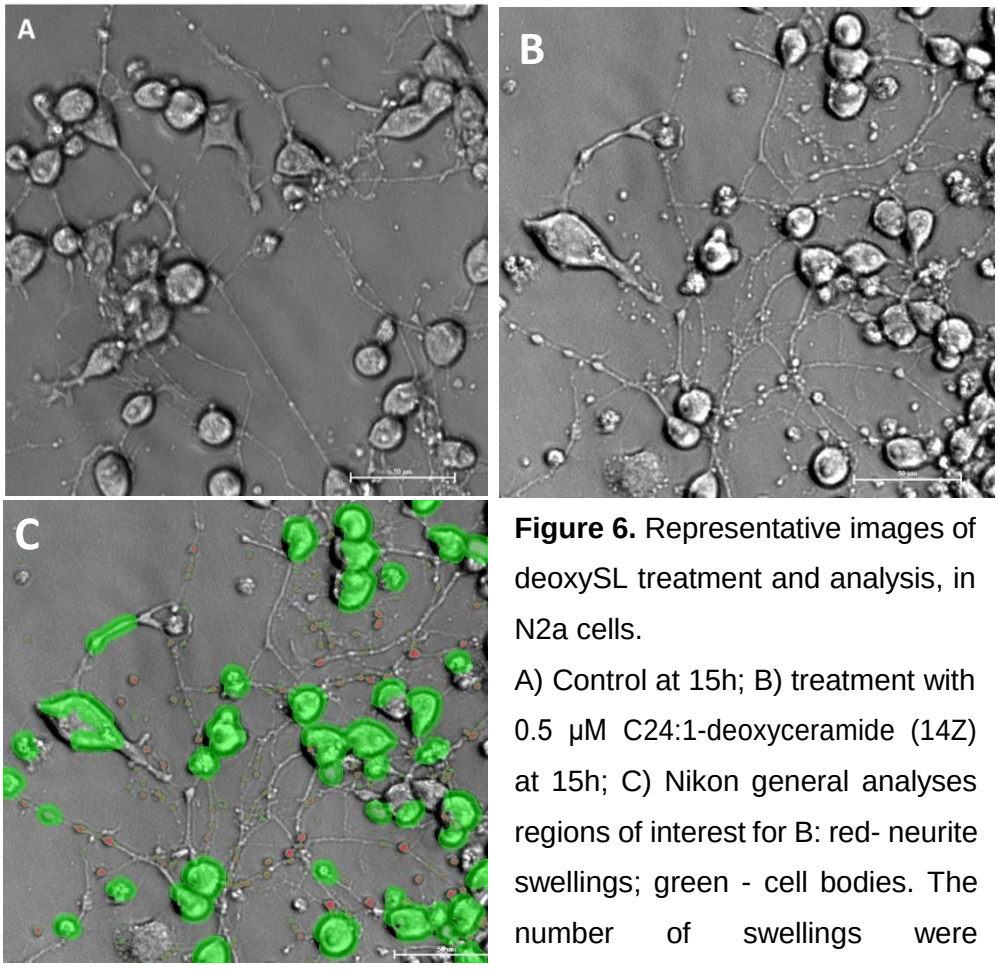
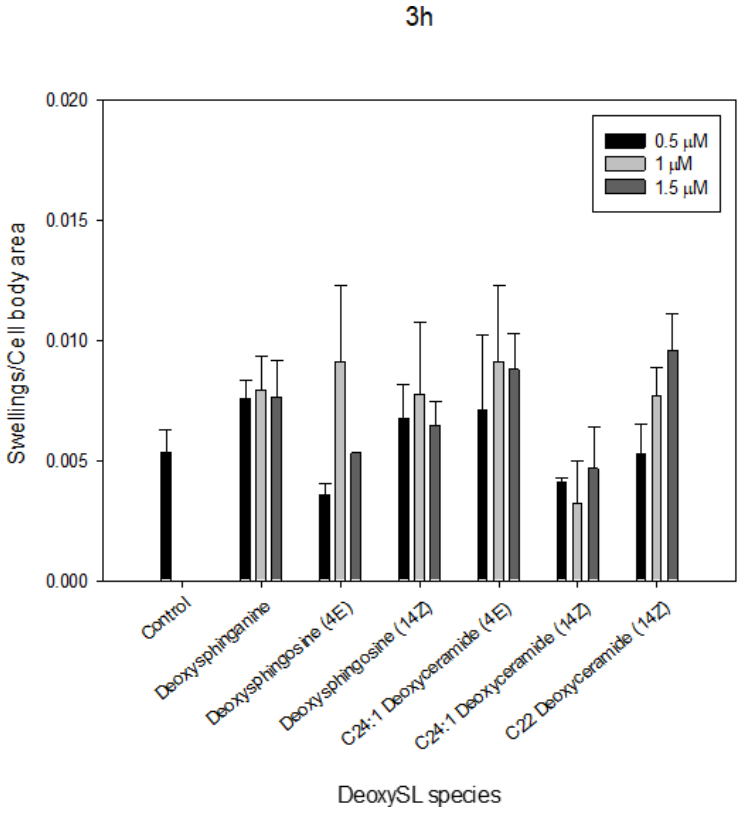
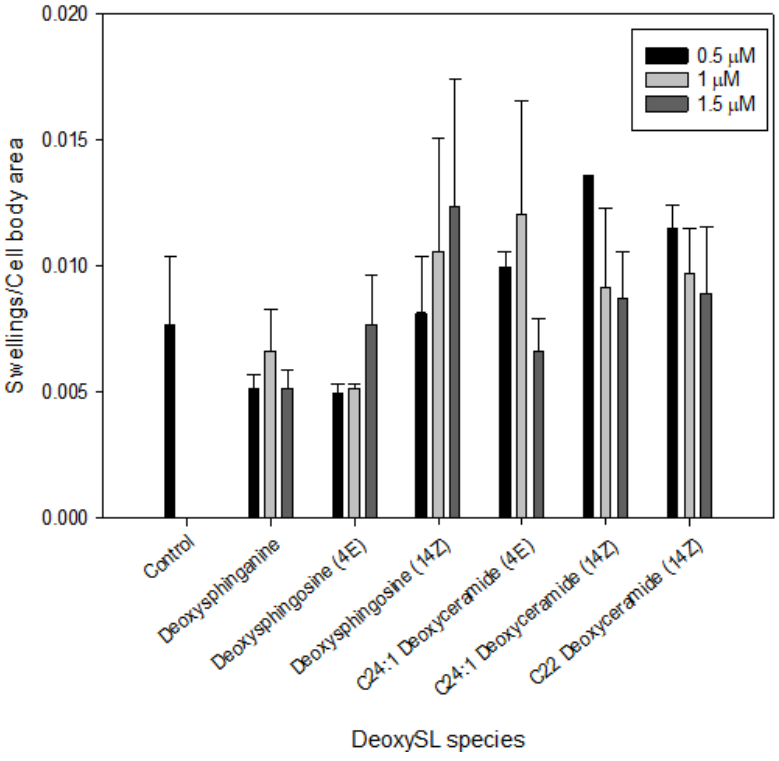


Figure 6. Representative images of deoxySL treatment and analysis, in N2a cells.
A) Control at 15h; B) treatment with 0.5 µM C24:1-deoxyceramide (14Z) at 15h; C) Nikon general analyses regions of interest for B: red- neurite swellings; green - cell bodies. The number of swellings were normalized to the cell body area.

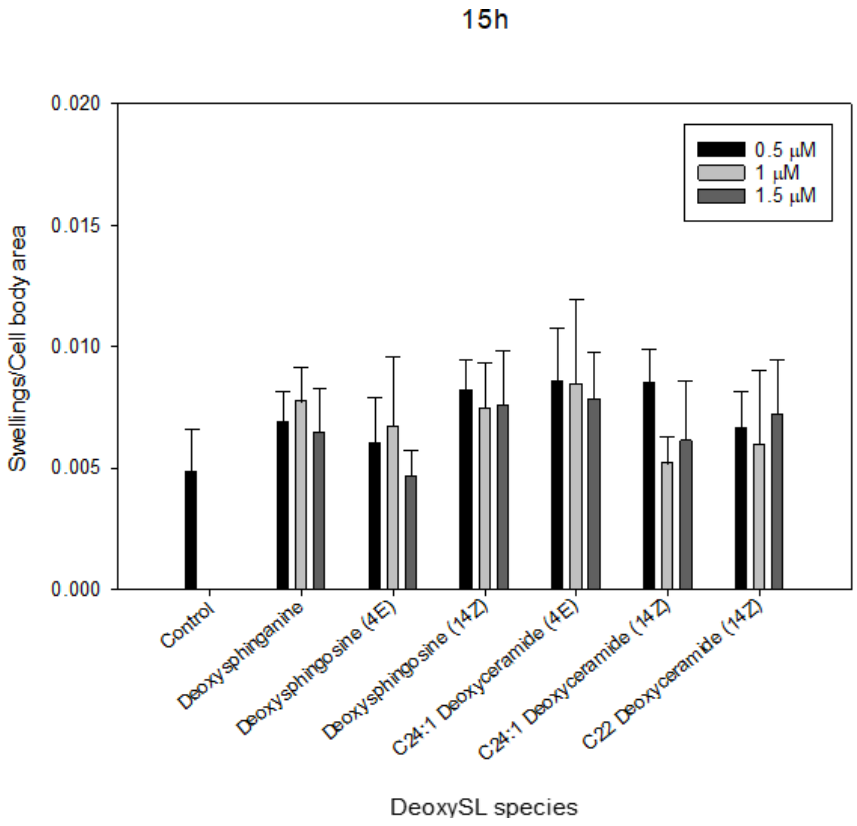
A)


3h
0.020
0.015
0.010
0.005
0.000
Swellings/Cell body area
0.5 µM
1 µM
1.5 µM
Control
Deoxysphinganine
Deoxysphingosine (4E)
Deoxysphingosine (14Z)
C24:1 Deoxyceramide (4E)
C24:1 Deoxyceramide (14Z)
C22 Deoxyceramide (14Z)
DeoxySL species

B)


7h
0.020
0.015
0.010
0.005
0.000
Swellings/Cell body area
0.5 µM
1 µM
1.5 µM
Control
Deoxysphinganine
Deoxysphingosine (4E)
Deoxysphingosine (14Z)
C24:1 Deoxyceramide (4E)
C24:1 Deoxyceramide (14Z)
C22 Deoxyceramide (14Z)
DeoxySL species

C)


15h
0.020
0.015
0.010
0.005
0.000
Swellings/Cell body area
0.5 µM
1 µM
1.5 µM
Control
Deoxysphinganine
Deoxysphingosine (4E)
Deoxysphingosine (14Z)
C24:1 Deoxyceramide (4E)
C24:1 Deoxyceramide (14Z)
C22 Deoxyceramide (14Z)
DeoxySL species

D)

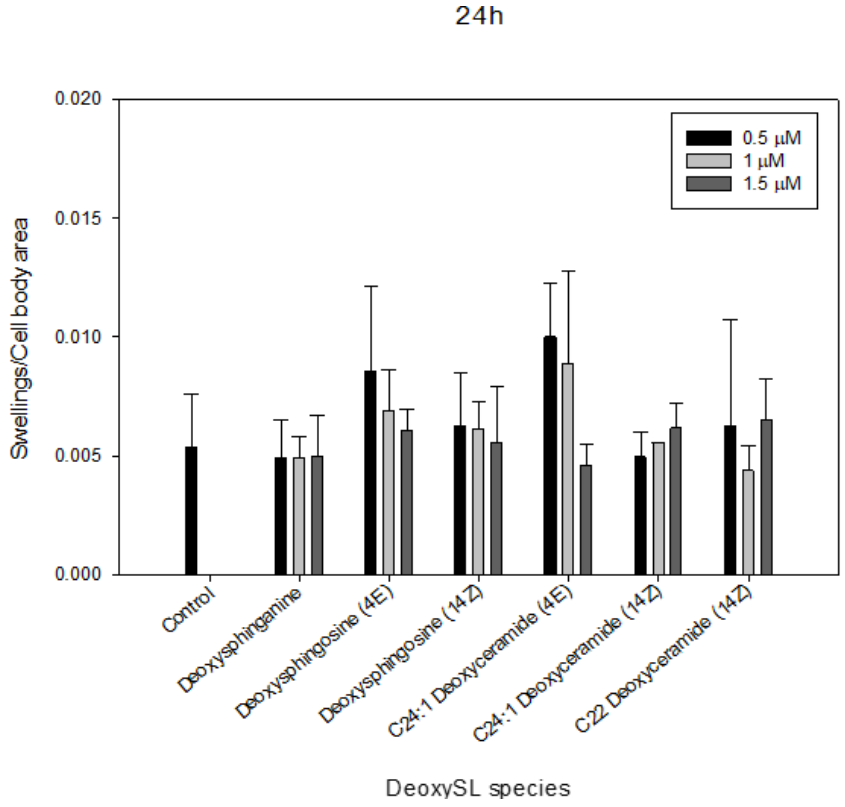

24h
0.020
0.015
0.010
0.005
0.000
Swellings/Cell body area
0.5 µM
1 µM
1.5 µM
Control
Deoxysphinganine
Deoxysphingosine (4E)
Deoxysphingosine (14Z)
C24:1 Deoxyceramide (4E)
C24:1 Deoxyceramide (14Z)
C22 Deoxyceramide (14Z)
DeoxySL species

Figure 7. Neurite swelling quantification in differentiated N2a cells treated with individual deoxySL. Increasing concentrations (0.5 µM, 1 µM, and 1.5 µM) of individual deoxySL species were used for treatment. The number of neurite swellings were counted at four time points. A) 3h post treatment; B) 7h post treatment; C) 15h post treatment, and D) 24h post treatment. Quantification of neurite swellings were performed on bright field images of individual treatment samples. Nikon General analyses software was used to count neurite swellings. Multiple technical repeats (2-6) were used to quantify the number of the neurite swellings for each species/concentration/time point. The statistical analyses were generated in STATISTICA 10.0 using Post Hoc tests, least significant difference (LSD) test.

6.4 In KCNR cells deoxySL cause rounding of the cell bodies

Continuing addressing the question of which deoxySL is the most toxic, KCNR cells were treated with 1 µM of deoxysphinganine, and 0.5 µM, 1 µM, and 1.5 µM of deoxysphingosine (4E), deoxysphingosine (14Z), C24:1 deoxyceramide (4E), C24:1 deoxyceramide (14Z), C22 deoxyceramide (14Z), analyzed at different time points (3h, 7h, 15h, 24h).

In KCNR cells, the treatment with 1 µM deoxysphinganine overtime resulted in morphological changes, non-treated cells presented a flat morphology. As a result of treatment with deoxySLs, the cells gradually appeared rounded **(Figure 8).** Other effects as a result of deoxysphinganine treatment were the loss of neurite projection and reduction of their complexity.

Treatment with 1 µM deoxysphinganine after 24h showed statistically significant changes in the circularity of the cell bodies compared to the control ($P < 0.001$) and to the rest of the deoxySL species tested ($P < 0.05$) **(**

Figure 9). The rest of the tested deoxySL did not show a statistically significant difference in circularity compared to the control. This result agrees with our cytotoxicity assay **(Figure 5)** that identified deoxysphinganine as the most toxic deoxySL.

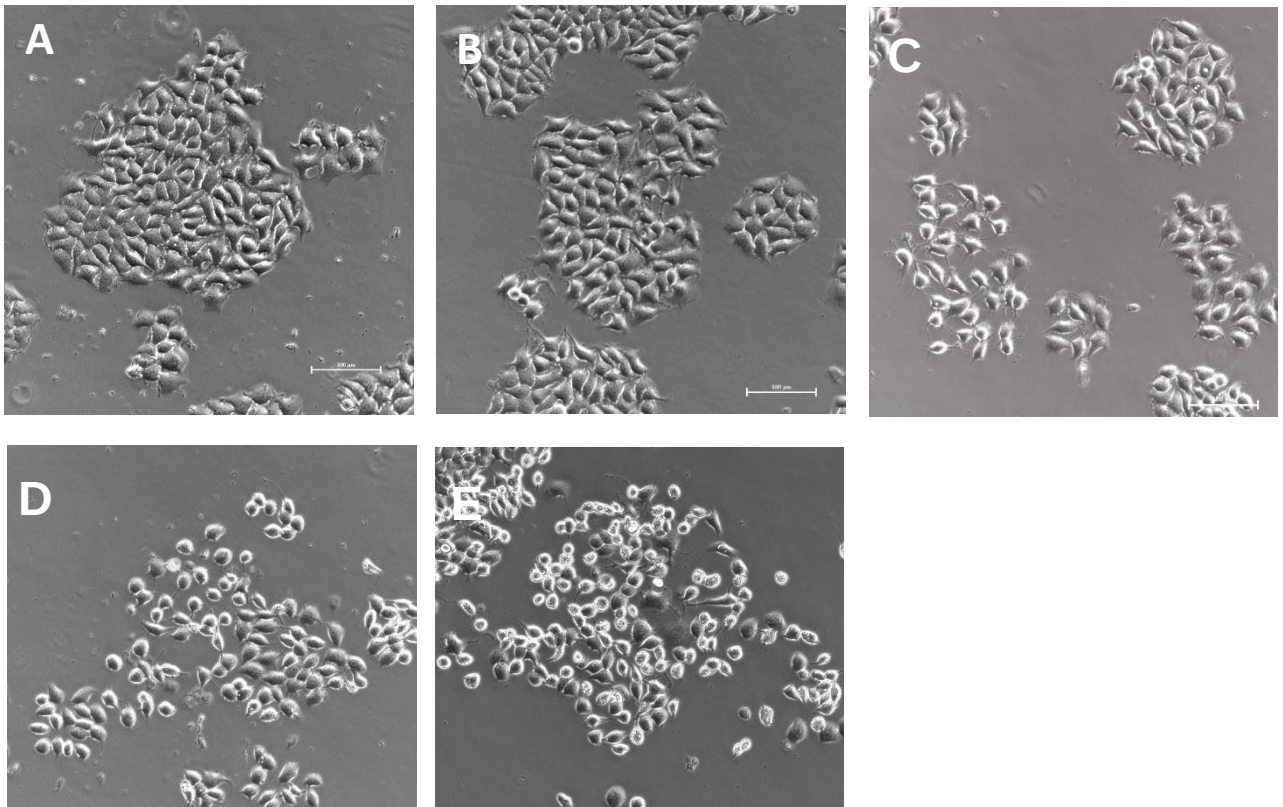


Figure 8. Treatment with 1-deoxysphinganine (1µM) of KCNR cells results in rounding of the cell bodies over time. Representative images of treatment with 1 µM of 1-deoxysphinganine. A) Control; B) 3h; C) 7h; D) 15h and E) 24h, after treatment.

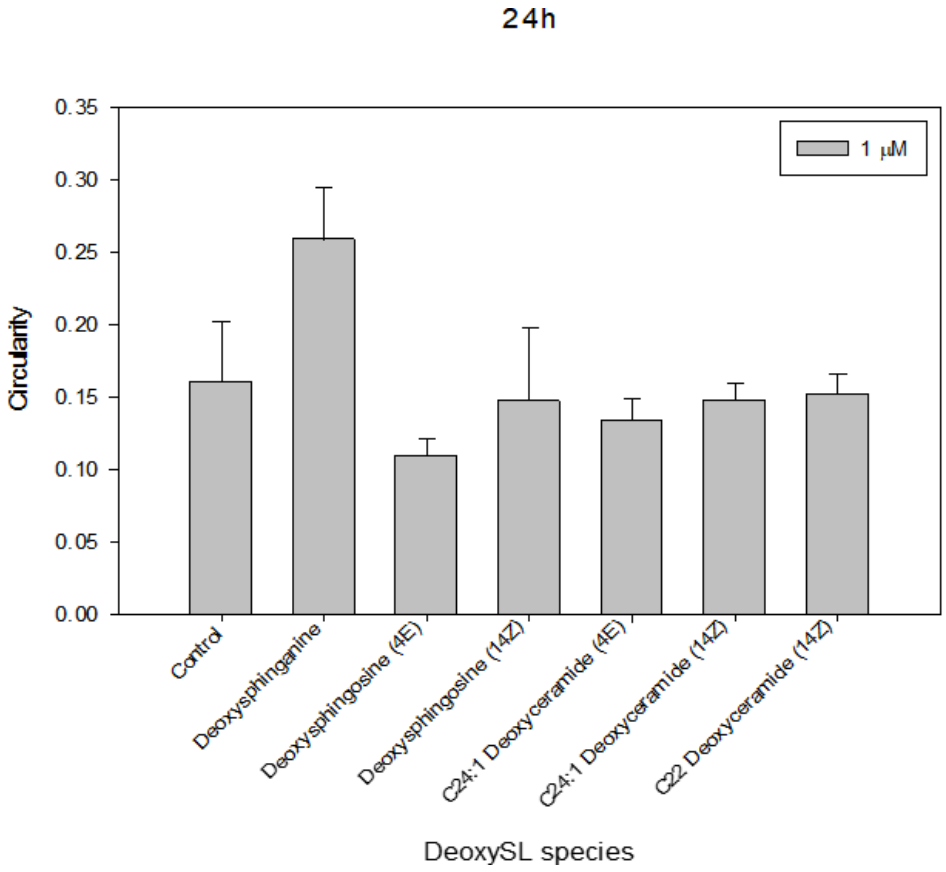


Figure 9. Treatment with deoxysphinganine in KCNR cells results in cell body rounding. KCNR cells were treated with 1 µM of individual deoxySL species for 24h. Circularity of the cell bodies was assessed on bright field images taken with a Nikon microscope. Technical repeats (2 to 6) were used to quantify the circularity for each species, 24h after

treatment. The statistical analyses were generated in Sigma plot 15.0 using multiple comparison by ANOVA ($P < 0.001$), and pairwise multiple comparison between treatment groups using the Bonferroni t-test ($P < 0.05$) and control ($P < 0.001$). The error bars represent standard deviation.

6.5 DeoxySL treatment causes actin re-organization

Results from KCNR rounding and previous research have shown actin alterations because of deoxySL treatment causing a decreased of stress fibers, actin accumulation on the perinuclear area and actin swellings (Cuadros R, 2000), (Zuellig RA, 2014), (Becker et al., 2020).

In order to visualize and monitor the deoxySL effect on actin filaments over time, KCNR cells were treated with deoxysphinganine, at 1.5 µM, labeled with 40 nM Sir-Actin for 3h before treatment with deoxySL, and monitored by fluorescence microscopy with time-lapse live-imaging. Our results show that at 7h and 13h post-treatment, there was a disruption of the actin stress fibers, and an accumulation of the label into the cortical region likely indicating assembling of cortical actin (**Figure 10)**.

Actin changes were also seen in primary DRG neurons treated with deoxysphinganine, deoxysphingosine (4E), deoxysphingosine (14Z), C24:1 deoxyceramide (4E), C24:1 deoxyceramide (14Z) and C22 deoxyceramide (14Z), at 0.5 µM, 1 µM and 1.5 µM (Data shown for 0.5 µM). DRG neurons were labeled with 75 nM Sir Actin and visualized after 6h and 11h after treatment, performing time-lapse fluorescence microscopy imaging with a Nikon microscope. Results showed bulbs or swelling formation, accumulation on the neurite tips, as well as movement along the neurite, increasing the manifestation over time **(Figure 11).**

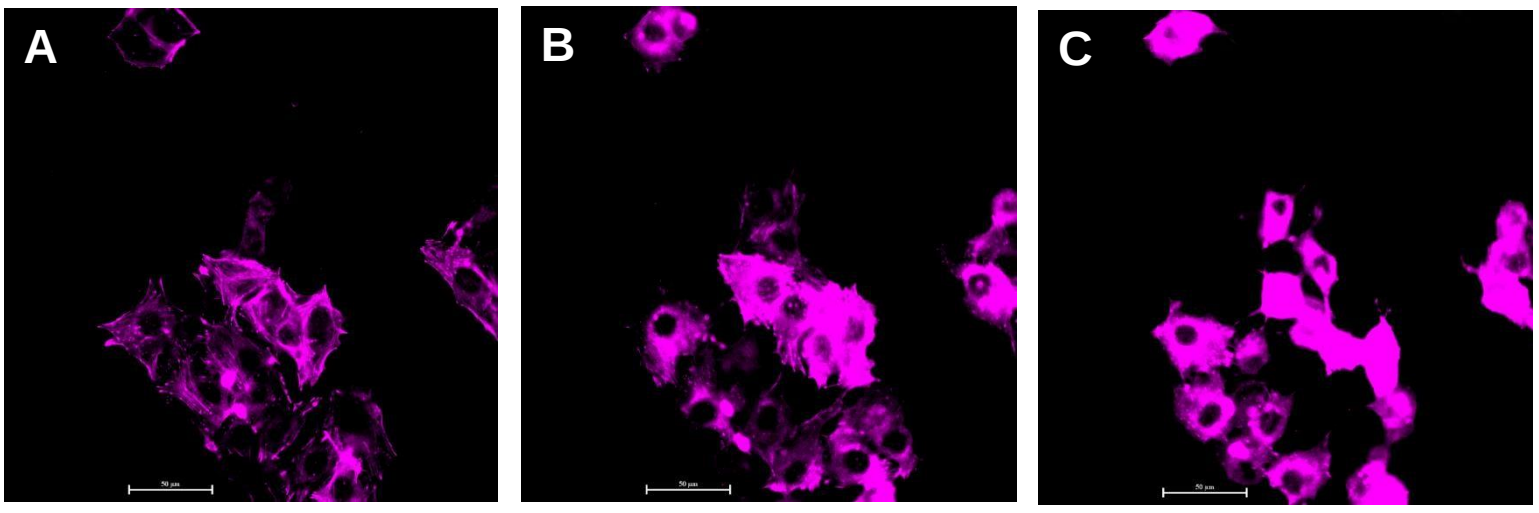


Figure 10. KCNR cells labeled with 40 nM Sir Actin for 3h and treated subsequently with 1.5 μM 1-deoxysphinganine for A) 0h; B) 7h; and C) 13h. The images were obtained by time-lapse fluorescence imaging with a Nikon microscope (Cy5 filter).

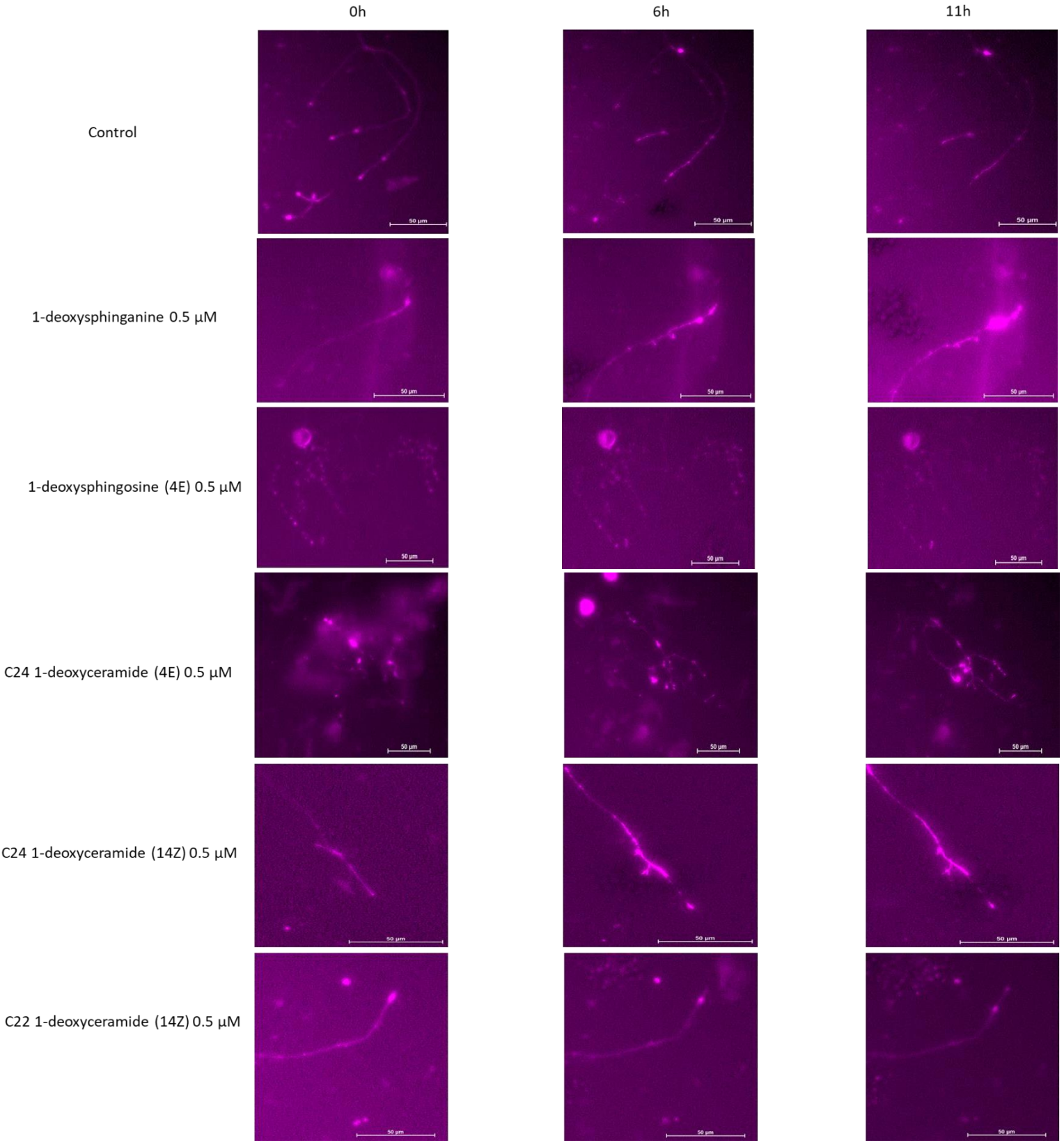

0h
6h
11h
Control
1-deoxysphinganine 0.5 µM
1-deoxysphingosine (4E) 0.5 µM
C24 1-deoxyceramide (4E) 0.5 µM
C24 1-deoxyceramide (14Z) 0.5 µM
C22 1-deoxyceramide (14Z) 0.5 µM
50 µm

Figure 11. DRG primary neurons labeled with 75 nM Sir Actin 5h before treatment with deoxySL. Deoxysphinganine, deoxysphingosine (4E), deoxysphingosine (14Z), C24:1 deoxyceramide (4E), C24:1 deoxyceramide (14Z) and C22 deoxyceramide (14Z), tested at 0.5 µM, 6h and 11h post treatment. The images were obtained by time-laps fluorescent imaging with Nikon microscope (Cy5 filter).

6.6 FTY720, a S1PRs functional antagonist, did not affect neurite swellings in N2a cells

FTY710 is a broader modulator of $S1PR_1$, $S1PR_3$, $S1PR_4$ and $S1PR_5$, which allow us to answer the question in a broader way, whether the deoxySL neurotoxic signaling is mediated by S1PRs.

N2a cells were treated with FTY720 in combination with 0.5 µM or 1 µM of 1-deoxysphinganine for 7h. The neurite swellings were quantified as described above. Our result showed that there were no changes in the reduction of swellings compared to deoxysphinganine treatment (P =< 0.89) **(**

Figure 12).

Treatment combination of deoxysphinganine 0.5 µM with 100 nM of FTY720 showed not significant differences compared to control or treatment with only deoxysphinganine 0.5 µM (P = 0.84). Increasing concentrations of deoxysphinganine 1 µM, in combination with 100 nM of FTY720, did not showed significant differences compared to deoxySL treatment (P = 0.89), but significant differences were seen compared to control and FTY720 100 nM treatment (P =< 0.009).

Nevertheless, these results allowed us to confirm deoxysphinganine toxicity, which resulted in neurite swelling formation. Deoxysphinganine at a concentration of 1 µM, showed significant differences compared to control (P = 0.01), and a dose response was evidenced for deoxysphinganine 0.5 µM and 1 µM.

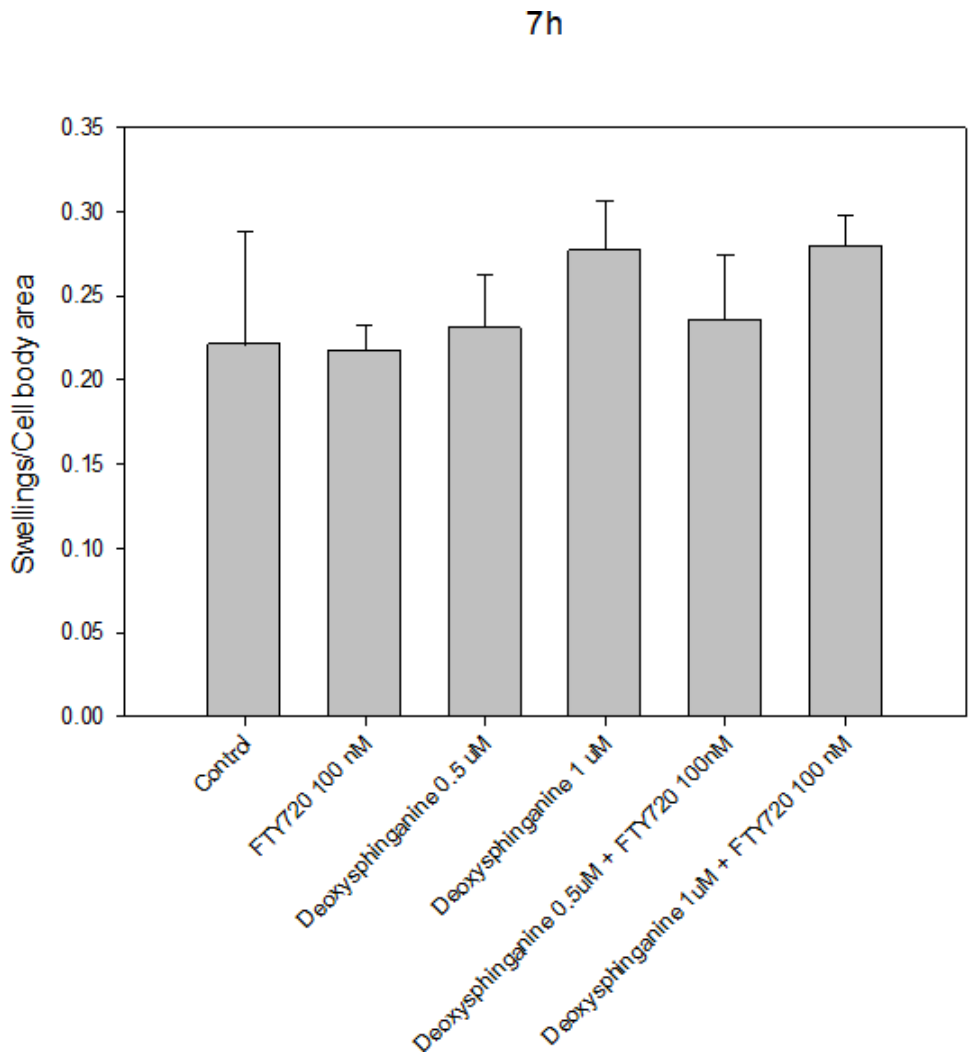


Figure 12. Effect of FTY720 on the neurite swellings in N2a cells. N2a cells were treated with a broad S1PR functional antagonist, FTY720, at 100nM for 7h, alone or in combination with 1-deoxysphinganine (0.5 μM or 1 μM). Neurite swellings were quantified on bright field images taken with a Nikon fluorescent microscope. Quantification was performed using Nikon General analyses software. Technical repeats (2-6) were used to quantify the number of the neurite swellings. The error bars represent standard deviation. Statistical analyses were performed by STATISTICA 10.0 software, using the least significant difference (LSD) test.

7. Chapter 7

7.1 Discussion

In this work, the neurotoxic effects of different species of deoxySL were analyzed using cytotoxicity assay and monitoring the morphological changes such as neurite swellings and rounding of the cells.

Of the 1-deoxysphingoid bases, 1-deoxysphinganine was the most cytotoxic in both studied neuroblastoma cell lines, KCNR and N2a (Fig. 5 A and B). In N2a cells, there was a dose-response to the tested deoxysphingoid bases, showing the most cytotoxic effects at 2 µM for 1-deoxysphinganine, and followed by 1-deoxysphingosine (4E) at 2 µM. However, this was not the case for 1-deoxysphingosine (14Z), where 2 µM treatment resulted in a reduction of cytotoxicity (Fig. 5A). In KCNR cells, the three tested 1-deoxysphingoid bases showed similar cytotoxicity when compared to each other (Fig. 5B). This can be interpreted as for the N2a cells, 7h of 2 µM exposure of deoxySL produced the more membrane damage, but for KCNR 16h of 2 µM treatment other damages could be happening in the cell, such as cell death, as it was observed during the followed-up microscopy and LDH results. Cytotoxicity and cell death introduced by 1-deoxysphinganine have been also shown by Günter et al (Güntert T, 2016), in primary neurons from mouse cerebral cortex, where 0.5 µM for 48h treatment showed cell death, and 2 µM for 24h also proved to be neurotoxic. Compared to our results, we were able to corroborate in neuroblastoma cell lines, that treatment with 0.5 µM of 1-deoxysphinganine resulted in cytotoxicity at earlier time points, 7h and 16h after treatment. However, at 2 µM we cannot corroborate that 1-deoxysphinganine cytotoxicity would increase up to 24h after treatment as Günter et al suggested. Based on our results in KCNR cells treated with 2 µM 1-deoxysphinganine (Fig. 5B) there was a reduction of cytotoxicity after 16h of treatment.

Additionally, there is into consideration that N2a and KCNR, even both being neuroblastoma cell lines, do not present an identical metabolism or biology. Analyses of our cytotoxicity assay results in N2a cells showed a dose-response for 1-deoxysphinganine and 1-deoxysphingosine (4E) treatments, but not for the treatment with 1-deoxysphingosine (14Z). As mentioned before, in KCNR cells, none of the deoxySL species showed an increase in the cytotoxicity at 2 µM. Those results can be attributed to the difference in the exposure time, since for KCNR cells were exposed longer (16h), or because unlike N2a cells, KCNR were not differentiated, resulted in continued proliferation of the control affecting the readout of toxicity, which is not the

case in the differentiated N2a cells. Despite the possible differences in cytotoxic mechanisms, both cell lines showed cytotoxicity, when exposed to deoxysphingoid bases.

In N2a cells, the neurotoxic effect of deoxySL was assessed by quantification of the neurite swellings. We observed significant fluctuation in the swellings number within the treatment groups. Depending on the time point or the concentration tested, a different species of deoxySL showed the highest number of neurite swellings. In addition, at the earlier time points, a general increase of the neurite swellings was evident for all the tested deoxySL species, whereas at later time points the number of neurite swellings did not follow a dose or time responses (Fig. 7 (B)). At the later time point (above 7h), almost all the tested deoxySL species showed a trend of reduction in the number of the neurite swellings compared to the earlier time points or to the control. One example is the result of 1-deoxysphinganine treatment (Fig. 7 (D)). This reduction of the number of the neurite swellings at the later treatment time points can be attribute to the loss of neurites, due to the long exposure of treatment. This result is also in agreement with the results with cytotoxicity assay (Fig. 5 (A)).

Our results in differentiated N2a cells, showed that treatment with individual deoxySL species led to the increase in neurite swellings formation either at a certain concentration, or at a certain time point (Fig 7. A, B, C and D), suggesting that neurite swellings are a transient toxic effect of deoxySL treatment in N2a cells. This also suggests that depending on the time point, different intracellular processes might be occurring as a response to the individual deoxySL, leading to neurite swellings.

Differences between the two positional isomers for 1-deoxysphingosine (4E) and 1-deoxysphingosine (14Z) were seen in the cytotoxicity results and in their ability to trigger the formation of neurite swellings.

There were also differences between the two 1-deoxysphingosine moiety positional isomers, (4E) and (14Z), in 1-deoxyceramides, suggesting that the position of the double bond not only has repercussions in the biophysical properties as it has been proved before, but also that their toxic effects can differ between deoxySL positional isomers (Regula Steiner, 2016), (Santos, 2021).

The neurotoxic effects of deoxySLs have been mostly studied for 1-deoxysphinganine (Wilson ER, 2018), (Penno et al., 2010), (Alecu et al., 2017). In primary neuronal cultures, the most characteristic toxic effects of 1-deoxysphinganine were reduction in neurite length, decrease in neurite outgrowth, mitochondrial fragmentation, and irregular

distribution of mitochondria (Penno et al., 2010), (Alecu et al., 2017). Those studies showed that the toxic effect of 1-deoxysphinganine is dose-dependent (Wilson ER, 2018), (Penno et al., 2010), (Alecu et al., 2017). In addition to 1-deoxysphinganine studies, a study with 1-deoxysphingosine (4E) in primary DRG neurons also showed neurite swellings, reduced neurite outgrowth, and neurite retraction (Becker et al., 2020).

The determination of whether a particular deoxySL species is responsible for the toxicity has become a challenging task. As (Haribowo et al., 2019) concluded in a yeast model, deoxySL toxicity depends on the total deoxySL levels. The authors concluded that toxicity of each individual 1-deoxySL species can vary depending on the conditions. This is in agreement with our results that toxicity of the individual 1-deoxySL species varies depending on the conditions or the assays it was tested. This was true for both, 1-deoxysphingoid bases and 1-deoxyceramides. However, our experiments were not designed to assess whether the overall toxicity is due to a sum of several species or by a specific deoxySL species.

One of the first research that showed that deoxySL treatment can lead to the alteration of actin fibers was by (Cuadros R, 2000). In this study the marine compound Spisulosine (1-deoxysphinganine), was shown to disrupt and reduce the stress fibers in Vero cells, leading to changing cell shape, i.e. cell bodies became rounded. Our results in KCNR cells showed a similar response to deoxySLs, presented by morphological changes like the loss of cells interconnections, reducing the neurites and its complexity (Fig. 10). Labeled KCNR cells with SiR Actin, an actin visualization dye, showed a re-organization of the actin, specifically, disruption of the stress fibers and presenting a more cortical appearance of the actin label (Fig. 10).

Cytoskeleton as a downstream target of deoxySL, have also been analyzed in other model systems, e.g. yeast, mammalian insulinoma and fibroblasts cell lines, primary cortical and DRG neurons. The observed changes included disruption of F-actin organization, neurofilament formation, reduction in actin fibers and actin concentration in the perinuclear area (Haribowo et al., 2019), (Penno et al., 2010), (Güntert T, 2016), (Zuellig RA, 2014), (Alecu et al., 2017), (Becker et al., 2020). Actin re-organization was also shown in our current study in the neurites of primary DRG neurons after deoxySL treatment (Fig. 11). We mostly observed neurite swellings, accumulation of actin at the neurite tips and actin dynamic movement along the neurite (data not shown). (Güntert T, 2016) described in primary cortical neurons, the loss of neurites. Additionally, (Becker et al., 2020), showed neurite swellings in primary DRG neurons treated with 1-

deoxysphingosine, and actin label movement towards the soma, instead of the neurite edges, concluding that actin transport is also disrupted due to the deoxySL treatment.

In the same study, DRG neurons were treated with S1P in combination with 1-deoxysphingosine, which showed a rescue in the number of neurite swellings, indicating that S1P signaling and deoxySL neurotoxicity can have interfering downstream targets. In order to address the question, if deoxySL neurotoxic signaling is through S1PRs, we tested the broad functional antagonist of S1PRs, FTY720, for its ability to attenuate the deoxySL toxicity. FTY720 is a functional antagonist of four of the five S1PRs, $S1PR_1$, $S1PR_3$, $S1PR_4$ and $S1PR_5$ (Mandala et al., 2002). Our results showed no reduction in the swellings, compared to neither control nor to 1-deoxysphinganine treatment (Fig 12). With these results we can only speculate whether the concentration tested was not sufficient to have an effect. It is likely that specific antagonists for each of the S1PRs need to be evaluated to address if deoxySL neurotoxic effects are mediated by the S1P signaling pathway.

FTY720 modulation of S1PRs has been object of study because its therapy potential due to its immunomodulatory property (Huwiler & Zangemeister-Wittke, 2018) (Huwiler & Zangemeister-Wittke, 2018). It has been successfully implemented as a drug for multiple sclerosis (Kandjani, 2023) and continues being studied for Alzheimer's Disease, (Jung, 2023), (Leßmann, 2023). In addition, other studies have target S1PR1 via FTY720 modulation to reduce neuropathic pain in patients under taxane and bortezomib chemotherapy (Janes et al., 2014), (Stockstill et al., 2018). The modulation of S1PRs has a potential to be a therapeutic target in neurodegenerative disease and it is worth studying if the deoxySL toxicity is through S1PR receptors.

8. Conclusions and recommendations

8.1 Conclusions

The neurotoxic effects of the individual deoxySL were evaluated under the parameters of cytotoxicity and morphological changes.

In both neuroblastoma cell lines, KCNR and N2a, the most cytotoxic deoxysphingoid base is 1-deoxysphinganine.

For the morphological evaluation, differentiated N2a cells treated with individual deoxySL resulted in neurite damage such as neurite swellings and neurite loss. The neurite swellings in differentiated N2a appeared to be a transient effect of deoxySL treatment. Morphological changes for KCNR cells due to the deoxySL treatment, resulted in cell rounding.

DeoxySLs affect actin in neuroblastoma cell lines and in primary DRG neurons. KCNR cells labeled for actin showed disruption of the actin stress fibers, suggesting actin re-organization. In the DRG neurons, deoxySL treatment caused neurite swellings, actin accumulation at the tips of the neurites, and abnormal movement along the neurites.

The FTY720 modulator treatment showed no rescue of the neuritic damage by deoxySLs, leaving inconclusive results whereas S1P signaling is a mediator of the deoxySL toxic effects.

Finally, the neurotoxic effects of individual deoxySL have been established for a broader range of individual deoxySLs, including three different 1-deoxyceramides and the two positional isomers of 1-deoxysphingosine. However, additional studies are still required to determine the most toxic individual deoxySL. Continued studies are required as, to further comprehend the biological and signaling mechanisms involved in deoxySL neurotoxicity. This is necessary to provide knowledge for future therapies that can attenuate neurotoxicity in diseases like in the case of TIPN, and other dieses where deoxySLs have shown toxicity.

8.2 Recommendations

In order to continue studying the neurotoxic effects of deoxySLs and their downstream mechanisms involved, it is considered pertinent to contemplate certain characteristics, described below.

While neuroblastoma cell lines resemble neuronal cells and are a useful model to initially test deoxySL, it is recommended to evaluate and quantify the neurotoxic effects of individual deoxySL in primary neuronal cells, such as DRG or motor neurons.

To further understand the cellular mechanisms of deoxySL toxicity and to be able to answer the question which species of deoxySL is the most toxic, it is recommended to include a lipidomics approach. A Lipidomcs approach can address the question whether to the toxicity is due to 1-deoxysphingoid base or if it has to be converted in 1-deoxydihydroceramide or 1-deoxyceramide to be toxic.

To further understand the effects of deoxySL on the actin structures, it might be appropriate to examine if a long time of exposure to the cells to SiR-Actin could be also toxic, which might compromise the quantification of toxicity of deoxySL in the live imaging experiments. As a possible alternative approach if necessary to include a fixation of the cells and the use of another fluorescence label of actin, such as Phalloidin. Additionally, it would be pertinent for the study of the microfilaments alterations in the cytoskeleton, to evaluate if the deoxySLs have a toxic effect in the microtubules.

More studies with other modulators of S1PR are strongly suggested, including using specific antagonist for the individual S1PRs in combination with deoxySL treatment and using silencing by siRNA specific for each S1P receptor before deoxySL treatment, to determine if the neurotoxic effects of deoxySL are through the S1P signaling.

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