Title

2-Acetyl-5-tetrahydroxybutyl imidazole (THI) protects 661W cells against oxidative stress

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Abstract

Purpose. Retinal degeneration and in particular Retinitis Pigmentosa (RP) is associated to Ceramide (Cer) accumulation and cell death induction. Cer and sphingosine-1-phosphate (S1P) belong to the sphingolipids class and exert a pro-apoptotic and pro-survival activity, respectively. Our aim is to target sphingolipid metabolism by inhibiting S1P lyase that regulates one of the S1P degradation pathway, to reduce retinal photoreceptor damage.

Methods. Murine 661W cone-like cell line was pretreated with THI, an inhibitor of S1P lyase and exposed to H_2O_2-induced oxidative stress. 661W cell viability and apoptosis were evaluated by Trypan Blue and TUNEL assay, respectively. Protein expression of mediators of the survival/death pathway (ERK1/2, Akt, Bcl-2, Bax) was analyzed by Western Blotting. RT-PCR was performed to establish HO-1 transcript changes and LC-MS analysis to measure Cer intracellular content.

Results. THI rescues inhibitory H_2O_2-effect on 661W cell viability and impairs H_2O_2-induced apoptosis by increasing Bcl-2/Bax ratio. THI administration counteracts the oxidative stress effects of H_2O_2 on 661W cells by activating Nrf2/HO-1 pathway, regulating ERK and Akt phosphorylation level and decreasing Cer intracellular content.

Conclusions. We conclude that sphingolipid metabolism manipulation can be considered a therapeutic target to promote photoreceptors survival.

Introduction

Photoreceptors are the most fragile cells in retina, due to their continuous exposition to light, to their high rate energy demand and constant turnover of the outer segment (Stone et al. 1999). Their degeneration represents the critical step of many severe retinal diseases (Wright et al. 2010) including Retinitis Pigmentosa (RP), which consists in a group of inherited retinal dystrophies. The common form of RP typically starts with night blindness culminating with the characteristic tunnel vision and, in many cases, with total loss of vision. Most of RP genetic mutations are related to rods, which degenerate during the first stage of the disease (Hartong et al. 2006) originating an oxidative bystander stress that leads to cone damage (Piano et al. 2013). This secondary degeneration of cones is all along considered the major target in RP prevention (Mustafi et al. 2009; Shelley et al. 2009). Apoptosis is the first death-process documented to be involved in RP mouse model (Portera-Cailliau et al. 1994) and it could be regulated, among others, by sphingolipids (SPLs) a class of structural membrane components with important signaling functions, recently correlated with retinal physiology and pathology (Acharya et al. 2003; Rotstein et al. 2010; Tuson et al. 2004) and suggested as a target for developing of new therapeutic strategies. Among these lipid mediators, ceramide (Cer) and sphingosine-1-phosphate (S1P) exert opposite effects: the former is a well-known pro-apoptotic intracellular messenger; the latter is involved in survival and anti-apoptotic pathways (Hannun and Obeid 2008). In addition to its pro-survival properties, S1P was seen to promote the overexpression of the transcriptional factor Nrf2 (Yasuo et al. 2013) that stimulates the production of detoxifying and antioxidant enzymes (Prestera et al. 1995).

Nrf2 is also known to protect human RPE (Retinal Pigment Epithelium) cells from H_2O_2-induced oxidative stress *via* Nrf2-mediated upregulation of the expression of enzymes involved in the pro-survival PI3K/Akt pathway (Li et al. 2013). Moreover, Cer *de novo* synthesis inhibition with Myriocin (Myr), a potent and selective inhibitor of serine palmitoyl transferase (SPT), the first limiting enzyme of the pathway, decreases photoreceptor degeneration and induces tissue morphology and neuronal functionality rescue in *rd10* mouse, a mouse model of RP (Strettoi et al. 2010).

We here demonstrated ~~that increased levels of S1P, obtained by pharmacological inhibition of its irreversible degradation through~~ that the administration of THI, known as an inhibitor of S1P lyase, promotes 661W cone cells resistance to H_2O_2 oxidative stress. 661W cone-derived cell line is particular suitable for the investigation of photoreceptors features due to the ability to respond to light stimulation and, upon light stress, to undergo cell death as shown for *in vivo* retina experiments (Kanan et al. 2007). Due to their easy handling, 661W were widely used in ophthalmology research, especially as a model of photoreceptors degenerations subjecting them to several kinds of stresses as light damage (Tanaka et al. 2011) and oxidative stress (Chen et al. 2012).We speculate that THI may offer therapeutic perspective in the prevention of bystander retina degeneration in genetic neurodegenerative diseases.

Materials and methods

Reagents and antibodies

H_2O_2 (hydrogen peroxide), THI (2-acetyl-4(5)-(1(R),2(S),3(R),4-tetrahydroxybutyl)-imidazole), Myriocin (Myr), fatty acid free Bovine Serum Albumin (BSA), Trypan blue dye, protease inhibitor cocktail and Hoechst 33258 dye were purchased from Sigma-Aldrich (MO, USA). S1P was purchased from Avanti Polar Lipids (AL, USA). Penicillin and streptomycin (Invitrogen) were purchased from Life Technologies Italia (Italy) meanwhile DMEM culture media, fetal bovine serum (FBS) and the chemoluminescence system LiteAblot from EuroClone Life Science Division (Italy). SYBR Premix Ex Taq (TliRNaseH Plus) was purchased from Takara Bio, Inc. (Japan) and RT-qPCR primers from Eurofins Genomics (Germany).

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

S1P solution preparation

S1P powder was diluted in methanol:water (95:5) to a final concentration of 1μM and 100nM, dried through a stream of dry nitrogen and stored according to the Avanti Polar Lipids protocol. To perform cellular treatments, dried S1P powder was resuspended in sterile fatty acid free BSA (4 mg/ml) and a 125μM S1P stock solution was obtained. The resuspended S1P powder was incubated for 30 minutes at 37°C, sonicated and vortexed until totally dissolved.

Cells and treatments

661W cells have been cloned from retinal tumors of transgenic mouse line expressing the SV-40 T antigen under control of the inter-photoreceptor retinal binding protein promoter (IRBP) (al-Ubaidi et al. 1992) and kindly provided by Dr. Muayyad Al-Ubaidi (University of Oklahoma Health Sciences Center, OK, USA). 661W cells were grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and seeded in Petri dishes (100mm) at $8x10^5$ cells/Petri in 10 ml or 6-well tissue culture plates at $2.5x10^5$ cells/well in 3 ml, according to the analysis performed. Twenty four hours after seeding, at about 60-70% confluence, medium was replaced and cells were pretreated with THI (75 μM), Myr (10 μM) or S1P (100 nM), for two, five and one hour, respectively, before adding H_2O_2 (1 mM) for further twelve hours. All treatments were performed in medium with serum. Cells were then collected according to the assay performed.

Trypan blue dye exclusion test

Trypan blue assay was used to evaluate the number of viable and dead cells after treatments. 661W cells were seeded in tissue culture Petri dishes (100mm) at $8x10^5$ cells/Petri. Cells were detached using 0.25% trypsin and resuspended in 2 ml of Phosphate buffered saline (PBS). 10 μl of 0.4% Trypan blue dye were added to 90 μl cell suspension aliquots and the dead blue-stained cells were counted using Bürker chamber under a MoticAE31 optical inverted microscope. The amount of alive and dead cells was expressed as percentage of the total cells number.

Western blotting

Protein expression level was analyzed by Western blotting. Treated and control 661W cells ($8x10^5$ cells/100mm Petri dish), were collected by scraping on ice in cold PBS-EDTA buffer, containing protease inhibitor cocktail and centrifuged at 800xg for 5 minutes at 4 °C. Supernatants were discarded, the pellets resuspended in 80 µl of the same buffer and a 15 µl aliquot of each sample, was used for total protein quantification (Bradford method). The remaining suspension was resuspended in Laemmli solution, boiled for 8 min and stored at -20°C. Equal amount of proteins (15 µg) was separated by SDS-electrophoresis and then immunoblotted as previously described (Signorelli et al. 2015).

The following primary antibodies at the indicated concentrations were used: 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), anti Nrf2 (1:1000 in TBS-T) and β-actin level (1:2000 in TBS-T-3% BSA). β-actin levels were quantified for data normalization. Bands were detected by chemoluminescence and specific bands intensity was quantified by Gel Doc 2000, with Quantity one software (Bio-rad gel doc system, Chemidoc XRS).

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

In order to measure SPLs intracellular amount, treated and control 661W cells ($8x10^5$ cells/100mm Petri dish) were scraped and collected as for Western blotting analysis. The obtained cellular pellets were either lyophilized or directly frozen at -80°C. SPLs extracts from untreated and treated cells, fortified with internal standards (N-dodecanoylsphingosine, N-dodecanoylglucosylsphingosine, N-dodecanoylsphingosylphosphorylcholine, C17-sphinganine (0.2 nmol each) and C17-sphinganine-1-phosphate (0.1nmol), were prepared and analyzed as reported (Merrill et al. 2005). The liquid chromatography-mass spectrometer consisted of a Waters Aquity UPLC system connected to a Waters LCT Premier orthogonal accelerated time of flight mass spectrometer (Waters, Millford, MA), operated in positive electrospray ionization mode. Full scan spectra from 50 to 1500 Da were acquired and individual spectra were summed to produce data points each 0.2 s. Mass accuracy and reproducibility were maintained by using an independent reference spray by the LockSpray interference. The analytical column was a 100 mmL 2.1 mm i.d., 1.7 mm C8 Acquity UPLC BEH (Waters). The two mobile phases were phase A: methanol; phase B: water, both contained 0.2% formic acid (v/v) and 2 mM ammonium formate. A linear gradient was programmed—0.0 min: 20% B; 3 min: 10% B; 6 min: 10% B; 15min: 1% B; 18 min: 1% B; 20 min: 20% B; 22 min: 20% B. The flow rate was 0.3 mL/min. The column was held at 30 °C. Quantification was carried out using the extracted ion chromatogram of each compound, using 50 mDa windows. The linear dynamic range was determined by injecting standard mixtures. Positive identification of compounds was based on the accurate mass measurement with an error <5 ppm and its LC retention time, compared to that of a standard (2 %).

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Apoptosis was determined by the TdT assay (In situ Cell Death Detection Kit, TMR Roche Diagnostics, Germany). After collection, treated and control 661W cells ($2.5x10^5$ cells/well) were washed in 1 ml cold PBS and centrifuged 5 min at 800xg at 4°C. The pellet was resuspended in 500 µl of 4% cold buffered formalin for 30 min and then processed as already described (Caretti et al. 2010). Images were acquired by a digital camera (DS-2MV; Nikon, Japan) and the number of TdT-labeled nuclei counted (8-10 random fields in a blinded procedure)(Terraneo et al. 2013). Results are expressed as number of TdT-labeled nuclei/total nuclei.

Ferric reducing antioxidant power (FRAP) assay

Cellular supernatants separated from pellets of treated and control 661W cells ($2.5x10^5$ cells/well) were used to perform FRAP assay according to the method of Benzie & Strain (Benzie and Strain 1999) with minor modifications, as previously reported (Signorelli et al. 2015). Aqueous solutions of $FeSO_4 \cdot 7H_2O$ (100–1000 μM) were used for the calibration and the results were expressed as FRAP value (μM Fe (II)) of the samples (Zarban et al. 2009), evaluating the total concentration of reduced Fe(II) normalized on the total protein content of each sample.

Real-time PCR (RT-qPCR)

To evaluate the influence of treatments on mRNA transcription, real-time PCR was performed on control and treated cells ($2.5x10^5$ cells/well). Total RNA was extracted with miRNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. Extracted RNA (1μg) was reverse transcribed in cDNA (Faioni et al. 2014). Each primer set was used at 200 nM in a final volume of 20 μl by using the SYBR Green system according to the protocol. Primer's sequences of target gene heme oxygenase-1 (HO-1) (Chen et al. 2012) were obtained from published studies.

HO-1	forward TCTATCGTGCTCGCATGAAC
	reverse CTGTCTGTGAGGGACTCTGG
GAPDH	forward AACTTTGGCATTGTGGAAGG
	reverse ACACATTGGGGGTAGGAACA

RT-PCRs were performed on a CFX96 Real-Time System (Bio-Rad, CA, USA). Results were normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) RNA. Relative quantification of gene expression was calculated using the 2-ΔΔCt method as previously described (Arocho et al. 2006).

Statistical analysis

GraphPad Prism software (GraphPad Software, CA, USA) was used for graph preparation and statistical analysis. Data were analyzed using one-way ANOVA followed by Bonferroni post hoc test for multiple comparisons. Differences between groups were considered statistically significant at values of $P<0.05$. Results are expressed as mean ± SD. Western blotting and TUNEL images are the most representative of at least three independent experiments.

Results

Sphingolipid metabolism is involved in the regulation of cone photoreceptor like cells degeneration induced by oxidative stress

To trace the involvement of SPLs in photoreceptors degeneration, modulation of Cer *de novo* synthesis was analyzed in an *in vitro* model of photoreceptor degeneration induced by oxidative stress. 661W cells were pretreated with either Myr (10 μM for 5 hours) an inhibitor of Cer *de novo* synthesis pathway or THI (75 μM for 2 hours) an inhibitor of S1P lyase. Cells were treated for further 12 hours with 1mM H_2O_2. We observed an overall increase of ceramide (figure 1a), significant for the two C18:0 and C20:0 species (Figure 1b). Myr reduced by more than 50% the total Cer content in Myr/H_2O_2 vs the H_2O_2-stressed group (from 1162,06 ± 81.95 to 490.18 ± 92.42 picomoles/mg protein; one-way ANOVA: $P = 0.016$) (Figure 1a) and significantly lowered the content of C18:0 and C20:0 acyl chain bearing ceramides in the same treatment groups (Figure 1b). Moreover, we observed 30% reduction of total Cer in THI pretreated with respect to H_2O_2-

stressed cells (Figure 1a: from 1162.06 ± 81.95 to 763.92 ± 46.68 picomoles/mg protein) and a significant decrease in the level of C18:0 and C20:0 acyl chain bearing ceramide (Figure 1b).

We thus confirmed that Cer synthesis is involved in retinal photoreceptors under stress condition *in vitro* as we previously demonstrated *in vivo* (Strettoi et al. 2010).

Sphingolipid metabolism modulation counteracts the oxidative stress effects on photoreceptor like cells

In order to evaluate 661W cells viability, Trypan blue exclusion assay was performed on cells under oxidative stress condition either pretreated or not with Myr (10 μM; 5 hours), S1P exogenously added (100 nM; 1 hour) or THI (75 μM; 2 hours). THI is known to increase S1P endogenous level by inhibition of S1P lyase that blocks one of its degradation pathways (Schwab et al. 2005). Myr, S1P and THI treatments allowed the recovery of cell survival, raising the percentage of alive cells from 75% (in untreated H_2O_2-stressed cells) up to 90% (in pre-treated H_2O_2-stressed cells) (Figure 2a; one-way ANOVA: $P = 0.003$). Myr, S1P and THI treatments reduced the percentage of dead cells from 25% to about 10% in H_2O_2-stressed *vs* pretreated cells, accordingly (Figure 2b; one-way ANOVA: $P<0.001$).

Since modulating the SPLs metabolites improved H_2O_2-stressed 661W cells survival, the antioxidant potential of the three compounds was investigated by means of the FRAP assay in culture media of 661W.

THI alone enhances by 30% the anti-oxidant potential of 661W with respect to untreated control cells. Myr pretreatment induced almost 2.5 folds increase in anti-oxidant potential, whereas S1P and THI almost two folds increase with respect to H_2O_2 treatment (Figure 3; one-way ANOVA: $P = 0.002$) highlighting that SPL modulation increases antioxidant-intrinsic power of photoreceptors as a survival response to counteract oxidative stress.

THI attenuates oxidative stress *via* activation of Nrf2/HO-1 pathway in cone photoreceptor like cells

Since THI recovered cell survival (Figures 2a and b), we investigated the molecular mechanism that ensures THI photoreceptor protection from oxidative stress and analyzed Nrf2/HO-1 pathway, being Nrf2 a key player of the antioxidant survival response. Accordingly with the anti-oxidant potential increase (Figure 3), THI treatment induced intracellular accumulation of the Nrf2 transcription factor in THI/H_2O_2-treated with respect to H_2O_2-stressed photoreceptors like cells (Figure 4a; one-way ANOVA: $P<0.001$) which in turn show higher expression of Nrf2 than the control untreated cells. Moreover, THI/H_2O_2-treated cells exhibited 20% increase in mRNA expression of the Nrf2 target gene HO-1 with respect to H_2O_2-stressed cells, indicating that Nrf2 regulated pathway is activated (Figure 4b; one-way ANOVA: $P< 0.001$). These data suggest that THI is able to initiate a pro-survival stress response implying transcription and activation of Nrf2-dependent pathway.

THI impairs apoptosis and induces pro-survival responses

To study the protective effect of anti-oxidant signaling activation, we investigated both apoptosis and pro-survival responses in 661W cone-like cell line. TUNEL-positive apoptotic cells were 70% of the total in H_2O_2-treated cells and decreased to 25% in THI/H_2O_2 cells (Figure 5a; one-way ANOVA: $P< 0.001$). Moreover Bcl-2/Bax ratio, a known index of anti-apoptotic effect (if >1), increased three folds in THI/ H_2O_2 *vs* H_2O_2-treated cells (Figure 5b; one-way ANOVA: $P< 0.001$).

Next, we studied the involvement of key mediators of survival/death pathway known to be correlated with sphingolipid metabolism modulation (German et al. 2015; Giussani et al. 2009). THI pretreatment of H_2O_2-treated cells, promoted 1.5

fold up-regulation of the pro-survival and stress sensors ERKs (one-way ANOVA: P=0.017) and 40% reduction of the phosphorylation of Akt at the Ser473 (one-way ANOVA: P<0.001) (Yang et al. 2006) with respect to H_2O_2-stressed photoreceptors (Figures 6a and b).

Discussion

Involving a variety of mutations, RP deserves a therapeutic approach aimed at addressing pathological mechanism that are downstream the mutation and that are shared among patients. Currently, many studies focus on mutation-independent ways to rescue photoreceptors neurodegeneration as, for example, electrical stimulation, pharmacological and nutraceutical approaches and administration of neurotrophic factors aimed at straightening survival and/or inhibiting death stimuli (Trifunovic et al. 2012). A broad variety of treatments has been employed: neurotrophic factors as NGF, BDNF, CNTF, PEDF and GDNF (Kolomeyer and Zarbin 2014), nutraceutical and antioxidant agents such as vitamin A and its derivatives (Perusek and Maeda 2013), docosahexaenoic acid (DHA) (Hoffman et al. 2014), lutein (Berson et al. 2010) and taurine (Froger et al. 2014).

The hypothesis underlying the present work is that, acting downstream the mutations and targeting those mechanism that are altered in all mutation variants (such as pro-survival response demise, inflammation and oxidative stress, apoptosis disposition, it may be possible to obtain effective therapeutic approach in RP. Within all the mediators that are able to determine photoreceptors loss or survival through regulation of death-salvage processes, a family of bioactive molecules, SPLs, is evidenced (Rotstein et al. 2010). This study aims to demonstrate the involvement of SPLs in photoreceptor death, suggesting the manipulation of these signalling molecules as potential therapeutic targets in retinal diseases. We previously demonstrated that Cer accumulation occurs in retina degeneration and that the inhibitor of Cer synthesis, Myr, has a therapeutic role in reducing or delaying photoreceptors apoptosis and visual loss (Strettoi et al. 2010).

THI inhibits pyridoxal kinases (Elsinghorst et al. 2015). Among SPL metabolizing enzymes, the rate limiting step of *de novo* synthesis of ceramide is catalysed by PLP dependant SPT. Nonetheless, Schwab et al. for the first time demonstrated that S1P abundance in lymphoid tissues of mice increased more than 100-fold after THI treatment and that it depends on ~~Sphingosine~~ S1P lyase inhibition (Schwab et al. 2005). Several publications followed this first evidence, demonstrating that the major effect of the compound is targeting S1P lyase activity and inducing S1P elevation (Gorshkova et al. 2013; Klyachkin et al. 2015; Nguyen-Tran et al. 2014; Ohtoyo et al. 2015). Although such strong evidences have been reported *in vivo*, no *in vitro* data are available at present on THI intracellular action.

~~Given the pro-survival activity of intracellular S1P, in opposition to Cer signalling, we here investigated the protective effects of inhibition of S1P lyase, the enzyme responsible for one of the two degradation pathways of S1P.~~

S1P is a well-known anti-apoptotic sphingolipid able to induce cell survival, proliferation and differentiation in several cell lines (Cieslik et al. 2015; Czubowicz and Strosznajder 2014). Nowadays, it is known that this enigmatic sphingolipid has a pivotal role in many pathologies such as cancer, atherosclerosis, inflammation, neurodegeneration, cardiovascular and respiratory affections (Maceyka et al. 2012). In rat retina neuronal cultures, S1P acts on photoreceptor progenitors, stimulating their proliferation and differentiation and preventing photoreceptor mediated-apoptosis death (Miranda et al. 2009). Given the pro-survival activity of intracellular S1P, in opposition to Cer signalling, we here investigated the potential pro survival activity of THI, known as an inhibitor of S1P lyase.

We exposed 661W cone-like cells to oxidative stress by means of H_2O_2 that results in a strong decrease of alive cells and in a parallel augment of dead cells at early time point. To understand the intracellular pathways involved, two well-known proteins with pivotal role in cells proliferation and survival, ERKs and Akt, were investigated. ERKs activation in retina was associated to protection against light injury in *in vivo* rat model (Liu et al. 1998), as well as in 661W cell culture under brief stimulation with H_2O_2. The time-response experiment showed a rapid activation of phosphorylated ERK1/2 within 5 minutes after exposure to H_2O_2 followed by a decrease of activated ERK1/2 expression from 5 to 30 minutes. This correlates with an initial survival response triggered by the cells and a final demise of the cells if the stress is sustained in time (Dong et al. 2012). Accordingly, we observed that 12 hours of H_2O_2 treatment causes cell death and ERKs

dephosphorylation. Another important player in survival responses is the Akt kinase whose activity is modulated during pathological situations (Toker and Marmiroli 2014) such as retinal degeneration. Akt activation was demonstrated to rescue rat retinal neurons from light-induced apoptosis (Mo et al. 2013). In 661W model, Mackey et al. showed that serum deprivation induced a rapid burst of H_2O_2, activating survival pathways and promoting Akt phosphorylation at Thr408 (Mackey et al. 2008). Later, the same group demonstrated that bFGF administration to 661W enhanced ROS production and again Akt phosphorylation occurred, even if on Ser473 (Farrell et al. 2011). The same phosphorylation was documented in H_2O_2-stressed RPE (Yang et al. 2006). According to the severity of the stress, Akt undergoes phosphorylation on different residues, being Ser473 phosphorylation associated to a brief stress meanwhile Thr408 corresponded to intense and prolonged stress (Yung et al. 2011). Our results confirmed that oxidative stress induced Akt activation that resulted in Ser473 phosphorylation. In our model, Akt activation persisted with respect to that of ERK, suggesting a potential regulation of Akt in the switching off of the ERK pathway. THI~~, inhibiting S1P lyase,~~ was able to reduce stress-induced blockade of proliferation. Moreover, THI exerted a protective effect on cell death, increasing the number of viable cells in H_2O_2 stressed samples.

In order to elucidate the THI protective mechanism, we investigated the effect of its pre-treatment on signalling mediators. As previously shown, DHA-induced protection of photoreceptors death was attributed to the increase of Bcl-2/Bax ratio likely induced by ERK/MAPK activation (German et al. 2006). Furthermore Bcl-2 overexpression rescues 661W cells from photo-oxidative stress (Crawford et al. 2001) and counteracts photoreceptors apoptosis in different retinal degenerations (Chen et al. 1996). Additionally, in Jurkat cells, S1P inhibition of the pro-apoptotic Bax and Bad activation, was supposed to be caused by MEK activation (Betito and Cuvillier 2006). In our hands, THI pre-treatment reduced apoptosis, decreasing the number of TUNEL-positive cells *vs* stressed cells only. Furthermore, Bcl-2 expression overtakes that of Bax, shifting their ratio equilibrium to survival stimulus and proving that ~~the inhibition of S1P degradation~~ THI pre-treatment has a role in preserving 661W cells from apoptosis. Moreover, THI showed an antioxidant activity against H_2O_2 stress. Although H_2O_2 alone induced accumulation of Nrf2 with respect to the control untreated cells, the combined treatment of THI and H_2O_2 significantly increased Nrf2 protein expression in stressed cells, thus supporting the hypothesis of a critical role of the transcription factor in cells protection. In a rat model of emphysema, S1P ability to overexpress Nrf2 against stress was previously shown (Yasuo et al. 2013). Nrf2 protected human RPE cells from H_2O_2-induced oxidative stress via Nrf2-mediated upregulation of the expression of Phase II enzymes involving the PI3K/Akt pathway (Li et al. 2013). Furthermore, HO-1 induction downstream Nrf2, protected retinal ganglion cells in diabetic retinopathy (Fan et al. 2012). In THI and H_2O_2 treated cells, HO-1 transcription was protracted in agreement with Nrf2 upregulation. We can conclude that THI is able to enhance antioxidant response thus promoting cell survival. Finally, knowing the apoptotic role of Cer accumulation in photoreceptors degeneration we investigated if THI was able to affect Cer intracellular concentration. Within an overall trend of counteracting ceramide accumulation upon stress, we observed a specific reduction of two acyl chain species , that is reproduced by both Myr and THI pretreatment, suggesting a specific role of this minor species in controlling cone-like cells survival. ~~We hypothesized that inhibition of Cer synthesis and increase of S1P degradation following THI pre-treatment, may be interconnected, in the intent of improving cell protection.~~

Our hypothesis is that THI exerts a protective mechanism, possibly via inhibiting S1P lyase and enhancing intracellular S1P , that is responsible of down-modulating ceramide acccumulation. Therefore in oxidative stress condition, THI may confer cell protection from ceramide mediated death.

THI is supposed to interact with PLP, the S1P lyase cofactor, blocking its normal involvement in enzymatic mechanism to degrade S1P (Bigaud et al. 2014). The recent identification of SphK-1 and SphK-2 as regulators of de novo Cer

synthesis (Maceyka et al. 2005), suggests the existence of a feedback action of S1P on Cer synthesis as previously demonstrated for phosphorylated long-chain sphingoid bases (van Echten-Deckert et al. 1997). THI inhibits pyridoxal kinases with competitive and mixed-type non-competitive behavior towards its two substrates, pyridoxal and ATP, respectively. Both S1P lyase and Serine Palmitoyl Transferase (SPT) use PLP coenzyme, thus the two enzymes are eligible for THI inhibition. Nontheless, the scarce effect on ceramide cell content of THI, proved that its activity goes far beyond the inhibition of ceramide synthesis and it is related to pro survival signaling known to be induced upon S1P increase. Oxidative stress induced apoptotic Cer generation which is reduced by THI and THI treatment favours survival and proliferation upon stresses in an *in vitro* model of photoreceptor degeneration by modulating ERK and Akt activation, Bcl-2/Bax equilibrium, Nrf2-HO-1 expression All these outcomes demonstrate and confirm sphingolipid biosynthetic pathway as a paramount target in therapeutic approaches for photoreceptor degeneration.

Figure legend

Fig. 1 Myriocin and THI antagonize H_2O_2-induced increase of Cer To analyze Cer involvement in the regulation of cone photoreceptors degeneration induced by oxidative stress, H_2O_2-stressed 661W cells (1 mM H_2O_2, 12 hours) were either pretreated or not with Myr (10 µM for 5 hours) or THI (75 µM for 2 hours). The effect of both THI alone (75 µM; 2 hours) and Myr alone (10 µM for 5 hours) on control cells was also reported. By means of LC-MS, lipid extracts were analyzed for total Cer (a) and C18:0/C20:0 acyl chain bearing Cer (b) that are the two Cer species significantly affected by both Myr and THI treatment. Cer amount (picomoles) were normalized on milligrams of proteins (picomoles/mg protein). Data are obtained from three individual experiments (independent culture and stimulation) and expressed as mean ± SD. Significance was evaluated by one-way ANOVA followed by Bonferroni post-test when significant. Panel a): *, $P < 0.05$ *vs* H_2O_2-stressed cells; **, $P < 0.01$ *vs* control untreated cells. Panel b): **, $P < 0.01$; ***, $P < 0.001$ *vs* control untreated cells; $^{\#}$, $P<0.05$; $^{\#\#}$, $P<0.01$ *vs* H_2O_2-stressed cells.

Fig. 2 Sphingolipid modulation preserves the survival of 661W cells against oxidative stress Cell viability was assessed by Trypan blue exclusion assay in H_2O_2-stressed 661W cells (1mM H_2O_2, 12 hours), either pretreated or not with Myr (10 µM; 5 hours), S1P exogenously added (100 nM; 1 hour) or THI (75 µM; 2 hours). The effect of THI alone (75 µM; 2 hours) on control cells was also reported. Percentage of alive (2a) and dead (2b) cells were calculated considering total cells as 100%. Data are obtained from three individual experiments (independent culture and stimulation) and values are expressed as mean ± SD. Significance was evaluated by one-way ANOVA followed by Bonferroni post-test when significant. Panel a) and b): *, $P < 0.05$; **, $P < 0.01$; ***, $P<0.001$ *vs* H_2O_2-stressed cells; $^{\#}$, $P<0.05$, $^{\#\#}$, $P<0.01$ *vs* control untreated cells.

Fig. 3 Sphingolipid modulation increases antioxidant-intrinsic power of photoreceptor like cells In order to investigate the antioxidant potential of Myr, S1P or THI, 661W cells supernatant was analyzed by the FRAP test assay in H_2O_2-stressed 661W cells (1mM H_2O_2, 12 hours), either pretreated or not with Myr (10 µM; 5 hours), S1P exogenously added (100 nM; 1 hour) or THI (75 µM; 2 hours). The effect of THI alone (75 µM; 2 hours) on control cells was also reported. Data are obtained from three individual experiments (independent culture and stimulation) and expressed as mean ± SD. Results are expressed as fold change of FRAP value (µmoles Fe(II)/µg proteins) *vs* untreated control cells. Significance was evaluated by one-way ANOVA followed by Bonferroni post-test when significant: *, $P < 0.05$ *vs* untreated control cells and **, $P < 0.01$; ***, $P < 0.001$ *vs* H_2O_2-stressed cells.

Fig. 4 THI enhances Nrf2 expression and HO-1 transcription to counteract oxidative stress To better understand the molecular mechanism underpinning stressed-photoreceptor rescue after THI treatment, Nrf2/HO-1 pathway was investigated. Cells were pretreated with THI (75 µM; 2 hours) and either stressed or not with 1 mM H_2O_2 for twelve hours. a) Densitometric analysis of Nrf2 protein bands (57kDa). Values were normalized on the corresponding β-actin (43kDa) and expressed as fold change *vs* untreated control. Western Blot uncut, labeled and clearly indicating the area which was used for Nrf2 detection can be found in Supplemental file 1. b) mRNA expression level of HO-1. Data were normalized on GAPDH housekeeping gene and expressed as fold change *vs* untreated control. Data are obtained from three individual experiments (independent culture and stimulation) and expressed as mean ± SD. Significance was evaluated by one-way ANOVA followed by Bonferroni post-test when significant. Panel a): *, $P < 0.05$ vs control untreated cells; **, $P < 0.01$ vs H_2O_2-stressed cells. Panel b): ***, $P < 0.001$ vs control untreated cells; **, $P < 0.01$ vs H_2O_2-stressed cells.

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Fig. 5 THI reduces photoreceptor like cells apoptosis degree Cells were pretreated with THI (75 µM) for two hours and either stressed or not with 1mM H_2O_2 for twelve hours. a) TUNEL-assay was performed to estimate the apoptosis. Images show TdT positive nuclei labeled with rhodamine (red) while all nuclei were counterstained with Hoechst 33258 (blue) and refer to the H_2O_2-stressed and the THI/H_2O_2-treated cell group. Scatter plots below represent the quantification of the apoptotic cells expressed as percentage of TdT-positive on total nuclei. 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). Western blot images are the most representative of three independent experiments. Western Blot uncut, labeled and clearly indicating the area which was used for Bcl2 and Bax detection can be found in Supplemental file 1. Scatter plots below report Bcl-2/Bax ratio as fold change *vs* untreated control. β-actin (43 kDa) represents the loading control for the above reported marker. Data are obtained from three individual experiments (independent culture and stimulation) and expressed as mean ± SD. Significance was evaluated by one-way ANOVA followed by Bonferroni post-test when significant. Panel a): *, $P < 0.05$ *vs* H_2O_2-stressed cells; **, $P < 0.01$ *vs* control untreated cells. Panel b): ***, $P < 0.001$ vs control untreated cells; $^{###}$, $P < 0.001$ vs H_2O_2-stressed cells.

Fig. 6 THI pro-survival effect involves ERK1/2 and Akt phosphorylation Cells were treated with 75µM THI for two hours and either stressed or not with 1mM H_2O_2 (for further 12 hours). a-b) Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies for (phosphorylated) p-ERKs, (total) t-ERKs (44 and 42 kDa), (phosphorylated) p-Akt and (total) t-Akt (60 kDa). Western blot images are the most representative of three independent experiments. Western Blot uncut, labeled and clearly indicating the area which was used for ERK and Akt detection can be found in Supplemental file 1. Scatter plots below report phosphorylated/total proteins ratio as fold change *vs* untreated control. β-actin (43 kDa) represents the loading control. Data are obtained from three individual experiments (independent culture and stimulation) and expressed as mean ± SD. Significance was evaluated by one-way ANOVA followed by Bonferroni post-test when significant. Panel a): *, $P < 0.05$ *vs* control untreated cells and **, $P < 0.01$ *vs* H_2O_2-stressed cells. Panel b): *, $P < 0.05$ *vs* H_2O_2-stressed cells and ***, $P < 0.001$ *vs* control untreated cells.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure 1 revised

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