Sphingolipid signaling as a target in photoreceptor degeneration: an *in vitro* model for therapeutic strategies in Retinitis Pigmentosa

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Ai miei genitori,
per essere, da sempre, il mio porto sicuro.

A Chico
per tutto l’amore con cui, quotidianamente,
riempie il nostro mondo.

Si diventa molto più fortil
là dove ci si spezza…
ABSTRACT

**Background:** Sphingolipids are a broad class of molecules with the double role of cell membranes components and intra/extra cellular signal mediators, controlling proliferation, differentiation, stress survival and apoptosis. Ceramide (Cer), the core of complex sphingo lipids, can undergo deacylation giving rise to sphingosine and sphingosine-1-phosphate (S1P). This latter exerts a pro-survival and proliferative activity, as opposed to Cer, which promotes cell cycle arrest and apoptosis. Retinal degeneration and in particular Retinitis Pigmentosa (RP) are associated to Cer accumulation and cell death induction. In a murine model of RP (rd10 mutant mice), it has been demonstrated that inhibition of Cer synthesis and accumulation, blocking serine palmitoyltransferase (SPT) with Myriocin, rescues retinal photoreceptors, especially cones, from degeneration.

**Aim:** Our aim is to target sphingolipid metabolism to reduce retinal photoreceptor damage, in a cone photoreceptors cell line. In particular, promoting S1P intracellular increase by S1Plyase inhibition and Cer depletion through SPT inactivation.

**Methods:** Murine 661W cone-like cell line were treated with 75µM 2-acetyl-4(5)-(1(R),2(S),3(R),4-tetrahydroxybutyl)-imidazole (THI), an inhibitor of S1Plyase, for 2 hours; next, cells were starved and treated with 1mM H₂O₂ for different times. Cell growth curve was determined by MTT assay and viability with Trypan blue. TUNEL assay and FRAP test were employed to verify, respectively, apoptosis degree and antioxidant-intrinsic power. Sphingolipid intracellular amounts were measured through LC-MS analysis. ERK1/2 and Akt/PKB phosphorylation, Bcl-2/Bax ratio and Nrf2 expression was evaluated by Western blotting with specific antibodies. Real time-PCR was performed to establish HO-1 and S1P receptors transcript changes upon THI and oxidative stress treatments. Exogenous S1P (100nM) and Myriocin (10µM) were also employed, respectively 1 hour and 5 hours before H₂O₂, to antagonize oxidative stress effect on photoreceptors. Lastly, we screened 30 new synthetic compounds to determine their ability in inhibiting SPT, through an enzymatic activity assay.

**Results:** We show that enhanced stability of S1P, obtained through THI administration, reduces inhibitory starvation and H₂O₂ effect on cell proliferation and viability. In particular, through THI ability to reverse stresses-induced ERK1/2 dephosphorylation and Akt phosphorylation on Ser473. We focused our investigations on oxidative stress, finding that THI counteracts H₂O₂-induced apoptosis increasing Bcl-2/Bax ratio and antioxidant-intrinsic power modulating Nrf2/HO-1 pathway. Furthermore, THI differentially induces S1P receptors transcript expression, showing the major effect on S1P4 and S1P5. In addition, low exogenous S1P improves 661W proliferation, viability and antioxidant response, whereas higher concentration leads to S1P receptors saturation. Myriocin treatment, either alone or in combination with THI, rescues photoreceptors from H₂O₂-induced oxidative stress and drastically reduces all Cer pools. Simultaneously, we selected three compounds with inhibitory activity on SPT, showing an IC50 values ranging from 17.71µM to 40.41µM.

**Conclusions:** We conclude that S1P stabilization and, in general, sphingolipid metabolism manipulation can be considered a therapeutic target in order to
promote photoreceptors survival under different stress conditions, such as oxidative stress.
RIASSUNTO

**Introduzione:** gli Sfingolipidi sono una vasta famiglia di molecole con il duplice ruolo di componenti strutturali di membrane e mediatori intra ed extra cellulari. Sono implicati nel controllo di proliferazione, differenziazione e sopravvivenza cellulare ma anche di fenomeni legati alla morte, tra cui l’apoptosi. Ceramide (Cer), alla base degli sfingolipidi complessi, può andare incontro a deacilazione generando sfingosina e sfingosina-1-fosfato (S1P). Quest’ultimo è considerato un mediatore di sopravvivenza e proliferazione cellulare, in opposizione a Cer, che promuove arresto del ciclo ed apoptosi. La degenerazione retinica ed, in particolare la Retinite Pigmentosa (RP) sono associate all’accumulo di Cer ed all’induzione di morte cellulare. L’inibizione della sintesi e dell’accumulo di Cer, inattivando la serina palmitoiltransferasi (SPT) attraverso l’utilizzo di Miriocina, è stata dimostrata essere coinvolta nel recupero morfologico e funzionale dei fotorecettori retinici, in particolare dei coni, in un modello murino di RP (mutante rd10).

**Scopo:** Il nostro scopo è di intervenire sul metabolismo degli sfingolipidi per ridurre il danno di fotorecettori retinici, attraverso l’utilizzo di una linea cellulare di fotorecettori coni. In particolare, promuovendo l’aumento di S1P intracellulare, tramite l’inibizione dell’enzima S1P liasi, e la riduzione di Cer attraverso l’inattivazione di SPT.

**Metodi:** le cellule murine 661W, una linea di fotorecettori (coni), sono state tratte con 2-acetil-4(5)-(1(R),2(S),3(R),4-tetraidrobutil)-imidazolo (THI) 75µM, un inibitore dell’enzima S1P liasi, per 2 ore; quindi, le cellule sono state sottoposte a depravazione di siero e trattate con H₂O₂ 1mM per differenti lassi di tempo. La curva di crescita di queste cellule è stata determinata attraverso il saggio MTT e la vitalità con Typan blue. Il saggio TUNEL e il FRAP test sono stati impiegati per verificare, rispettivamente, il grado di apoptosi e il potere antiossidante intrinseco dei fotorecettori. I contenuti intracellulari di sfingolipidi sono stati misurati attraverso l’analisi LC-MS. La tecnica del Western blotting, attraverso l’impiego di anticorpi specifici, è stata utilizzata per valutare la fosforilazione di ERK1/2 e Akt/PKB, il rapporto tra Bcl-2/Bax e l’espressione proteica di Nrf2. Attraverso Real time-PCR, le modulazioni nell’espressione dei trascritti di HO-1 e dei recettori di S1P sono stati valutati in seguito a stress ossidativo e trattamento con THI. S1P esogeno (100nM) e Miriocina (10µM) sono stati impiegati, rispettivamente 1 ora e 5 ore prima H₂O₂, per antagonizzare l’effetto dello stimolo ossidativo sui fotorecettori. In fine, abbiamo esaminato 30 composti di nuova sintesi per determinare la loro capacità inibitoria su SPT, attraverso un saggio di attività enzimatica.

**Risultati:** i nostri risultati mostrano che l’aumentata stabilità di S1P, ottenuta tramite l’utilizzo di THI, è in grado di ridurre l’effetto inibitorio sulla proliferazione e la vitalità cellulare, dato dalla depravazione di siero e dal trattamento con H₂O₂. Il pretrattamento con THI, infatti, antagonizza la de-fosforilazione di ERK1/2 e la fosforilazione di Akt su Ser473, indotte dagli stimoli di morte utilizzati. Abbiamo, quindi, focalizzato la nostra ricerca sugli effetti dello stress ossidativo, evidenziando che THI si contrappone all’induzione dell’apopstosi tramite l’aumento del rapporto Bcl-2/Bax e influenza il potere antiossidante intrinseco dei fotorecettori modulando la via di segnale di Nrf2 e HO-1. Inoltre, THI aumenta l’espressione dei recettori di S1P, mostrando un effetto maggiore sui recettori S1P4 e S1P5. Inoltre,
abbiamo osservato che basse concentrazioni di S1P migliorano la proliferazione, la vitalità e la risposta antiossidante di cellule 661W, mentre alte concentrazioni portano alla saturazione dei recettori. Il pretrattamento con miriocina, sia da solo che in combinazione con THI, protegge i fororecettori dallo stress ossidativo e riduce drasticamente i livelli intracellulari di tutte le specie di Cer. Parallelamente, abbiamo selezionato tre composti con attività inibitoria su SPT, determinando i rispettivi valori di IC50 che sono risultati essere compresi tra 17,71µM e 40,41µM. 

**Conclusioni:** possiamo quindi concludere che la stabilizzazione di S1P e, più in generale, l’azione sul metabolismo degli sfingolipidi è da considerarsi un target terapeutico per promuovere la sopravvivenza fotorecettoriale in differenti condizioni di stress, tra cui lo stress ossidativo.
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SIMBOL LIST
3-KdhSph 3-ketodihydrosphingosine
3-KSR 3-ketosphinganine reductase
ABCC1 ATP binding cassette C1
AC (aCDase) Acid Ceramidase
adRP Autosomal dominant RP
Akt/PKB Akt/Protein kinase B
AMD Age-related macular degeneration
arRP Autosomal recessive RP
ASMase (aSMase) Acid sphingomyelinase
Bax BCL (B Cell Lymphoma)-Associated X
BDNF Brain derived neurotrophic factor
BH3 Bcl-2 homology 3
C1P Ceramide-1-phosphate
CA Carnosic acid
CAPE Caffeic acid phenethyl ester
Cer Ceramide
Cer.s Ceramides
CERK Ceramide kinase
CERK-L Ceramide kinase-like protein
CerS Ceramide synthase
CerSs Ceramide synthases
CERT Ceramide transport protein
CFTR Cystic fibrosis transmembrane regulator
cGMP Cyclic guanosine monophosphate
ChR2 Channelrhodopsin-2
CNTF Ciliary neurotrophic factor
CPE Ceramide phosphoethanolamine
CRalBP Cellular retinaldehyde binding protein
DES Dihydroceramide desaturase
DHA Docosahexaenoic acid
dhCer Dihydroceramide
dhCerS Dihydroceramide synthase
dhCers Dihydroceramides
dhCerSs Dihydroceramide synthases
dhSph Dihydrosphingosine
EDG Endothelial differentiation gene
ER Endoplasmatic reticulum
ERG Electroretinography
ERK Extracellular signal-regulated kinases
ES Electrical stimulation
GalCer Galactosyl Ceramide
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GCase Glucocerebrosidase
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<tr>
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<tr>
<td>GCS</td>
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<tr>
<td>GDNF</td>
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<tr>
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<td>Mitogen-activated protein kinase</td>
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<td>PDE</td>
<td>Phosphodiesterase</td>
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<tr>
<td>PE (EA1P)</td>
<td>Phosphoethanolamine</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment epithelium derived factor</td>
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<tr>
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<td>Protein kinase C</td>
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<td>Pyridoxal 5'-phosphate</td>
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<td>PM</td>
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<tr>
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<tr>
<td>PRE</td>
<td>Purple rice bran extract</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
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<td>Retinal ganglion cells</td>
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<td>RHO</td>
<td>Rhodopsin</td>
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<td>Reactive oxygen and nitrogen species</td>
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<td>RP</td>
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<td>Retinal pigment epithelium specific protein 65kDa</td>
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<td>S1PRs</td>
<td>Sphingosine-1-phosphate receptors</td>
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<td>SD</td>
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<td>Sphingomyelin</td>
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<td>Sphingosine</td>
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<td>SPLs</td>
<td>Sphingolipids</td>
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<tr>
<td>SPP</td>
<td>Sphingosine-1-phosphate phosphatase</td>
</tr>
<tr>
<td>SPT</td>
<td>Serine palmitoyltransferase</td>
</tr>
<tr>
<td>sSMase</td>
<td>Secretory sphingomyelinase</td>
</tr>
<tr>
<td>TES</td>
<td>Transcorneal electrical stimulation</td>
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<tr>
<td>THI</td>
<td>2-acetyl-4(5)-(1(R),2(S),3(R),4-tetrahydroxybutyl)-imidazole</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<td>XLRP</td>
<td>X-linked RP</td>
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1. INTRODUCTION
1.1 Retina

The retina, or eye nervous tunic, is placed in the posterior segment of the eye, between the vitreous body and the vascular tunic. It is a multilayer structure mostly comprised of neural cells and crossed by Müller cells, representing the retinal glia.

It is the neural portion of the eye and belongs to the central nervous system, although its peripheral anatomic position. It structurally arises from the diencephalon, which is connected through the optic nerve, that refolds originating the optic vesicle, and consequently the optic cup: whose inner wall corresponds to the retina whereas the outer becomes the pigment epithelium (RPE). The epithelium is made up of a tight-junction, connected, single cell layer in close contact with the external retinal neurons, photoreceptors, and separated from the choroid by the Bruch's membrane, the blood-retina barrier. RPE apical membrane and processes surround the light-sensitive outer segments of photoreceptors establishing a tight structural and functional relationship. Because of high melanin content, it represents a sort of shield for entering light; furthermore, its thigh relation with photoreceptors involves it in several metabolic activities with critical relevance in photoreceptors survival, such as: release of neurotrophic factors and nutrients and daily phagocytosis of outer segments to prevent retinoids derivates and oxidized lipid accumulation, in RPE cells also visual cycle occurs [1].

Five are the typologies of neurons present in the retina. Starting from the pigment epithelium we find: photoreceptors, the light-sensitive cells; bipolar cells and ganglion cells (RGCs), whose axons bundle to form the optic nerve. The nervous signal arises in photoreceptors after light stimulus and goes through this three-neurons chain, crossing the optic nerve, directly to the visual cortex in the central nervous system. The other two kinds of neural cells are the horizontal and the amacrine; they are, respectively,
responsible for lateral interactions of photoreceptor-bipolar cells and bipolar-ganglion cells synapses, originating the so-called ribbon synapses. The neural retina can be divided into six layers distinguished by the kind or the structure of cells that it houses: photoreceptors outer segments layer; outer nuclear layer, with photoreceptors nuclei; outer plexiform layer, where photoreceptors make synapse with bipolar and horizontal cells; inner nuclear layer, containing bipolar, horizontal and amacrine nuclei; inner plexiform layer, where there are the synapses of photoreceptors or amacrine cells with ganglion cells and finally ganglion cell layer, occupying from their nuclei (Fig. 1.1) [2].

**Fig. 1.1: Schematic representation of neural retinal cell layers.** Modified from: [www.webvision.med.utah.edu](http://www.webvision.med.utah.edu)

### 1.1.1 Photoreceptors

The photoreceptors are the retinal neurons, where the phototransduction cascade is activated. Their cellular structure could be described dividing it into two major parts: an outer segment, specialized in detecting photons signal and constituted of stacks of disk membranes in which cromophores
are localized, and an inner segment, containing the nucleus and other cellular organelles necessary for cell metabolism.

Photoreceptors can be distinguished in two classes: rods and cones. Their complementarity is described in several traits in which they differ: number, retinal distribution, structure, function and sensitivity to light.

Due to their sensitivity to light, rods, with their slim and protracted structure, are able to detect a single incoming photon and therefore their function consists in allowing vision under dark-dim conditions at night (scotopic vision). On the other hand, cones, robust conical-shaped and shorter than rods, are up to 100-fold less sensitive than rods but show an extraordinary ability in adjusting their sensitivity and are fundamental for colour perception in our visual image (photopic vision). Their specific and opposite roles, bring to rods saturation in bright light and to inactivation of cones in darkness. [3]. They differ also in number and retinal distribution, cones are mainly concentrated, organized in a hexagonal mosaic, in the central area, the fovea, and do not exceed 6-7 million of cells. On the other hand rods are distributed all across the retina and, even if they maintain a regular architecture, they do not follow a real defined distribution. Rods are much more numerous, they can reach a total of up to 120 million of cells [4].

### 1.1.2 Phototransduction

Phototransduction is the signal transduction pathway whereby incoming light is transformed in electrical response. The key proteins starting this mechanism are retinal chromophores, rhodopsin in rods and three different wavelength sensitive-opsins in cones, long wavelength sensitive for red (L-cones), middle wavelength sensitive for green (M-cones) and short wavelength sensitive for blue (S-cones). Absorption of a photon by retinal cromophores cause the isomerization of 11-cis retinal to all-trans retinal and the start of the phototransduction cascade. This conformational change
brings to the dissociation of the prostatic group from the opsin, that binds and stimulates a GTP binding protein, transducin, inducing the activation of its $\alpha$-subunit. The activated transducin subunit binds to the enzyme cGMP phosphodiesterase (PDE) that gradually hydrolyzes cytoplasmatic cGMP and leads to the closure of cGMP-gated channels on the plasma membrane (PM) (Fig. 1.2). These channels maintain a steady inward current in darkness (“dark current”) and depolarize the cell sufficiently (dark membrane potential at $\sim 30$ mV) to sustain synaptic-transmitter (glutamate) release. The light-induced closure of the cGMP-gated channels produces a reduction of positive ions concentration ($Na^+$ and $Ca^{++}$) inside the outer segment and a consequent membrane hyperpolarization. The generated electric current crosses the photoreceptor body up to the synaptic terminal, reducing or stopping glutamate release and secondary neurons inhibition [5].

**Fig. 1.2: Schematic representation of phototransduction cascade.** Original source: Yau et al 2009

Along with phototransduction, visual cycle occurs in RPE photoreceptors-adjacent cells. This fundamental process implicated in cromophores recycling, covers an important role, not only in guaranteeing a prompt
transformation of all-\textit{trans} retinal back in 11-\textit{cis} retinal, necessary for an efficient response to light, but also in protecting photoreceptors against bis-retinoids and adducts possible accumulation [6].

\textbf{1.1.3 Retinal degenerations: Retinitis Pigmentosa}

Due to its complexity, the retina is exposed to several kind of degenerations, generally distinguishable by the first kind of cell layer affected but often extended to others retinal structures. This underlies how critically important is the equilibrium between all cells that constitute the retina. Some of the most studied pathologies are linked below.

Age-related macular degeneration (AMD), in which photoreceptors placed in the macula, the central area of the retina, start to degenerate in later-life, seems to be related both with neovascularization and RPE dysfunctions that leads to the accumulation of extracellular deposits between epithelial cells and Brunch’s membrane. This induces the block of nutrients and regulatory substances fluxes, which are vital for RPE and photoreceptor survival. Glaucoma, characterized by an elevation of intraocular pressure (IOP), shows a degeneration of RGCs and optic nerve head, rapidly translated in loss of visual fields. Diabetic retinopathy is tightly connected with neovascularization, caused by hyperglycaemia-induced microvascular occlusion. As well as in AMD, new vessels invade RPE and branch out inside the neuroretina [7]. Retinitis Pigmentosa (RP) is one of the most common forms of inherited pathologies that affects retinal tissues and, probably, the most complex and heterogeneous studied. RP includes a group of inherited retinal disorders, in which loss of photoreceptors and retinal pigment deposits are observed. It, characteristically, starts with rod loss, linked to poor night vision, and then evolves in progressive cone degeneration causing the reduction of mid-peripheral field of vision. Still-active area, gradually, shrinks into a small central region corresponding to
macular cones and defining the typical tunnel vision described in these kinds of pathologies. Final-point patients lose the central vision in later-life and can reach legal and, at last, total blindness. RP could be classified in three wide groups: simple, when only eye dysfunctions are identified; syndromic, in the case of, also, non-ocular disease are implicated and systemic, related to pathologies in which retinal degeneration becomes less relevant than other organs involvement. Some of the most studied syndromes linked to RP are Usher’s syndrome, Bardet-Biedl syndrome, Leber’s congenital amaurosis and Refsum’s disease [8] [9].

1.1.3.1 Genetic mutations

Three are the main kinds of inherited transmission in RP, almost of monogenic mutations (deletions, insertions or substitutions): autosomal-dominant (20-25%), autosomal-recessive (15-20%) and X-linked (10-15%). The residual percentage is represented by non-genetic factors, non-Mendelian inheritance (for example mitochondrial or de novo mutations) and complex inheritance. Some patients present digenic RP (one modification in RDS gene, encoding peripherin, and another in ROM1 gene, encoding retinal outer segment membrane protein 1) [10]. Since 1984, when the first polymorphism was associated to RP [11], more than 50 genes, linked to non-syndromic RP, were mapped and identified (Tab. 1.1).
<table>
<thead>
<tr>
<th>RP Category</th>
<th>Total Genes and Loci</th>
<th>Identified Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autosomal dominant (adRP)</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Autosomal recessive (arRP)</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>X-linked (XLRP)</td>
<td>5</td>
<td>2</td>
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</tbody>
</table>

**Tab. 1.1: Number of genes associated to non-syndromic retinitis pigmentosa.** Original source: [https://sph.uth.edu/retnet/sum-dis.htm#D-graph](https://sph.uth.edu/retnet/sum-dis.htm#D-graph)

Genetic mutations resulting in photoreceptors degeneration can affect genes implicated in several functions: renewal and shedding of outer segment, as mutation in peripherin/RDS gene, one of the earliest protein linked to RP [12, 13]; visual transduction cascade, as mutated phosphodiesterase catalytic subunits α (PDE6A) and β (PDE6B) and five different mutations of rod cGMP-gated channel, both kind of mutations related to arRP; metabolism of retinol, as mutation in cellular retinaldehyde-binding protein (CRalBP) or in retinal pigment epithelium protein (RPE65) [14]. One of the most studied retinal protein is the rhodopsin (RHO), the first found to be mutated in RP [15] and the responsible for almost 25% of adRP cases. Many different mutations, divided in six classes, are related to this protein giving rise to patients extensive clinical heterogeneity [16]. Among all numerous mutations causing arRP one is particularly relevant for our study. In RP26, an autosomal recessive locus, it was identified a mutation linked to CERKL gene, encoding a modified ceramide kinase-like protein able to augment the sensibility of retinal cells to apoptotic stimuli. This outcome determines the first link between human genetic modification of sphingolipid metabolism and the onset of retinal degeneration [17].
1.1.3.2 Mechanisms of photoreceptors degeneration

Photoreceptors vulnerability is particularly enhanced by their peculiar physiology and biochemistry, keeping them on a fine line between survival and death, where any defect can be crucial in defining their fate. The first factor that subjects photoreceptors to risks is light exposure. In presence of oxygen, the ionizing effect of light is boosted and reactive oxygen and nitrogen species (RONS) are generated especially by photosensitizing molecules, such as retinoids. Both retinoid derivates and RONS excessive accumulation can cause photoreceptor degeneration. Another source of vulnerability in photoreceptors is represented by their lipid-rich outer segment membranes, especially for very-long-chain polyunsaturated fatty acid (PUFA). PUFAs oxidation gives rise to highly reactive electrophilic aldehydes, including malondialdehyde (MDA) that starts destructive free radical chain reactions. Moreover, retinal protein mutations, especially linked to RHO, cause protein misfolding activating the unfolded protein response (UPR) in the endoplasmatic reticulum (ER). All this events can similarly subject photoreceptors to oxidative stress which represents one of the most studied death stimulus to be counteracted in retinal degenerations [18].

When genetic mutations or external factors initiate one, or more, of those mechanisms, photoreceptors death processes are activated. Which kind of intracellular pathway is, exactly, responsible in leading photoreceptors to degenerate, is still unclear. However, apoptosis is the first death-process documented to be involved in RP mouse model [19]. There is still no doubt of apoptotic events in retinal degeneration, even if many studies report conflicting evidences and show that complex crosstalk and interconnected signals are involved. It is now accepted that apoptosis of photoreceptors can occur through both a caspase-dependent and independent via [20]. Many outcomes maintain that caspases participate in cone photoreceptor
apoptosis, although neither pharmacological inhibition nor genetic manipulation of caspase cascade are able to definitely rescue photoreceptors [21]. The involvement of other proteases linked to apoptotic cell death is investigated. In particular, calpains and caspases involvement in starved-661W cells is studied, showing that calpain-mediated pathway is independent and parallel with respect to caspase activation [22]. As upstream regulators of survival or apoptosis cascade, Bcl-2 family members too are taken into consideration and become the centre of an intriguing issue. On one hand, several studies highlight the importance of Bcl-2 as anti-apoptotic molecule [23] especially, supporting it by successful Bcl-2 overexpression in rescue 661W cells from death [24]. On the other hand, some evidences show Bcl-2 failure in promote salvage signaling [25]. In any case, an increased body of evidence suggests that apoptotic mitochondrial pathway is involved in caspase-dependent or independent apoptosis of retinal cells [26]. At the same time, in a model of retinal detachment, it is showed that apoptosis of photoreceptors can occur through both extrinsic-Fas receptor mediated and intrinsic-mitochondrial cytochrome-c dependent activation [27]. All this studies on photoreceptors apoptosis prove the involvement of a variety of woven, parallel and sometimes contradictory pathways and draw attention, as well, to non-apoptotic mechanisms [28], in particular, pointing up autophagy or its co-occurrence with apoptosis [29]. Within all the mediators able to determine photoreceptors loss or survival through regulation of the abovementioned death-salvage processes, a family of bioactive molecules, sphingolipids (SPLs), is evidenced [30]. In particular, Ceramide (Cer), Sphingosine (Sph) and Sphingosine-1-phosphate (S1P), that play an active role in regulating cell death, survival and differentiation.
1.1.3.3 Therapeutic approaches in RP

To preserve photoreceptors by RP-induced degeneration, many therapeutic strategies have been developed across the years, defining also a systematic classification depending on the degeneration stage (Fig. 1.3) [31].

![Therapeutic approaches in Photoreceptor Degeneration](image)

**Fig. 1.3: Schematic representation of therapeutic approaches on the basis of photoreceptor degeneration stage.** Original source: Trifunović et al 2012.

Ideally, the best way to fight a genetic disease is to correct the causative gene defect. Unfortunately this is not always feasible in RP due to, at least, two main causes: first of all, it should require an early disease diagnosis, allowing to act on genetic mutation in live cells, instead RP is recognized in patients with secondary cone degeneration already started and with all rod photoreceptors almost dead; secondly, a high efficiency to identify the gene of interest has a crucial importance and it is not easy to obtain given the wide heterogeneity of RP mutations. Nevertheless, experimental approaches to gene therapy in RP treatment have advanced in recent years. For instance, in arRP one of the most studied is the MERTK gene that encodes for the human receptor tyrosine kinase MER. MERTK located in the RPE mediates the association between these cells and the
photoreceptor outer segments. Regarding adRP, RHO and RDS/peripherin are the mutated proteins more studied. In both cases the interest is focused on a double gene therapy strategy consisting in siRNA-mediated suppression together with gene replacement [32].
Currently, many studies focus on mutation-independent ways in rescuing photoreceptors as, for example: retinal transplantation, retinal prostheses, retinal optogenetic therapy, electrical stimulation and neuroprotection through neurotrophic factors, pharmacological treatments and nutraceutical approaches. Replacement of degenerated photoreceptors with healthy transplanted tissue or cells, is a well studied practice [33]. In the last years retinal prostheses has become more and more interesting, especially with the development of new biocompatible materials and nanotechnologies [34]. The most promising device designed in this moment is Alpha-IMS, a wireless subretinal implant made up of a 1500-pixel multiphotodiode array, which combines, better than others devices, clinical availability, vision restoration potential and long-term biocompatibility. Clinical trials for this intraocular photodiode implant have already demonstrated increased patient independence by mimicking natural vision under control of eye movement [35]. Retinal optogenetic strategy, in contrast to traditional gene therapy, employ light to control genetically targeted groups of cells. It acts from the inside of the cells, using light sensors to induce ganglion or bipolar cells to become reactive to light. Once activated from light, the sensors create an electrical current that can stimulate the cell [36]. For example, channelrhodopsin-2 (ChR2), a photosensitive cation-selective channel that contains an all-trans-retinal chromophores, is used in a mouse model of RP. When transduced on RGCs, it restores visual responses in mice [37]. Another potential therapeutic approach is low electrical stimulation of the eye that in theory releases neurotrophic substances. Recently, transcorneal electrical stimulation (TES) in RP patients has improved the function of retinal cells, as measured by electroretinography (ERG), and the visual field
Neuroprotection can be employed in two different directions: to straighten survival stimuli and/or to stop death induction. It can consist in a broad variety of treatments: neurotrophic factors [39], as NGF, BDNF, CNTF, PEDF and GDNF; nutraceutical and antioxidant agents, as, for example, vitamin A and its derivatives [40], docosahexaenoic acid (DHA) [41], lutein [42] and taurine [43].

1.1.4 661W: a cone-like cell line

Many kinds of retinal cell lines are available for in vitro studies. Some of the most employed are: ARPE-19, human retinal pigment epithelium [44]; RGC-5, rat retinal ganglion cells [45]; R28, rat glial-neuronal cell line [46]. One in particular is suitable for the investigation of photoreceptors features and behaviours: 661W cell line. It is a cone-derived cell line, transformed by Dr. Muayyad Al-Ubaidi’s group (Department of Cell Biology, University of Oklahoma Health Science Centre, Oklahoma City, U.S.). 661W were cloned from retinal tumours of transgenic mouse line expressing the SV-40 T antigen under control of the interphotoreceptor retinal binding protein promoter (IRBP) [47]. Cells express cone but not rod specific antigens: SV40 T antigen, blue and green pigments, transducin, and cone arrestin. Immunocytochemical detection of blue and green opsins showed distribution throughout the cell, the nucleus included. However, these cells did not express rod-specific antigens, such as opsin and arrestin or rod- and cone-specific proteins such as phosducin, peripherin/RDS, and ROM1. Furthermore, the cells did not express RPE65, a cone and RPE specific protein [48]. 661W cell line is unique in its ability to respond to light stimulation and upon light stress it undergoes cell death, as showed for in vivo retina experiments (www.otd.ou.edu) [49]. Due to their easy handling and distinctive traits, 661W are widely used in ophthalmology research, especially as a model of photoreceptor degenerations subjecting them to
several kinds of stresses. In particular: light damage [49, 50], serum deprivation [22] and oxidative stress by means of several compounds, for example: hydrogen peroxide [51] [29], sodium nitro-prusside [52], sodium iodate [53] and paraquat [54]. They are also employed to investigate retinal cells inter-relations [55-58], cellular toxicity of new treatments [59], [60], and, in general, to better understand death and salvage pathway of cone photoreceptors.

1.1.4.1 Rescuing treatments on stressed 661W cells

Since 661W cone-derived cell line has been recognized as a precious in vitro mean for studying photoreceptors, worldwide scientific works have incremented over years. In particular, the investigation on rescuing treatments represents one of the more examined field, ranging from electrical stimulation to a whole series of different antioxidant agents and nutraceutical compounds. Electrical stimulation (ES) has been shown to promote survival on light-damaged photoreceptor through diminished microglial activation and fortified Müller cells reactive gliosis, which may have great potential in ameliorating photoreceptor damage. ES might change the activation status of microglia and Müller cells by altering transmembrane voltage-gated ion channel activity suggesting that factors of microglia or Müller cells, that can be changed by electrical fields of different strengths, must be involved in this process [61]. DHA, that is the most polyunsaturated fatty acid present in retinal neurons [62], has been demonstrated to rescue 661W cells from oxidative stress, reducing calpain activation, cytochrome c release and caspase-3 action [63]. Unoprostone isopropyl (unoprostone) is a docosanoid, known to bear neuroprotective effects and to activate large conductance Ca^{2+}-activated K^{+} (BK) channels. Unoprostone protects photoreceptors and RPE cells against oxidative- and light-induced damage by a mechanism different from antioxidant activity. In
particular, the protective effect of unoprostone is mediated by BK channel activation, probably on those existing in mitochondria, reducing the stress-induced mitochondrial damage [64]. Purple rice bran extract (PRE), derived from a coloured variety of *Oryza sativa L.* contains three anthocyanins (cyanidin 3-O-glucoside, peonidin 3-O-glucoside, and cyanidin 3-O-gentiobioside) that are supposed to be its active components. PRE inhibition of reactive oxygen species (ROS) production in visible light irradiated-661W cells was demonstrated and proposed to occur by inhibition of p38 and/or JNK activation [50]. Carnosic acid (CA), present in rosemary extract, crosses the blood–brain barrier to exert neuroprotective effects. Recently, it was found to promote survival of 661W photoreceptors and ARPE-19 cells, against H$_2$O$_2$-induced cell death. Accounting for this effect, CA induced nuclear translocation of Nrf2 and then activated transcription of phase II genes [65]. Mandal’s group performs several studies on 661W evaluating the effects of antioxidant agents, especially through food-derived substances. Curcumin, a naturally occurring yellow pigment, isolated from the rhizomes of the plant *Curcuma longa*, was tested for its beneficial properties on 661W stressed with H$_2$O$_2$. Curcumin pretreated cells respond to oxidative stress activating cellular regulatory system such as NF-κB, Akt, Nrf2 and growth factors, which in turn inhibit cellular inflammatory responses and protect photoreceptors [66]. Caffeic acid phenethyl ester (CAPE), an active component of honeybee propolis, was found to activate the antioxidant gene expression pathway in H$_2$O$_2$-challenged 661W cells and in rat retinas, enhancing ERG responses [51]. Interestingly, they evidences that CAPE-altered retinal fatty acid composition probably contributed to the higher ERG responses, confirming the involvement of lipids in optimal retinal function as previously demonstrated [67].
1.2 Sphingolipids

In 1884 J.L.W. Thudichum, a german-born physician and biochemist, wrote “A Treatise on the Chemical Constitution of Brain”. Here he described his studies on isolation and characterization of brain constituents and, for the first time, the backbone sphingolipid: sphingosine. He chose this name such as a parallelism of this enigmatic molecule and a mysterious mythological figure: the Sphinx. Across the years, many other sphingolipids have been described until a real family of bioactive molecules have been defined [68].

1.2.1 Structure

Sphingolipids are a board and heterogeneous class of bioactive molecules, characterized by a common eighteen carbon amino-alcohol backbone, on which different biochemical modifications occur, giving rise to many metabolites. They are amphipathic molecules distinguished for relative hydrophilicity/hydrophobicity degree, strongly influencing individual intracellular localization and portability. In particular, hydrophilic region is represented by the sphingoid backbone, eventually linked to a charged headgroup, meanwhile lipidic portion is composed by N-linked acyl chains, with different numbers of carbons and insaturations. The simplest member, which actually corresponds to the common backbone, is Sph. It can undergo different modifications on C1 and C2 functional groups, in particular, acylation on C2-amino group that discriminates two big categories: sphingolipids with one or two hydrophobic chains. Different acyl chains are added through different ceramide synthases (CerS.s) that consequently generate respective Ceramides (Cer.s). Contemporarily, complexity degree can augment because of reactions on the C1-hydroxyl group. Sphingomyelin (SM) arises from the binding of phosphocholine,
phosphorylation gives ceramide-1-phosphate (C1P), glycosylation, with glucose or galactose, creates the family of complex glycosphingolipids (GSLs). Finally, when acylation does not occur, phosphorylation on the hydroxyl group leads, for example, to S1P [69] (Fig. 1.4).

Fig. 1.4: Structures of principal sphingolipids.

Sphingolipids, as fundamental elements of lipid rafts, structurally constitute and functionally influence cellular membranes. Furthermore, they are intracellular messengers actively involved in cell functions regulation. Their heterogeneity is reflected also in their functions, insomuch as, relative species equilibrium is decisive in directing cell fate, towards death or survival pathways. The most representative example is the dualism between Cer, a death effector, and, in contrast, S1P that promotes cell proliferation and survival [68].

1.2.2 Sphingolipid metabolism

Two major pathways collaborate in the synthesis of sphingolipids: de novo and salvage pathway. The first one starts and ends with non-sphingolipid molecules and is characterized by an enter point, serine palmitoyl transferase (SPT), which gives rise to the first sphingoid-based member of
the family, and an exit point, sphingosine-1-phosphate lyase (SPL) that allow the irreversible degradation of S1P. *De novo* synthesis, occurring in ER, begins with SPT catalysis of cytosolic serine and palmitoyl-CoA condensation, to give 3-ketodihydrosphingosine (3-KdhSph). Since it is reduced to dihydrosphingosine, (dhSph) by 3-ketosphinganine reductase (3-KSR), and acylated by (dihydro)ceramide synthases ((dh)CerSs) with various length acyl chains, to give a wide variety of (dihydro)ceramides ((dh)Cer.s). By means of molecular oxygen and then NAPDH aid, dihydroceramide desaturase (DES) introduces a double bound in the C4-C5 position of dhCer and generates Cer [69].

Cer is now ready to eventually undergo different modifications: phosphorylation (ceramide kinase), glycosylation (glucosyl or galactosyl ceramide synthases) or condensation with a phosphocholine, deriving from phosphatidylcholine (PC), to generate SM (via sphingomyelin synthesis). The latter two pools of sphingolipids represent the main source of Cer deriving from the salvage pathway, which implies its release from complex sphingolipids. Cer recycling synthesis takes place in the acidic subcellular compartments, the late endosomes and the lysosomes. Here specific exohydrolases and Acid sphingomyelinase (ASMase) degrade GSLs and SM to Cer that, in turn, is hydrolyzed by acid ceramidase (AC) to Sph able to exit the lysosomes. SM breakdown can occur also at the PM level by the action of Acid or neutral SMase depending on which side of the membrane the reaction happens and here again Cer is converted to Sph. At this point, all the Sph produced can explicate its intracellular functions or undergo to sphingosine kinases (SphK1-2) phosphorylation to generate S1P. In turn, S1P can act as second messenger, extracellular ligand or be metabolized. Two are the enzymes that can transform S1P, one is sphingosine-1-phosphate phosphatases (SPP) that regenerates Sph and the other one is sphingosine-1-phosphate lyase (SPL) that catalyzes the irreversible
degradation of S1P to phosphoethanolamine (PE, EA1P) and hexadecenal (Hex) (Fig. 1.5) [70].

Fig. 1.5: Summarized pathways of sphingolipid metabolisms. Original source Mullen et al. 2012

1.2.3 Subcellular compartmentalization

Depending on their chemical nature, sphingolipids are free to move in cytoplasmatic and extracellular spaces or are relegated into biological membranes, and linked to specific mechanisms of transport to leave their site of generation. Precisely for this reason, the roles of many sphingolipids are tightly connected with specific subcellular localization of their synthesis enzymes. In particular, their mobility is based on the number of hydrophobic chains and on the charge at neutral PH. Cer, with two aliphatic chains and neutral headgroup, can flip-flop membranes but is unable to move in cytoplasm. It reaches the Golgi apparatus through two different ways depending on which metabolite is required: by the action of transfer protein CERT, if SM generation is needed or using vesicular transport if glucosylceramide (GluCer), or in general GSLs, must be synthesized.
Vesicular transport is also involved in the mobilization of Cer hydrophobic derivate species, SM and GSLs, to the PM, from where they can be addressed to lysosomes for entering the endosomal salvage pathway. Due to more hydrophilic nature, sphingolipids with just one aliphatic chain are soluble in cytosol and can freely move among intracellular membranes. Sph, which does not present any charge, is able to flip-flop membranes meanwhile, its phosphorylated metabolite, S1P, is linked to specific transporters to pass the lipid bilayer [68]. In particular, it is demonstrated that S1P traffic is linked to ATP binding cassette (ABC) members, on one hand ABCC1 that allows its exit [71] and on the other hand cystic fibrosis transmembrane regulator (CFTR), implicated in S1P influx [72].

1.2.4 Ceramide

Cer is one of the most studied sphingolipids and represents the central point of the entire family, it is the backbone of complex members and plays a strategic role in the membrane structure and in the activation of a wide variety of intracellular pathways, depending on cell type and extracellular stimulus. Cer synthesis is catalyzed by a family of ER-localized enzymes, CerS.s, that differ in their selectivity for acyl-CoAs based on the length of the acyl chain. CerS family is composed by six members (CerS1-6) that are differently expressed in tissues and differently react to stimuli, generating the particular Cer specie requested to face the incoming situation [73]. Cer involvement in many cellular process, such as growth arrest, differentiation, apoptosis and senescence makes the regulation of its synthesis and degradation an important target in several pathologies. Depending on the length of the fatty acid added to the sphingoid base, Cer.s can be constituted by 14 to 28 carbons chains, defining several different species involved in different kind of signaling [74]. Cer.s synthesis, that can occur both through de novo and salvage pathway, is induced in
response to stress stimuli such as cytokines (TNF, Fas, nerve growth factor), “environmental” stresses (heat, UV radiation, hypoxia/reperfusion) and chemotherapeutic agents [75]. The intracellular pathways activated by Cer are shown to be directly linked with the ceramide-activated protein phosphatases PP1 and PP2A [76], which is demonstrated to be involved in many Cer.s-induced effects, such as dephosphorylation/inactivation of anti-apoptotic proteins including PKC, Akt and Bcl-2 [77] but also activation of apoptosis inducers as Bax [78]. Cer is also able to activate lysosomal protease cathepsin D [79], kinase suppressor of Ras as mediator of its effects on Ras, Raf, and ERKs [80] and the transcription factor NF-kB through PKC action [81].

1.2.5 Sphingosine-1-phosphate

Since its recognition as an active promoter of cellular growth and survival [82], S1P gains and keeps a central position in several investigations. Nowadays, it is known that this enigmatic sphingolipid has a pivotal role in many pathologies [83] such as cancer, atherosclerosis, inflammation, neurodegeneration, cardiovascular and respiratory affections [83]. It is also involved in tissue and organs development [84], in particular in cardiogenesis [85], limb development [86], neurogenesis [87] and angiogenesis [88]. S1P signaling pathways activation is a very intriguing issue, in particular, attention is drawn on identification and especially discrimination between its effect as second intracellular messenger and/or extracellular receptors ligand [89]. In order to clarify which way is involved in different S1P effects, many studies have been developed, in particular employing pertuxiss toxin as an inhibitor of S1P-specific G protein-coupled receptors (S1PR.s). As intracellular messenger, S1P plays critical roles in calcium regulation [90] and in apoptosis inhibition [91], in particular as anti-apoptotic effector by means of many intracellular pathways. For instance,
intracellular-S1P is demonstrated to influence MAPK family members, which are known to modulate apoptosis both inducing its activation through JNK, inhibited by S1P, and, on the contrary, stopping the death process by ERK double effect on cytochrome c release. In fact, ERK is able to act upstream inactivating pro-apoptotic Bcl-2 family members and downstream inhibiting caspase cascade and its activation is improved by increased intracellular S1P levels [92]. SphK-S1P is also able to counteract apoptosis activating Akt, in hepatocytes [93]. Finally, it is demonstrated that intracellular synthesis of S1P is involved in NF-κB pathway activation, against TNFα effect [94].

On the other side, also S1P action through its surface receptors occupy a pivotal role in cell fate determination, similarly activating the same signaling pathways with respect to those depending on intracellular S1P. It is demonstrated that exogenous S1P can reduces Bax proteins levels in a lymphoblastoma cell line and antisense plasmids against its receptors counteract S1P-anti-apoptotic effect [95]. S1P activation of survival markers, such as ERK, are investigated in hepatoma cells stably expressing two of the five enzymes [96] and in endothelial cells where pertussis toxin blocks this ERK phosphorylation [97]. Furthermore, in this latter cellular type antisense for the first receptor attenuates S1P inhibition on Cer-induced apoptosis [98].

1.2.5.1 Sphingosine-1-phosphate kinases

S1P complexity already starts in its synthesis enzyme, indeed the phosphorylation of Sph is catalyzed by SphK, which exists in two isoforms, SphK-1 and SphK-2 [99] [100]. It is well known that SphK-1 is involved in cell growth induction and apoptosis inhibition [101]. SphK-1 resides in cytoplasm and, after ERK phosphorylation, translocates to the PM [102], where its substrate, PM-associated sphingosine, is located. Generated S1P
can activate intracellular signaling or, due to the PM proximity, easily exits the cell and interacts with its cell surface receptors. SphK-2 also needs to be activated by ERK phosphorylation [103] but, saved this trait, it deeply differs from its close relative in localization, function and influence on Cer contents. Primarily, SphK-2 is found at the level of internal membranes, in particular at ER, mitochondria and nucleus and surprisingly it promotes apoptosis [104]. Which mechanism is behind its action is still unclear but, rather than to its synthesized S1P receptor interaction, it is supposed to depend on an intrinsic characteristic of the enzyme aminoacidic sequence. It is demonstrated that SphK-2 contains a 9-amino acid sequence reminiscent of the Bcl-2 homology 3 (BH3) domain present in “BH3 domain-only” proteins that, it is known to be responsible of their interaction with pro-survival Bcl-2 family members and in consequent induction of apoptosis [105]. Despite that, it has recently been demonstrated that pharmacological inhibition of SphK-2 augments cancer cells sensitivity to apoptosis [106] and its knockout abolishes preconditioning-induced tolerance in ischemia/reperfusion injury [107]. Another important difference between these two enzymes is the influence on apoptotic Cer.s intracellular amounts: SphK-1 induces a decrease of Cer.s, meanwhile SphK-2 promotes their synthesis [99].

All these outcomes open and keep alive an intriguing issue on the correlation between S1P functions and the enzyme isoform that catalyzes sphingosine phosphorylation, hence its site of creation.

1.2.5.2 Sphingosine-1-phosphate receptors

S1P-related cellular effects were associated to its function as intracellular second messenger until the discover of pertussis toxin ability in inhibiting some of its effect in fibroblasts [108]. Since then the outcome of S1P extracellular effects, mediated by surface receptors (S1PRs), were
investigated and deepened over years. In 1998, S1P was identified as a ligand of endothelial differentiation gene 1 (EDG1) product, a GPCR [109] and today the family has expanded with other four members (S1P1-5/EDG) that are accepted to be activated by extracellular S1P [110]. S1PRs add a further degree of complexity to the signaling pathways of this enigmatic sphingolipid for, at least, two reasons: their coupling to different kind of G proteins, activating respective pathways (Fig. 1.6), and their differential tissue and cell lineages expression, that can change depending on the stimulus received and/or the developmental stage. Furthermore, it is demonstrated, especially through investigation on S1P1, that S1PRs can be induced to internalize after acute agonist exposure. Two pathways of internalization are evidenced, the physiological agonist pathway which sees S1P-induced temporary internalization followed by intact receptor recycled to the PM where signaling can restart and superphysiological agonist pathway, linked to S1P-receptors potent ligands such as FTY720-P, in which ubiquitin pathway is activated and internalized receptors degrade in lysosomes [111].

S1P1/EDG1, the first discovered and the most well-studied of the family, is widely expressed in tissues and involved in T- and B- lymphocyte egress from lymphoid organs [112] also in inflammation [113] and tumorigenesis [114]. Like S1P1, S1P2/EDG5 shows a widespread expression. It is involved in increasing vascular permeability, through disruption of adherens junctions between endothelial cells [115] and upregulated by hypoxia in the retina inducing defective neovascularization and inflammatory response increasing cyclo-oxygenase-2 [116]. S1P2 plays pivotal role also in development, immunity, neural functions and cancer [117]. S1P3/EDG3, due to its coordination with S1P1 and S1P2, is barely studied alone. It is shown to play a role in immune reactions [118], in multiple sclerosis [119] and inflammation [120]. S1P4/EDG6 is highly expressed in lymphocytic and hematopoietic cells even if its role in immune modulation is still unclear.
The last receptor and less investigated of the family is S15/EDG8. It shows some traits of difference against the others receptors. It is especially studied in brain and of particular interest is its differential activation in oligodendrocytes depending on their developmental stage. In fact, it mediates process retraction and impedes migration of immature cells whereas regulates survival of mature ones through Akt activation [122]. It is also involved in maintenance of the human brain endothelial barrier, in inhibiting inflammation by NF-κB activation [123] and in cancer [124].

**Fig. 1.6:** Schematic representation of different S1PRs G-protein depending pathways. Original source: [www.caymanchem.com](http://www.caymanchem.com)
1.2.6 The entrance and the exit of the pathway: SPT and SPL

As described above, de novo sphingolipid synthesis starts and ends with no-sphingoid molecules and the two reactions, that respectively initiate the biosynthesis and terminate the catabolism of Cer, are catalyzed by two essential pyridoxal 5'-phosphate (PLP)-dependent enzymes: SPT and SPL (Fig. 1.7). They share a similar mechanism of action, which initiates with the replacement of lysine (K265 in SPT and K311 in SPL), in the Schiff base formed with the aldehyde group of PLP, with serine or S1P. The resulting external aldimine undergo to different modifications; in particular, in SPT it is subjected to a nucleophilic attack on Cα by palmitoyl-CoA thioester and, after the formation of two quinonoid intermediates and other two external aldimines, it becomes 3-KdhSph, causing the internal aldimine restoration. On the other hand, in SPL the external aldimine gives Hex after a retro-aldol cleavage, forms a quinonoid intermediate, another external aldimine and finally release PE to allow the re-formation of the resting state internal aldimine [125].

SPT is the first enzyme of the biosynthesis, localized in ER membrane-associated complex. It is composed by 3 subunits: LCB1, LCB2 and LCB3. The last one is recently characterized [126] and recognized as an isoform of LCB2. The latter two, due to their PLP-binding, are recognized as catalytic subunits, instead LCB1 relation with PLP is still to be clarified [127]. SPT expression is modulated by different stimuli [128] such as endotoxins or cytokines [129] and UVB irradiation [130].

SPL is a homo-oligomer, situated at the ER membrane with the active site exposed to the cytosol. Its activity is tightly controlled at different levels such as transcriptional, epigenetic and post-translational [131]. Due to its function as sphingolipid pathway gatekeeper and S1P level regulator it is considered an important target for several therapeutic strategies. At the moment, it is especially studied in autoimmune diseases, due to its role in
modulating lymphocytes trafficking; in fact it is widely demonstrated that SPL inhibition causes lymphopenia linked to S1P-gradient modification [132]. Furthermore, SPL expression regulation is suggested to be involved in inflammation, cancer [133], metastatic tumours [134] and protective effect against cytotoxic therapy and radiation [135].

Fig. 1.7: X-ray structure of SpSPT (Sphingomonas paucimobilis) and StSPL (Symbiobacterium thermophilum). Dimeric structure of SpSPT and StSPL, represented in cartoon and ribbon with N- and C- termini indicated. PLP appears in ball-and-stick in the same color of the corresponding subunit. Original source: Bourquin et al. 2011

1.2.6.1 Sphingolipids pathway inhibitors: Myriocin and THI

Modification of sphingolipids levels, especially through the manipulation of critical enzymes of their biosynthetic pathway, is the basis for the study of increasing number of diseases, leading to the development of many enzymatic inhibitors or activators.

In the case of SPT, Myriocin represents a specific tool to powerfully inhibit de novo synthesis. Myriocin is a natural product that was firstly isolated for its antibiotic and antifungal activity from Myriococcum albomyces [136] and Mycelia sterilia (Thermozymocidin) [137], and some years later also from Isaria sinclairi (ISP-I) [138]. In 1995, its in vitro inhibition of cell growth was demonstrated to derive by SPT activity inhibition [139]. Across the years, Myriocin has been employed in a huge number of researches on: cancer
Introduction

[140] [141, 142], RP [143], multiple sclerosis [144], obesity [145] atherosclerosis and cardiomyopathies [146]. Recently, its mechanism of action has been clarified: it is defined to act as a “suicide inhibitor”. Myriocin reacts with PLP to form an external aldimine that decomposes after 16 hours producing a long chain aldehyde, able to bind the active site lysine and irreversibly inactivate the enzyme [147].

SPL can be inhibited by different compounds: among these, there is 2-Acetyl-5-tetrahydroxybutyl imidazole, THI [148-150]. THI derives from ammonia caramel colour III, a food colorant belonging to the big family of ammonia caramel colours (AC) and is employed in beers, sauces and biscuits. THI biological effects were firstly evidenced in 1988, in rats, defining it as a negative modulator of circulating lymphocytes. Interestingly it was observed that deficiency of pyridoxine (Vitamin B6, whom PLP is the active form) in the diet was shown to augment THI-induced lymphopenia [151]. The same year another group analyzed THI effect on mouse and observed that THI is able to selectively induce lymphopenia also in this animal model [152]. In the following years, many studies focused on THI toxicity and on the importance of vitamin B6 in counteracting THI effect, until 1992 when a human trial, on individuals with a marginal deficit in vitamin B6, demonstrated that THI, as others ACs, did not induce any effect on blood and lympha parameters [153]. In 2005, for the first time, THI action was associated to the inhibition of SPL. Schwab’s group demonstrated that S1P abundance in lymphoid tissues of mice increased more than 100-fold after THI treatment and that it depended on SPL inhibition, especially given to the ability of vitamin B6 to tightly influence THI and other SPL inhibitors activity [154] [155]. Recently, it has been demonstrated that it has a cardioprotective effect on ischemia/reperfusion injury on murine heart [156], it abrogates eosinophilic inflammation in a mouse model of allergic rhinitis [157] and it is involved in the maintenance of energy metabolism in dystrophic mouse [158].
1.2.7 Sphingolipids in the eye

The first direct evidence of a correlation between sphingolipids and photoreceptors degeneration arrived in 2003 from Acharya’s group [159]. By lowering Cer levels, they succeeded in rescuing photoreceptors in a *Drosophila* model of retinal degeneration. This outcome linked two important fields of research giving rise to many further, *in vitro* and *in vivo*, investigations. Some years later, on rat retinal neuronal cultures, C$_2$-Cer treatment triggered photoreceptor apoptosis, meanwhile Cer synthesis inhibition counteracted oxidative stress-induced death [160]. Similarly on 661W cells, oxidative stress, produced with sodium nitroprusside, stimulated Cer accumulation that is reduced through a SMase inhibitor [161]. Furthermore, sphingolipids result crucial also for their role as membrane structural component, in fact membrane domains enriched of sphingolipids are associated with critical photoreceptor membrane channels and manipulation of the sphingolipid pathway promotes their delocalization and dysfunction [162]. Interestingly, in a mouse model of RP, rd10, the inhibition of Cer synthesis slows retinal degeneration and preserves photoreceptors structure and function [143]. Regarding human retinal degeneration, the connection with sphingolipids was demonstrated with the finding of a mutation in a Cer kinase-like protein and arRP [17]. On the other side, S1P effects were studied in both rescue of degenerating photoreceptors and developmental retina. Apoptosis of rat retinal neuronal cells was induced by means of oxidative stress and counteracted by S1P addiction [30]. Moreover, GDNF is demonstrated to induce Sphk1 expression and translocation to the PM and, in addition, its positive effect on photoreceptors is blocked by S1P inhibition. Finally, S1P is able to increase proliferation of photoreceptor progenitors and then stimulates their differentiation [163].
2. RATIONALE
Due to their complexity, photoreceptors are the most fragile cells among those composing the retina especially due to their continuous exposition to light, high rate energy demand and constant outer segment turnover [164]. Their degeneration is the critical step of many severe retinal pathologies [18], such as RP [8]. Nevertheless, most of RP genetic mutations are related to rods, that indeed degenerates in the first stage of the disease, the following secondary cone degeneration is all along considered the major target in RP prevention [165], as also declared by Wright, in 1997: “Keep the cones alive and some 1,5 million people worldwide will see...” [166]. In fact, while rods death leads to night blindness, allowing an almost normal life, cones loss prevents diurnal vision and represents a tragic invalidating consequence of the pathology.

Given the critical role of sphingolipids in retinal degeneration [30] and recent evidences that Cer metabolism modulation can recover photoreceptors [143], particularly cones [167], in a murine model of RP, we investigated if both prevention of Cer formation by treating cells with Myriocin and most of all increasing the endogenous level of the pro-survival S1P, by inhibition of its degradation, can promote cones resistance to stress and save their functionality.

In order to demonstrate our hypothesis, we employed an *in vitro* model of cone-like cells and mimicked retinal degeneration stress by treating them with oxidative agents or depriving them of growth factors, the two conditions affecting cones upon rods death in RP retina.

We intend to demonstrate that not only impairment of Cer formation delays cones death but also the increase of endogenous S1P triggers a survival response that is aimed at overcoming oxidative stress. If our hypothesis is confirmed, combination of sphingolipid metabolism inhibitor can be envisaged as powerful therapy against RP retinal degeneration.
3. AIMS
This study aims at demonstrating the involvement of sphingolipids in photoreceptor degeneration, focusing on:

1) Endogenous accumulation and signaling of S1P as a mean to rescue photoreceptors from stress induced death.

2) Combination of S1P intracellular increase and Cer de novo synthesis inhibition to obtain additive effect on photoreceptor survival upon stress.
4. MATERIAL AND METHODS
4.1 Reagents and antibodies

H₂O₂ (hydrogen peroxide), THI (2-acetyl-4(5)-(1(R),2(S),3(R),4-tetrahydroxybutyl)-imidazole), Myricin, fatty acid free Bovine Serum Albumin, bovine serum albumin (BSA), MTT dye, Trypan blue dye, protease inhibitor cocktail, leupeptin, Pyridoxal-5′-phosphate, L-Serine, Palmitoyl coenzyme A and Hoechst 33258 dye were purchased from Sigma-Aldrich (St. Louis, Missouri USA). Sphingosine-1-phosphate (S1P) was purchased from Avanti Polar Lipids. Penicillin and streptomycin (Invitrogen) were purchased from Life Technologies Italia (Monza, Italy). DMEM culture media, fetal bovine serum (FBS) and the chemoluminescence system LiteAbLot were purchased from EuroClone Life Science Division (Milan, Italy). SYBR Premix Ex Taq (Tli RNaseH Plus) was purchased from Takara Bio, Inc. (Japan), RT-qPCR primers from Eurofins Genomics (Germany) and L-[³H(G)]-Serine from PerkinElmer, Inc. (Massachusetts, USA).

Primary antibodies were purchased from: p-Akt (#9271) and t-Akt (#9272) Cell Signaling; p-ERK1/2 (sc-7383), t-ERK1/2 (sc-93), Bcl-2 (sc-7382), Bax (sc-7480) and Nrf2 (sc-722) Santa Cruz; β-actin (A5316) Sigma. Horseradish peroxidase (HRP)-conjugated secondary antibody were from Jackson Laboratories (Bar Harbor, Maine, USA).

4.2 Cell cultures

4.2.1 661W

Cone-derived cell line, 661W cells, were provided by Dr. Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Science Center, Oklahoma City, U.S.). 661W were cloned from retinal tumours of transgenic mouse line expressing the SV-40 T antigen under control of the inter-photoreceptor retinal binding protein promoter (IRBP) [47]. 661W
growth doubling time is of ~24 hours in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution as antibiotics. They were maintained in a sterile and controlled atmosphere incubator at 5% CO₂, 95% humidity at 37 °C. Experiments were performed in 96 (3x10^3 cells in 0,2 ml), 6 (2,5x10^5 cells in 3 ml) multiwell plates and 100 mm Petri dishes (8x10^5 cells in 6 ml) starting treatments after about 24 hours, however when cells reached a 60%-70% confluence.

4.2.2 MDA-MB-231
Human breast cancer cell line MDA-MB-231, from America Type Culture Collection (ATCC), Rockville, MD, USA, was chosen as source of Serine-Palmitoyl Transferase (SPT) enzyme. Cells were maintained at 5% CO₂, 95% humidity at 37 °C in DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin solution as antibiotics. Cells were seeded in 150mm Petri dishes (3x10^6 cells in 24 ml) and left to grow for at least 3 days, however until 80-90% confluence was reached, before collected them for microsomal membranes fraction extraction, due to the localization of the studied enzyme at this level.

4.3 Cells treatments
661W cells were stressed through hydrogen peroxide (H₂O₂ 1mM) and serum starvation (1% FBS DMEM). They were pre or cotreated with the following compound: S1P (1µM and 100nM), solubilized in a methanol:water (95:5) solution, dried through a stream of dry nitrogen and stored according to the Avanti Polar Lipids protocol. Before use, S1P was resuspended in sterile fatty acid free Bovine Serum Albumin (BSA, 4 mg/ml), to make a 125 µM S1P solution and incubated for 30 minutes at 37°C, sonicated and vortexed occasionally. THI (75 µM) and Myriocin (10 µM) were both solved in dimethyl sulfoxide (DMSO).
The same procedure was followed prior to all assays: cells were seeded and left to grow for about 24 hours in 10% FBS/1% penicillin/streptomycin DMEM. Before treatments, the media was replaced with 10% or 1% FBS DMEM, depending on the kind of stress, always without antibiotics, and appropriate compound was added. At proper time point, they were collected and processed according to different assays.

4.4 3-(4,5-dimethyl-2-ia)-2,5difenilitetrazolio bromide (MTT) assay

Cell growth curve was performed, evaluating cell proliferation by MTT assay at 12, 24 and 48 hours after stress. Cells were seeded in 96-well tissue culture plates at 3x10^4 cells/well. At every time point, 20 µl of MTT solution (5 mg/ml) were added in each well and 661W cells were incubated, in darkness, at 37°C for 4 h. Media was removed, 100 µl of DMSO was added and the optical density (O.D.) was measured at 540 nm with a microplate reader (Sunrise Tecan microplate reader). Identical concentrations were tested in six-repetitions and the assay performed at least three times. Results are expressed as proliferation index fold change with respect to time zero (T0), against time.

4.5 Trypan blue dye exclusion test

Trypan blue assay was used to evaluate the number of viable cells after treatments. 661W cells were seeded in tissue culture Petri dishes (100mm) at 8x10^5 cells/Petri, treated and after 12 hours, counting was evaluated in experimental and control groups. For that, cells were detached using 0.25% trypsin, for 5 minutes at 37°C, collected and resuspended in 2 ml of Phosphate buffered saline (PBS) solution, 90 µl aliquots of this cell suspension were added to 10 µl of 0,4% Trypan blue dye (1:10). Next, live and dead cells were counted using Bürker chamber under a MoticAE31
optical inverted microscope. Live and dead cells were reported as both number of cells and percentages over the control.

4.6 Western blotting

Protein expression levels were analyzed by Western blotting. Cells, seeded at $8 \times 10^5$ in each tissue culture Petri dishes (100 mm), were treated and, after 12 hours, collected by scraping on ice in cold PBS-EDTA buffer, containing protease inhibitor cocktail. Then samples were centrifuged at 800g for 5 minutes at 4 °C, supernatants were discarded and pellets resuspended in 80 ul of the same buffer. A 15 ul aliquot, for every sample, was used for total protein quantification ($\mu$g/ul), through Bradford method \[168\]. The remaining suspension was added with Laemmli solution \[169\], boiled for 8 min and stored at -20°C. Proteins (15ug) were separated by SDS-PAGE with different concentrations acrylamide gels, depending on investigated protein size (10% for ERKs, Akt and Nrf2, 15% for Bcl2 and Bax. Proteins were transferred onto a nitrocellulose membrane with and unspecific binding sites were blocked by one hour of 5% dry skimmed milk in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) For immunoblotting, membranes were incubated at 4°C overnight with primary antibodies: anti p-ERK1/2 (1:500 in TBS-T-3% BSA), anti t-ERK1/2 (1:500 in TBS-T-3% BSA), anti p-Akt (1:1000 in TBS-T-3% BSA), anti t-Akt (1:1000 in TBS-T-3% BSA), anti Bcl-2 (1:200 in TBS-T), anti Bax (1:200 in TBS-T) and anti Nrf2 (1:1000 in TBS-T). $\beta$-actin levels (anti $\beta$-actin 1:2000 in TBS-T-3% BSA) were quantified for Nrf2 amounts normalization and as loading control for all others markers analyzed as protein levels ratio: ERK1/2 and Akt (phosphorylated on total form), Bcl-2 and Bax (Bcl-2, anti-apoptotic, on Bax, pro-apoptotic, marker). Membranes were incubated with the proper horseradish peroxidise (HRP) conjugated secondary antibody diluted 1:10000 in TBS-T-5% dry skimmed milk or TBS-T, depending on the
primary antibody. Bands detection was obtained by chemoluminescence and X-ray film exposure. Specific bands intensity was quantified by Gel Doc 2000, with Quantity one software (Bio-rad gel doc system, Chemidoc XRS). Levels of proteins were expressed in different ways, see respective figure captains for clarifications.

4.7 Liquid chromatography-tandem mass spectrometry (LC–MS) analysis

In order to measure sphingolipids intracellular amounts, cells were collected and total protein fractions were quantified following the protocol applied for Western blotting analyses. The obtained cellular pellets were lyophilized or directly frozen at -80 °C. Sphingolipids extracts from untreated and treated cells, fortified with internal standards (N-dodecanoylsphingosine, N-dodecanoylglucosylsphingosine, N-dodecanoylsphingosyl phosphorylcholine, C17-sphinganine (0.2 nmol each) and C17-sphinganine-1-phosphate (0.1 nmol), were prepared and analyzed as reported [170]. S1P amount was measured without adding internal standards.

4.8 Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Apoptosis degree was evaluated by means of TUNEL test, trypsinated cells were washed in 1 ml cold PBS and centrifuged 5 min at 1000 rpm at 4° C. The pellet was resuspended in 500 µl of 4% cold buffered formalin for 30 min, washed in PBS, centrifuged (1000 rpm, 5 minutes, 4 °C), resuspended in 50 µl PBS and stratified on slides (Superfrost Plus-Menzel Gmbh and CoKG, Braunschweg, Germany). After drying at room temperature, slides were maintained at –20 °C until use. Apoptosis was determined by the TdT assay (In situ Cell Death Detection Kit, TMR Roche Diagnostics,
Mannheim, Germany) in an inverted fluorescence microscope (40x magnification) Axiovert25 CFL (Zeiss, Göttingen, Germany) equipped for the detection of rhodamine (filter set 15, excitation band pass 546 nm, emission low-pass 590 nm). Nuclei were stained with the karyophilic dye Hoechst 33258 (250 ng/ul) for 3 minutes at room temperature in the dark, followed by rinsing twice in PBS and coverslipping. Slides were examined using a filter for Hoechst staining (filter set 02, excitation band pass 365 nm, emission low-pass 420 nm). Images were acquired by a digital camera (DS-2MV; Nikon, Tokyo, Japan) and the number of TdT-labeled nuclei counted (8-10 random fields in a blinded procedure). Results are expressed as number of TdT-labeled nuclei/total nuclei.

4.9 Ferric reducing antioxidant power (FRAP) assay

Cellular supernatants were separated from pellets and used to perform FRAP assay according to the method of Benzie & Strain [171] with minor modifications. Briefly, to prepare the FRAP solution, 100 ml of acetate buffer 300 mM, adjusted to PH 3.6 with acetic acid, were mixed with 10 ml of ferric chloride hexahydrate 20 mM dissolved in distilled water and 10 ml of 2,4,6-tris(2-pyridyl)-s-triazine 10 mM dissolved in HCl 40 mM. 100 µl of sample were added to 3 ml of a freshly prepared FRAP solution in glass test tubes in triplicate and the absorbance was measured at 593 nm after 5 minutes of incubation at 37 °C against a blank of acetate buffer. Aqueous solutions of FeSO₄·7H₂O (100–1000 µM) were used for the calibration and the results were expressed as FRAP value (µM Fe (II)) of the samples [172].
4.10 Real-time PCR (RT-qPCR)

To evaluate the influence of treatments on mRNA synthesis, real-time PCR was performed. 661W cells were seeded in 6-well tissue culture plates at 2.5x10^5 cells/well and treated. After 12 hours, total RNA was extracted with miRNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA was quantified by fluorometry with Qubit (Life Technologies Italia) and about 1 ug of total RNA was reverse transcribed in cDNA. The reaction was performed in a final volume of 20 µl starting with an incubation of 45 minutes at 37 °C and continuing for others 45 minutes at 42 °C to induce the degradation of eventual secondary RNA structures. RT-q Polymerase chain reaction amplification of the respective genes were performed with the SYBR Premix Ex Taq and set up in a total volume of 20 µl, with 200nM forward and reverse primers through the SYBR Green system according to the protocol. For the amplification, the following primers were used (5’->3’orientation). Primer’s sequences of target genes, heme oxygenase-1 (HO-1) [51] and S1PRs (S1P1, S1P2, S1P3, S1P4 and S1P5) [173], were obtained from published studies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>HO-1</td>
<td>TCTATCGTGCTCGCATGAAC</td>
<td>CTGTCTGTGAGGGACTCTGG</td>
</tr>
<tr>
<td>S1P1</td>
<td>AAATGCCCCAACGGGAGACTCTG</td>
<td>TTGCTGCGGGCTAAATTCCATGC</td>
</tr>
<tr>
<td>S1P2</td>
<td>CCCAACTCCGGGACATAGA</td>
<td>ACAGCCAGTGTTGGTTTTG</td>
</tr>
<tr>
<td>S1P3</td>
<td>TCAGTGGGTCATCATGCTGG</td>
<td>CAGGTCTTTCTGACCTTC</td>
</tr>
<tr>
<td>S1P4</td>
<td>AAGACCAACGGGTGTATGG</td>
<td>TCAGCAGGGTGTTAGTGC</td>
</tr>
<tr>
<td>S1P5</td>
<td>GCCTGGGTGCTACTGCTACAG</td>
<td></td>
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</tbody>
</table>
Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad). Amplification of the house-keeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the 2-DeltaDeltaCt method as previously described [174].

4.11 Serine palmitoyltransferase (SPT) activity assay

Microsomal membranes fraction was collected through MDA-MB-231 homogenization in a specific extraction buffer (Hepes PH 7.4 25 mM, EGTA PH 7.8 5 mM, NaF 50 mM, leupeptin 10 µg/ml and trypsin inhibitor 10 µg/ml) and centrifugation at 4070 rpm for 10 minutes at 4 °C. After Bradford protein assay, the enzymatic assay was performed incubating 300 µg of total proteins at 37 °C for 30 minutes in the reacting buffer (Hepes PH 8.3 100 mM, EDTA PH 7.4 2.5 mM and DTT 5 mM) containing Pyridoxal-5'-phosphate 50 µM as enzymatic cofactor and L-[³H(G)]-Serine (1 mM, 2 µCi specific activity 26 Ci/mmol), L-Serine (1 mM) and Palmitoyl coenzyme A (200 µM) as substrates. Counts per minute (CPM) were measured with β-counter (Packard Tri-Carb Liquid Scintillation Analyzer, model 2900TR) after washing three times the organic phase with bi-distilled water, dividing the two phases (hydrophilic and organic) with various centrifugations, drying up the samples overnight at 39 °C and resuspending them in scintillation cocktail. Results are expressed as percentage of enzymatic activity with respect to the control (DMSO) ± Standard Deviation, assuming
boiled enzyme as zero. Only for most interesting compounds, IC50 was obtained through a concentration-response curve performed calculating the enzymatic activity ± Standard Deviation at different concentrations.

4.12 Statistical analysis

Data on 661W cells are provided as means ± SEM. Statistic significance was evaluated for all data, choosing the best method depending on the number of analyzed groups: paired two-tailed Student’s t-test for two-group comparison and one-way analysis of variance (ANOVA) for multiple-group comparison, with Bonferroni post hoc test when P<0.05. Only results with P<0.05 were considered statistically significant. All statistical computations were carried out with InStat 3.01 (GraphPad Software, San Diego, California, USA). Western blot and TUNEL images are the most representative of at least three independent experiments.
5. RESULTS
5.1 \( \text{H}_2\text{O}_2 \) and serum deprivation inhibit proliferation and viability in cone photoreceptors

661W cells were challenged through 1mM \( \text{H}_2\text{O}_2 \) or starved in 1% serum medium (serum deprivation, SD) for 12, 24 and 48 hours and cellular proliferation was assessed by MTT assay. Oxidative stress induced 30% inhibition of cellular proliferation within the first 12 hours of treatment, whereas SD induced 50% proliferation inhibition in 48 hours (Fig. 5.1A and B). Both treatments induced a three folds increase of cell death, measured by Trypan blue assay, after 12 hours (Fig. 5.1C).

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Fig. 5.1: Oxidative stress and serum deprivation affect in vitro cone photoreceptors proliferation and viability.** Cells were treated with 1mM \( \text{H}_2\text{O}_2 \) or starved with 1% FBS DMEM for 12, 24 and 48 hours. A-B) Cell proliferation was evaluated by MTT assay. Data are expressed as fold change vs time zero (T0) against time. C) Photoreceptors viability was assessed through Trypan blue exclusion assay after 12 hours of stresses. The number of live and dead cells was showed in graphs. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through ANOVA followed by Bonferroni multiple comparisons post test, when \( P<0.05 \). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), n.s. not significant. SD: Serum deprivation.
In order to identify the mechanism underpinning cell arrest and death, we studied the involvement of key mediators of survival/death signaling. Stress activated kinases ERK1 and 2 are known to be phosphorylated to promote survival. In H$_2$O$_2$ or SD 12 hours treated 661W cells, ERKs were significantly dephosphorylated, in agreement to the above reported effect on cell death induction (Fig. 5.2A). Akt phosphorylation on Ser 473, was shown to be enhanced under acute, but sub-lethal oxidative stress in retinal pigment epithelial cells [175]. 661W cells, treated with 1mM H$_2$O$_2$ or SD for 12 hours exhibited an increase of Akt phosphorylation on Ser 473: six folds and four folds respectively for H$_2$O$_2$ and SD (Fig. 5.2B).

**Fig. 5.2: Oxidative stress and serum deprivation modulate ERK1/2 and Akt phosphorylation.** Cells were treated with 1mM H$_2$O$_2$ or starved with 1% FBS DMEM for 12 hours. A-B) The effect of 12 hours of 1mM H$_2$O$_2$ or 1% SD treatment was studied on ERKs and Akt phosphorylation. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies for p-ERKs, t-ERKs (44 and 42 kDa), p-Akt and t-Akt (60 kDa). Value of densitometry quantification of phosphorylated forms were normalized on corresponding total forms and the ratio was expressed as fold change vs control (Ctr), untreated cells; β-actin (43 kDa) was showed as loading control. Western blot images are the most significant among those quantified. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through ANOVA followed by Bonferroni multiple comparisons post test, when P<0.05. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant. SD: Serum deprivation.
5.2 THI rescues photoreceptors from both stresses, without affecting unchallenged cells.

THI is an inhibitor of SPL, leading to S1P cell accumulation. S1P is a pro-survival inducer which was shown to counteract Cer induced cell death when its intracellular levels raise above the baseline. In order to rescue photoreceptors from stress induced cell death, we treated 661W cells with 75 µM THI, either 2 hours before H₂O₂-stimulus or in cotreatment with SD. We monitored cellular proliferation at 12, 24 and 48 hours, and observed a slight but significant recovery in THI cell survival, visible already at 12 hours and more evident over time (Fig. 5.3A and B). After 12 hours of treatments, we observed that the percentage of live cells augmented almost of 25% and the number of dead cells lowered almost three folds in both stresses (Fig. 5.3C and D).
Fig. 5.3: THI attenuates stresses effects on 661W cells. 75µM THI was added to cells, two hours before 1mM H₂O₂ and directly with 1% FBS DMEM (SD). Cells were analyzed after 12, 24 and 48 hours from stress induction. A-B) Cell proliferation was evaluated by MTT assay. Data are expressed as fold change vs time zero (T0), against time. C-D) Photoreceptors viability, in presence of THI, was assessed by Trypan blue exclusion assay after 12 hours of stress. Percentages of live and dead cells were calculated considering total cells as 100%. Data are obtained from three independent experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant. SD: Serum deprivation

Once again, we investigated a possible involvement of ERKs and Akt pathways: THI always antagonized death-stimuli, by increasing activation of ERK by 1.5 folds against H₂O₂ and two folds against SD (Fig. 5.4A) and by inducing a 35-40% reduction of Ser473 Akt phosphorylation (Fig. 5.4B).
Fig. 5.4: THI counteracts stresses effect on ERK1/2 an Akt phosphorylation. 75µM THI was added to cells, two hours before 1mM H₂O₂ and directly with 1% FBS DMEM. THI rescue action was studied on ERKs and Akt phosphorylation. A-B) Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies for p-ERKs, t-ERKs (44 and 42 kDa), p-Akt and t-Akt (60 kDa). Value of densitometry quantification of phosphorylated forms were normalized on corresponding total forms and the ratio was expressed as fold change vs stress (H₂O₂ or SD); β-actin was showed as loading control. Western blot images are the most significant among those quantified. Data are obtained from three independent experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant. SD: Serum deprivation.
We also evaluated THI influence on unstressed photoreceptor viability, showing that the enzymatic inhibitor did have no significant effects on survival per se (no change in live and dead cells ratio: Fig. 5.5).

**Fig. 5.5: THI does not influence photoreceptors viability.** Percentages of live and dead cells were evaluated after 12 hours of 75μM THI, considering total cells as 100%. Data are obtained from three independent experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test, when P<0.05. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
5.3 H$_2$O$_2$ and SD influence Cer contents in different ways

We investigated how stress influenced Cer total and single species amount, differing for length and saturation of the amide linked acyl chain. Liquid-chromatography coupled to mass-spectrometry analysis (LC-MS) was performed on total lipids extracts of 661W cells, after 12 hours of stress. H$_2$O$_2$ did not significantly change total Cer but elicited its action modulating single species amount. On one hand, it significantly augmented long-chains Cer.s: 1.5 folds N-C$_{16}$ acyl chain, 2.5 folds N-C$_{18}$ acyl chain, 3 folds N-C$_{20}$ acyl chain species; on the other hand, a 50% decrease was showed for N-C$_{24}$ acyl chain Cer, the very long chains species analyzed (Fig. 5.6A).

We also studied SD effect on Cer species discovering that it never caused Cer amounts raise but instead it decreased them. This is in accordance with the starvation shut down of any synthesis activity and/or autophagy promotion. Significant decrease was observed only for the total Cer.s, reduced of about 30% with respect to the control (Fig. 5.6B).
Results

Fig. 5.6: Oxidative stress and serum deprivation change cellular Cer.s amount. Cells were stressed with 1mM H$_2$O$_2$ and 1% FBS DMEM for 12 hours. A-B) Lipid extracts were analyzed for the quantification of total and six single Cer species (from N-C$_{14}$ to N-C$_{24}$ acyl chain Cer.s) by means of LC-MS. Cer amounts (picomoles) were normalized on milligrams of proteins and showed values were expressed as fold change vs control (Ctr), untreated cells. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant. SD: serum deprivation.

5.4 THI lowers Cer contents in H$_2$O$_2$-challenged photoreceptors

Given the antithetic role of Cer and S1P, we focused our study on ceramide-inducing oxidative stress, since SD did not alter Cer level. Two hours of 75µM THI pretreatment, followed by 1mM H$_2$O$_2$ for 12 hours, reduced Cer both as total level (25%) and almost all the single species. Regarding shorter-chain members, reductions were: 30-35% for N-C$_{14}$ acyl chain and N-C$_{18}$ acyl chain and 50% for N-C$_{20}$ acyl chain species. THI significantly acted also on longer Cer, lowering of 30-35% both N-C$_{22}$ acyl chain and N-C$_{24}$ acyl chain elements (Fig. 5.7).

Fig. 5.7: THI counteracts Cer H$_2$O$_2$-induced raise. Cells were pretreated with THI for two hours and stressed with 1mM H$_2$O$_2$. After 12 hours, lipid extracts were analyzed for the quantification of total and six different Cer.s (from N-C$_{14}$ to N-C$_{24}$ acyl chain Cer.s) by means of LC-MS. Cer amounts (picomoles) were normalized on milligrams of proteins and showed values were expressed as fold change vs H$_2$O$_2$. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
5.5 THI and H$_2$O$_2$ oppositely modulate S1P intracellular content

Due to THI function as inhibitor of S1PL, we investigated S1P amounts changes, through LC-MS measurement, on treated and untreated 661W cells and we observed low but significant modulation induced both by THI and H$_2$O$_2$. In particular, THI promotes the accumulation of S1P with respect to the control (Fig. 5.8A) and, conversely oxidative stress induces a 40% decrease of its amount (Fig. 5.8B). Finally, we were not able to determine a significant change when 661W cells are pretreated with the inhibitor and then stressed with H$_2$O$_2$ (Fig. 5.8C).

**Fig. 5.8: THI and H$_2$O$_2$ act on S1P amounts.** Cells were pretreated with THI for two hours and stressed with 1mM H$_2$O$_2$. After 12 hours, lipid extracts were analyzed for the quantification of S1P by means of LC-MS. S1P amounts were normalized on milligrams of proteins and showed values were expressed as fold change against Ctr or H$_2$O$_2$ depending on the treatment evaluated: A) THI vs Ctr; B) H$_2$O$_2$ vs Ctr and C) THI/H$_2$O$_2$ vs H$_2$O$_2$. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
5.6 Oxidative stress increases apoptosis in 661W cells and activates Nrf2/HO-1 pathway

To study the outcome of oxidative stress and above reported signaling in cone like cell line, we investigated on apoptosis in Cer accumulating cells after H$_2$O$_2$ treatment. First, we performed TUNEL assay after 12 hours of 1mM H$_2$O$_2$ and obtained a 50% apoptosis above the baseline control cells, as evidenced in the corresponding immunofluorescence images below (Fig. 5.9A). Next we investigated apoptosis mechanism and analyzed the cytosolic presence of the pro-survival Bcl-2 and the pro-apoptotic Bax. The relation between these two proteins, both Bcl-2 family members, is crucial in cell fate determination, indeed, while Bax-Bax homodimer induces apoptosis, Bcl-2-Bax heterodimer promotes survival signals [176]. The shift of their ratio expresses an ongoing apoptosis path, dominant Bcl-2 saves cells from death, whereas major Bax expression triggers to apoptosis. We performed Western blotting to quantify Bcl-2 and Bax. The ratio Bcl-2/Bax, observed under H$_2$O$_2$ treatment was decreased about four folds with respect to the control (Fig. 5.9B).
**Fig. 5.9: Oxidative stress induces apoptosis in photoreceptors.** Cells were stressed with 1mM H$_2$O$_2$ for 12 hours. A) TUNEL-assay was performed to estimate the apoptosis degree. Images show TdT-positive nuclei labeled with rhodamine (red) and counterstained-Hoechst 33258 nuclei (blue). Apoptotic cells percentage was expressed as average number of TdT-positive nuclei on total amount of nuclei present. B) Apoptosis was also investigated by quantification of Bcl-2/Bax ratio. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies for Bcl-2 (26 kDa) and Bax (23 kDa). The two proteins were quantified by densitometry and their ratio was calculated and reported in graph as fold change vs control (Ctr) untreated cells; β-actin (43kDa) was showed as loading control. Western blot images are the most significant among those quantified. Data are obtained from three experiments, values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.

H$_2$O$_2$ toxicity is exerted by ROS induction within the cell. Intracellular survival response consists in the formation of antioxidant molecules that are released in culture medium. Their concentration can be evaluated by means of FRAP (Ferric reducing antioxidant power) test, which measures their ferric reducing power. H$_2$O$_2$-treated 661W cells revealed a 40% decrease of antioxidant-intrinsic power, after 12 hours treatment (Fig. 5.10A). A key player of the antioxidant survival response is Nrf2. This latter is a transcriptional factor whose ubiquitination and proteolysis is impaired under oxidative stress leading to its transcriptional activation of ARE-dependent genes, among which ROS defence related proteins, such as HO-1 [177]. To better understand the ongoing pathway, we investigated Nrf2/HO-1 activation in 661W cells treated with 1mM H$_2$O$_2$. We performed a Western blotting quantifying Nrf2 at 12 hours and we noticed an initial increase of Nrf2 activation (Fig. 5.10B).
Results

Fig. 5.10: Oxidative stress induced Nrf2 expression in 661W. Cells were stressed with 1mM H₂O₂ for 12 hours. A) Antioxidant compounds release in supernatant was analyzed by the FRAP test and expressed in FRAP value (µmoles Fe(II)/µg proteins) as fold change vs control (Ctr), untreated cells. B) Activation of antioxidant molecular pathways was studied through the densitometric analysis of Nrf2 Western blotting bands (57kDa) from total cell lysates. Values were normalized on the corresponding β-actin (43kDa) and expressed as fold change vs control (Ctr) untreated cells. Data are obtained from three experiments, values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.

Finally, we analyzed HO-1 mRNA content by Real-Time PCR (RT-qPCR) at 12, 24 and 48 hours and a potent induction (ten folds increase) of HO-1 mRNA transcription was observed at the first time point. HO-1 transcript level decreases over time, side by side with cell demise (Fig 5.11).

Fig. 5.11: Oxidative stress strongly upregulates HO-1 transcript expression at early time points. 661W were stressed with 1mM H₂O₂ for 12, 24 and 48 hours and mRNA expression level of HO-1 was analyzed by RT-qPCR. Data were normalized on GAPDH housekeeping gene and expressed as fold change vs control (Ctr), untreated cells. Data are obtained from three experiments, values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
5.7 THI counteracts H$_2$O$_2$-induced apoptosis, eliciting its antioxidant action through additional activation of Nrf2 pathway

To study the potential of THI and S1P accumulation and signaling in reducing oxidative-stress induced death in cone-like cells, we pretreated 661W cells with THI 75 µM for two hours and then treated them with H$_2$O$_2$ 1mM for 12 hours and studied cell death by TUNEL assay. TUNEL-stained cells reached 70% in H$_2$O$_2$ and decreased to 30% in THI/H$_2$O$_2$, as shown in the immunofluorescence images below (Fig. 5.12A). When looking at the molecular pathway involved, the Bcl-2/Bax ratio resulted increased by three folds in THI-protected cells, with respect to H$_2$O$_2$-treated cells (Fig. 5.12B).

**Fig. 5.12: THI reduces photoreceptors apoptosis degree.** THI Cells were pretreated with THI for two hours and stressed with 1mM H$_2$O$_2$ for 12 hours. A) TUNEL-assay was performed to estimate the apoptosis degree. Images show TdT-positive nuclei labeled with rhodamine (red) and counterstained-Hoechst 33258 nuclei (blue). Apoptotic cells percentage was expressed as average number of TdT-positive nuclei on total amount of nuclei present. B) Apoptosis was also investigated by quantification of Bcl-2/Bax ratio. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies for Bcl-2 (26 kDa) and Bax (23 kDa). Ratio of densitometry quantifications were calculated and reported in graph as fold change vs H$_2$O$_2$, β-actin (43 kDa) was showed as loading control. Western blot images are the most significant among those quantified. Data are obtained from three experiments, values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
Antioxidant-induced activity of THI was observed by FRAP analysis and appeared to be two folds augmented against stressed 661W, in collected-colture media (Fig 5.13A). We also investigated if THI protection was due to modulation of endogenous survival response. Indeed we observed a further increase in Nrf2 accumulation and activation in THI/H2O2-treated cells with respect to 12 hours H2O2-stressed photoreceptors (Fig 5.13B).

Fig. 5.13: THI increases antioxidant-intrinsic power of photoreceptors and potentiates Nrf2 expression. Cells were pretreated with THI for two hours and stressed with 1mM H2O2 for 12 hours A) Antioxidant compounds release in supernatant was analyzed by the FRAP test and expressed in FRAP value (µmoles Fe(II)/µg proteins) as fold change vs H2O2. B) Activation of antioxidant molecular pathways was studied through the densitometric analysis of Nrf2 protein bands (57kDa). Values were normalized on the corresponding β-actin (43kDa) and expressed as fold change vs H2O2. Data are obtained from three experiments, values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
Correspondingly, the antioxidant survival response was enhanced at 12 hours and, in addition, it was prolonged and improved as showed by the maintenance of high levels of HO-1 transcript up to 48 hours (Fig 5.14). This observation is in accordance with THI recover from death, starting at 12 hours and within the same time frame (see above, Fig. 5.3A).

Fig. 5.14: THI enhances HO-1 transcription, over time, against oxidative stress. Cells were pretreated with THI for two hours, stressed with 1mM H₂O₂ for 12 and 48 hours and mRNA expression level of HO-1 was analyzed by RT-qPCR and data were normalized on GAPDH housekeeping gene and expressed as fold change vs H₂O₂. Data are obtained from three experiments, values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
**5.8 S1P receptors expression in 661W cells**

Due to the scarcity of studies on S1P signaling in 661W cells, we could not find any evidence of S1P-receptors expression in this cell line. S1P is known to act as endogenous signaling mediator but also to be secreted by specialized pump and act in a paracrine fashion, via GPCR receptors engagement. Therefore we analyzed the expression, by RT-qPCR, of the known five isoforms of S1PRs in 661W cell line. We showed that the entire family of S1PRs was expressed in our cells, pointing out an almost decreasing linearity from the first to the last member (Fig. 5.15).

**Fig. 5.15: S1PR1-5 in photoreceptors.** mRNA expression levels of all five S1PRs were analyzed by RT-qPCR, data were normalized to the endogenous GAPDH control gene and expressed as fold change vs the first receptor (S1PR1). Data are obtained from three experiments, values are expressed as average ± SEM.
5.9 THI and H₂O₂ influence S1P receptors transcripts expression

To better understand the role and the power of THI on stressed photoreceptors, we evaluated the mRNA transcript modulation on 661W cells in presence of THI and H₂O₂-treatment. After THI stimulation the expression of the last two receptors, S1P4 and S1P5, was strongly upregulated, reaching about a four folds increase, instead of S1P1, S1P2 and S1P3 that did not sense THI-effect (Fig. 5.16).

![Fig. 5.16: THI modulates S1P4 and S1P5 mRNA expression.](image)

On the contrary, 12 hours of oxidative stress produced a significant decrease of S1P3, S1P4 and S1P5 mRNA, in particular it halved their amounts. H₂O₂ did not have the same effect on S1P1 and S1P2 transcript expression and especially it did not affect S1P2 mRNA at all (Fig. 5.17)
Fig. 5.17: Oxidative stress affects S1P3, S1P4 and S1P5 mRNA expression. Cells were challenged with 1mM H$_2$O$_2$ for 12 hours. mRNA expression levels of all five S1PRs was analyzed by RT-qPCR, data were normalized on GAPDH housekeeping gene and expressed as fold change vs control (Ctr), unstressed cells. Data are obtained from three experiments, values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.

We observed that THI cotreatment did not significantly change S1P2 and S1P3 transcript amounts with respect to that of oxidative stressed cells. Meanwhile, S1P1 mRNA was weakly incremented, S1P4 and S1P5 mRNA were highly upregulated showing a four folds and an eight folds increase respectively (Fig. 5.18).

Fig. 5.18: THI counteracts H$_2$O$_2$ effect on S1P1, S1P4 and S1P5 mRNA expression. Cells were pretreated with THI for two hours and then with 1mM H$_2$O$_2$ for 12 hours. mRNA expression levels of all five S1PRs was analyzed by RT-qPCR, data were normalized on GAPDH housekeeping gene and expressed as fold change vs H$_2$O$_2$. Data are obtained from three experiments, values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
5.10 Extracellular S1P rescues photoreceptors from oxidative stress

Due to the above reported evidence of THI modulation of S1P1-5 receptors expression, we decided to investigate a possible paracrine action for the endogenously produced S1P in 661W rescue. Therefore we analyzed the effect of exogenously administered S1P. Cells were pretreated with 1µM and 100nM S1P for 1 hour, before oxidative stimulus, cell proliferation was assessed after 12, 24 and 48 hours through MTT assay. First we observed a strict dependence of receptor signaling on agonist concentration, as expected for GPCRs regulated by agonist inactivation. Whereas 1µM S1P did not trigger protection against stress (Fig. 5.19A), S1P low concentration, 100 nM, reduced H₂O₂ induced arrest of proliferation, measured by MTT, at 12, 24 and 48 hours (Fig. 5.19B). To analyze cell viability, Trypan blue exclusion assay was performed after 1 hour of 100nM S1P pretreatment and 12 hours of oxidative stress, percentage of live and dead cells was determined with respect to total cell number. The number of alive cells increased 25% and, at the same time, dead cells were diminished about three folds (Fig. 5.19C).
Results

**Fig. 5.19: Extracellular S1P counteracts H$_2$O$_2$ action.** 1µM (A) and 100nM (B) S1P was added to cells, one hour before 1mM H$_2$O$_2$ and cells were analyzed after 12, 24 and 48 hours from stress. Cell proliferation was evaluated by MTT assay. Data are expressed as fold change vs time zero (T0), against time. C) Photoreceptors viability, in presence of S1P, was assessed with Trypan blue exclusion assay after 12 hours of stress. Percentages of live and dead cells were calculated considering total cells as 100%. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.

To assess a possible antioxidant-induced power of extracellular S1P against H$_2$O$_2$, FRAP assay was performed in collected-colture media. FRAP value obtained from S1P pretreated cells was significantly higher (two folds) in comparison with those from stressed photoreceptors (Fig. 5.20).

**Fig. 5.20: Exogenous S1P improves antioxidant-intrinsic photoreceptors power.** 100nM S1P was added to cells, one hour before 1mM H$_2$O$_2$ and cells were analyzed after 12 hours from stress. Antioxidant compounds release in supernatant was analyzed by the FRAP test and expressed in FRAP value (µmoles Fe(II)/µg proteins) as fold change vs H$_2$O$_2$. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
5.11 Myriocin strongly affects Cer amounts as single treatment and with THI, both in unstressed and stressed cells

S1P derives from the rupture of Cer amide linkage and the release of sphingosine and fatty acid. Sphingosine is immediately phosphorylated to avoid toxicity. Thus S1P may derive either from ceramide neo-synthesis or from ceramide release from sphingomyelin hydrolysis. In order to trace a possible contribution of ceramide de novo synthesis under oxidative stress, 661W cells were pretreated with Myriocin, an inhibitor targeting the first and rate limiting step of ceramide biosynthesis pathway.

THI did not affect significantly Cer amounts except for the shorter specie studied: N-C\textsubscript{14} acyl chain. Myriocin, in single treatment but also in combination with THI, reduced four times total ceramides and downregulated all studied species: 50% reduction in N-C\textsubscript{14} acyl chain and N-C\textsubscript{18} acyl chain species, 60% in N-C\textsubscript{20} acyl chain specie, 70% in N-C\textsubscript{16} acyl chain and N-C\textsubscript{22} acyl chain species and 90% in N-C\textsubscript{24} acyl chain specie (Fig. 5.21)

Fig. 5.21: Myriocin drastically decreases Cer.s amounts. 10\textmu M Myriocin and 75\textmu M THI were added to cells. After 12 hours, lipid extracts were analyzed for the quantification of total and six single Cer species (from N-C\textsubscript{14} to N-C\textsubscript{24} acyl chain Cer.s) by means of LC-MS. Cer.s amounts (picomoles) were normalized on milligrams of proteins and values were expressed as fold change vs control (Ctr), unstressed cells. Data are obtained from three experiments and expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
Results

Pretreatment of 661W with Myriocin, before oxidative stress with H₂O₂, induced a 50% reduction in Cer.s, affecting all fatty acyl species. As expected, THI combination with Myriocin did not alter Cer.s profile with respect to Myriocin only (Fig. 5.22).

Fig. 5.22: Myriocin antagonises H₂O₂-induced increase of Cer.s. 10µM Myriocin and 75µM THI were added to cells, five and two hours before 1mM H₂O₂. After 12 hours, lipid extracts were analyzed for the quantification of total and six single Cer species (from N-C14 to N-C24 acyl chain Cer.s) by means of LC-MS. Cer.s amounts (picomoles) were normalized on milligrams of proteins and values were expressed as fold change vs H₂O₂. Data are obtained from three experiments and expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
5.12 Myriocin and Myriocin-THI treatments counteract oxidative stress effect on photoreceptors.

In order to evaluate Myriocin effects on 661W cells, Trypan blue exclusion assay was performed, pretreatment with 10µM Myriocin for five hours before the addition of H₂O₂, or THI 75µM (2 hours) and then oxidative stress was carried on for other 12 hours. 75% inhibition of survival was obtained with H₂O₂ only (Fig. 5.23). Myriocin and Myriocin/THI treatments insured a recover of survival up to 90% and 95%, respectively, after oxidative stress. Accordingly, dead cells ratio lowered almost five times with both treatments.

Fig. 5.23: Myriocin and Myriocin-THI recover 661W cells viability from oxidative stress. 10µM Myriocin and 75µM THI was added to cells, five and two hours before 1mM H₂O₂. Cells were analyzed after 12 hours. Photoreceptors viability, in presence of inhibitors, was assessed with Trypan blue exclusion assay. Percentages of live and dead cells were calculated considering total cells as 100%. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through ANOVA followed by Bonferroni multiple comparisons, when P<0.05. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
Results

Antioxidant-induced effect of Myriocin and THI on H$_2$O$_2$ stress was evaluated by means of FRAP assay, in culture media. Both Myriocin and Myriocin/THI induced two folds increase in antioxidant factors release with respect to H$_2$O$_2$ (Fig. 5.24).

![Graph showing antioxidant-induced activity against H$_2$O$_2$.](image)

**Fig. 5.24: Myriocin and Myriocin-THI have antioxidant-induced activity against H$_2$O$_2$.** 10µM Myriocin and 75µM THI were added to cells, five and two hours before 1mM H$_2$O$_2$. Antioxidant compounds release in supernatant was analyzed by the FRAP test and expressed in FRAP value (µmoles Fe(II)/µg proteins) as fold change vs H$_2$O$_2$. Data are obtained from three experiments and values are expressed as average ± SEM. Significance was analyzed through Student t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
5.13 New synthetic compounds: SPT inhibitors

In order to characterize new compounds with the same activity of Myriocin in inhibiting SPT, the rate limiting enzyme of the ceramide synthesis pathway, a number of compounds have been synthesized as potential inhibitors. Due to SPT internal membranes localization, enzymatic activity was evaluated in enriched fractions of microsomal membranes obtained from MDA-MB 231 cells. This cell line was chosen for its high proliferation rate and also basing on our laboratory experience, however it was previously employed to study SPT *in vitro* activity [178]. The enzyme inhibitory activity of 30 new compounds, developed from different backbone structures, was tested *in vitro*. We considered Myriocin incubation with microsomal membranes-SPT extract as positive control and DMSO incubation (vehicle of each compound) as 100% of SPT activity. At first, inhibitors were screened at a fixed concentration of 50µM, only the most promising molecules were selected and tested at scalar concentration to determine their IC50 value.

From the first step screening, we found three active inhibitors on SPT: DC9, DC14 and DC80, that lowered the percentage of SPT activity to 5.68%, 12.58% and 29.90% respectively. Therefore, we measured their IC50, resulting in: DC9 17.71µM, DC14 19.42µM and DC80 40.41µM (Tab. 5.1).
<table>
<thead>
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<th>Compound name</th>
<th>SPT activity ± SD (% vs DMSO)</th>
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<tr>
<td>A7</td>
<td>139.35 ± 21.73</td>
</tr>
<tr>
<td>A9</td>
<td>56.17 ± 3.24</td>
</tr>
<tr>
<td>DC6</td>
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<td><strong>DC9</strong></td>
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<tr>
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Tab. 5.1: Comparative analysis of the 30 compounds tested for SPT. Synthetic compounds were tested on SPT activity at 50µM and their inhibition power was indicated as percentage of SPT activity considering DMSO incubation as 100%. Three of them, that showed a remaining percentage of enzymatic activity under 30%, were analyzed to determine their IC50. Data are obtained from three experiments and values are expressed as average ± SD.

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<tr>
<th>Compound name</th>
<th>IC50 ± SD (µM)</th>
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<tr>
<td>DC9</td>
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</tr>
<tr>
<td>DC14</td>
<td>19.42 ± 4.98</td>
</tr>
<tr>
<td>DC80</td>
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6. DISCUSSION
This study aims at demonstrating the involvement of sphingolipids in photoreceptor death, suggesting the manipulation of this signaling molecules as potential therapeutic targets in retinal diseases. In particular, we worked on a stressed cone-like cell line as a model of cone photoreceptors degeneration. In RP, cones photoreceptors degeneration is secondary to rods damages and it occurs mainly as bystander effect. This makes this process suitable for therapeutic approaches aimed at reducing the potency of stress insults on viable cones.

In spite of rods degeneration and considering the importance of cones preservation to allow minimal daily vision, the purpose of our study is to increase intracellular S1P concentration through the inhibition of SPL, the enzyme responsible for one of the two degradation pathway.

The cone-like cell line, 661W, is a unique *in vitro* model of photoreceptors, derived from mouse retinal tumours [47]. In the intent to investigate the possibility to activate salvage pathways of these photoreceptors, we subjected 661W cells to two different kind of stress: oxidative stress by means of H$_2$O$_2$ and ischemia-like condition throughout serum deprivation (SD). Due to their different nature, these two stresses produced distinct effects, evidenced in cell proliferation measurement over time. In Fig.5.1A and B, we showed, on one side, how H$_2$O$_2$ effect was pronounced causing apoptosis in a large part of the population whereas a small fraction of cells remained viable and proliferating, proportionally to the control rate. On the other side, we observed that SD progressively reduced cellular proliferation rate over time. Hence the two stress are sensed differently and may activate diverse stress responses: oxidative stress can be considered an acute and intense stimulus, partially overcome by a small number of resistant and growing cells; SD fails to select resistant cells, and slowly affects the entire population because of continuous lack of necessary nutrients, such as growth factors.
First of all, we considered cellular viability at the 12 hours time point (Fig. 5.1C). Both stresses resulted in a strong decrease of live cells and in a parallel augment of death at this early time point. To understand the intracellular pathways involved, we chose two well known proteins with pivotal role in cells proliferation and survival, ERKs and Akt. The ERK/MAPK pathway activation is often linked to proliferation and survival in both in vitro and in vivo models [179]. Particularly ERKs activation in rat retina was associated to protection against light injury [180], as well as in 661W cell colture under short stimulation with H₂O₂ (30 minutes). Although an initial survival response is triggered by the cells, if stress is sustained in time, cell demise will finally follow [181]. Accordingly, we observed that 12 hours of H₂O₂ treatment or SD cause cell death and ERKs dephosphorylation (Fig. 5.2A). Another important player in survival responses is the kinase Akt/PKB, whose activity is modulated during pathological situations [182] such as retinal degeneration, indeed Akt activation was demonstrated to rescue rat retinal neurons from light-induced apoptosis [183]. Cotter’s group showed, in 661W, that serum deprivation induced a rapid but detectable burst of H₂O₂, activating survival pathways and promoting Akt phosphorylation, at Thr408 [184]. Years later the same group demonstrated that bFGF administration to 661W enhanced ROS production and again Akt phosphorylation occurred, even if on Ser473 [185], the same phosphorylation was documented in H₂O₂-stressed RPE [175]. Another group demonstrated that, in a different stress and cellular model, the site of Akt phosphorylation changed depending on the severity of the stress: Ser473 phosphorylation was associated to an early stress meanwhile Thr408 corresponded to intense and prolonged stress [186]. Our results confirmed that oxidative stress, as well as SD, induced Akt activation that resulted in Ser473 phosphorylation (Fig. 5.2B). In our model Akt activation persisted with respect to that on ERK suggesting a potential regulation of Akt in switching off of the ERK pathway.
S1P is a well-known anti-apoptotic sphingolipid able to induce cell survival, proliferation and differentiation in several cell lines and tissues [83]. Furthermore, S1P was recognized to participate in photoreceptors protection against oxidative stress [163]. In order to investigate a potential role of S1P in rescue 661W from death-induced stimuli, we treated cells with THI, SPL inhibitor, at the aim of increasing the endogenous amount of S1P. Inhibitory effect of this compound is mainly used in studies on autoimmune disorders. Due to its ability to accumulate S1P, THI induces its gradient deregulation that consequently leads to lower levels of circulating peripheral lymphocytes [150]. In our study we treated cells with THI 2 hours prior to H$_2$O$_2$ (1mM) and contemporarily with SD. THI pretreatment was able to reduce stresses inhibition of proliferation from 12 up to 48 hours of treatment (Fig. 5.3A and B). Moreover THI exerted a protective effect on cell death, increasing the number of viable cells in both starved and H$_2$O$_2$ stressed samples (Fig. 5.3C and D). To analyze the correlation between intracellular S1P and survival intracellular signaling [187], the activation state of ERK and Akt was analyzed under THI action. In both cases the inhibitor was able to counteract stress effects, on one side by inducing the activation of switched-off ERK 1 and 2 (Fig. 5.4A), on the other side by decreasing stress-induced phosphorylation on Akt (Fig. 5.4B). Confirming a potential cross-talk between these two pivotal survival regulators, in which one regulates the other depending on the stress severity and likely also on the site of Akt phosphorylation.

By comparing the two stresses, serum deprivation did not effectively killed cells but rather stopped them slowing their proliferation. Such a difference was also evidenced in Cer species quantification. Considering that Cer is a well-known apoptotic signaling mediator whose cellular content increases upon stress [188] and that its involvement in oxidative stress-induced apoptosis was also documented in photoreceptors [52], we evaluated Cer
Discussion

content by LC–MS analysis and we observed different profile of Cer.s pools under the two stresses. \( \text{H}_2\text{O}_2 \), which is a direct and immediate ROS mediated damage inducer, causing mitochondria failure and triggering a rapid apoptotic response, induced an augment in long-chain Cer (16:0, 18:0, 20:0 and 22:0), known as pro-apoptotic species [73]. At the same time, it produced a decrease in the very long-chain Cer 24:0. Given that Cer 16:0 and 24:0 are the most abundant species, the total level of Cer resulted unchanged overall. (Fig. 5.6A). SD did not induce any augment in Cer content, on the contrary we observed that all the species were reduced (Fig. 5.6B), according to the necessity to slow down all energy consuming processes, including sphingolipids synthesis.

The evidences that oxidative stress directly influences sphingolipid pathway and, in particular, Cer which is the hub of all the biosynthetic chain, led us to focus our investigation mainly on this kind of stress. Before deepening in biochemical mechanisms, we investigated the influence of THI pretreatment on \( \text{H}_2\text{O}_2 \)-stressed photoreceptors, observing that the inhibitor had a significant effect on Cer quantities and succeeded in lowering, not only almost all Cer species analyzed, but also total Cer, because of its indiscriminate action upon different chain-length species (Fig. 5.7).

Furthermore, we investigated S1P amounts modulation under THI and \( \text{H}_2\text{O}_2 \) treatment. Intracellular S1P detection is not easily feasible in comparison with others sphingolipids, given both its low concentration inside the cells and its rapid degradation by means of SPL and phosphatases, although we succeeded in evidencing significant modulation of S1P. In particular, on one side THI treatment promoted S1P raise in photoreceptors (Fig. 5.8A) and on the other side oxidative stress induced its decrease (Fig. 5.8B). Whereas the inhibitor pretreatment on stressed cells did not produce a sufficient effect, indeed S1P increase against
challenged photoreceptors resulted to be not significant, likely due to abovementioned difficulties in its detection (Fig. 5.8C).

Due to the fact that pro-death Cer species increased under oxidative stress and declined in the presence of THI, we investigated apoptosis by TUNEL assay observing, as expected, that the percentage of apoptotic photoreceptors increased after 12 hours of H$_2$O$_2$ (Fig. 5.9A). Next, we analyzed and compared the protein expression of two Bcl2 family members with opposed roles: Bcl2, an anti-apoptotic marker, and Bax, a pro-apoptotic one [189]. Apoptosis decrease during retinal development [190] or pharmacological impairment of apoptosis in retina [191] was previously demonstrated to associate with pro-death Bcl-2 proteins content.

Our data, in line with literature, showed a decrease in Bcl2/Bax ratio translating in a predominant relative amount of Bax that could be associated to the apoptosis degree observed with the immunofluorescence test (Fig. 5.9A and B).

By means of FRAP test, a commonly employed method to investigate plasma reducing power [192], we observed that 12 hours of H$_2$O$_2$ decreased the amount of reduced iron, that in turn is associated with a diminished release of antioxidant molecules in the culture medium and hence in augmented oxidative stress in cells (Fig. 5.10A).

In order to elucidate the mechanism underlying photoreceptors responses to oxidative stress, we investigated the activation of the nuclear factor erythroid 2 (NFE2)-related factor 2, Nrf2. The latter is a transcription factor with a fundamental role in cell resistance to oxidative stress. In basal condition its activity is suppressed by Keap1 protein (Kelch-like ECH-associated protein 1) through a continuous ubiquitination-proteasomal degradation that is suspended by the action of oxidants and electrophiles, via modification of Keap1 critical cysteine thiols groups and release of Nrf2 [177]. Nrf2 role in 661W cells protection to oxidative stress was previously
observed [193]. Similarly, our data showed a weak however significant increase of Nrf2 expression after H$_2$O$_2$ treatment (Fig. 5.10B). When Nrf2 is activated it translocates into the nucleus, where its binding to antioxidant-responsive elements (AREs), stimulates the transcription of phase II genes encoding detoxifying and antioxidant enzymes, such as heme oxygenase-1 (HO-1) [194]. HO-1 protective induction was previously documented in 661W cells [66] [51]. In order to identify a possible involvement of HO-1 activation, in Fig. 5.11, we analyzed its transcript and observed that an increased transcription is associated with early survival response to stress, but after 24 and 48 hours HO-1 mRNA levels fall down in association with cell death progression.

In order to elucidate THI protective mechanism, we investigated the effect of its pretreatment on the above studied signaling mediators. As previously shown, DHA-induced protection of photoreceptors death was attributed to the increased of Bcl2-Bax ratio likely induced by ERK/MAPK activation [195]. Furthermore Bcl2 overexpression was demonstrated to rescue 661W cells from photoxidative stress [24] and to counteract photoreceptors apoptosis in different retinal degenerations [196]. Additionally, in Jurkat cells, S1P inhibition of the pro-apoptotic Bax and Bad activation, was supposed to be caused by MEK activation [197].

In our hands, THI pretreatment was able to reduce apoptosis, decreasing the number of TUNEL-stained cells with respect to only stressed cells (Fig. 5.12A). Simultaneously Bcl2 expression augmented up to overtake that of Bax, shifting their ratio equilibrium in favour to survival stimulus and proving that the inhibition of S1P degradation has a role in preserving 661W cells from apoptosis (Fig. 5.12B). Moreover, THI showed an antioxidant activity against H$_2$O$_2$, augmenting the FRAP value (Fig. 5.13A) and further stabilizing Nrf2 protein in stressed cells (Fig. 5.13B), thus supporting the hypothesis of a critical role of the transcription factor in cells protection. In THI and H$_2$O$_2$ treated cells, HO-1 transcription was protracted, in
accordance with Nrf2 stabilization. Whereas after 48 hours of H$_2$O$_2$ HO-1 transcription was repressed, in THI pretreated cells the transcript is still present at high levels, likely as a part of the THI induced survival response, the higher difference was at 48 hours (Fig. 5.14). We can conclude that one of the downstream effect of THI is to strengthen the action of detoxification enzymes. This may be imputable to an indirect S1P antioxidant induction against oxidative stress, resulting in an enriched response of detoxifying pathways. Similarly, in a rat model of emphysema, S1P ability to overexpress Nrf2 against stress was previously showed [198], in addition Nrf2 was showed to protect human RPE cells from H$_2$O$_2$-induced oxidative stress via Nrf2-mediated upregulation of the expression of phase II enzymes involving the PI3K/Akt pathway [199]. Furthermore HO-1 induction protected retinal ganglion cells in diabetic retinopathy [200], even if it was also demonstrated that decreased expression of HO-1 was observed in treatments with molecules that acted as direct scavengers against H$_2$O$_2$ [201].

Given S1P duality as intracellular messenger and extracellular ligand and its well-know ability to exit the cell through specific transporters [202], we hypothesized that THI, by means of S1P accumulation, could interact or modulate S1PRs. To the best of our knowledge, the expression of S1PRs has not been proven in 661W cell line. Analyzing S1P1-5 mRNA we confirmed their expression in our cellular model and underlined different and proportional decreasing degree of receptor’s expression. In particular, S1P1 resulted to be the more abundant member, followed by S1P2, whereas S1P3-5 showed the lowest expression (Fig. 5.15). S1PRs belong to the family of GPCR proteins, they are coupled to different G-proteins and, for this reason, their influence on cells behaviour widely changes [110].
Preliminary data on THI effect on S1PRs expression showed an interesting tendency in a preferential modulation of S1P4 and S1P5. THI alone increased both mRNA receptors and did not significantly alter S1P1-3, especially for what concerned the second receptor (Fig. 5.16). In contrast, oxidative stress had a negative influence on transcript amounts, powerfully acting on S1P3-5, as emerged from Fig. 5.17. THI pretreatment on stressed photoreceptors strongly overexpressed S1P4 and S1P5 and showed a weak however significant activity also on S1P1 (Fig. 5.18). In conclusion S1P2 resulted to be the only member of S1PRs family, in 661W, that was affected neither by THI nor by oxidative stress effects. All these observations, although they must be further confirmed, have to be considered interesting and significant in the global vision for putative THI purpose in visual and, in particular, neural-retinal pathologies, opening the possibility of new therapeutic strategies. Therefore, we distinguished a double THI effect in antagonizing oxidative stress: firstly, its action on cell proliferation, viability, apoptosis and oxidant power and, secondly, its ability to modulate S1PRs expression in unstressed and stressed photoreceptors.

Given the interesting modulation of S1PRs in our in vitro model, we investigated the involvement of exogenous S1P in 661W cells rescue from oxidative stress. Cells were pretreated for 1 hour with S1P at 1µM and then challenged with H$_2$O$_2$ 1mM for 12, 24 and 48 hours. Exogenously added S1P did not show significant cellular proliferation improvement against oxidative stress (Fig. 5.19A). Being GPCR, S1PRs underwent desensitization via temporary or β-arrestin-induced internalization upon massive engagement by respectively S1P or its potent agonists [111]. As a matter of fact, lowering exogenous S1P concentration to 100nM, we obtained a remarkable rescue from cellular proliferation arrest upon oxidative stress (Fig. 5.19B). We confirmed that low S1P concentration increased cell viability after 12 hours of oxidative stress, (Fig. 5.19C). In line
with previous investigations, we measured FRAP value after S1P treatment obtaining an augment in antioxidant induced power respect to stressed cells (Fig. 5.20).

To strengthen our hypothesis on the pivotal role of sphingolipids in photoreceptors fate and on the importance of their biosynthetic pathway manipulation, as a tool in contrasting retinal degeneration, we focused on Cer modulation employing a potent inhibitor of its de novo synthesis, Myriocin. This inhibitor was, previously, employed in vivo by our group demonstrating that inhibition of Cer de novo synthesis is able to rescue photoreceptors from degeneration, in a RP murine model [143]. We hypothesized that inhibition of Cer generation and increase of S1P intracellular content may be combined in the intent of improving cell protection. At this aim cells were treated with Myriocin (10 µM) also in the presence of THI (75 µM) for 5 hours and then H₂O₂ (1mM) was added for other 12 hours. LC-MS analysis confirmed that Myriocin drastically reduced all Cer species (Fig. 5.22), suggesting that de novo synthesis of Cer is majorly involved in oxidative stress in 661W. Unexpectedly, the combination of the two inhibitors failed to allow additive protection from cell death (Fig. 5.23); moreover it did not increase the production of antioxidant molecules (Fig. 5.24) under H₂O₂ treatment. This data lead to hypothesize that S1P protection and Cer induced apoptosis signal through pathways that are somehow interconnected, as indicated by THI modulation of some Cer species.

THI is supposed to interact with PLP, the SPL cofactor, blocking its normal involvement in enzymatic mechanism to degrade S1P [149]. This hypothesis is supported by the evidence of vitamin B6 opposition to THI effect [154]. In our case, due to the pivotal role of PLP in SPT activity, this should be an explanation for decreased Cer amounts that we observed in
THI pretreated cells, even if significant modulation was registered only after oxidative stimulus (Fig. 5.21). This last observation together with the recent hypothesis defining SphK-1 and SphK-2 as regulators of de novo Cer synthesis [99], suggest the existence of a feedback action of S1P on Cer synthesis as previously demonstrated for phosphorylated long-chain sphingoid bases [203]. Oxidative stress induced apoptotic Cer generation. However, in THI pretreated cells, accumulated S1P modulates Cer, triggering their decrease. Both hypothesis look to the de novo pathway rather than to the salvage one, especially if we consider THI similitude to Myriocin in the indiscriminately way to induce Cer amount modulations.

The pivotal role of Myriocin SPT inhibition in physiological and pathological states was demonstrated. Given the high cost of this inhibitor, we screened 30 new synthesized compounds as putative SPT inhibitors. We were able to evidenced three molecules that led to a residue SPT activity equal to, at least, 30%. The IC50 of these new inhibitors was calculated (Tab. 1). Even if Myriocin effect remains incomparable, the identification of putative new structures leaves the way open to further investigations. An important association is underlined between some functional groups and the increase of SPT activity. To date, this trend is noticed in 4 compounds (A7, DC6, DC11 and DC12). This analysis is still ongoing in our laboratories.
7. CONCLUSIONS
Conclusions

In conclusion S1P endogenous increase, obtained with THI treatment, favours survival and proliferation upon stresses in an *in vitro* model of photoreceptor degeneration. S1P effect, against oxidative stress, is linked to some crucial intracellular pathways involved in cell fate regulation. Indeed, it modulates ERK and Akt activation, Bcl-2/Bax equilibrium, Nrf2-HO-1 expression and reduced intracellular Cer amounts (Fig. 7.1).

![Fig. 7.1: Conclusion scheme. THI accumulation of S1P induces decrease of apoptotic Cer and activates intracellular survival pathways.](image)

From our preliminary data, we can also conclude that THI-induced increase of S1P influences S1PRs expression. In addition, to confirm a supplementary role of extracellular S1P, we show that low exogenous S1P counteracts the inhibition of photoreceptors proliferation and viability induced by oxidative stress. Moreover, the cotreatment with inhibitors of sphingolipid synthesis and degradation, reducing Cer and increasing S1P intracellular content, is able to ensure survival in response to oxidative stress, even if the hypothesized additive effect was not observed. All these outcomes together demonstrate and confirm sphingolipid biosynthetic pathway as a paramount target in therapeutic approaches for photoreceptor degeneration.
8. REFERENCES


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- The manuscript on thesis topic is in preparation.

Presentations on international events:

- The Association for research in vision and ophthalmology (ARVO) annual meeting, Seattle (WA, USA), May 5-9 2013. Poster presentation: “Inhibition of S1P degradation rescues 661W cells from oxidative stress”
- Sphingolipid Club Meeting 2013, Assisi (PG, Italy), June 27-30 2013. Oral presentation: “Inhibition of S1P degradation rescues 661W cells from oxidative stress”
RINGRAZIAMENTI

Un ringraziamento speciale è per il Professor Riccardo Ghidoni, per avermi seguito in questo percorso, per essere stato una tutore di dottorato ma soprattutto un punto di riferimento e una guida.

Inoltre vorrei ringraziare la Dott.ssa Paola Signorelli, per avermi aiutato costantemente a destreggiarmi nei meandri della biochimica degli sfingolipidi e non solo.

Un “grazie particolare”, va alla Dott.ssa Anna Caretti, per esserci sempre stata, per non essersi mai stancata di ascoltarmi anche quando gli interrogativi, scientific e non, si ripetevano incessamente.

Ovviamente, un “Grazie” è per tutto il laboratorio del 9° piano dell’Ospedale San Paolo di Milano, per avermi accolto e coccolato sin dall’inizio di questa avventura.: Nadia, Paola B., Rita P., Marco T., il Prof Michele Samaja e il Prof. Enzo Santaniello. In particolare, vorrei ringraziare tutte le bimbe e Andrea con cui ho condiviso gioie e dolori di questi anni milanesi.

Inoltre ringrazio la Prof.ssa Gemma Fabriàs, la Prof.ssa Josefina Casas e il loro laboratorio (iQAC, CSIC Barcellona, Spain) per il support tecnico ed il prezioso contributo. Infine vorrei ringraziare il Prof. Macchia e il suo gruppo dell’Università di Pisa per la proficua collaborazione di questi anni.