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SAHA is neuroprotective in in vitro and in situ models of retinitis pigmentosa

Nathan R. Perron,[1] Cécile Nasarre,[2] Mausumi Bandyopadhyay,[2] Craig C. Beeson,[1] Baerbel Rohrer[2,3]

[1]Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, SC; [2]Department of Ophthalmology, Medical University of South Carolina, Charleston, SC; [3]Research Service, Ralph H Johnson VA Medical Center, Charleston, SC

Purpose: Recent reports linking HDAC6 to mitochondrial turnover and neurodegeneration led us to hypothesize that an inhibitor such as Vorinostat (suberoylanilide hydroxamic acid, SAHA) may reduce mitochondrial damage found in retinitis pigmentosa (RP), a progressive neurodegenerative disease of the eye. Here we tested the efficacy of SAHA for its ability to protect photoreceptors in in-vitro and in-situ models of RP. As the stressor, we focused on calcium overload. Calcium is one of the main drivers of cell death, and is associated with rod loss in the rd1 mouse retina, which harbors a mutation in the Pde6b gene similar to that found in human patients suffering from autosomal recessive RP.

Method: Murine photoreceptor cell line (661W) were exposed to agents that led to calcium stress. Cell survival and redox capacity were measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, real-time changes in cellular metabolism were assessed using the Seahorse Biosciences XF24 analyzer, and mitochondrial fission-fusion using imaging. In-situ, neuroprotection was assessed in RPE/retina organ cultures of the rd1 mouse. SAHA effects on cell survival were compared in 661W cells with those of the specific HDAC6 inhibitor tubastatin A, and those on protein acetylation by Western blotting.

Results: In stressed 661W cells, SAHA was found to increase cell survival that was associated with improved mitochondrial respiration and reduced mitochondrial fission. The protective effects of SAHA were also observed on photoreceptor cell survival in whole retinal organ explants of the rd1 mouse. Even though tubastatin A was ineffective in increasing cell survival in 661W cells, HDAC6 activity was confirmed in 661W cells after SAHA treatment with protein acetylation specific for HDAC6, defined by an increase in tubulin, but not histone acetylation.

Conclusions: SAHA was found to protect mitochondria from damage, and concomitantly reduced photoreceptor cell death in cell and organ cultures. The lack of activity of tubastatin A suggests that there must be an additional mechanism of action involved in the protective mechanism of SAHA that is responsible for its neuroprotection. Overall, SAHA may be a useful treatment for the prevention of photoreceptor degeneration associated with human RP. The results are discussed in the context of the effects of inhibitors that target different classes and members of the HDAC family and their effects on rod versus cone survival.

Retinitis pigmentosa (RP) is a genetically linked neurodegenerative disease of the eye that affects more than a million people worldwide. Although RP begins with a loss of peripheral vision, the disease can ultimately lead to complete blindness. Photoreceptor cell damage in RP can occur as a result either of elevated Ca^{2+} levels or from reactive oxygen species (ROS) [1], which primarily originate from mitochondrial respiration [2]. Elevation of intracellular calcium in RP acts on the mitochondria and leads to additional ROS formation, triggering apoptotic cell death pathways. For instance, one of the human mutation loci causing RP is the gene for rod phosphodiesterase (*PDE6;* Gene ID 5158, OMIM 180072), which catalyzes the formation of guanosine monophosphate (GMP) from cyclic GMP (cGMP). Cyclic GMP is the ligand for the cGMP-gated Na^{+} and Ca^{2+} cation channels in the plasma membrane. These channels control the membrane potential during signal transduction. In RP, however, the mutated *PDE6* is unable to hydrolyze cGMP, causing a buildup of cGMP that leads to an abundance of open channels and Ca^{2+} influx [3]. Excess intracellular Ca^{2+} increases ROS production and uptake of Ca^{2+} by mitochondria, leading to mitochondrial swelling and the mitochondrial permeability transition, ultimately resulting in apoptosis [4]. The pharmacological PDE6 inhibitor, 3-isobutyl-1-methylxanthine (IBMX), can be used to mimic these processes in vitro by elevating cGMP levels in the same way as RP [5].

Despite the genetic heterogeneity of RP (at least 45 genes have been attributed to RP), there is a shared link in the pathways involved with photoreceptor cell death: the mitochondria. Oxidative stress has been implicated as the main trigger for photoreceptor cell death [6]. It also has been suggested that retinas with degenerating photoreceptors

Correspondence to: Bärbel Rohrer, Department of Ophthalmology, Medical University of South Carolina, 167 Ashley Ave, SEI 614, Charleston, SC 29425. Phone: (843) 792-5086; FAX: (843) 792-1166; email: rohrer@musc.edu

(the main oxygen-consuming cells of the retina) are slightly hyperoxic [7], and the outer-retina environment is also highly oxygen rich [8]. Additionally, mitochondrial dysfunction leading to proton leak within retinal cells can generate ROS [2]. Finally, retinal iron has been shown to accumulate with age [8] and is strongly linked to retinal degenerative diseases [10]. Chelatable iron is thought to originate primarily from lysosomes, but superoxide has also been shown to release iron from ferritin [10]. Often, this chelatable iron translocates to the mitochondria [10], and when iron overload or misregulation in the retina occurs concomitantly with increased ROS production, iron can participate in the Fenton reaction, generating hydroxyl radical (•OH), and leading to a vicious degenerative cycle.

As there are currently no treatment options for RP, and no therapies approved by the U.S. Food and Drug Administration (FDA) that address the primary underlying causes, we hypothesize that agents which protect against the early stages of mitochondrial damage can increase the life span of photoreceptors in RP.

Histone acetylation is a key biologic regulator of gene expression in eukaryotic organisms [11]. Histone acetyl transferases (HATs) covalently link acetyl groups from acetyl Co-A to terminal amine groups of lysine residues on histones [12]. The histone deacetylases (HDACs) remove acetyl groups that were added by HATs. Modifying histones with the addition of acetyl groups has been shown to result in increased transcription, leading to increased protein expression, whereas decreased levels of acetylation silence gene expression and protein synthesis. Although HATs and HDACs were initially thought to be only histone modulators, they are now known to act on several targets, including signaling molecules [13].

Histone deacetylase 6 (HDAC6) belongs to the family of class II HDACs. It is found in the cytoplasm and plays several important roles, such as misfolded protein degradation [14], cell migration [15], autophagy [16], and mitochondrial trafficking and turnover (i.e., mitophagy) [17]. Inhibition of HDAC6 dramatically increases acetylation of tubulin, implicating tubulin as a major substrate of HDAC6 [18]. Increased tubulin acetylation in neurons has been shown to recruit kinesin-1 [19], which is the motor protein required for transport of mitochondria within axons [20]. In addition, HDAC6 inhibition reportedly reversed the transport deficit in a Huntington's disease model by increasing vesicular transport of brain-derived neurotrophic factor (BDNF), another kinesin-1 cargo protein [21], and reversed axonal loss in a mouse model of Charcot-Marie Tooth disease [22], suggesting that HDAC6 inhibition may be a viable neuroprotective strategy. These recent reports linking HDAC6 to mitochondrial turnover and neurodegeneration led us to the hypothesis that an HDAC6 inhibitor, such as SAHA (also known as suberoylanilide hydroxamic acid, Vorinostat™, or Zolinza™), may be protective of mitochondrial damage and the pathologies associated with mitochondrial damage and abnormal mitochondrial turnover and distribution, as found in RP, Alzheimer disease, Parkinson disease, Huntington's disease, amyotrophic lateral sclerosis, and other neurodegenerative diseases [23]. SAHA is an FDA-approved drug known to inhibit HDACs 1, 2, 3, and 6 with high potency (K_i about 1.0–10 nM) [24]. Genes that are most upregulated by HDAC inhibition encode for the proteins $p21^{WAF1}$, E2F-1, p53, Apaf-1, as well as additional proteins that precede apoptosis, which rationalizes the classical use of HDAC inhibitors for treatment of cancer [25]. However, healthy cells are relatively resistant to HDAC inhibition-induced cell death, and neurons, which cannot actively replicate to replace lost cells, have sophisticated mechanisms of survival in place [26]. For example, the proapoptotic p53 reportedly does not induce apoptosis in postnatal cortical neurons [27].

We examined whether SAHA can protect mitochondria from damage and concomitantly reduces photoreceptor cell death in cell and organ cultures as a potential treatment for the prevention of photoreceptor degeneration associated with human RP.

METHODS

Mouse 661W cells: Mouse retina-derived 661W photoreceptor cells were generously provided by Dr. Al-Ubaidi (University of Oklahoma) [28] and expanded in T75 flasks in DMEM + 10% fetal bovine serum (FBS). Cells were confirmed to contain cone-specific proteins, UV opsin and GNAT2, with immunohistochemistry, using verified antibodies [29].

Calcium cytotoxicity screen: The 661W cells were seeded into 96-well plates using DMEM supplemented with 5% FBS, grown to confluency for 48 h, followed by a media exchange with DMEM + 1% FBS. HDAC inhibitors were added in concentrations of 0.1–10 µM, followed by the addition of the calcium ionophore A23187 (final concentration 1 µM and 3% dimethyl sulfoxide, DMSO) 1 h later. After 24 h, the cells were analyzed for redox capacity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT dye (1 mg/ml; Sigma-Aldrich; St. Louis, MO) was added for overnight incubation. The resulting dye crystals were dissolved in 50 µl of 20% sodium dodecyl sulfate (SDS) in 0.01 M HCl, and the absorbance measured at 570 nm (background wavelength 650 nm) using a SpectraMax 190 spectrophotometer (MDS Analytical Technologies, Toronto,

Canada). A23187 at 1 µM decreased the formazan dye signal by about 50% at 24 h [30].

Seahorse assay: Oxygen consumption rate (OCR) measurements were performed using a Seahorse Bioscience (Agilent; Santa Clara, CA) XF24 instrument as previously reported [31,32]. Cells were plated in the XF24 plates, and before the experiment was run, the growth medium was replaced with 700 µl of bicarbonate-free DMEM buffer. The buffer contained $CaCl_2$ (1.8 mM), $MgCl_2$ (0.6 mM), KH_2PO_4 (0.5 mM), KCl (6.3 mM), Na_2HPO_4 (0.5 mM), NaCl (135 mM), glucose (5.6 mM), 1 mM glutamine, minimum essential medium (MEM) amino acids solution, MEM non-essential amino acids, MEM vitamin solution, penicillin/streptomycin, 1% bovine serum albumin (BSA, factor V fatty-acid-free), 1% FBS, and insulin (100 nM). Experimental plates were treated with vehicle control (0.5% DMSO) and 0.01–1.00 µM of SAHA. OCR measurements were obtained every 5 min, collecting three measurements per well at baseline and three measurements upon injection of the protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 1 µM) which collapses the proton gradient across the inner mitochondrial membrane. The OCR was normalized to the amount of protein present on the plates using the bicinchoninic acid assay [31].

Imaging: The cells were plated on glass-bottom six-well slides. The cells were stained with nonyl acridine orange (NAO) as recommended by the manufacturer ((TermoFisher; Waltham, MA)) in phenol-red-free DMEM and examined with confocal microscopy (Leica TCS SP2 AOBS; Leica, Bannockburn, IL) using the same laser settings for all experiments.

Western blotting: Cell culture lysates were separated with electrophoresis on a 10% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA), and the proteins were transferred to a nitrocellulose membrane. The membranes were probed with polyclonal antibodies for acetylated tubulin (AcTub) and histones (AcHH3 and 4) as previously described [33]. Tubulin and glyceraldehyde 3-phosphate dehydrogenase (GADPH) were provided as controls. Acetylated tubulin, tubulin, acetylated-HH4, acetylated HH3, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The density of the bands was analyzed and normalized against GAPDH control using the Alpha Innotech Fluorchem 9900 imaging system while running Alpha Ease FC software 3.3 (Alpha Innotech, San Leandro, CA).

Rd1 mouse organ cultures: Rd1 mice were generously provided by Deborah Farber (UCLA, Los Angeles, CA) [34] and housed in the MUSC Animal Care Facility under a 12 h:12 h light-dark cycle, with access to food and water ad libitum. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the MUSC Animal Care and Use Committee. All chemicals used for organ cultures were tissue-culture grade and were purchased from Invitrogen (Carlsbad, CA). Retina-RPE cultures were grown using the interface technique according to published protocols [35-37] with modifications [29]. Briefly, eyes were incubated in 1 ml of media containing cysteine (0.035 mg) and papain (20 Units) at 37 °C for 15 min, which allows for removal of the retina with the RPE attached from the remainder of the eyecup. To flatten the tissue, relaxing cuts were made into the retina-RPE sandwich followed by transfer of the RPE-layer face-down to the upper compartment of a Costar Transwell chamber (ThermoFisher) in a drop of neurobasal medium (Invitrogen). Nutrients and compounds were provided to the cultures using neurobasal media supplemented with 1% N1 and 2% B-27 supplements in the lower compartment. The cultures are grown at 37 °C (5% CO_2, balanced air, 100% humidity), with the media replaced every 2 days. After completion of the experiment, the retina cultures were fixed in 4% paraformaldehyde (PFA), and the tissues cryoprotected in 30% sucrose, frozen in TissueTek O.C.T. (Fisher Scientific; Waltham, MA), cut into 14 µm cryostat sections, and stained with toluidine blue. Rows of photoreceptors were counted in four regions of the retina, and an average was obtained across the retina [38].

Statistics: For data consisting of multiple groups, one-way ANOVA followed by Fisher's post hoc test ($p<0.05$) was used, and post-hoc tests were run only if F achieved $P<0.05$; single comparisons were analyzed by t test analysis ($p<0.05$), using Prism (GraphPad, San Diego CA) and StatView (SAS Institute, Cary NC) software.

RESULTS

SAHA improves NAD(P)H-dependent cellular oxidoreductase enzyme activity and mitochondrial function and structure in an HDAC6-independent mechanism: We explored the ability of SAHA to protect photoreceptor neurons in in vitro and in situ models of RP. First, the 661W photoreceptor cell line was treated with the Ca^{2+}-ionophore A23187 (1 µM) to mimic the increased Ca^{2+} influx that occurs in RP [3,30], resulting in an approximate 50% reduction in signal in the MTT assay. This assay has been used for high throughput drug screening to assess cell protection. As the assay assesses NAD(P)H-dependent cellular oxidoreductase enzymes, it provides a measure of mitochondrial function and metabolic activity [39]. Two HDAC inhibitors known to target HDAC6, SAHA (which inhibits HDAC classes I and II, including HDAC6)

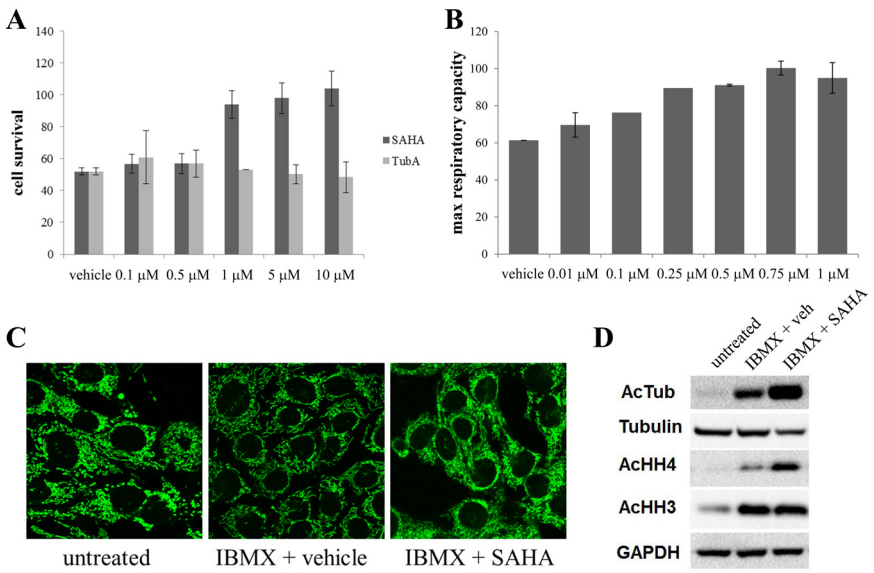

Figure 1. Neuroprotective effects of SAHA in 661W photoreceptors. **A**: Two different HDAC6 inhibitors, one non-selective (Vorinostat; suberoylanilide hydroxamic acid, SAHA) and the other selective (tubastatin A, TubA), were tested for their effect in protecting 661W photoreceptor cell survival and increasing NAD(P)H-dependent cellular oxidoreductase enzyme activity using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell death was induced by the calcium ionophore A23187, selecting a dose that reduces the MTT assay readout by 50% (1 µM). The MTT readout could be improved upon in the presence of SAHA, but not tubastatin A over a 2 log unit dose range. **B**: The same dose range of SAHA was tested using a high-resolution respirometric XF Seahorse assay examining the maximal respiratory capacity (FCCP response). The oxygen consumption rate (OCR), a direct correlate of ATP reduction, demonstrated that application of 3-isobutyl-1-methyl-xanthine (IBMX) reduced the OCR by about 40%, whereas SAHA was able to prevent that decline over the same dose range as in (**A**). **C**: IBMX (600 µM) was shown to induce fragmentation in 661W cells stained with 10-N-nonyl acridine orange (NAO), an acridine orange derivative that marks the inner mitochondrial membrane in whole cells. SAHA treatment prevented mitochondrial fission, resulting in a fused phenotype. **D**: Cell extracts of 661W cells were probed for acetylated tubulin (AcTub) and histones (AcHH3 and 4) using western blotting. Tubulin and glyceraldehyde 3-phosphate dehydrogenase (GADPH) were provided as controls. The typical signature for HDAC6 was elucidated, stress increased tubulin and histone acetylation, but only tubulin acetylation, and not histone acetylation, was further increased by SAHA. Data are shown as mean ± standard deviation (SD; n = 3–12), or representative images are shown.

and tubastatin A (which targets only HDAC6) were chosen to further investigate a potential role for HDAC6 in neuroprotection. Both compounds were added for 1 h pre-A23187, and the inhibitor and the stressor were left in the medium for 24 h, after which the MTT assay was performed. Interestingly, over the dose range investigated, 0.1–10 µM, SAHA was found to increase cell survival and oxidoreductase enzyme activity, whereas tubastatin A was ineffective (Figure 1A). Thus, for the remaining experiments, we focused on SAHA.

Next, as MTT-based cell protection is linked to mitochondrial function, mitochondrial respiration was assessed using the Seahorse Biosciences extracellular flux assay to study 661W cells under stress in the presence or absence of the HDAC inhibitor, as we have described previously [31]. After a 1 h pretreatment period with SAHA, 600 µM of PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) was introduced, and the OCRs were measured after 24 h [31]. OCR measurements upon injection of the FCCP (1 µM), which collapses the proton gradient across the inner mitochondrial membrane and increases the maximum electron transport chain oxygen consumption (termed the maximum respiratory capacity), were examined. The XF data showed that 600 µM IBMX caused approximately a 40% decrease in respiratory capacity after 24 h treatment in the 661W cells (Figure 1B). One hour pretreatment with SAHA followed by IBMX insult protected the 661W cells (Figure 1B), providing maximum protection of 96% at 1 µM, similar to the dose response identified for the MTT assay.

Previous studies demonstrated that SAHA significantly decreases the expression of mitochondrial fission protein Fis1, reduces the translocation of Drp1 to the mitochondria, and results in a fused phenotype [40,41]. We used confocal microscopy (Leica) to visually assess the mitochondrial fusion or fission state in the cells stained with NAO, a fluorescent marker of the inner mitochondrial membrane. Results showed that 24 h IBMX treatment (600 µM) caused a shift

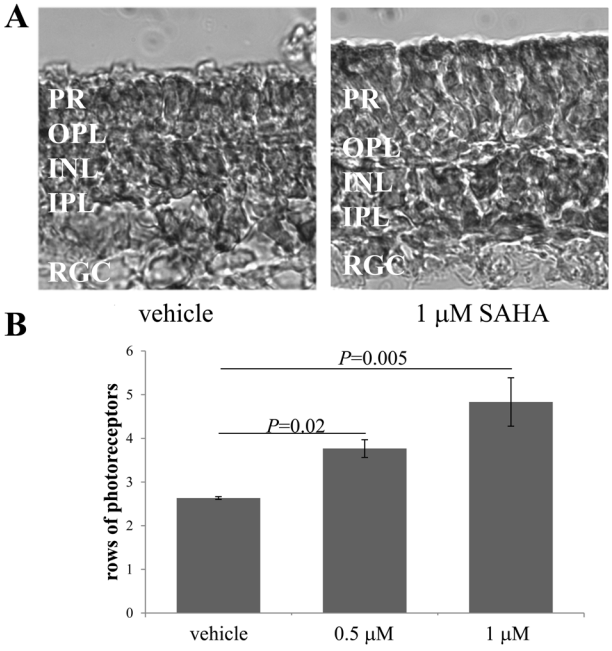

Figure 2. Neuroprotective effects of SAHA in *rd1* organ cultures. *Rd1* retina-RPE sandwich organ cultures were grown for 10 days from postnatal day 10 to 20, during which the majority of photoreceptors degenerate in vehicle-treated samples. Vorinostat (suberoylanilide hydroxamic acid, SAHA) was found to reduce the amount of cell loss in a dose-dependent manner. **A**: Representative samples of retina sections are shown. **B**: Cell counts over ten locations across the explant culture document a twofold increase in average cell numbers with 1 µM SAHA. Data are shown as mean ± standard error of the mean (SEM; n = 3–4), or representative images are shown.

toward mitochondrial fission, but pretreatment with 1 µM SAHA reversed that trend, resulting in a fused mitochondrial network, similar to that of untreated control cells (Figure 1C).

Finally, HDACs act as dual deacetylases for tubulin and histones, but HDAC6 was found to consistently induce elevated levels of acetylated α-tubulin only, and not histones [42]. Western blotting was used to identify the effect of SAHA on acetylation of tubulin and histones H3 and H4 (Figure 1D) [43]. Tubulin and histone H3 and H4 acetylation were all increased by IBMX stress, which was further augmented by SAHA for tubulin, but not histones H3 and H4.

SAHA improves photoreceptor cell survival in rd1 mouse organ cultures: We demonstrated that SAHA, but not another HDAC6-specific inhibitor (tubastatin A), protects photoreceptor neurons in an in vitro a model of RP. To further show that protection of mitochondria correlates to protection of photoreceptors, we chose to use the *rd1* mouse, which is genetically homologous to the disease etiology observed in some cases of human RP [44]. The *rd1* mouse is an ideal model of human RP and loses rod photoreceptors due to apoptosis in a reproducible manner beginning at post-natal day (P) 8 with none left after 4 weeks [44]. In addition, excessive HDAC activation, as evidenced by a lack of histone (and other proteins) acetylation, was found to be critically involved in the photoreceptor neurodegeneration pathway of this mouse [45]. Retina-RPE explant cultures were grown on Transwell plates according to our published methods [29], supplying fresh SAHA at every media exchange. Retinas were grown in culture between days 10 and 21, the time of active photoreceptor cell death in this model, followed by fixation, sectioning, and staining with toluidine blue to count surviving photoreceptors (Figure 2A) [38]. Photoreceptor cell survival was improved significantly in a dose-dependent manner in retinas receiving SAHA throughout the course of the experiment compared to the untreated *rd1* control retinas (Figure 2B).

DISCUSSION

Taken together, the results show that (1) the cell survival and redox capacity of calcium-stressed 661W cells are improved upon the application of SAHA, but not the specific HDAC6 inhibitor tubastatin A. (2) The improvement in the MTT assay by SAHA was mirrored by the improvement in the maximum respiratory capacity of the 661W cells over the same dose range. (3) SAHA prevented stress-induced mitochondrial fission. (4) SAHA increased photoreceptor cell survival in the *rd1* organ culture model. Finally, (5) tubulin and histone H3 and histone H4 acetylation were elevated by IBMX-induced stress, which could be ameliorated by SAHA only for tubulin, whereas histone H3 and H4 acetylation remained elevated. These results suggest that one of the effects of SAHA is to inhibit HDAC6 specifically, but as the specific HDAC6 inhibitor tubastatin A did not increase cell survival, there must be other, non-HDAC6-inhibitory modes of action that are responsible for SAHA's neuroprotective effects.

Oxygen consumption in photoreceptors is extremely high due to their high ATP demand [46], and photoreceptors have been shown to use oxidative phosphorylation and glycolysis for ATP production [47]. According to Nicholls and colleagues, "neuronal mitochondria are subjected to a variable ATP demand, depending primarily on their excitation pattern, and it is critical that the electron transport chain has sufficient capacity to supply protons to the ATP synthase" [48]. When mitochondria are damaged by chronic stress, such as from elevated Ca^{2+}, ROS, or elevated iron levels, or all three, their respiratory capacity is diminished. Ultimately, these cells can no longer meet the energy demands required to cope with ongoing stress and eventually die, most likely through apoptosis [49]. The inability to handle chronic calcium and the ensuing ROS stress is one of the likeliest causes of photoreceptor cell death in RP. Therefore, increasing the available energy or maintaining mitochondrial homeostasis or both has been an ongoing research topic for the identification of therapeutics for RP [30,50]. SAHA was shown to achieve all the milestones of a compound that increases cell survival by increasing mitochondrial energy production and stabilizing the mitochondrial network in vitro, as well as increasing cell survival in an organ culture of a mouse model of RP that genotypically and phenotypically models the human condition of PDE6b-dependent RP.

HDAC inhibitors have been tested in several different models and ocular diseases. HDAC inhibition has been shown to restore RPE function in hyperglycemia, involving HDAC6 activity [51]. In retinal ganglion cells, hypoacetylation associated with ischemic injury was shown to involve an increase in HDAC1/2 activity [52], and suppressing HDAC activity with valproic acid (VPA) was shown to protect those cells from ocular-hypertensive stress [53]. In RP, VPA was used in a prospective trial; however, patients treated with oral VPA for autosomal dominant RP had worse visual field outcomes than patients treated with placebo [54]. However, VPA has many side effects and interactions, including a black box warning for hepatotoxicity, pancreatitis, and fetal abnormalities, with toxicity of VPA most likely being due to its interference with mitochondrial beta-oxidation. Beta-oxidation is the catabolic process to generate acetyl-CoA from fatty acids the substrate for the citric acid cycle, and NADH and FADH2, coenzymes used in the electron transport chain of oxidative phosphorylation. Thus, if HDAC inhibition is involved in RP, a safer inhibitor might be SAHA.

However, the mechanism by which SAHA protects photoreceptor mitochondria from damage is not known. Our studies suggest that it is upstream or coincident with regulation of mitochondrial fission and fusion, but may not proceed through inhibition of HDAC6 as originally hypothesized. Overall, we have shown that there is a link between protection against mitochondrial damage and preservation of photoreceptor cells in isolated cells. If the link between the MTT assay and mitochondrial respiration extends to the protection in *rd1* organ cultures, it suggests that the cell survival mechanism does not depend solely on HDAC6 inhibition, as tubastatin A, a unique HDAC6 inhibitor, did not result in protection against cell death. SAHA is a class I/II HDAC inhibitor, and HDACs from both classes are present in the rodent retina [52]. SAHA is a hydroxamate-based HDAC inhibitor that has recently been shown to protect neurons from oxidative stress via a catalase-like mechanism [55]. In addition, HDAC inhibitors have been shown to induce HSP70, which can protect neurons from many different stressors, in part by preventing apoptosome formation [56]. Finally, treatment of neuroblastoma cells with HDAC inhibitors trichostatin A and VPA upregulated PGC-1α expression, a regulator of mitochondrial biogenesis [57].

Additional studies have examined the involvement of HDAC inhibition in general and HDAC6 inhibition in particular, in photoreceptor cell protection. These studies revealed important insights into HDAC inhibition in rods and cones. General HDAC inhibition, as is provided by SAHA, can also be achieved using trichostatin A, a class I (HDAC1, 2, 3), class IIa (HDAC4, 7, 9), and class IIb inhibitor (HDAC6). Trifunovic and coworkers used trichostatin A in the cone photoreceptor function loss-1 (cpfl1) mutant mouse retina in vitro in retinal explants and in vivo after a single intravitreal injection [58] and showed significant cone protection. The authors speculated on the role of HDAC6 in that model, because trichostatin A prevented cone degeneration but in addition, improved aberrant cone migration. Migration might be affected by the effect of HDAC6 on the acetylation of α-tubulin required for stability of microtubules in migrating cells [59]. This delay in cone degeneration by trichostatin A was confirmed in additional RP models, *rd1* and *rd10* mice [60]. As the authors did not want to examine the effect of rods on cone survival, but instead, the effect of HDAC inhibition on cone survival only, a late time point, beyond rod survival, was chosen to start the treatment. However, increased rod survival in the context of general HDAC inhibition was shown in the *rd10* organ culture. The *rd10* mutation affects the same gene as the *rd1* mouse used in the present study but has a slower rate of degeneration. After 14 days in culture, treatment with 1 µM SAHA every other day, the same treatment paradigm and concentration we used, led to a fivefold increase in the number of surviving *rd10* rod photoreceptors [61]. Overall, the effects of general HDAC inhibition suggest

that rod and cone degeneration can be reduced by increasing acetylation on target proteins.

Tubastatin A, a specific HDAC6 inhibitor, was used in two relevant publications. Results by Leyk and colleagues [62] showed in 661W cells that tubastatin A protects against oxidative stress induced by 200 µM H_2O_2. In addition, tubastatin A improved visual function in vivo as assessed with the optokinetic response assay in the dye^{ucd6} zebrafish model of inherited blindness. The mutation in these fish affects a subunit of the vacuolar ATPase complex. The same group followed up this observation and extended the analysis on tubastatin A to photoreceptor degeneration in the *rd10* retina. Interestingly, tubastatin A improved cone but not rod survival [63]. In summary, the results discussed suggest that specific HDAC6 inhibition specifically improves cone survival in vivo. The results in 661W cells have shown that tubastatin A protects against oxidative stress [62], but not calcium stress (see Figure 1A), and an HDAC6-specific effect could be identified in SAHA-treated, IBMX-stressed cells (see Figure 1D), a stressor that increases intracellular calcium and oxidative stress [5]. In this context, cone Ca^{2+} levels are involved in photoreceptor cell death in primary (cpfl1) but not in secondary (*rd1*) cone degeneration [64], and elevated calcium is one of the key drivers of rod degeneration in *rd1* and *rd10* mice [65].

Thus, it will be of great interest in future experiments to identify the specific HDAC activity required for the protective effect of SAHA or trichostatin A, the target pathway and/or protein acetylated in the process, or potential HDAC-independent factors that might play a role in the therapeutic effects for rod survival.

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